Assessing the safety of medications during pregnancy using multi-omics and a neuronal differentiation model of early human neurodevelopment

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Til Aurora og Selma

CONTENTS

ACKNOWLEDGEMENTS ... *V*
SUMMARY ... *VII*
SAMMENDRAG ... *IX*
LIST OF PUBLICATIONS ... *XIII*
ABBREVIATIONS ... *XIV*
CHAPTER 1: INTRODUCTION ... *1*
1.1 Medications during pregnancy ... 1
1.1.1 Pregnant women and the use of medications ... 1
1.2 Human embryogenesis ... 1
1.2.1 Nervous system development ... 3
1.3 Epigenetic mechanisms in embryonic development ... 6
1.3.1 Post-translational histone modifications ... 7
1.3.2 DNA methylation ... 8
1.3.3 Chromatin accessibility ... 11
1.3.4 Epigenetic patterns during embryonic development ... 12
1.4 Epigenetic dysregulation and disease ... 14
1.4.1 Neurodevelopmental teratogens and disorders ... 15
1.4.2 Teratogenicity of common medications during pregnancy ... 17
1.5 Paracetamol and pregnancy ... 18
1.5.1 Properties and metabolism of paracetamol ... 18
1.5.2 Paracetamol in pregnancy ... 20
1.5.3 Paracetamol exposure in early development of animals ... 21
1.5.4 Prenatal exposure to paracetamol and neurodevelopmental outcomes in human studies ... 22
1.5.5 Paracetamol and DNA methylation ... 24
1.5.6 Potential mechanisms for effect of paracetamol on neurodevelopment ... 25
1.6 SSRIs and pregnancy ... 25
1.6.1 Properties and metabolism of citalopram ... 25
1.6.2 Citalopram in pregnancy ... 26
1.6.3 Citalopram exposure during early animal development ... 28
1.6.4 Prenatal exposure to SSRIs and neurodevelopmental outcomes in human studies ... 29
1.6.5 SSRIs and DNA methylation ... 31
1.7 Limitations of common methods used to study medication safety during pregnancy ... 32
1.7.1 Case reports ... 32
1.7.2 Epidemiological studies ... 32
1.7.3 Animal studies ... 33
1.7.4 In vitro studies ... 34
1.8 Stem cells and neuronal differentiation ... 35
1.8.1 Stem cell types and properties ... 35
1.8.2 In vitro neural differentiation of hESCs as a model for neurodevelopmental toxicity ... 36
CHAPTER 2: AIMS, HYPOTHESIS AND OBJECTIVES OF THE STUDY ... *39*
CHAPTER 3: METHODOLOGICAL CONSIDERATIONS ... *41*
3.1 Neuronal differentiation protocol ... 41
3.2 Choice of cell line ... 42
3.3 Choice of medication concentrations and metabolites ... 42
3.3.1 Paracetamol ... 42
3.3.2 Citalopram ... 43

3.4 Choice of endpoint omics outcomes 43

CHAPTER 4: SUMMARY OF RESULTS *45*

4.1 Paper I: Robust neuronal differentiation of human embryonic stem cells for neurotoxicology 45

4.2 Paper II: A multi-omics approach to visualize early neuronal differentiation from hESCs in 4D 45

4.3 Paper III: Multi-omics approach reveals dysregulated genes during hESCs neuronal differentiation exposure to paracetamol 46

4.4 Paper IV: Citalopram exposure of hESCs during neuronal differentiation identifies differentially expressed genes involved in neuronal development and depression 47

CHAPTER 5: DISCUSSION *50*

5.1 What does the neuronal differentiation model reflect? 50

5.2 What does the neuronal differentiation model not reflect? 51

5.2.1 Neurodevelopmental outcomes 51

5.2.2 Fetal-maternal interactions 51

5.2.3 Genetic and familial vulnerability 52

5.2.4 Later pregnancy stages 52

5.2.5 Metabolites 53

5.3 Interpretation of findings 54

5.3.1 How to make sense of the large amount of acquired data? 54

5.3.2 How to relate the results to the MoBa study? 55

CHAPTER 6: FUTURE DIRECTIONS *57*

REFERENCES *58*

PAPER I *79*

PAPER II *99*

PAPER III *133*

PAPER IV *209*

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SUMMARY

Background: Pregnant women are susceptible to common diseases and comorbidities, requiring medical intervention that may involve pharmaceuticals. Medications taken during pregnancy may reach the developing fetus and interfere with neurodevelopmental processes, which can impact the risk of disease later in life. Epigenetic modifications essential for cellular differentiation and fetal neurodevelopment have been proposed as mechanisms for this link. Prenatal medication exposures may impact these epigenetic modifications, modulating long-term adverse effects on brain structure and function. Epidemiological studies have found associations between commonly used medications during pregnancy, altered epigenetic patterns in cord blood and adverse neurodevelopmental outcomes in the children. However, the causative nature of medication-induced epigenetic changes and their role in disease development are uncertain. The lack of safety data for drugs taken during pregnancy limits the understanding of how these medications impact fetal neurodevelopment and complicates the assessment of medication risks and benefits during pregnancy. The aim of this thesis was to evaluate the effect of paracetamol and citalopram on early neuronal differentiation.

Methods: First, this thesis presents a 20-day in vitro protocol for neuronal differentiation of human embryonic stem cells (hESCs) to generate a robust model for drug exposure. Second, a molecular timeline of gene expression and epigenetic patterns at four timepoints during neuronal differentiation were constructed using a multi-omics approach of bulk RNA-sequencing (RNA-seq), DNA methylation (DNAm), single-cell RNA-sequencing (scRNA-seq) and single-cell ATAC-sequencing (scATAC-seq). Third, the paracetamol-induced changes in gene expression and epigenetic modifications in hESCs and during neuronal differentiation was assessed using this multi-omics approach. Additionally, integration of chromatin opening with promoters of active genes to identify potential regulatory regions and DNAm was linked with modulation of gene expression. Fourth, the changes to cell-type composition, time- and dose-effects of citalopram exposure to hESCs during neuronal differentiation was assessed using DNAm, RNA-sequencing and single-cell RNA-sequencing analyses.

Results: The protocol for neuronal differentiation of hESCs consisted of three stages: neural induction, self-pattering and neuronal maturation stage. The protocol was optimized for time-course of neuropharmacological exposures. The multi-omics characterisation of these cells identified decreased expression of pluripotency markers and increased expression of neuronal

markers from Day 0 to 20. DNAm changes correlated with gene expression changes during neuronal differentiation, and scRNA-seq analysis identified heterogeneous populations of progenitors, mature neurons, and immature neurons with telencephalic signatures. Exposure to therapeutic concentrations of paracetamol to hESCs undergoing neuronal differentiation induced transcriptional and DNAm changes in genes involved in neuronal development. scRNA-seq analysis identified subtle differences in cell composition between the paracetamol-exposed cells and control cells at Day 20 and changes in several major cellular processes. Integration of the scATAC-seq and scRNA-seq data at Day 20 revealed changes in chromatin opening and transcriptional activity linked to putative regulatory regions associated with paracetamol exposure. We also found a dose-dependent increase in DNAm levels at significant sites in paracetamol-exposed cells compared to controls, and the differential methylation was linked to differential expression. An overlap was identified between dysregulated genes in paracetamol-exposed differentiating cells and differentially methylated genes in cord blood of children with ADHD exposed to paracetamol during pregnancy. Finally, exposure to therapeutic concentrations of citalopram to hESCs undergoing neuronal differentiation induced a dose-dependent effect of citalopram exposure on the number of DEGs but did not affect DNAm levels. scRNA-seq revealed a citalopram-induced increase in pseudotime values, indicating enhanced neuronal maturation. Time- and dose-effects of citalopram were identified in genes involved in neuronal development, brain function, depression and therapeutic mechanisms.

Discussion: Using the hESC neuronal differentiation model and multi-omics to investigate the effects of paracetamol or citalopram exposure on early human neurodevelopment revealed dysregulation of genes involved in key neuronal differentiation processes at bulk and single-cell RNA levels. Paracetamol exposure identified transcriptional dysregulation suggesting potential developmental delays during early neuronal differentiation. Further, differential gene expression, DNAm and chromatin accessibility were identified for genes associated with neuronal maturation, neurogenesis, synaptogenesis, neurotransmitter signalling and genes implicated in neurodevelopmental disorders, suggesting potential mechanistic pathways for paracetamol. Changes in epigenetic modifications linked to gene expression and the overlap with differentially methylated genes in the cord blood of children with ADHD exposed long-term to paracetamol during pregnancy indicates paracetamol-induced epigenetic regulation of gene expression programmes in early brain development. Citalopram exposure identified time- and dose-dependent effects expression of genes involved in neurodevelopment,

axon guidance, neuronal maturation, synaptic transmission and stress response, providing important insights into the potential molecular mechanisms underlying the effects of citalopram exposure on early brain development. The dose-dependent effects on gene expression, coupled with the absence of significant DNA methylation changes, suggest the involvement of alternative gene regulatory mechanisms. Moreover, citalopram had an effect on genes linked major depressive disorders and potential antidepressant therapeutic mechanisms. These molecular alterations may contribute to the observed effects of citalopram on early brain development and potentially influence neurodevelopmental outcomes. Overall, results from paracetamol and citalopram exposure offer important mechanistic insights and potential translational implications. However, an in vitro hESC neuronal differentiation model does not fully reflect the complexity of in vivo neurodevelopmental processes. Further, these studies does not account for neurodevelopmental outcomes, metabolites, fetal-maternal interactions or genetic vulnerability.

Conclusion: The findings of this thesis provide valuable insights into the effects of paracetamol and citalopram exposures on DNAm, chromatin opening and gene expression during early neuronal differentiation. Future studies aiming to determine the causality of paracetamol- and citalopram-induced epigenetic and transcriptional changes and their role in disease development, may facilitate more accurate assessments of the risks and benefits of these medications during pregnancy.

SAMMENDRAG

Bakgrunn: Gravide kvinner er sårbare for vanlige sykdommer og komorbiditeter som krever medisinsk behandling, inkludert farmasøytiske midler. Legemidler som tas under graviditeten kan nå fosteret og forstyrre nevroutviklingsprosesser, noe som kan påvirke risikoen for sykdom senere i livet. Epigenetiske endringer, som er essensielle for cellulær differensiering og fosterets nevroutvikling, har blitt foreslått som mekanismer for denne sammenhengen. Prenatal medikamenteksponering kan påvirke disse epigenetiske mekanismene og modulere langvarige uønskede effekter på hjernestruktur og -funksjon. Epidemiologiske studier har funnet sammenhenger mellom medikamenter som er vanlig å bruke under graviditet, endrede epigenetiske mønstre i navlestrengsblod og ugunstige nevrologiske utfall hos barna. Årsakssammenhengen mellom medikamentinduserte epigenetiske endringer og betydningen for sykdomsutviklingen er ukjent. Medikament kan ikke bli testet på foster under utvikling,

dermed er vurderingen risiko og fordeler av medikamentbruk under graviditet vanskelig. Målet med denne avhandlingen var å studere effekten av paracetamol og citalopram på tidlig nevronal differensiering av humane embryonale stamceller (hESCs).

Metoder: Denne avhandlingen presenterer en 20-dagers in vitro protokoll for nevronal differensiering av hESCs som en modell for tidlig human nevroutvikling. Genekspresjon og epigenetiske modifikasjoner ble kartlagt ved fire tidspunkter under nevronal differensiering ved hjelp av en en multi-omic metode som inkluderer målinger av RNA-sekvensering (RNA-seq), DNA-metylering (DNAm), enkeltcelle RNA-sekvensering (scRNA-seq) og enkeltcelle ATAC-sekvensering (scATAC-seq). Deretter ble paracetamol-induserte endringer i genekspresjon og epigenetiske modifikasjoner i hESCs under nevronal differensiering vurdert ved bruk av den samme multi-omic metoden. I tillegg ble dataene integrert for å identifisere genekspresjon assosiert med kromatinåpning eller DNAm. Til slutt ble endringer i celletype-sammensetning, tid- og dose-effekter av citalopram-eksponering for hESCs under nevronal differensiering vurdert ved hjelp av DNAm, RNA-seq og scRNA-seq.

Resultater: Protokollen for nevronal differensiering av hESCs bestod av tre stadier: nevronal induksjon, selv-mønstring og nevronal modning. Protokollen ble optimalisert for nevrofarmakologiske målinger. Karakteriseringen ved bruk av multi-omic metode av disse cellene identifiserte en nedregulering av pluripotensmarkører og en oppregulering av nevronale markører fra Dag 0 til 20. Endringer i DNA-metylering korrelerte med endringer i genekspresjon under nevronal differensiering, og enkeltcelle RNA-sekvenseringsanalyse identifiserte heterogene cellepopulasjoner av progenitorer, modne nerveceller og umodne nerveceller med molekylære signaturer fra telencephalon. Eksponering av paracetamol i hESCs under nevronal differensiering induserte endringer i transkripsjon og DNA-metylering i gener involvert i nevronal utvikling. Enkeltcelle sekvenseringsanalyse identifiserte subtile forskjeller i cellekomposisjonen mellom paracetamol-eksponerte celler og kontrollceller ved Dag 20, samt endringer i flere viktige cellulære prosesser. Integrering av scATAC-seq- og scRNA-seq-data ved Dag 20 identifiserte endringer i kromatinåpning og transkripsjonsaktivitet forbundet med paracetamoleksponering. Vi fant også en doseavhengig økning i DNAm-nivåene i signifikante seter i paracetamol-eksponerte celler sammenlignet med kontroller, samt en sammenheng mellom differensiell metylering og differensiell genekspresjon. Dysregulerte gener i paracetamol-eksponerte celler under differensiering overlappet med gener med differensiell metylering i navlestrengsblod hos barn med ADHD eksponert for paracetamol under

graviditeten. Eksponering av citalopram i hESCs under nevronal differensiering identifiserte en dose-effekt på antall differensielt uttrykte gener, men påvirket ikke DNAm-nivåene. scRNA-seq avdekket en citalopram-indusert økning i pseudotidverdier, noe som indikerer fremmet nevronal modning. Tids- og doseeffekter av citalopram ble identifisert i gener involvert i nevronal utvikling, hjernefunksjon, depresjon og virkningsmekanismer for terapeutisk effekter.

Diskusjon: Ved å bruke en hESC-modell for nevronal differensiering og multi-omic-analyser til å undersøke effekten av legemiddeleksponering på tidlig human nevroutvikling, ble dysregulering av gener involvert i viktige nevronale differensieringsprosesser identifisert på både global og enkeltcelle RNA-nivå. Paracetamoleksponering identifiserte transkripsjonell dysregulering som kan indikere mulige forsinkelser i utviklingen under tidlig nevronal differensiering. Videre ble differensiell genekspresjon, DNAm og kromatin-åpenhet identifisert i gener knyttet til nevronal modning, nevrogenese, synaptogenese, nevrotransmitter-signalering og gener involvert i nevrologiske utviklingsforstyrrelser, noe som antyder potensielle virkningsmekanismer for paracetamol. Endringer i epigenetiske modifikasjoner knyttet til genekspresjon, og overlapp med differensielt metylerte gener i navlestrengsblodet til barn med ADHD eksponert for paracetamol under lang tid, indikerer at eksponering av paracetamol mulig kan føre til epigenetisk regulering av genekspresjon i tidlig hjernutvikling. Citaloprameksponering identifiserte tid- og dose-effekter på genuttrykk knyttet til nevrologisk utvikling, aksonveiledning, nevronal modning, synaptisk overføring og stressrespons, noe som gir viktige innsikter i de potensielle molekylære mekanismene som ligger til grunn for effektene av citaloprameksponering på tidlig hjernutvikling. Dose-effekter på genekspresjon, kombinert med fravær av signifikante endringer i DNA-metylering, antyder involvering av alternative genreguleringsmekanismer. Citalopram påvirket også gener knyttet til depresjon og potensielle terapeutiske virkningsmekanismer av antidepressiva. Disse molekylære endringene kan mulig medvirke til de observerte effektene av citalopram på tidlig hjernutvikling og potensielt påvirke nevroutviklingsutfall. Samlet sett gir disse resultatene viktige mekanistiske innsikter og potensielle implikasjoner for translasjonell forskning. En in vitro hESC-nevronal differensieringsmodell kan imidlertid ikke gjenspeile kompleksiteten i in vivo nevroutvikling. Modellen tar heller ikke hensyn til nevroutviklingsutfall, metabolitter, fetale-maternelle interaksjoner eller genetisk sårbarhet.

Konklusjon: Funnene i denne avhandlingen gir verdifulle innsikter i effektene av paracetamol- og citaloprameksponering på epigenetiske modifikasjoner og genekspresjon under tidlig nevronal differensiering av hESCs. Fremtidige studier som tar sikte på å fastslå årsakssammenhengen mellom paracetamol- og citalopram-induserte epigenetiske og transkripsjonelle endringer samt deres rolle i sykdomsutvikling, kan bidra til en bedre vurdering av medikamenters risiko og fordeler under graviditet.

LIST OF PUBLICATIONS

Paper I:

Samara A*, Falck M*, Spildrejorde M, et al. Robust neuronal differentiation of human embryonic stem cells for neurotoxicology. STAR Protoc. 2022;3:101533. doi:10.1016/j.xpro.2022.101533

Paper II:

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Paper III:

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Paper IV:

Spildrejorde M, et al, Citalopram exposure of hESCs during neuronal differentiation identifies differentially expressed genes involved in neuronal development and depression. *Manuscript.*

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ABBREVIATIONS

5caC	5-carboxylcytosine
5fC	5-formylcytocine
5hmC	5-hydroximethylcytosine
5mC	5-methylcytosine
AD	antidepressant
ADHD	Attention-Deficit/Hyperactivity Disorder
AM404	*N*-arachidonoylphenolamine
ASD	Autism Spectrum Disorder
BDNF	brain-derived neurotrophic factor
bp	base pairs
BP	biological process
Cit50-400	cells exposed to 50-400 nM citalopram
COX	cyclooxygenase
CpG	cytosine-phosphate-guanine
CRE	cis regulatory element
CSF	cerebrospinal fluid
DEGs	differentially expressed genes
DMCs	Differentially methylates CpGs
DMGs	differentially methylated genes
DNAm	DNA methylation
DNBC	Danish National Birth Cohort
DNMTs	DNA methyltransferases
DT	developmental toxicity
EB	embryoid body
ECVAM	European Centre for the Validation of Alternative Methods
ESCs	embryonic stem cells
ESTn	neural embryonic stem cell test
EWAS	epigenome-wide association study
FGF	fibroblast growth factor
HATs	histone acetyltransferases
HDACs	histone deacetylates
hESCs	Human embryonic stem cells
ICM	Inner cell mass
iPSCs	Induced pluripotent stem cells
K	lysine
MBP	methyl-CpG-binding protein
mESCs	Mouse embryonic stem cells
mEST	Mouse embryonic stem cell test

MM test	Rat micromass test
MoBa	Norwegian Mother, Father and Child cohort
NAPQI	*N*-acetyl-*p*-benzoquinone imine
NDDs	neurodevelopmental disorders
NDT	neurodevelopmental toxicity
NES cells	neuroepithelial stem cells
NPCs	neural progenitor cells
NSAIDs	non-steroidal anti-inflammatory drugs
NSCs	neural stem cells
OCT4	octamer-binding transcription factor 4
OTC	over the counter
P100-200	cells exposed to 100/200 μM Paracetamol
PCR	polymerase chain reaction
PSCs	pluripotent stem cells
PTM	post-translational modifications
RA	retinoic acid
RNA-seq	RNA sequencing
RG	radial glia
scATAC-seq	single cell assay for transposase-accessible chromatin with sequencing
scRNA-seq	single cell RNA sequencing
SOX2	sex determining region Y box 2
SSRI	selective serotonin reuptake inhibitor
T	thymine
TET	ten-eleven translocation
TF	transcription factor
TGFβ	transforming growth factor β
TSS	transcription start sites
TRPV1	vanilloid receptor subtype 1
VPA	valproic acid
WEC test	Rat whole embryo culture assay

CHAPTER 1: INTRODUCTION

1.1 Medications during pregnancy

1.1.1 Pregnant women and the use of medications

Common diseases that affect the general population, for example cardiovascular disorders, influenza and other infectious diseases, seizure disorders, migraine and psychiatric disorders, also affect pregnant women. Moreover, pregnant women have an increased risk of comorbidities such as gestational diabetes, hyperemesis, hyperthyroidism and hypertension. These pre-existing and pregnancy-related conditions often require pharmaceutical intervention. In addition, a substantial number of women become pregnant unintentionally[1], and may be exposed to medications prior to finding out. While unnecessary exposure to medications should be minimized, avoiding treatment for serious illness could potentially be more harmful for both the mother and the developing child than the medication used to treat the condition.

Despite the need for medications during pregnancy there is a large gap in knowledge on how they affect the developing child. Of the 468 medications that were approved by the US Food and Drug Administration between 1980 and 2000, 91% had an unknown teratogenic risk[2], with an average time of 27 years from approval to pregnancy risk classification[3]. Due to the exclusion of pregnant women in clinical trials for ethical reasons, safety data is difficult to obtain. Moreover, the extensive physiological changes in pregnant women can affect pharmacokinetics of medications, thus data derived from studies on non-pregnant woman may lead to wrongful interpretation in the context of pregnant women[4]. This poses a challenge for weighing the benefits of pharmaceutical interventions in pregnant women against the potential risks to the developing fetus. Nevertheless, 81% of women use either over the counter (OTC) or prescription medications at least once during pregnancy[5].

Embryonic and fetal developmental periods are highly susceptible to potential teratogens, including medications[6]. Thus, medications taken at different times during pregnancy could affect different developmental processes[6].

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1.2 Human embryogenesis

Embryogenesis starts with fertilization, where the genetic material of an ovum and spermatozoon fuse together forming a zygote (Fig. 1). This event is followed by cleavage, a

series of mitotic divisions resulting in undifferentiated cells called blastomeres. At the 4/8-cell stage maternally stored mRNA and proteins are replaced by the activation of embryonic transcription programme. Then, starting between the 8- and 16-cell stage, the process of compaction and polarization follows. The blastomeres bind tightly together and obtain apicobasal polarity[7]. At embryonic day 5, the developing structure is called a blastocyst, comprised of trophectoderm tissue (precursors of the placenta) surrounding the inner cell mass (ICM) and a fluid-filled cavity called blastocoel[7].

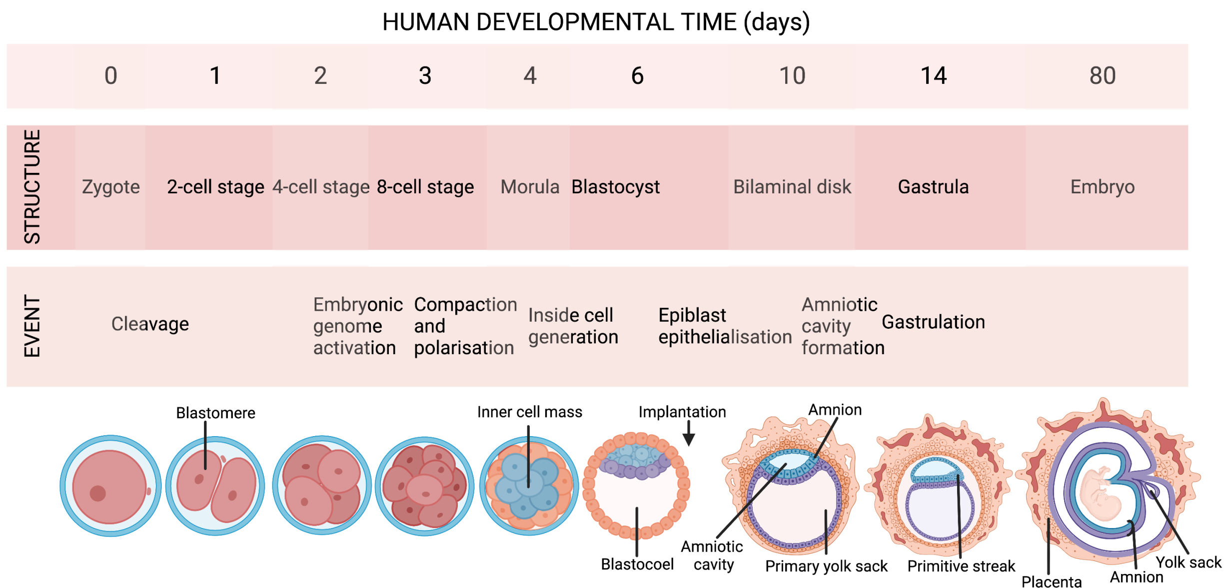

Figure 1. Development of the human embryo. The zygote undergoes cleavage into undifferentiated blastomeres. Then the morula forms, where the initial cell fate decision into trophectoderm and ICM take place. By embryonic day 5 the blastocele cavity has formed, resulting in the blastocyst comprised of trophectoderm, hypoblast and epiblast tissue. Morphological transformation and epiblast epithelialization results in a bilaminar disk primed for gastrulation. Gastrulation starts at embryonic day 14, establishing the three germ layers: ectoderm, endoderm and mesoderm. Tissues derived from trophectoderm, hypoblast and epiblast are shown in orange, purple and blue respectively. Adapted from *Shahbazi* (2020)[7] with permission. Created with BioRender.com.

The ICM gives rise to pluripotent epiblast and extra-embryonic hypoblast, which are the precursors of the embryo and yolk sac, respectively. At this stage, the blastocyst implants into the uterine lining and subsequently goes through a morphological transformation to form the bilaminar disc which has now acquired a dorsal/ventral axis[8]. The epiblast cells in this bilaminar disk differentiates into extra-embryonic amniotic epithelial cells which serve as a membrane outlining the amniotic cavity, or remains pluripotent epithelium[7]. Gastrulation starts at embryonic day 14 with the formation of the primitive streak, which establish the cranial/caudal

axis. The epiblast cells then lose their pluripotency and commit to the mesoderm, endoderm or ectoderm lineages.

Endoderm and mesoderm originate from epiblast cells that undergo epithelial-to-mesenchymal transition and subsequently migrate through the primitive streak[7]. Endoderm is the precursor of cells found in the respiratory system, thyroid, liver, pancreas and intestines. Mesoderm is the precursor of muscle, connective tissue, bone, cartilage, kidney, reproductive organs, subcutaneous layer of the skin, circulatory and lymphatic systems, microglia and adrenal cortex[8]. Ectoderm originates from the remaining outer layer of epiblast cells that do not go through the primitive streak and generates the nervous system and epidermis[7].

1.2.1 Nervous system development

Primary neurulation, neural tube formation leading to the formation of the brain and spinal cord, occurs between embryonal weeks 3 and 4 (Fig. 2)[9]. At the end of gastrulation, dorsal mesoderm forms the notochord, a crucial structure for initiation of neurulation. Signals from the notochord cause the overlaying ectoderm layer to thicken and form the neural plate. The cells that encompass the neural plate undergo neural induction, resulting in neuroepithelial stem (NES) cells[10]. Next, the neural plate folds inward starting from the center of the embryo and progress outward in cranial and caudal direction, forming the neural fold surrounding the neural groove[11]. Further, the neural fold fuse together resulting in the cerebrospinal fluid-filled neural tube, the precursor of the spinal cord and brain[9]. Neural crest cells detach from the fusing grooves and compose a layer separate from the surface ectoderm and the neural tube. These migratory and multipotent cells will eventually form the peripheral nervous system, spinal and cranial nerves, melanocytes, bone and cartilage[12]. The surrounding surface ectoderm becomes the outer layer of the skin.

As the embryo develops, the neural tube forms three distinct segments: the forebrain, midbrain and hindbrain (Fig. 3). By embryonic week 5, telencephalon and diencephalon have developed from the forebrain, mesencephalon has developed from the midbrain and the metencephalon and myelencephalon from the hindbrain[13]. In the adult brain, the telencephalon becomes the cerebrum, the major control center for sensory perception, motor function, emotions, language, learning and memory. The diencephalon develops into the hypothalamus, epithalamus and thalamus. Mesencephalon, metencephalon and myelencephalon forms three distinct regions of

the brainstem: midbrain, pons and medulla oblongata, respectively. Moreover, the cerebellum develops from metencephalon[13].

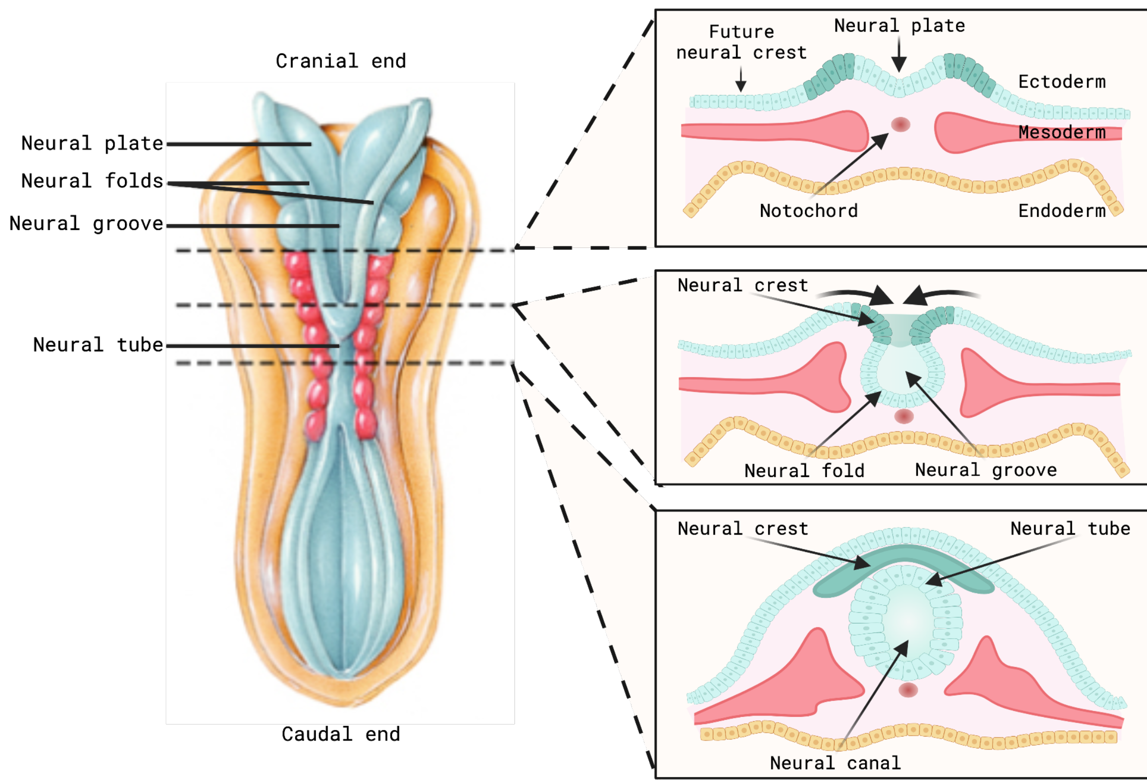

Figure 2. Primary neurulation. *Left panel:* Dorsal view of the embryo at approximately embryonic day 22. Stippled lines show cross-sections viewed in *right panels* and represent the progressive closure of the neural tube. The notochord initiates thickening of ectoderm to generate the neural plate. Folding inward starts in the center of the embryo forming a neural groove. The neural fold fuse together forming the neural tube towards the caudal end and neural crest cells towards the dorsal end of the ectoderm. Modified from *Sadler* (2005) [11] with permission. Created with BioRender.com.

The cells in the developing brain acquire distinct identities according to their spatial positions through a process called neural patterning[14]. Neural patterning is defined by morphogen gradients along the anterior-posterior and dorsal-ventral axis, which mediate region-specification of NES cells to neural progenitor subtypes by initiating local transcriptional networks (Fig. 3)[15]. The anterior-posterior axis is coordinated by signals from Wnt, retinoic acid (RA) and fibroblast growth factor (FGF) morphogens. Wnt morphogens control the forebrain, midbrain, hindbrain and anterior spinal cord segmentation in a dose-dependent manner[16], while RA and FGF morphogens control spinal cord segmentation[17]. Moreover, the dorsal-ventral axis is coordinated by Wnts, bone morphogenic proteins (BMPs) and sonic

hedgehog (SHH). Increasing concentrations of Wnt and BMPs lead to more dorsal identities, whereas SHH causes ventralization[15].

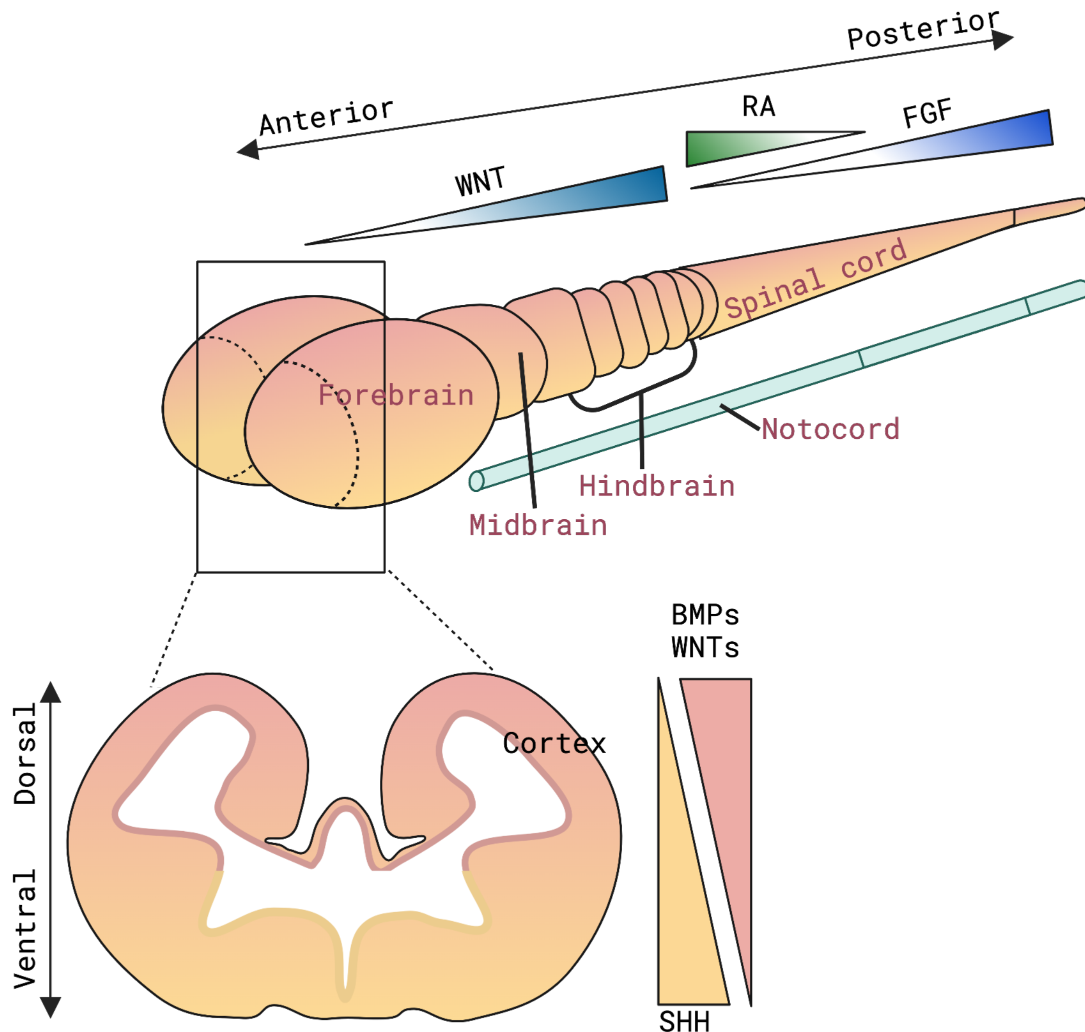

Figure 3: Neural patterning. *Top panel:* The anterior-posterior axis is coordinated by Wnt, RA and FGF morphogens. The segmentation of the forebrain, midbrain, hindbrain and anterior spinal cord is controlled by the Wnt-gradient, whereas spinal cord segmentation is controlled by RA and FGF gradients. *Bottom panel:* The dorsal-ventral axis is coordinated by opposing gradients of Wnt and SHH morphogens. Adapted from *Tao and Zhang* (2016) [15] with permission. Created with BioRender.com.

At the cellular level, neurodevelopment entails a series of tightly regulated events. NES cells undergo extensive proliferation followed by the onset of neurogenesis, where they differentiate to radial glia (RG) cells[18]. RG cells are self-renewing progenitors of neuronal, glial and oligodendroglial lineages, in addition to acting as a scaffold for migrating neurons[18]. Redundant cells are eliminated by apoptosis[19]. RG cells undergo asymmetrical divisions, resulting in new RG cells and primitive, committed neurons, neuroblasts[20]. Neuroblasts then

migrate from their sites of origin to their final destination in the developing cortex and cerebellum. Here, dendritic growth, synaptogenesis, maturation and finally myelination occur[19]. Disruption of any of these finely tuned processes can singly or in combination cause minor or severe malformations and functional abnormalities contributing to NDDs[9].

1.3 Epigenetic mechanisms in embryonic development

Cell specification during embryonic development, including neurodevelopment, is regulated by epigenetic mechanisms. Historically, there have been multiple definitions of epigenetics[21]. Here, epigenetics will be referred to as the study of mitotically heritable chemical modifications to DNA and DNA associated proteins that regulate gene expression patterns without changes to the DNA sequence itself[22,23]. Epigenetic modifications mediate cellular memory which is essential to cellular differentiation during normal embryonic development and for maintaining cell identities throughout adult life[24].

Epigenetic transcriptional control should be seen in context with chromatin, the dynamic structural organization of the genome in complex with proteins which condenses DNA in the cell nucleus (Fig. 4). In the chromatin complex, 145 base pairs (bp) DNA is coiled around histone octamers consisting of two copies of four histone proteins (H2A, H2B, H3 and H4), collectively called a nucleosome[25].

Chromatin can be highly condensed in the form of heterochromatin associated with transcriptional silence, or more loosely bound as euchromatin associated with active transcription[25]. A linker histone, histone H1 interacts with the entry and exit point of the nucleosome and promote chromatin compaction. Epigenetic mechanisms that are involved in cellular memory and interact to regulate the cell-type specific, dynamic state of chromatin are DNA methylation (DNAm) and post-translational modifications (PTM) of histones[26]. The sum of these chemical modifications comprises the epigenome of a cell, which is established by intrinsic signals from the genome and may be influenced by extrinsic signals from the environment. Thus, epigenetic alterations can be viewed as a link between the environment and changes in gene expression resulting in a particular phenotype.

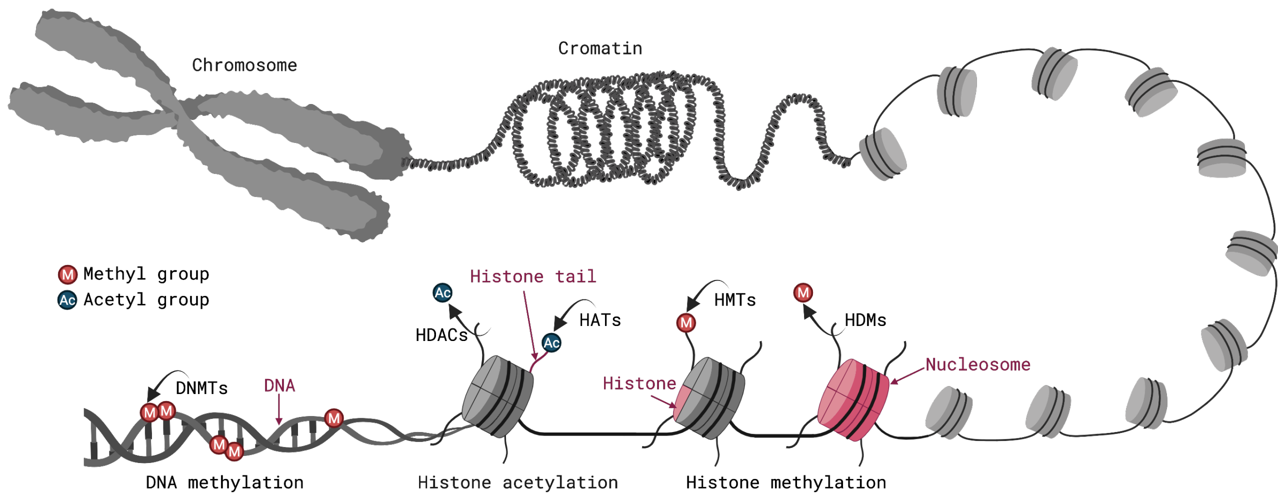

Figure 4. Epigenetic factors involved in transcriptional regulation. The basic functional unit of chromosomes is chromatin, which consist of coiled up DNA in complex with nucleosomes. Two copies of each histone H2A, H2B, H3 and H4 together with approximately 145 bp of DNA constitute one nucleosome. Regulation of gene expression is dependent on the accessibility of DNA to the transcription machinery, which is affected by post-translational histone modifications, such as histone methylation and acetylation, and DNAm. Tightly packed heterochromatin is associated with transcriptional silencing, whereas euchromatin is more loosely bound and associated with active transcription. HDMs, histone demethylases; HMTs, histone methyltransferases; HDACs, histone deacetylases; HATs, histone acetyltransferases; DNMTs, DNA methyltransferases. Adapted from Joosten *et al*. (2018)[27] with permission. Created with BioRender.com.

1.3.1 Post-translational histone modifications

Amino acids at the N-terminal tails, C-terminal tails and non-tail regions of histone proteins can be covalently modified after translation, although it occurs most frequently at N-terminal tails[28]. These modifications include methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, citrullination, glycosylation and ADP ribosylation and are involved in the regulation of gene expression through their recruitment of regulatory proteins and by altering chromosome structure[29]. Moreover, histone PTMs are essential for the regulation of other cell processes such as DNA replication, DNA repair, cell cycle control and DNA condensation during cell division[28].

Histone acetylation is catalyzed by histone acetyltransferases (HATs) and is reversed by histone deacetylates (HDACs)[29]. Histone acetylation occurs on lysine residues and is generally associated with transcriptional activity, whereas histone deacetylation is associated with transcriptional silence[25]. For example, acetylation of histone H3 lysine 9 (H3K9ac), H3K14ac

and H3K27ac are found at enhancers (cis regulatory elements (CREs) which interact with regulatory proteins to enhance the transcription target genes) and transcription start sites (TSS) of active genes[28]. In contrast, histone methylation acts as both repressive and activating signals depending on the degree of methylation and the position in the histone. Histone methylation is catalysed by histone methyltransferases (HMTs) and demethylated by histone demethylases (HDMs) and occur on lysine, arginine and histidine residues[28]. Moreover, there is an additional level of complexity in that lysine residues may exhibit mono-, di- or tri-methylation and arginines may be mono- or di-methylated in a symmetrical or asymmetrical manner[30]. For example, histone H3 lysine 4 mono-, di- and tri-methylation (H3K4me/me2/me3), H3K9me, H3K27me and H3K36me/me3 are considered active chromatin marks, whereas H3K9me3, H3K27me3 and H4K20me3 are considered marks of repressed chromatin[31].

Histone PTMs affect chromatin accessibility by altering histone-histone and DNA-histone interactions, creating binding sites for chromatin modifiers and influencing nearby occurrences of PTMs[31]. For example, acetylation and phosphorylation decrease the positive charge of histones, thereby resulting in weaker DNA-histone interactions and subsequent decompaction of the chromatin structure and transcriptional accessibility[29]. The various histone modifications and the interplay between them results in different effects on gene regulation, and they are reversible, dynamic and tightly regulated[29].

1.3.2 DNA methylation

Methylation of DNA occurs by covalent binding of methyl-groups (CH_3) to the fifth carbon of cytosine, generating 5-methylcytosine (5mC). In mammals, DNAm mainly takes place on cytosine-phosphate-guanine (CpG) dinucleotides, but is also on non-CpGs in embryonic stem cells (ESCs) and adult brain[32]. In the human genome there are approximately 29 million CpGs of which 65-80% are methylated[33]. DNAm is involved in regulation of gene expression, genomic imprinting, X-chromosome inactivation, chromosomal stability and genomic defense against transposable elements[34]. DNAm patterns are cell-type specific, mitotically heritable and are important for lineage commitment and cellular memory during development.

The establishment of DNAm is catalyzed by DNA methyltransferases (DNMTs). DNMT1 is responsible for maintaining global DNAm patterns[35]. Immediately after replication the DNA is hemimethylated: the parental strand is methylated while the newly synthesized daughter strand is unmethylated. The multidomain protein UHRF1 (ubiquitin-like, containing PHD and RING

finger domains 1) recognizes H3K9me3 and hemimethylated DNA, and recruits DNMT1 to the replication fork by ubiquitylating K14, K18 and K23 on histone H3[36]. Then, DNMT1 methylates the hemimethylated DNA, ensuring restored symmetry and inheritance of DNAm patterns through cell division[36]. The DNMTs responsible for *de novo* DNAm are DNMT3A and DNMT3B in conjunction with the regulatory factor DNMT3L. DNMT3L interacts with unmethylated H3K4 and recruits two DNMT3A and one DNMT3B to form a tetramer capable of establishing new DNAm groups[37].

DNA demethylation can occur via passive or active processes. Passive demethylation is the result of lack of maintenance of DNAm after DNA replication, diluting the methylation groups over consecutive rounds of cell divisions[38]. In contrast, active demethylation occurs by enzymatic modification or removal of the methyl group catalyzed by ten-eleven translocation (TET) enzymes[38].

CpG dinucleotides are generally underrepresented in the mammalian genome, thought to be due to the high mutation rate of 5mC which, via spontaneous deamination forms thymine (T). The exception from this underrepresentation can be found in CpG-rich regions defined as CpG islands (CGIs), which are often unmethylated in germline cells and consequently stably passed on to the next generation[35]. CGIs remain primarily unmethylated in somatic cells. Furthermore, CGIs are found at two-thirds of mammalian promoter regions and TSSs, but can also be found in gene bodies and intergenic regions[34]. DNAm at CGI promoters is well known as a transcriptional repressive epigenetic signal through recruitment of transcriptionally repressive methyl-CpG-binding proteins (MBPs), interfering with TF binding and nucleosome positioning (Fig. 5)[39]. In contrast, actively transcribed genes typically have unmethylated CGI promoters and are associated with H3K4me3[34]. This allows promoter binding of CXXC domain-containing activator complexes, while methylation-sensitive TFs bind to unmethylated enhancers (Fig. 5)[39]. However it is becoming clear that the function of DNAm varies with the context in which it is found[40]. DNAm can also facilitate transcriptional activation through TFs that preferentially bind to methylated sequences (Fig. 5)[34]. The activity of genes with CGI promoters can be regulated in a flexible manner by polycomb repressive complex 2-mediated H3K27 methylation, whereas long-term stabilization of silencing can be achieved with DNAm. Examples of DNAm-based silencing include X-chromosome inactivation, genomic imprinting and genes specific to germline cells[34].

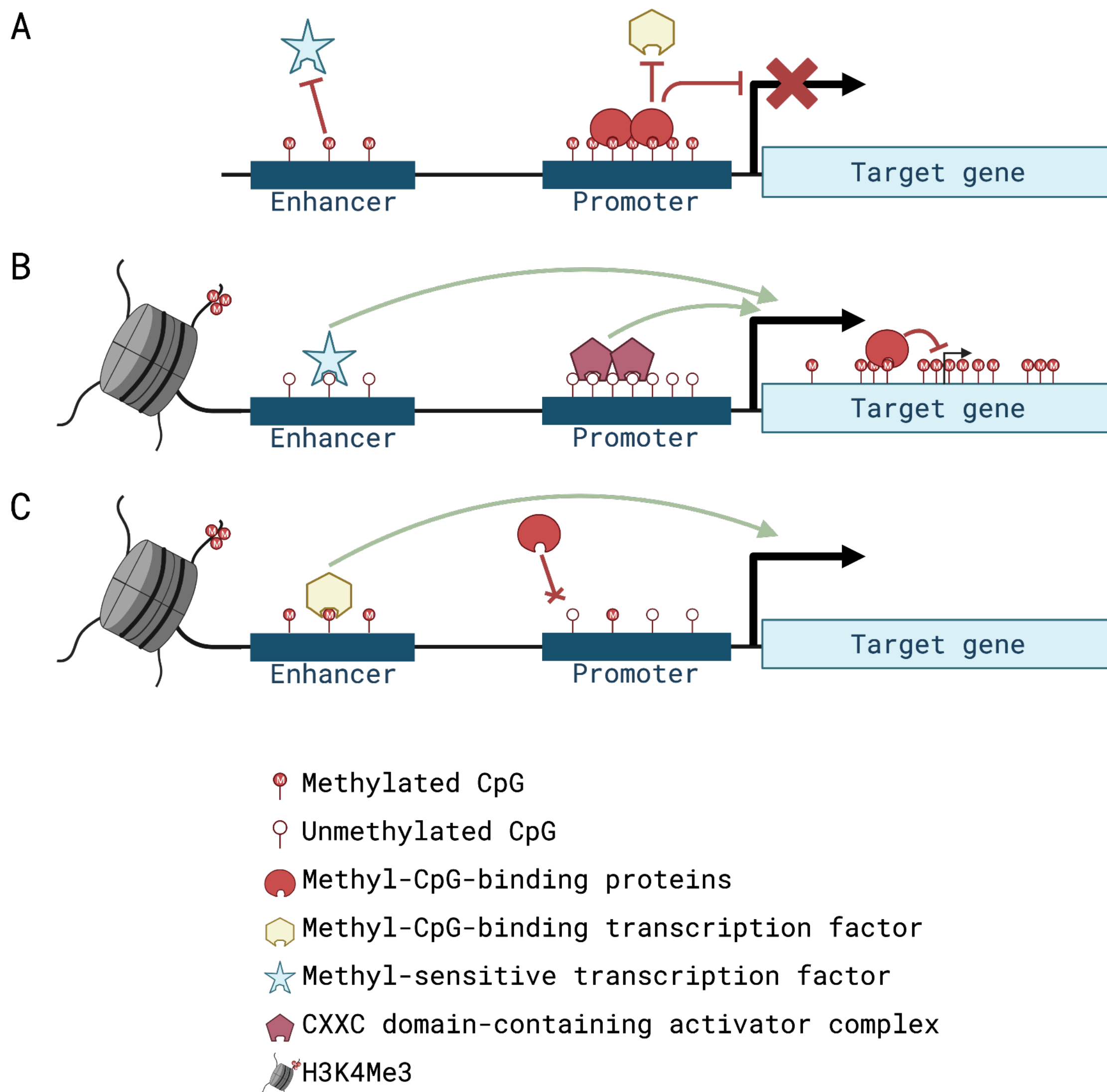

Figure 5. Transcriptional regulation by DNAm. A) Genes with methylated CGI promoters are repressed through the binding of repressive methyl-CpG-binding proteins (MBPs). Methylated enhancers can also block the binding of TFs that are sensitive to methylation. B) Unmethylated CGI promoters are associated with trimethylated H3K4 (H3K4me3) and accessible to binding of CXXC domain-containing activation complexes. Moreover, transcription factors bind to unmethylated enhancers to initiate transcription. Gene activation is not sensitive to gene body methylation, although it is proposed to block transcription initiation of repetitive DNA elements. C) Genes with methylated low density CpG promoters do not interact with MBPs and can be transcribed in some cases. Methylation-specific transcription factors can bind to methylated enhancers associated with H3K4me3. Adapted from *Spruijt and Vermeulen* (2014)[39] with permission. Created with BioRender.com.

Gene bodies typically have a low density of CpGs which are methylated in a tissue-specific manner. In contrast to CGI promoter methylation, gene body methylation is not associated with

transcriptional repression[34]. Despite being bound by MBPs, similar to CGIs at TSS, methylation at gene body CGIs is positively correlated with transcription, suggesting that silencing of genes is due to interference with transcriptional initiation but not elongation in mammals[40]. Although the function of gene body methylation is not yet fully understood, two hypotheses have been proposed. First, gene body methylation is thought to contribute to chromosomal stability by silencing intragenic cryptic promoters of repetitive DNA elements, including retrotransposons and LINE1 elements[40]. Second, gene body methylation may be involved in regulation of splicing. There is significantly higher methylation levels in exons compared to introns[41]. Moreover, constitutive exons are more highly methylated than alternative exons[42]. Thus, DNAm may facilitate splicing through methylation-sensitive regulatory mechanisms. For instance, DNAm prevents binding of CTCF protein which is involved in exon inclusion by slowing down RNA polymerase II elongation[43].

The function of DNAm at intergenic regions and regulatory elements such as enhancers and insulators, is not yet fully elucidated. Generally, enhancers have a lower CpG density than promoters, and methylation levels are more dynamic and cell type-specific than promotor methylation[44]. Whereas enhancer DNAm levels are inversely correlated with transcriptional activity similarly to promoter methylation levels, their activity is also dependent on several other factors[44].

1.3.3 Chromatin accessibility

Physical accessibility of DNA sequences for transcriptional machinery is controlled by chromatin opening, a dynamic process influenced by various factors, such as histone modifications, chromatin remodelling complexes, DNAm and transcription factors (TFs)[45]. TFs are key regulators of this process either by competing with histones and other chromatin-binding proteins, by recruiting chromatin remodellers or by opening chromatin directly, to modulate nucleosome occupancy and regulate DNA access[45]. The interaction between TFs and the accessibility landscape of a cell type is reciprocal, chromatin accessibility influences TF binding, and consequently transcription of genes.

Dysregulation of chromatin opening can disrupt gene expression programs, and has been implicated in NDDs[46–48].

1.3.4 Epigenetic patterns during embryonic development

Cellular differentiation is regulated by epigenetic mechanisms. After fertilization, the epigenome is reprogrammed to restore totipotency before new cell type-specific patterns are established. This critical developmental period of major epigenetic reprogramming and cell fate decision events may be particularly susceptible to alterations by environmental influences.

Genome-wide DNA demethylation occurs in early preimplantation embryos (Fig. 6), subsequently reaching the lowest levels of DNAm at the early blastocyst stage[49]. During reprogramming, the paternally inherited genome undergoes active demethylation mediated by TET3, where 5mC is oxidized to form 5hmC, 5fC and 5caC. The levels of oxidation derivatives of 5mC then gradually decrease through passive DNA replication-dependent dilution[49]. In contrast, the maternally inherited genome is protected from TET3-mediated demethylation, and is subsequently passively demethylated during zygotic cleavage due to the exclusion of the maintenance enzyme DNMT1 from the nucleus[34]. Following reprogramming, around the time of implantation, de novo DNAm mediated by DNMT3A and DNMT3B occur rapidly to establish the embryonic methylation pattern[49]. During differentiation de novo methylation and demethylation of the DNA take place in a dynamic manner to ensure lineage commitment[50]. However, approximately 20% of CpGs escape this global wave of demethylation[51]. The CpGs which retain methylation inherited from gametes are located mainly in imprinting control regions and evolutionary young retrotransposons[34].

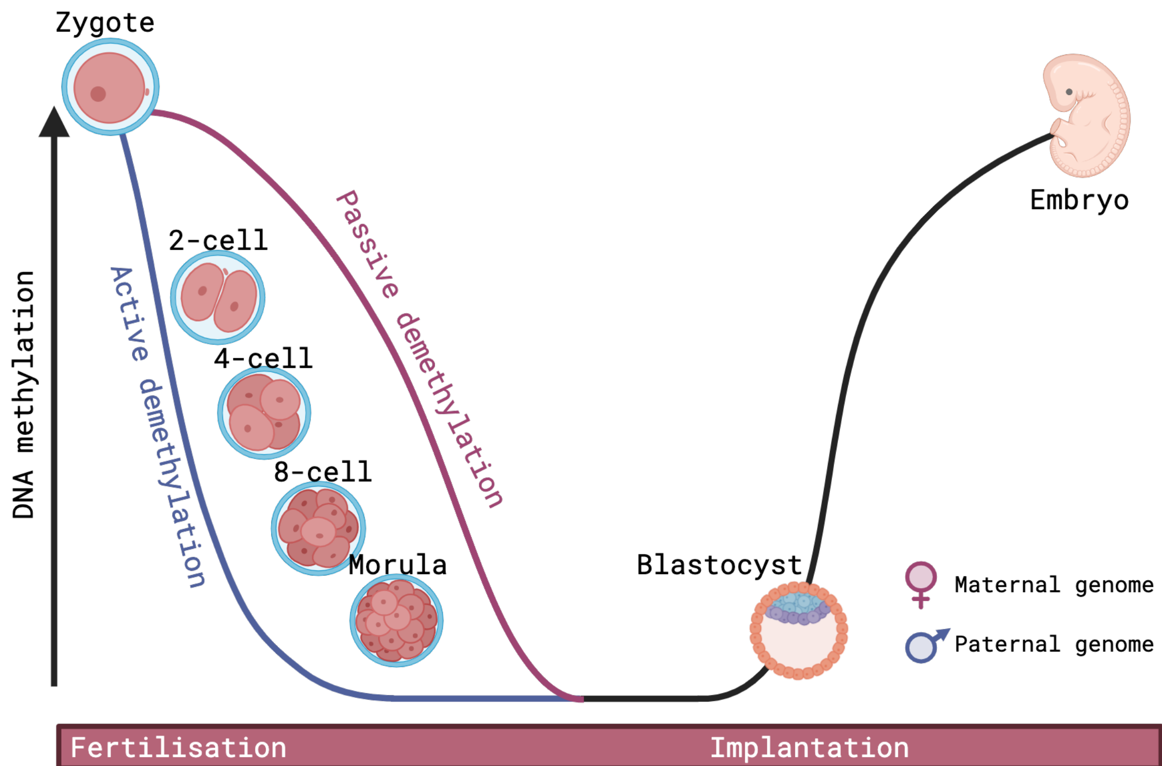

Figure 6. DNAm reprogramming during embryonic development. After fertilization, global DNAm is erased. The maternal pronuclear genome undergoes passive demethylation, whereas the paternal pronuclear genome undergoes active demethylation by TET enzymes. At the blastocyst stage, de novo methylation by DNMT3A and DNMT3B occur to reestablish methylation patterns. Adapted from *Zeng and Chen* (2019)[49] with permission. Created with BioRender.com.

In ESCs, low-density CpG promoters that are associated with tissue-specific genes are mostly methylated, whereas CGI promoters that are associated with developmental genes are generally unmethylated[52]. This methylation status is dynamic and changes during differentiation. Upon commitment to neural lineages, genes related to pluripotency and non-neural lineages gain DNAm at their promoters contributing to their long-term silencing. In contrast, some distal regulatory elements related to neural genes undergo DNA demethylation[53].

Compared to DNAm, less is known about the major changes in global chromatin organization and PTMs of histones in the preimplantation embryo. The reprogramming of histone PTMs involves a series of extremely complicated and highly controlled locus-specific events which is not yet fully understood[54]. The histone marks of parentally inherited genomes are characterized by asymmetry in the zygote and through the 4-cell stage, and it is believed that this asymmetry is necessary for epigenetic reprogramming. Protamines in the paternal

pronucleus are replaced by maternally inherited histones genome-wide in the zygote[55]. The maternal pronucleus contain PTMs resembling those of somatic cells, whereas the paternal pronucleus is distinguished by a hypomethylated chromatin state thought to permit increased accessibility to the paternal genome from the late zygote stage[56]. In the maternal pronucleus, histone methylation marks that are typical features of heterochromatin such as H3K9me3, H3K27me3, H3K64me3 and H4K20me3 are lost by the 4-cell stage[54,56]. Moreover, the loss of repressive marks is consistent with the timing of embryonic genome activation from the 4-cell stage[55]. Histone methylation marks are then progressively re-established, with a delayed detection tri-methylation compared to mono-methylation marks in early development[56].

In ESCs it has been shown that non-transcribed genes encoding developmental regulators can be kept in a poised state, where their promoters carry opposing PTMs including the active marks H3K9ac and H3K4me3 and the repressive mark H3K27me3[57]. In response to developmental signals during differentiation, promoters of tissue-specific genes lose the H3K27me3 mark and become de-repressed[53]. For example, genes encoding TFs involved in neural development, including *PAX6*, *NESTIN*, *SOX1*, *ASCL1* and *NKX2.2*, carry these opposing histone marks in ESCs. H3K27me3 is then lost during neural differentiation, resulting in gene activation[58].

1.4Epigenetic dysregulation and disease

Aberrant epigenetic regulation of gene expression may cause altered phenotypes and ultimately lead to disease. This is evident from the fact that many Mendelian disorders are caused by mutations in genes encoding epigenetic regulators[59]. Genetic variants in genes encoding major regulators of DNAm, including DNMT and TET genes, can cause neurological disorders, immunodeficiencies and cancers[34].

Epigenetic dysregulation has also been implicated in NDDs. Genetic variants in the X-linked *MECP2* gene (encoding the MBP methyl CpG-binding protein 2, MeCP2) causes Rett Syndrome, a progressive NDD characterized by intellectual disabilities, ataxia, autism and seizures[60]. MeCP2 functions as a global repressor of transcription by binding to methylated CGIs and recruiting HDACs, and disruption of this process can cause aberrant gene expression[61]. Genetic variants in *MECP2*[62] and other MBPs, including *MBD5*[63,64], *MBD3* and *MBD4*, are also indicated as risk factors for ASD, which is a common comorbidity of Rett syndrome[65]. Additionally, genome-wide association studies (GWAS) and have identified

variations *DNMT3A* and in genes encoding chromatin remodelers, including *CDH8/CTNNB1, CHD2* and *ARID1B*, associated with increased risk of ASD[63,64]. Collectively, this indicates the importance of epigenetic regulation to NDDs.

1.4.1 Neurodevelopmental teratogens and disorders

Teratogens are substances that disrupt fetal development and cause physical or functional deficits in the child following exposure during pregnancy. In the 1960s it was discovered that thalidomide, at the time an OTC medication taken worldwide against sleeplessness and morning sickness, caused severe birth defects in thousands of children[66]. The extent of this scandal has fortunately not been repeated, however other medications have been reported to have severe teratogenic effects in children exposed in utero. Isotretinoin, a treatment for severe acne, when used during pregnancy was shown to cause congenital craniofacial, cardiac and thymic defects and neurocognitive impairment[67]. Exposure to valproic acid (VPA), a treatment for epilepsy, bipolar disease and prevention of migraines, has been associated with an increased risk of congenital defects in developing children, including neural tube defects, heart defects, cleft palate, limb defects and genitourinary defects[68]. Moreover, prenatal exposure to the anticonvulsant topiramate has been associated with autism spectrum disorder (ASD) and intellectual disabilities[69]. Other commonly used medications used by pregnant women that have been linked to congenital abnormalities are lithium[70], used to treat mental illness, the blood thinner warfarin[71] and tetracyclines[72], used to treat bacterial infections. These medications are considered to be major teratogens. As mentioned, however, there are numerous medications for which safety data is scarce or lacking completely. Studies where long-term and subtle outcomes of medication exposure are investigated, including neurodevelopmental outcomes, are few and far between and often underpowered.

Medications that cross the placenta and the blood-brain barrier may interfere with normal fetal neurodevelopment. Most medications may reach the embryo via passive diffusion[73], or even active transportation, and thus have the potential to perturb neurodevelopment. Normal neurodevelopment involves complex processes such as neural differentiation, proliferation, migration, synaptogenesis, apoptosis, myelination, synaptic pruning and development of the blood-brain-barrier[74]. Disruption of these events may result in long-term adverse effects in central nervous system function[75]. Thus, this critical developmental period which continues during the entire pregnancy, is highly sensitive to environmental influences[6]. This is also

evident from the observations that some well-defined neurotoxins such as lead and methylmercury affect the developing brain at lower concentrations than the adult brain[76].

Several environmental compounds have been associated with neurodevelopmental toxicity (NDT) in children exposed in utero. Prenatal exposure to organophosphates, a group of pesticides present in food, water and soil which inhibit acetylcholinesterase activity[77], have been linked to ASD[78]. Polybrominated diphenyl ethers are additive flame retardants which have been associated with decreased motor function, attention, cognitive scores and altered behaviour[79,80]. Further, bisphenol A (a component of polycarbonate plastic), air pollutants, maternal alcohol consumption, smoking, malnutrition and infection are also associated with adverse neurodevelopmental outcomes[75,78,81–86]. Some of the pathophysiological mechanisms proposed to be involved in the neurotoxic effect of these compounds are oxidative stress, immune system dysregulation, altered neurotransmitter systems and thyroid hormone disruption[75].

Disruption of brain development can result in a variety of conditions defined as neurodevelopmental disorders (NDDs), such as attention-deficit/hyperactivity disorder (ADHD), ASD, intellectual disabilities, learning disabilities, epileptic encephalopathies and schizophrenia[87]. NDDs affect 17% of US children aged 3 to 17 years, and the prevalence has increased over the last two decades[88]. ASD, is characterized by challenges in social interaction, communication and restricted interests and behaviours[89]. ADHD, characterized by hyperactivity, inattention and impulsivity, is the most common NDD with a prevalence of 5% worldwide[90]. Although ADHD is highly heritable[91], environmental factors such as maternal stress, anxiety, depression, smoking and alcohol consumption, prenatal exposure to organophosphates, polychlorinated bisphenyls, bisphenol A, methylmercury and lead have been associated with ADHD[75,92–94]. Despite the differences in clinical presentation, ADHD shares both genetic[95] and environmental[75] risk factors with ASD.

The causes of NDDs are highly complex and poorly characterized, however the interplay between heterogeneous genetic factors, environmental influences and epigenetic factors are proposed to be the major contributors to NDD aetiology[96]. Considering the enormous socioeconomic burden and the individual burden NDDs represent, it is crucial to identify and minimize the environmental risk factors. Therefore, it would be valuable to obtain pregnancy

safety data, including long term neurodevelopmental effects, for medications commonly used by pregnant women.

1.4.2 Teratogenicity of common medications during pregnancy

The theory of developmental origins of adult-onset disease postulates the idea that an organism is capable of adapting to environmental signals during development, thus affecting risk of disease later in life, including NDDs[97]. This risk may be modulated through alterations of epigenetic patterns. Since epigenetic modifications are mitotically heritable, they provide a potential mechanism for long-term changes in gene expression after withdrawal of the exposure.

Developmental plasticity is supported by a number of epidemiological studies showing that maternal stress and nutrition status is associated with diseases such as obesity, diabetes, mental illness and childhood NDDs in the child[93,98–101]. Animal studies have shown that prenatal and early postnatal environmental factors such as maternal behaviour[102], heavy metals[103], pharmaceuticals[104], endocrine disruptors[105] and low-dose radiation[106] can cause altered epigenetic modifications and modulate disease susceptibility. For example, perinatal exposure to methylmercury in mice is associated with altered behaviour and long-term silencing of the *BDNF* promoter by DNA hypermethylation, increased H3K37me3 and decreased H3 acetylation[107]. However, whether these changes in epigenetic patterns are mediators, modifiers or consequences of disease is largely unknown (Fig. 7).

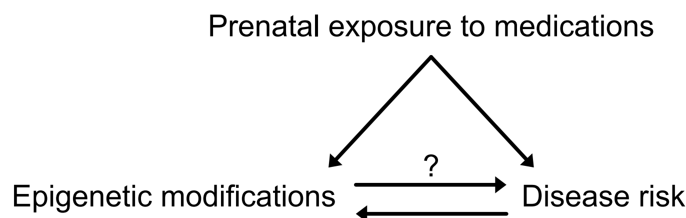

Figure 7. Are medication-induced epigenetic changes causative or a consequence of disease? Prenatal exposure to medications is associated with changes in epigenetic patterns and disease risk. However, it is unknown whether the increased disease risk is caused directly by medication exposure or mediated via epigenetic modifications. Furthermore, we do not know if these epigenetic changes mediate increased disease risk or if they are a consequence of disease mechanisms.

A range of prenatal environmental exposures in humans are associated with altered DNAm in cord blood, including heavy metals[108–110], bisphenol A[111], air pollutants[112], maternal

smoking[113,114], antiepileptic medications[115,116], methadone[117] and paracetamol[118]. Prenatal exposure to maternal smoking is a well-studied example and has been associated with a number of adverse effects in the child, such as low birthweight, asthma, behavioural problems and NDDs, including ASD and ADHD[119–121]. Smoking-induced DNAm changes have been found in several fetal and newborn tissues, including cord blood, placental tissue, peripheral blood, lung tissue and cortical plate, and can persist into childhood and adolescence and modulate risk of disease[119].

Prenatal environmental exposures may have a direct effect on the epigenome by altering DNAm or PTM patterns, or indirectly by activating TFs or other regulatory proteins. The antiepileptic and teratogen VPA functions as an HDAC inhibitor, directly causing histone hyperacetylation and subsequent demethylation of specific genes[122]. Moreover, epigenetic etiologies are often implicated when prenatal exposure to medications cause long-term gene expression changes which can lead to phenotypic alterations in the offspring. Thalidomide, isotretinoin, selective serotonin reuptake inhibitors (SSRIs) and cannabis are examples of such medications[123–125]. However, future studies are warranted to confirm the potential role epigenetic mechanisms have in mediating the medication-induced effects and disease susceptibility.

The study of epigenetic side-effects of medications, especially in context of prenatal exposures, may contribute to the understanding of underlying disease etiologies, subsequently establishing and reducing risk factors for NDD. In utero exposure to medications which affect the epigenome during neurodevelopment pose a potential risk to the unborn child. However, safety data remains lacking or inconclusive for the majority of medications commonly used during pregnancy. In this thesis, the focus is on two medications that are frequently taken by pregnant women: the analgesic paracetamol and the antidepressant citalopram.

1.5 Paracetamol and pregnancy

1.5.1 Properties and metabolism of paracetamol

Paracetamol, also called acetaminophen, is an OTC medication used world-wide to treat pain and fever. It is a compound with low molecular mass (151 g/mol) capable of passive diffusion though membranes[126]. Paracetamol is the first line treatment for pain and fever in all age groups, including children, pregnant women and nursing women[126].

The metabolism of paracetamol is complex and results in a number of metabolites. After therapeutic doses, the two major metabolic fates of paracetamol are paracetamol-glucuronide and paracetamol-sulfate conjugates, which are readily excreted by the kidneys[127]. A small proportion (< 5%) is also secreted as unchanged paracetamol. Moreover, paracetamol can be oxidized to form the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI). NAPQI is detoxified by binding to glutathione and excreted by the kidneys as paracetamol-cysteine and paracetamol-mercapturic acid conjugates. Whereas only small levels of NAPQI are formed at therapeutic doses, the sulfation and glucuronidation pathway gets saturated at supratherapeutic doses and the proportion of oxidized paracetamol increases causing an hepatotoxic effect[127]. Additionally, a small proportion of paracetamol undergo deacetylation to form *p*-aminophenol. In mice, *p*-aminophenol is transported to the spinal cord and brain where fatty acid amide hydrolase catalyze the conjugation with arachidonic acid to form the bioactive metabolite *N*-arachidonoylphenolamine (AM404)[128]. More recently, AM404 was also detected in human cerebrospinal fluid (CSF)[129]. The different pathways of paracetamol metabolism change during pregnancy and with age in children. In pregnant women paracetamol half-life was lower compared to non-pregnant women due to enhanced glucuronidation and oxidation[130]. In children, the sulphation pathway dominates from birth, whereas the glucuronidation pathway is mature from about two years of age[131].

The mechanism of action of paracetamol remains a subject of controversy[127]. The proposed primary pathway by which paracetamol mediates its analgesic effect is through inhibiting the synthesis of prostaglandins, which are important mediators of inflammation, pain and fever[127]. Prostaglandin synthesis from arachidonic acid is catalysed by cyclooxygenase-1 (COX-1) and COX-2. COX-2 catalyses the synthesis of prostaglandins from endocannabinoids[126]. Paracetamol acts as a competitive substrate of the COX-enzymes under low peroxide conditions and inhibits both COX-1 and COX-2, although the major effect appears to be on COX-2[126]. It has been proposed that paracetamol influences the descending serotonergic antinociceptive pathway[131]. Paracetamol may act as a pro-drug, with the metabolite AM404 involved in the analgesic effect. AM404 is a strong activator of the vanilloid receptor subtype 1 (TRPV1) and an inhibitor of endogenous cannabinoid reuptake. Moreover, AM404 has been found to inhibit COX-1 and COX-2[132]. In rats, the analgesic effect of paracetamol was lost by inhibiting AM404 activity[132]. Analgesic action through cholinergic systems, central noradrenergic systems and nitric oxide pathways has also been proposed[126].

Paracetamol is distributed in the body without binding to plasma proteins or tissues, thus concentrations found in vivo can be directly correlated to concentrations used for in vitro studies[126]. Following a standard therapeutic dose in adults (1 g four times per day), the peak plasma concentration has been found to be between 11-30 mg/L (70-200 μM) depending on how it is administrated, whereas the average plasma concentration over time is around 2 mg/L (13 μM)[126,133].

1.5.2 Paracetamol in pregnancy

Paracetamol is the first-choice treatment for pain and fever during pregnancy and has historically been considered safe to use. Reported paracetamol consumption during pregnancy varies from 4-81%, with the lowest levels in Saudi Arabia and highest levels in France[134]. Between 1997 and 2004, ~65 – 70% of US and Canadian expectant mothers reported paracetamol use at least once[135]. The intake of paracetamol was highest in the first trimester (54%) and decreased slightly by the second (51%) and third (48%) trimesters[135]. It was also shown that the intake of paracetamol during pregnancy increased by almost 30% from 1976 to 1990s before stabilizing[135]. This finding is in line with a Norwegian study from 2010, where 67% of pregnant women reported using paracetamol[136]. A study from 2012 reported that 48% of pregnant women from Europe, North America, South America and Australia used paracetamol at some point during pregnancy[5]. Paracetamol intake was higher in Northern Europe (62%) than in Western Europe (52%) and Eastern Europe (27%)[5]. Similar results have been found in several recent studies in different geographic areas and time periods[137–139]. Overall, the global prevalence of paracetamol consumption during pregnancy is high.

Paracetamol crosses the placenta. In term pregnancies, comparable peak paracetamol concentrations and half-life was shown in maternal blood compared to cord blood, indicating that maternal paracetamol concentrations can be used as estimate for fetal exposure[140]. Furthermore, a study using an ex vivo placenta perfusion model showed that fetal concentrations reached 96% of the maternal concentrations within 3.5 hours during maternal-to-fetal transfer of paracetamol[73]. It was also shown that the metabolites paracetamol-glucuronide and paracetamol-sulphate crosses the placenta, albeit at a lower rate[73].

Paracetamol readily crosses the blood-brain barrier and has been detected in human cerebrospinal fluid (CSF), thus it has the potential to affect neurodevelopment[141–143]. In children

given 15 mg/kg paracetamol intravenously, CSF concentrations ranged from 1.3-18 mg/L (8-120 μM) depending on time after injection[143]. At the maximum CSF concentration, the concentration ratio (CSF/plasma) was 0.95[143]. Prior to the establishment of placental circulation at embryonal Day 17-20[144], the fraction of paracetamol that reaches the developing embryo does so via passive diffusion[73].

1.5.3 Paracetamol exposure in early development of animals

The effect of developmental exposure to paracetamol has been studied in several species. In zebrafish (*Danio rerio*), wild-type and *lphn3.1* knockdown (ADHD-model) embryos were exposed to sub-toxic concentrations of paracetamol either for two hours or six days and locomotor activity was measured. Neither wild-type nor *lphn3.1* knockdowns exposed to paracetamol reported increased hyperactivity[145]. In contrast, studies in rodents have demonstrated the risk of offspring adverse neurodevelopmental outcomes following prenatal or early postnatal exposure to paracetamol at doses mirroring human therapeutic doses. In mice, paracetamol exposure on postnatal day 3 and 10 (corresponding to the beginning of the third trimester and time around birth in human pregnancies), but not day 19 (corresponding to a 2-year-old child), resulted in increased spontaneous behaviour, decreased habituation capability and reduced special learning[146,147]. These paracetamol-induced effects were not dependent on gender[147]. Moreover, another study in mice found a decreased number of neurons in the hypothalamus in male offspring following prenatal paracetamol and aniline (environmental pollutant and precursor of paracetamol) exposure. As adults, the offspring exhibited reduced sexual behaviour and territorial display[148].

In rats, prenatal exposure to high doses of paracetamol caused a reduction in the number of fetal germ cells and altered the timing of germ cell development[149]. Long-term prenatal and postnatal exposure to paracetamol at clinically relevant doses, modulates neurotransmission in the medulla oblongata and cerebellum of rat offspring[150,151]. Work from the same group demonstrated altered spatial memory, social behaviour and decreased motor function in the paracetamol-exposed offspring[152]. Another model assessing paracetamol exposure at embryonic day 15-19, showed changes in gene expression for genes involved in immune- and inflammatory responses, proliferation and differentiation[153].

Taken together, the evidence indicates adverse reproductive and neurodevelopmental outcomes following paracetamol exposure in rodents. Whether these results are relevant to humans is unknown.

1.5.4 Prenatal exposure to paracetamol and neurodevelopmental outcomes in human studies

Paracetamol exposure during pregnancy has been associated with cerebral palsy[154], language delay[155], childhood asthma[156] and cryptorchidism[157] in children. A number of cohort studies published in the last decade have examined the relationship between prenatal exposure to paracetamol and long-term adverse neurodevelopmental outcomes in children. In 2013, a sibling-controlled analysis of 2919 same-sex sibling pairs of the Norwegian Mother, Father and Child cohort (MoBa) found an increased risk of adverse neurodevelopmental outcomes in children exposed to paracetamol during pregnancy[158]. Specifically, paracetamol exposure for 28 days or more was associated with increased externalizing and internalizing behavioural problems, higher activity levels, poor gross motor development and communication at three years of age. The analysis was adjusted for confounders such as fever, infections, back pain and co-medication use during pregnancy[158].

Another study of 51200 children in MoBa reported that long-term (>28 days) exposure to paracetamol during pregnancy was associated with a moderately increased risk of communication problems and motor milestone delay in 18 month old children[159]. In contrast to the sibling-controlled study, an association with activity problems and externalizing behaviours was not found. A suggested explanation for this was that these behaviours are more difficult to observe at 18 months compared to 3 years of age. In a follow-up study from MoBa, which included 32934 children, only associations with internalizing behaviour problems remained at 5 years of age[160].

A prospective study of more than 60000 children in the Danish National Birth Cohort (DNBC) found that paracetamol exposure during pregnancy was associated with ADHD-like behavioural problems in children at age 7, increased risk for hyperkinetic disorder (a severe form of ADHD) and use of ADHD medications[161]. These risk associations increased with the frequency of paracetamol use during pregnancy. Potential confounders adjusted for included maternal inflammation, infection and smoking during pregnancy, maternal mental health problems, age and the childs birth weight and gestational age, none of which affected the results.

In contrast, a study from the same group found associations between prenatal exposure to paracetamol and an increased risk of ASD with hyperkinetic symptoms, the association was not found in other ASD cases suggesting a specific impact on hyperactive behaviour problems[162]. In line with the above-mentioned findings, an analysis of 871 children in the Auckland Birthweight Collaborative Study cohort, where children exposed to paracetamol prenatally had an increased risk of ADHD and ADHD-like behaviours at age 7 and 11[163].

Recently, several additional cohort studies have been published supporting the previous paracetamol-induced risk associations[164–168]. A study of 7797 mothers along with children and partners in the Avon Longitudinal Study of Parents and Children found that prenatal paracetamol exposure was associated with higher odds of the child having conduct problems, hyperactivity symptoms and emotional symptoms at age 7[165]. These associations were not found for maternal postnatal or partner´s use of paracetamol, indicating that the associations found for maternal prenatal use cannot be explained by unmeasured behavioural or social factors related to paracetamol use. A study of 2644 children in a Spanish birth cohort reported that prenatal paracetamol exposure was associated with an increased risk of hyperactivity and impulsivity symptoms at 5 years of age[166]. Additionally, an increased risk of ASD symptoms in paracetamol-exposed males compared to paracetamol-exposed females was found. Another study assessed attention and executive function in a sub-sample of 1491 children at age 5 in the DNBC[167]. The prenatally paracetamol exposed children had poorer metacognitive skills and attention function compared to unexposed children. Further, a trimester-specific effect was observed in the association between paracetamol exposure and poor attention function, with the strongest association found for exposure during the first trimester. The same group also found an association between maternal paracetamol use during pregnancy and lower IQ in the child at age 5, whereas when paracetamol was used to treat maternal fever the effect appeared to be compensatory[168].

The aforementioned analyses were adjusted for a range of potentially confounding effects. However, parental symptoms of ADHD and unmeasured familial factors such as genetic background was not accounted for, with the exception of genetic background in the sibling-controlled study[158]. Expectant mothers with impulsive personality traits are associated with higher odds for paracetamol consumption during pregnancy, which could potentially influence the risk of ADHD in the child[169]. Addressing this issue, a study of 112973 children from MoBa, including 2246 with an ADHD diagnosis, found that prenatal paracetamol exposure was

associated with an increased ADHD diagnosis in the children, and the association remained after adjusting for parental ADHD symptoms, indication of paracetamol use, maternal symptoms of depression and other factors[170].

To date, a causal relationship between prenatal paracetamol exposure and ADHD/ADHD-symptoms in children has not been established. Three meta-analyses have been published, and despite the heterogeneity in study designs and outcome measures, they agree that paracetamol consumption during pregnancy increases the risk of ADHD by 25-35 %[171–173]. This risk increased with long-term exposure. Of note, quantitative bias analysis of these data have suggested that the increased risk of ADHD may be due to unmeasured confounders[173]. Overall, adverse neurodevelopmental effects of paracetamol have been found independently in several different cohorts using different exposure and outcome measures and adjusting for a wide variety of confounding factors.

1.5.5 Paracetamol and DNA methylation

A study from our group used a sample of 383 cord blood samples from MoBa to conduct an epigenome-wide association study (EWAS) to assess whether long-term prenatal exposure (≥ 20 days) was associated with an ADHD diagnosis and DNAm differences[118]. No significant differences in DNAm were found when comparing the groups exposed to paracetamol alone or diagnosed with ADHD alone with controls (no paracetamol and no ADHD). In contrast, long-term prenatal exposure to paracetamol was associated with DNAm differences in children with an ADHD diagnosis compared to controls (6211 CpGs) and the no paracetamol/ADHD control group (193 CpGs). This result implies that individuals that are susceptible to ADHD respond differently to prenatal paracetamol exposure than controls, and this response may be mediated at least partly by epigenetic mechanisms. The differences in methylation were found in genes involved in oxidative stress, neural transmission and olfactory sensory pathways[118]. The differences in methylation were found in genes involved in oxidative stress, neural transmission and olfactory sensory pathways[111]. Other EWAS have identified differentially methylated CpGs associated with prenatal paracetamol exposure in placental tissue and blood, adding to the evidence of paracetamol-induced epigenetic differences[174–176].

1.5.6 Potential mechanisms for effect of paracetamol on neurodevelopment

The mechanism by which paracetamol may interfere with neurodevelopment is unknown, however several hypotheses have been proposed. First, paracetamol may influence the immune system and induce oxidative stress[177]. This hypothesis supported by the EWAS from MoBa that found associations between long-term paracetamol exposure during pregnancy and DNAm changes in genes related to oxidative stress and neural transmission in children with ADHD[118]. Second, paracetamol could interact with the endocannabinoid system which is important for neurodevelopment[178]. Third, paracetamol may interfere with brain-derived neurotrophic factor (BDNF)[146]. BDNF is important for several neurodevelopmental processes and is involves in learning, memory and attention, and interacts with the endocannabinoid system[179,180]. Fourth, paracetamol could disrupt normal neurodevelopment by disrupting maternal hormones[178] or maternal-fetal interactions via the placenta[174]

1.6SSRIs and pregnancy

SSRIs comprise a class of antidepressants (ADs) that act by inhibiting reuptake of 5-HT from the synaptic cleft of the pre-synaptic cell, thus restoring extracellular 5-HT levels and increasing serotonergic neurotransmission[181]. Depression and anxiety disorders have been associated with impaired serotonergic neurotransmission[182]. In the adult brain, serotonin (5-HT) regulates stress responses, cognition, attention, emotion, nociception, sleep and arousal[183]. During development however, 5-HT acts as a trophic factor and plays a crucial role in regulation of processes such as cell growth, differentiation, migration, myelination, synaptogenesis and pruning[183]. Consequently, there is a possibility that early life SSRI-exposure and subsequent changes in 5-HT signalling could affect important developmental pathways.

1.6.1 Properties and metabolism of citalopram

SSRIs are generally well-tolerated, and include medications such as fluvoxamine, paroxetine, sertraline, fluoxetine, citalopram and escitalopram. In addition to being the first-line treatment of depressive disorders, SSRIs are used to treat other psychiatric illnesses such as obsessive-compulsive disorder, anxiety, panic disorders and social phobias[184].

Citalopram (MW = 405 g/mol) is a commonly used SSRI with relatively few side effects and high clinical efficiency[182,185]. It has high bioavailability (~80-100%) and a half-life of approximately 35 hours in healthy adults. Moreover, 80% of citalopram is protein-bound and

it is widely distributed in peripheral tissues[182]. Citalopram is a highly specific inhibitor of 5-HT reuptake, whereas its effect on dopamine and noradrenaline reuptake is negligible[186]. Furthermore, citalopram is a racemic, bicyclic compound consisting of two enantiomers: R-citalopram and S-citalopram[181]. The major effect of citalopram has been attributed to the S-enantiomer, thus leading to the development of another SSRI, escitalopram, where S-citalopram is the active ingredient. In vitro, S-citalopram was shown to be 167 times more potent than R-citalopram[187]. The ratio of S-enantiomer/R-enantiomer in citalopram varies from 0.32 to 1.25[186].

The major metabolite of citalopram is CYP2C19-catalyzed R/S-demethylcitalopram, which is further demethylated to R/S-didemethylcitalopram. Oxidation and deamination of citalopram results in R/S-citalopram *N*-oxide and citalopram propionic acid derivatives, respectively. These metabolites are excreted by the kidney. In addition, about 12-23% is excreted as unchanged citalopram[181]. The metabolites of citalopram (and escitalopram) do not cross the blood-brain barrier readily, and the concentrations in plasma are much lower than the parent compound, suggesting that their contribution toward the clinical effect is negligible[182].

The recommended citalopram dosage varies between 10 and 60 mg per day, and the reported mean peak plasma concentration at steady state is 126 μg/L (311 nM) which is reached 2 to 4 hours after intake of 40 mg per day[186]. It has been shown that there is a linear relationship between citalopram dosage and steady-state citalopram concentrations, although no correlation had been found between clinical effect and plasma concentrations[186].

1.6.2 Citalopram in pregnancy

Depression or depressive symptoms are reported in ~15% of pregnant women[188]. Untreated depression may cause adverse effects in the child, including cognitive, emotional and behavioural problems, as well as adverse health effects in the mother[189]. SSRIs have been used since the early 1990s and is the first-choice class of antidepressants during pregnancy in many countries. Reported prevalence of SSRI use during pregnancy varies with country and populations, estimates range from 1-7% in European countries and 5-8% in North America[190–194]. Data from the US showed that among the 6.2% SSRI-exposed pregnant women the most common medication was sertraline (2.1% of pregnancies) and the least common medication was citalopram (0.8% of pregnancies). Antidepressant use during pregnancy increased more

than 2-fold from 1999 to 2003[192]. This finding is supported by data from other populations[190,195,196].

A study looking at pregnancies in European countries from 2004 to 2010 found that 4.5% were prescribed SSRIs, ranging from 1.2% in parts of Italy to 4.5% in Wales[194]. Citalopram and fluoxetine were the most prescribed SSRIs in Denmark and UK, whereas in the Netherlands and Italy it was paroxetine[194]. Comparable results were found in a study of pregnancies in Nordic countries between 2008 and 2012. 3.3% of pregnant women were exposed to SSRIs, ranging from 1.8% in Norway, 3.7% in Sweden and Denmark, and 7.0% in Iceland[193]. Exposure rates were highest in the first trimester and decreased in the second and third trimester. The most commonly used SSRIs were sertraline in Iceland and Sweden, citalopram in Denmark and escitalopram in Norway[193]. Another study reported that 0.9% of pregnant women in MoBa used SSRIs, of which citalopram/escitalopram was the most frequently used (0.5% of pregnancies)[197]. This study also supported a decline in prevalence of SSRI use in the second and third trimester.

CYP2C19, the enzyme responsible for the primary metabolism of citalopram, is highly genetically polymorphic, and thus varies in its pharmacokinetic function. CYP2C19 activity has been shown to be reduced during pregnancy, affecting citalopram clearance[198]. Citalopram readily crosses the placenta. One study measuring levels of antidepressants in cord blood at birth after a daily dose from 20-40 mg per day, found citalopram concentrations ranging from <10-36 μg/L (<25-89 nM)[199]. Metabolites of citalopram was measured to <10-11 μg/L (< 32-36 nM). The mean cord/maternal concentration ratio was 0.7 (range 0.17-1.42)[199]. A similar study, where median citalopram dose was 20 mg per day, reported citalopram concentrations ranging from 20-50 μg/L (49-123 nM) and metabolite concentrations ranging from 9-23 μg/L (29-74 nM) in cord blood[200]. The mean cord/maternal concentration ratio was measured as 0.83 (range 0.77-0.86), which was higher compared to other antidepressants. At day three, citalopram still persisted in infant blood[200]. A third study found citalopram cord blood concentrations after median daily dose of 20 mg ranging from 5-62 μg/L (12-153 nM) and a median cord/maternal concentration ratio of 0.78 (range: 0.46-1.66)[201]. Considerably higher citalopram concentrations were found in amniotic fluid compared to maternal serum (mean ratio 1.8), providing evidence of fetal exposure of citalopram through ingestion and re-ingestion of amniotic fluid in addition to cross-placental exposure[201].

SSRIs are able to cross the blood-brain barrier to reach their site of therapeutic action. A study analyzing the distribution pattern of citalopram found serum concentrations ranging from 28-279 μg/L (69-689 nM) and corresponding CSF concentrations ranging from 13-96 μg/L (32-237 nM) after a median daily intake of 20 mg[202]. The mean CSF/serum concentration ratio was 0.35, whereas when only accounting for the unbound fraction of serum citalopram (20 %) the relationship was reversed and the ratio was calculated to be 1.77. This indicates easy transport of citalopram over the blood-brain barrier and residence time in the CSF[202]. Overall, citalopram/escitalopram are commonly prescribed medications during pregnancy and readily cross the placenta and blood-brain barrier, and subsequently have the ability to affect the developing child.

1.6.3 Citalopram exposure during early animal development

Long-term safety data for SSRIs from animal studies are limited. Some studies did not find any significant effect on prenatal exposure of SSRIs on learning and memory[203,204], whereas others found changes in behaviour, brain structure and function in the offspring[205,206]. For citalopram specifically, perinatal exposure of mice resulted in adult altered emotional behaviour[207]. In rats, perinatal exposure to citalopram decreased the expression of 5-HT transporter in fibers of the hippocampus[208]. Structural and functional differences have been found in the corpus callosum, reminiscent of findings in ASD patients. As juveniles, these rats exhibited impaired social behaviour and response to novelty[209]. Reduced social behaviour was also found in two additional studies of prenatal and early postnatal citalopram-exposed rats, paralleling ASD-symptoms in humans[206,210]. Learning deficits, impaired memory, decreased sexual behaviour, compulsivity, anxiety-like and depressive-like behaviour were also observed in rats exposed to citalopram during early development[206,210–213].

Escitalopram is a newer medication compared to citalopram and studies of the effect on early development are scarce. Since escitalopram consist of the S-enantiomer of citalopram one would expect similar effects. However, escitalopram exposure in rats throughout gestation did not find effects on memory, learning, anxiety-like behaviour or hippocampus gene expression in male offspring[214]. In female offspring, prenatal escitalopram exposure resulted in fewer social interactions and increased expression of 5-HT receptors in the amygdala as adolescents but not as adults[215]. Female mice which were treated with escitalopram neonatally exhibited increased depressive-like behaviour and interrupted sleep patterns as adults[216].

A meta-analysis which pooled together data from perinatal exposure to different SSRIs in mice and rats found overall reduced activity and exploration behaviour, more passive stress coping style and decreased sensory processing efficiency in the offspring. Subgroup analysis also found that animals which were exposed postnatally (corresponding to the third trimester in humans) to SSRIs were less social and had poorer learning and memory skills compared to control animals[217]. This meta-analysis was limited by the varying quality of the individual studies included, confined by poor reporting of blinding, randomization and outcome measures, which potentially could introduce bias and hinder accurate interpretation. Additionally, the use of animal models with generally higher dosing regimens compared to humans, different route of administration and half-lives of compounds may not adequately reflect the clinical situation in humans, limiting the generalizability of findings.

1.6.4 Prenatal exposure to SSRIs and neurodevelopmental outcomes in human studies

The effect of prenatal exposure to SSRIs on the developing child is conflicting, particularly studies of individual medications, as opposed to SSRIs as a group, and long-term neurodevelopmental outcomes. Whereas AD do not seem to be major teratogens, they may increase the risk of poor neonatal adaption symptoms, including feeding difficulties, reflux, sleep disorders, restlessness and excessive crying[189].

When studying long-term outcomes of SSRI-use during pregnancy, given that maternal mood and depression disorders may possibly also result in adverse neurodevelopmental outcomes in the child[218], a major challenge is to separate between the effects of medication exposure and the underlying depression, as well as the severity of depression symptoms. Pregnant women who continue using antidepressants during pregnancy may be more severely depressed than untreated mothers, but at the same time, they may experience fewer depression symptoms due to treatment. Additionally, the adverse effects seen in children of mothers with depressive disorders may be confounded by genetic or familial factors, e.g., poor parenting[219]. Individual studies vary considerably in study size, exposure measures, outcome measures and confounding factors that are accounted for, thus reporting inconsistent results.

Some studies have reported long-term behavioural outcomes of prenatal SSRI exposure such as increased depressive symptoms, internalizing behaviour and anxiety symptoms after adjusting for maternal psychiatric illness[220–222]. However, one follow-up study indicated that

the association with anxiety symptoms could be due to familial confounders after paternal characteristics also were accounted for [223]. Another study reported that more severe neonatal adaption symptoms following prenatal SSRI-exposure was associated with increased social-behavioural disturbances in 3 year old children[224]. Furthermore, a sibling-controlled study found an association between prenatal AD (mostly SSRIs) exposure and increased risk of anxiety in 3 year old children[225].

There are several meta-analysis of prenatally AD- or SSRI-exposed children which have revealed an increased risk of ADHD and ASD compared to controls when accounting for measured confounders only[226–229]. However, few of the individual studies included in these metanalyses accounted for severity of maternal depression and paternal characteristics. When correcting for paternal AD use the association with ADHD diminished, whereas the association with ASD remained albeit somewhat reduced, suggesting that familial confounding plays a role in the observed risks[226,230,231]. Furthermore, sibling-controlled studies found no significant associations between prenatal AD-exposure and ASD or ADHD, supporting that familial confounding factors may be important[226,228]. Several studies found associations between AD use before pregnancy and increased risk of ADHD and ASD of similar magnitude as reported for use during pregnancy[226,228,232]. These results indicate that confounding by indication could account for the observed risks of prenatal use of AD. Finally, a recent study from MoBa of 6395 children of women with clinical depression/anxiety during pregnancy reported no substantial differences in ADHD risk between AD-exposed group compared to the non-medicated group at 5 years of age, whereas a modest increase in ADHD risk was reported at 7-9 years of age[233].

For citalopram and escitalopram, only a few studies have been conducted. One study followed pregnant women during pregnancy and lactation, assessing weight and neurodevelopmental outcomes (classified as normal/abnormal) in the children up to one year of age. No difference in neurologic status was found between the citalopram exposed children and control infants[234]. Another study of 396 pregnant women reported higher risk of poor neonatal adaption following citalopram-exposure, similar to other SSRIs, whereas long-term outcomes were not measured[235]. A study of prenatal exposure to escitalopram reported significantly lower birth weight compared to controls, whereas long-term outcomes were not measured[236]. Overall, the impact of prenatal SSRI-exposure on long-term neurodevelopmental outcomes remains uncertain.

1.6.5 SSRIs and DNA methylation

Studies elucidating associations between SSRI use during pregnancy and DNAm changes in cord blood are few and of varying quality. None of the studies investigated the effect of individual medications as opposed to AD or SSRIs as a group. One recent EWAS using 450k Illumina BeadChips found that prenatal AD exposure was associated with DNAm differences at 130 CpGs in cord blood in 479 children[237]. One CpG which mapped to *ZNF575*, had significantly lower methylation levels in AD-exposed compared to controls, was replicated in an independent cohort and persisted into early childhood[237]. In contrast, another EWAS using the same platform found no associations between prenatal SSRI-exposure and differences in DNAm[238]. This study included cord blood from only 58 children and did not correct for cell-type composition. Using bisulphite pyrosequencing, CpGs mapping to *COL7A1*, *NFKB2*, *SLC6A4*, *FKBP5* and *DNMT3A* were significantly differentially methylated in SSRI-exposed children compared to controls[238]. In two additional EWAS conducted using the 27k Illumina platform, a comparison was made between 151 AD-exposed children and 50 controls[239], as well as between 19 AD-exposed children and 25 controls[240]. These studies identified differentially methylated CpGs associated with *TNFRSF21*, *CHRNA4*[239], *CYP2E1*, *EVA1* and *SLMAP*[240]. Further, the association with *CYP2E1* was replicated with bisulphite pyrosequencing using cord blood samples from 42 children. *CYP2E1* DNAm levels were negatively correlated with maternal depressive symptoms in children exposed to SSRIs, but not in unexposed children, suggesting a maternal depression-medication interaction[240].

Other bisulphite pyrosequencing-based candidate gene studies found somewhat conflicting results. Whereas one study associated prenatal AD exposure to decreased methylation levels at one CpG mapped to *SLC6A4*[238] (encoding a serotonin transporter), another study reported associations with increased methylation levels at 6 CpGs mapping to the same gene[241]. A third study found no associations between prenatal SSRI-exposure and DNAm at *SLC6A4*[242]. DNAm of *NR3C1* (encoding glucocorticoid receptor) and *BDNF* (encodes brain-derived neurotrophic factor), genes involved in neurotransmitter activity and neural differentiation respectively, was not associated with prenatal exposure to antidepressants[238,243–245]. DNAm levels of a CpG mapping to the glucocorticoid receptor co-regulator *FKBP5*, was associated with prenatal exposure to SSRIs in cord blood[238], but not in placental tissue[246].

One clinical trial has assessed the effect of escitalopram in adult patients with major depressive disorder, comparing treatment responders and non-responders[247]. There were 2571

differentially methylated CpGs in escitalopram responders compared to non-responders, some of which overlapped with differentially expressed genes, supporting a role of epigenetic factors in treatment response. Moreover, in vitro exposure to 50 μM citalopram induced DNAm changes in genes related to proliferation and cell cycle regulation, neurodevelopment, depression nucleic acid metabolism and small molecule biochemistry in HEK293 cells[248].

The above-mentioned DNAm studies are heterogenous in relation to study sample and size, assessment of maternal mental health disorders and antidepressant exposure, confounding adjustment, sample size and methylation assessment platforms. Inconsistent results from these studies increases the difficulty in interpreting the effect of prenatal antidepressant-exposure on the developing child, and in assessing whether an epigenetic etiology is involved in the potential adverse outcomes.

1.7 Limitations of common methods used to study medication safety during pregnancy

Since pregnant woman are usually excluded from randomized control trials, safety data for pregnancy outcomes is normally obtained from case reports, epidemiological studies, animal studies and in vitro cellular studies.

1.7.1 Case reports

Case reports are useful for reporting novel or rare observations. However, one cannot infer causality or generalize from this type of study. Inherently, incidence, rates and ratios in a population cannot be quantified in case reports. In addition, as case reports are retrospective, they are subject to recall bias[249].

1.7.2 Epidemiological studies

Epidemiological studies, for example cohort and case-control studies, are powerful for identifying human teratogenic medications. Some advantages of cohort studies are that exposure status is determined before outcomes, and multiple outcomes can be investigated simultaneously. Contrary to case reports, cohort studies can identify population incidence and risk associations, although rarely clear-cut cause-effect relationships. Similar to case reports, however, recall bias may be an issue with retrospective epidemiological studies[250]. Confounding factors, such as maternal disease, age and smoking status, are major contributors

to bias in cohort studies, and well-designed studies with sufficient numbers of participants are required to limit these. Ideally, the teratogenic potential of medications would be discovered before exposure in pregnant women, although historically that has not been the case as most human teratogens have been identified epidemiologically or with case reports[251].

1.7.2.1 EWAS

EWAS investigates the relationship between DNAm patterns and various phenotypic traits or diseases. EWAS analyse large-scale DNAm data obtained from high-throughput technologies to identify differentially methylated sites or regions associated with specific phenotypes[252]. By assessing DNAm across the entire genome, EWAS can be used for the identification of potential biomarkers or therapeutic targets, predicting disease risk and assessing medication response. However, it is important to consider the limitations of EWAS. Firstly, the cross-sectional nature of these studies limits the ability to establish causal relationships between DNA methylation and phenotypes. Second, the interpretation of EWAS results is complex due to the high dimensionality of data, potential confounding factors, and issues of multiple testing correction. Third, the sample size and tissue specificity can impact the reproducibility and generalizability of findings[253]. Despite these challenges, EWAS have yielded valuable insights into the epigenetic aetiology of diseases and phenotypic variations.

1.7.3 Animal studies

All medications are tested for reproductive safety using animal studies prior to obtaining a market authorization. Animal studies are useful as they can be performed without the presence of disorder, thereby excluding confounding by the underlying condition. However, the results are limited by interspecies translatability, and human teratogenic effects cannot be excluded even when the medication is deemed safe in animals. There are many pharmacokinetic differences between species, including how medications are absorbed, distributed and metabolized, and this presents an issue when choosing exposure dosages appropriate to human clinical setting[254]. There are also species differences in gestational length, brain complexity and behaviour, adding to the challenge in extrapolating data obtained from animal studies to humans[255]. The mean positive predictability rate in animals was found to be 56% for medications classified as teratogens in humans, a rate only slightly higher than what would have been observed with random variability[256]. According to the test guidelines established by the organization for Economic Co-operation and Development (OECD), at least two generations

of animals are required for evaluation of developmental toxicity (DT)[257]. Thus, animal studies are time-consuming, labor-intensive, expensive, and raise ethical concerns regarding animal welfare[258]. Alternative methods should be explored according to the 3R concept for animal use (reduction, refinement, and replacement).

1.7.4 In vitro studies

In vitro teratogenicity studies are cheaper, easier to perform, allow a better controlled environment than in animal studies, and reduces the ethical constraint of animal welfare. There is a wide variety of in vitro platforms used to investigate the mechanistic effects of potential teratogens on cellular or tissue levels: immortalized cell lines, primary cell cultures, organ cultures, mammalian and non-mammalian primordia and embryos. These platforms have varying degrees of ability and reproducibility in predicting teratogens in human pregnancies and only focus on immediate outcomes[256]. Three such platforms have been validated by the European Centre for the Validation of Alternative Methods (ECVAM): the mouse embryonic stem cell test (mEST), rat micromass (MM) test and the rat whole embryo culture assay (WEC test)[259]. The accuracy in predicting embryotoxic compounds was shown to be 78, 70 and 80%, respectively[259]. Of these tests the mEST does not require the sacrifice of pregnant animals and is the most widely used[260]. However, endpoints such as cell death, growth retardation and malformations may not be relevant for developmental neurotoxicity, and where minor functional deficits relating to changes in cellular communication, positioning or differentiation may be sufficient to cause adverse outcomes.

The use of omics-based methods, i.e. genomics, epigenomics, transcriptomics, proteomics and metabolomics, with mEST or other DT assays has contributed to a greater understanding of teratogenic mechanisms of action and effect on functional biological processes during differentiation[261]. Although a neural embryonic stem cell test (ESTn) showed promising predictive results[262], species-specific differences limit the interpretation of the results to humans. To try to overcome this, human embryonic stem cells (hESCs) can be used. DT testing using hESCs have been limited by difficulties in cultivation and differentiation and requires considerable expertise, and robust and standardized protocols. However, there have been many advances in recent years (discussed in chapter 1.2.3).

1.8 Stem cells and neuronal differentiation

1.8.1 Stem cell types and properties

Stem cells are characterized by their potential to proliferate indefinitely and to differentiate into different cell types[263]. There are several categories of stem cells based on their developmental potency, the capacity of stem cells to differentiate into specialized cell types of different lineages. First, totipotent stem cells have the highest developmental potency and can give rise to all cell types of an organism, including embryonic and extra-embryonic tissues such as the placenta[263]. Second, pluripotent stem cells can differentiate into all cell types of the three primary germ layers but cannot form extra-embryonic tissues. Third, multipotent stem cells have a restricted developmental potential and can differentiate into multiple cell types within a specific germ layer or tissue lineage. Fourth, unipotent stem cells have the lowest developmental potency and can only differentiate into a single cell type within a specific tissue type[263].

Stem cells may also be categorised based on their origin: somatic stem cells, ESCs and induced pluripotent stem cells (iPSCs). Somatic stem cells are multipotent lineage-committed progenitors that can be found in many body tissues, where their function is to renew postnatal and adult tissue in which they reside[264]. For instance, neural stem cells can be found in the hippocampus and olfactory bulb of the adult brain[265].

ESCs are pluripotent cells derived from the ICM of a blastocyst, which have potential to differentiate into cells of the three primary germ layers in the developing embryo[263]. In vivo, their pluripotent state is transient, whereas it can be maintained by specific factors in vitro. ESC pluripotent property is regulated by the TFs octamer-binding transcription factor 4 (OCT4; encoded by *POU5F1*), sex determining region Y box 2 (SOX2) and NANOG[266]. These factors function together in regulatory loops to sustain self-renewal and suppress differentiation by the co-occupation of regulatory elements in the genome, including their own promoters[267]. In addition, several signalling pathways are important for the maintenance of pluripotency in hESCs[268–270]. Transforming growth factor β (TGFβ)/activin/nodal pathway co-regulates pluripotency genes though activin/nodal-activation of SMAD2 and SMAD3[271]. SMAD2 and SMAD3 then form a complex (SMAD2/3), which is transported to the nucleus with the help of the co-SMAD SMAD4, and binds to OCT4 and NANOG. Activation of the Wnt signalling pathway causes downstream stabilization of β-catenin and inactivation of T-cell factor 3, a

repressor of NANOG and other ESC-specific TFs[272]. The FGF signalling pathway plays a role in hESC pluripotency, both via downstream mitogen-activated protein kinase and by acting in synergy with the TGFβ/activin/nodal pathway and the Wnt pathway[270].

Finally, the third group of stem cells, iPSCs, are somatic cells that are reprogrammed to a pluripotent state with the TFs OCT4, SOX2, kruppel-like factor 4 and c-myc[273]. The development of iPSCs enables the generation of disease- and patient-specific stem cells while circumventing the ethical challenge of using human embryos.

1.8.2 In vitro neural differentiation of hESCs as a model for neurodevelopmental toxicity

Due to the intrinsic differentiation capacity of pluripotent stem cells (PSCs), they can be used to model early development. There are currently a large number of protocols which differ in culturing conditions and duration, describing the generation of a range of neural subtypes from PSCs, with applications such as disease-modeling, treatment of neurodegenerative diseases, medication screening and NDT testing. A widely used method of neural induction is through formation of embryoid bodies (EB), multilayered cell aggregates from which endoderm, mesoderm and ectoderm lineages can be generated[274]. This platform in combination with OMICs approaches has been used as a proof-of-principle to test compounds such as cytosine arabinoside, methylmercury, thalidomide and RA for NDT[275–279]. However, EB are highly heterogeneous in both cell-type composition and size[280], thus limiting their reproducibility and suitability for NDT testing.

Other strategies for neural induction rely on co-cultivation of PSCs and stromal feeder cells or selective survival conditions, with their respective limitations being poorly defined culture conditions adding additional complexity, and lengthy, low yield differentiation[280–282]. Neural induction by selective survival conditions combined with a targeted transcriptomics approach has been used for NDT testing of the anticonvulsants VPA and carbamazepine[283,284]. Although, promisingly, neural markers were differentially expressed in exposed cells compared to control cells, these protocols come with inherent issues, including cell heterogeneity and assay variability, that were not properly addressed.

In 2009, Chambers *et al*. proposed a method for monolayer neural induction using the small molecules noggin and SB431542[285]. The presence of the BMP inhibitor noggin prevents SMAD1/5/8-signalling[286], while TGFβ-pathway inhibitor SB431542 prevents SMAD2/3-signalling[287], causing perinuclear redistribution of SMAD4, the obligate co-SMAD involved in pluripotency maintenance. This dual-SMAD inhibition protocol was shown to efficiently induce neural conversion of hESCs to neural stem cells (NSCs) with a transient *PAX6*-expressing anterior phenotype capable of forming neural rosettes[285]. However, the resulting cell populations presented as heterogeneous neural cells of different developmental stages, thus the persistence of cell heterogeneity from hESC differentiation remains challenging in the context of NDT testing.

Since Chambers *et al.* published the dual-SMAD inhibition protocol, significant efforts have been made to improve the efficiency and create long-term self-renewing NSCs by using various morphogens, and extended to generate different endpoints and subtypes of neural cells[288], e.g., motor neurons[289], GABAenergic neurons[290] and dopaminergic neurons[291]. Neural induction by small molecule inhibitors is now the preferred method of ectoderm differentiation due to its simplicity and reliability, despite heterogeneous cell populations. Exposure to potentially teratogenic compounds during neural induction by dual-SMAD/small molecule inhibitors has been used as a model to test for NDT. While applying this model system to compounds such as VPA[292,293], silver nanoparticles[294], the traditional Chinese medicine NeuroAiD[295] and colchicine[293] have shown promising results, there are limitations to these studies. The outcome of a neural induction protocol is sensitive to the quality of start-point PSCs, seeding densities[285], lab techniques (pipetting techniques, time cells spent in room temperature when changing media and passaging cells etc.), coating quality of growth surface and fluctuating incubation conditions. Thus, results vary between labs, different experiments, well-to-well and even within-well (different developmental stages or fates) and is dependent on highly trained personnel. In addition, when using OMICs-approaches to detect targets of NDT, variation in cell type composition between conditions may create bias and should be taken into account.

In vitro neural differentiation of hESCs can recapitulate the temporal changes of in vivo embryonic neurodevelopment. Cell types generated from hESCs in vitro resemble cell types found in in vivo development (Fig. 8)[18], thus providing a platform for an increased understanding of cellular events, gene regulation and signalling pathways involved in NDT. Nevertheless, in vitro NDT testing is an experimental simplification of in vivo development

and fetal compound exposure. Maternal-fetal interactions, such as the supply of nutrients and hormones, placental function and maternal metabolism are lacking in this model, potentially resulting in biased results[296]. Other limitations include the lack of three-dimensional tissue structures, specific cell-cell interactions and extracellular environment found in vivo[18]. However, the hESC platform does provide an opportunity to study NDT on relevant cell types, which cannot be studied in vivo in humans.

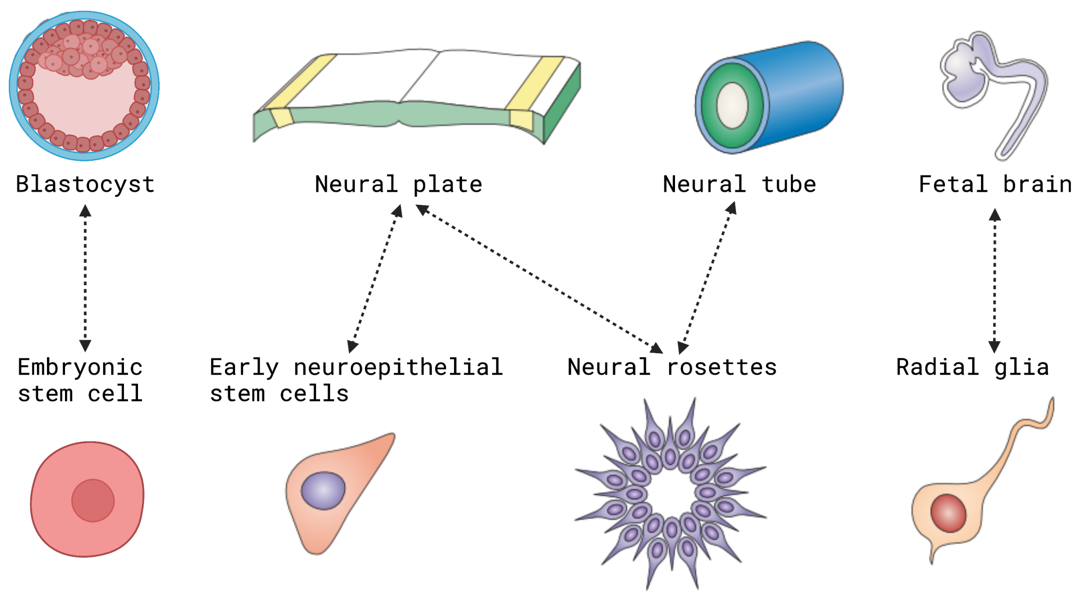

Figure 8. Correlation between in vivo neurodevelopmental structures and in vitro-derived neural stem cells. Embryonic stem cells (ESCs) are derived from the ICM of a blastocyst. ESCs undergoing neural induction can generate early neuroepithelial stem cells resembling the cells of the neural plate [285]. Neural stem cells forming radially organized columnar epithelial cells are termed neural rosettes, which resemble cells of the neural plate [297] forming the neural tube [298]. Radial glia-like neural stem cells resemble radial glia cells found in the fetal brain [299]. Adapted from *Conti and Cattaneo* (2010)[18] with permission.

CHAPTER 2: AIMS, HYPOTHESIS AND OBJECTIVES OF THE STUDY

The aim of the work presented in this thesis was to assess the effect of selected medications used by pregnant women, i.e., paracetamol and citalopram, on gene expression, DNAm and chromatin structure using neuronal differentiation of hESCs as a model of early human neurodevelopment.

The rationale for conducting this study was

i) epidemiological studies have linked paracetamol and citalopram exposure during pregnancy to adverse neurodevelopmental outcomes in the child (chapter 1.4.2, 1.5.4, 1.6.4) and

ii) EWAS have found associations between paracetamol and citalopram exposure during pregnancy and changes in epigenetic patterns in children with adverse neurodevelopmental outcomes (1.5.4, 1.6.4). This thesis presents functional studies on medication exposure during human ESC neuronal differentiation to identify potential causal or mechanistic factors involved.

The main hypothesis is that exposure to paracetamol and citalopram cause epigenetic and/or gene expression changes during neuronal differentiation (Fig. 9).

The specific objectives were to:

1. Establish a neuronal differentiation protocol of hESCs optimized for the study of early human brain development and neurotoxicology studies.
2. Characterize the transcriptional and epigenetic profiles of the cells derived from neuronal differentiation at multiple intermediate timepoints using a multi-omics approach.
3. Study the effect of exposure to paracetamol and citalopram on gene expression and epigenetic profiles during neuronal differentiation.

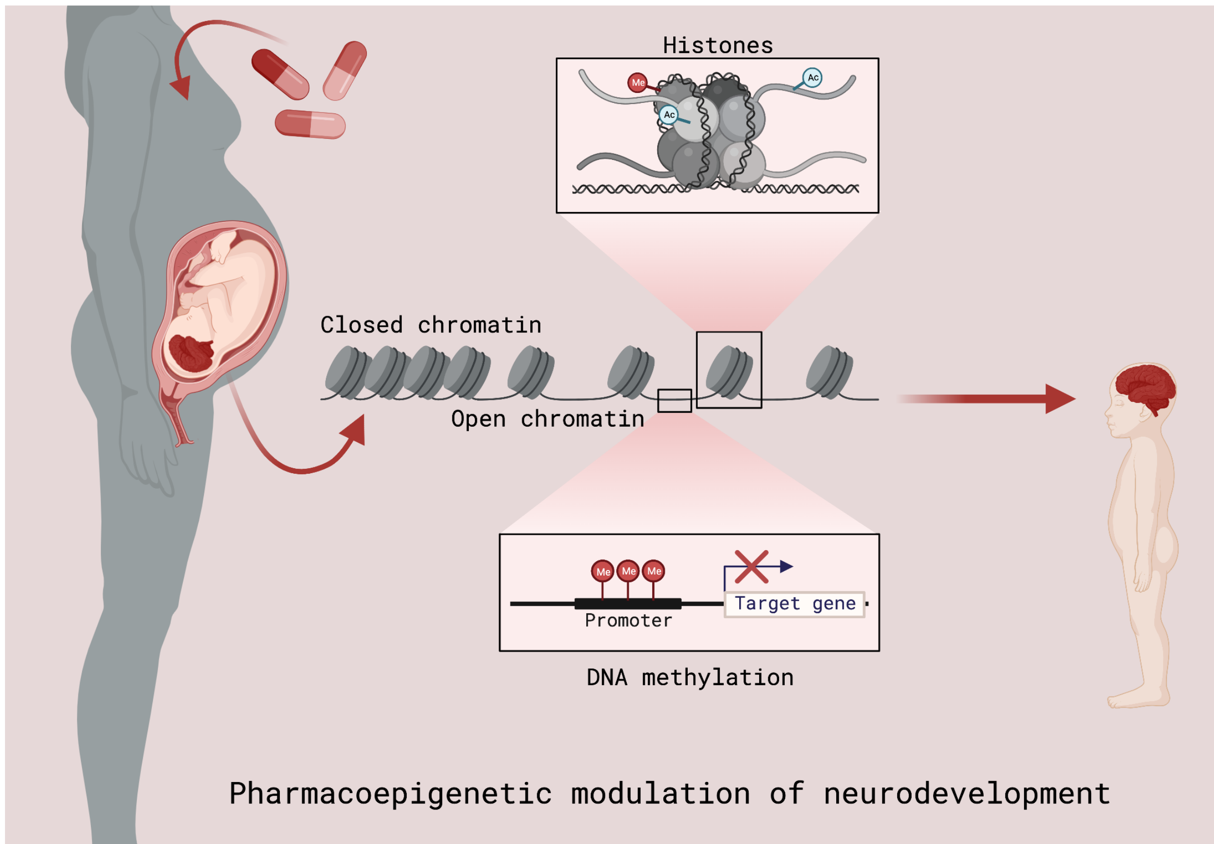

Figure 9. Pharmacoepigenetic modulation of neurodevelopment. Exposure to medications during pregnancy may reach the fetal brain and alter epigenetic and/or gene expression patterns, potentially causing adverse long-term neurodevelopmental outcomes in the developing child. Created with BioRender.com.

CHAPTER 3: METHODOLOGICAL CONSIDERATIONS

The current study uses an in vitro neuronal differentiation protocol optimized for medication exposure as a model of early human neurodevelopment. Multi-omics endpoint outcomes, including both single-cell and bulk methods, and integrative analyses were used to study the effect of paracetamol and citalopram in the model.

3.1 Neuronal differentiation protocol

As described in chapter 1.2.3, many neural induction protocols already exist, some of which have been used for toxicity studies. Before developing the protocol described in Paper I[300], we made many attempts to set up two previously described protocols[301,302]. However, we experienced significant reproducibility issues related to cell morphology and viability. The reason for the lack of success may be partly due to inexperience with stem cell culturing and differentiation at this stage, but also old equipment (fluctuating incubator temperature and CO_2 levels, manual hypoxia chambers) and inherent properties of the protocol such as media replacement every second day and cell splits by ratio instead of cell counts.

We needed to optimize a differentiation protocol for neurotoxicology studies, resulting in Paper I[300]. In brief, the protocol consists of three stages. At Day 0, hESCs are seeded as single cells at 17K cells/cm^2 on geltrex-coated plates and maintained in E8 medium. At Day 1, Stage I neural induction is initiated by replacing the E8 media with neural induction medium containing small molecule inhibitors LDN-193189, SB431542 and XAV939. The medium was replaced daily. At Day 7, cells are reseeded at 130K cells/cm^2 on plates sequentially coated with polyornithine, fibronectin and geltrex. Stage II, the self-patterning stage, starts at Day 7 and requires daily media changes of neuronal self-patterning medium containing B27 supplement. At Day 13, Stage III neuronal maturation is initiated. Cells are reseeded at 130K cells/cm^2 on plates sequentially coated with polyornithine, fibronectin and geltrex, and neuronal maturation medium containing B27, FGF2 and EGF is replaced daily until the end of the protocol at Day 20.

The main advantages compared to other neural induction protocols are: 1) media is replaced every day to avoid stability and degradation issues of nutrients and compounds of interest, 2) At split days cells were counted instead of using fixed split ratios ensuring equal cell numbers

across wells, thus reducing well-to-well variation, 3) A self-pattering phase (part II of the protocol) ensuring that cells recover after split and reducing cell death, 4) Substrate-coating conditions are optimized for cell adhesion.

3.2 Choice of cell line

At the start of the project, the hESC line H9 (WA09, WiCell Research Institute, Inc., Madison, WI, http://www.wicell.org) was used. H9 was originally chosen because it is a well-known and frequently used cell line[303]. However, H9 was prone to spontaneous neurulation during maintenance culturing, resulting in issues with reproducibility and well-to-well variation. For this reason, H9 was replaced with HS360[304] (KIe009-A, Karolinska Institutet Human Embryonic Stem Cell Bank, Stockholm, Sweden) for the toxicology studies. HS360 is a male (46, XY) cell line which was easy to maintain at the stem cell stage, and has previously shown to be able to differentiate toward neural lineages[305].

3.3 Choice of medication concentrations and metabolites

3.3.1 Paracetamol

We wanted to examine paracetamol at concentrations physiologically relevant to long-term exposure in vivo. Since paracetamol

1) crosses the placenta,
2) crosses the blood-brain barrier and
3) does not significantly bind to proteins,

maternal plasma/serum and cord blood concentrations can be used as estimates for the amount of paracetamol that reaches the developing fetal brain (see chapter 1.5.2). Concentrations of 100 μM and 200 μM were chosen to cover some of the range found in vivo, corresponding to one intermediate and one high peak plasma concentration[126,133].

Considering the metabolites of paracetamol, it was decided not to include these in the exposure experiments. It is not known if paracetamol's metabolites are responsible or contribute to the potential effects seen in studies of animal and human early development (see chapter 1.5.3-1.5.4). A cocktail containing both paracetamol and metabolites was considered, though it would be difficult to determine relevant concentration ratios. Additionally, there were practical issues with the solubility of the different metabolites, creating problems such as too high solvent concentrations in the media, different solvents and need for solvent controls, whereas

paracetamol was solved in distilled H_2O and added fresh to media every day throughout the protocol to recapitulate in vivo long-term exposure. Considering these issues, and that the differentiation protocol was labour intensive and expensive, we prioritized paracetamol.

3.3.2 Citalopram

For citalopram, the therapeutic concentration range in plasma is considered to be 150-340 nM[306]. However, studies have reported even wider ranges (see chapter 1.6.2), which vary with dosages and between individuals. Additionally, factors such as protein binding of citalopram, crossing over the placental and blood-brain barrier require consideration. Thus, estimating the amount of citalopram reaching the fetal brain is difficult. We opted to expose the differentiating cells to 100 nM, 200 nM and 400 nM citalopram to cover the concentrations in the therapeutic range. In addition, cells were exposed to 50 nM to cover the lower-end of citalopram concentrations found in cord blood. Citalopram was solved in distilled H_2O and added fresh to the media every day throughout the protocol to recapitulate in vivo long-term exposure.

Metabolites of citalopram were not included in the study. The reason for this decision was due to the metabolites crossing placenta and blood-brain barrier to a lesser degree than citalopram, and they are thought to have a negligible contribution to the clinical effect. Moreover, the time, cost and complexity of the experiments was reduced.

3.4 Choice of endpoint omics outcomes

The epigenetic and gene expression profiles of the hESCs and cells undergoing neuronal differentiation were characterized and compared at several timepoints using a multi-omics approach. Specifically, we used bulk RNA sequencing (RNA-seq) and DNAm analysis, and single-cell RNA sequencing (scRNA-seq) and single cell assays for transposase accessible chromatin sequencing (scATAC-seq) to create a molecular timeline of early neuronal differentiation and the effects of paracetamol and citalopram exposure. Using a multi-omics approach we gain insight into both bulk and cell type-specific gene expression, and the potential DNAm and chromatin regulatory mechanisms.

Epigenetic modifications serve as an important bridge between environmental factors and phenotypes. To study if medication exposure during neuronal differentiation affected epigenetic patterns, DNAm analysis using Illumina methylationEPIC microarray was

performed. The methylationEPIC microarray provides a cost-effective method to study approximately 850 000 CpG sites distributed across the genome[307]. As mentioned in Chapter 1, an association between paracetamol exposure during pregnancy and DNAm changes in cord blood has been identified[118]. Thus, we used DNAm analysis to compare the present study with the MoBa cord blood study.

To study whether changes in DNAm were associated with gene expression changes, we performed bulk RNA-seq. RNA-seq is the standard method to study gene expression changes and, given sufficient sequencing coverage, has the power to detect gene expression differences between groups even for lowly expressed genes[308].

Each cell type has a distinct epigenetic and gene expression profile. The obtained values from bulk RNA-seq and DNAm methods represent the average expression or DNAm of a cell population and does not allow deconvolution of cell-type composition. Thus, using these methods we cannot elucidate if the medication-induced changes are due to differences in cell type composition or direct effects of the medication exposure. At the time the laboratory work was conducted for this thesis, established single-cell methods for DNAm were not available to us. However, the Chromium platform (10x Genomics) was used to capture the gene expression profiles of individual cells and the heterogeneity of cell populations. This was crucial, as all neuronal differentiation protocols results in somewhat heterogeneous cell populations. In contrast to RNA-seq however, scRNA-seq data have relatively low sequencing coverage per cell. Thus, if RNA-seq was not used, the differences in lowly expressed genes could have been missed.

Finally, scATAC-seq was used to study condition-specific chromatin opening at single-cell resolution and integrated with scRNA-seq to identify areas of chromatin that is involved in regulating gene expression. The single-cell data are available in open-access webtools. For reproducibility of data analysis, all code has been made available on Github.

CHAPTER 4: SUMMARY OF RESULTS

4.1 Paper I: Robust neuronal differentiation of human embryonic stem cells for neurotoxicology

Paper I is a methods paper published in STAR Protocols, describing a 20 day protocol for 2D neuronal differentiation of hESCs towards heterogenous population of telencephalic progenitors and neurons[300]. The protocol is optimized for compound neurotoxicology and early-brain differentiation studies.

The neuronal differentiation protocol has optimized cell-seeding densities and coating conditions for high cell viability, which makes it suitable for single-cell omics studies. To reduce compound instability and degradation when conducting neurotoxicology studies, we changed the medium daily. Moreover, the protocol provides daily representative brightfield images for morphology assessment and qPCR analysis of pluripotency and neuronal differentiation markers. Pluripotency markers *NANOG* and *POU5F1* were highly expressed at Day 0 and decreased significantly from Day 7. Neuronal developmental markers *SOX2*, *OTX2*, *FOXG1*, *NEUROD1*, *VIM*, *TUBB3* and *MAP2* increased from Day 7 or Day 13.

4.2 Paper II: A multi-omics approach to visualize early neuronal differentiation from hESCs in 4D

In Paper II, the neuronal differentiation protocol from Paper I was utilized and derived cells were characterized using a multi-omics approach, creating a molecular timeline of an in vitro model of early brain development[309]. The aim of this study was to characterize and describe the epigenetic and gene expression profiles of hESCs (Day 0) and cells undergoing neuronal differentiation at Day 7, 13 and 20. The results from this study are important for understanding early events of cell fate commitment during neurodevelopment.

First, using RNA-seq, we identified differentially expressed genes between the four time points. After Day 0, we found decreased expression of pluripotency genes and increased expression of genes involved in neuronal differentiation and maturation. Stagewise, the highest number of changes occurred at the neural induction stage (Day 0 to Day 7). Furthermore, the neural induction stage also had the highest number of DNAm changes. Similar to the temporal gene expression changes across neuronal differentiation, we found DNAm changes in genes involved in neurogenesis and brain development. Integration of bulk gene expression and DNAm

revealed CpG sites predicted to regulate gene expression. We also found that non-CpG DNAm levels decreased during differentiation.

Gene expression and open chromatin of individual cells, cell heterogeneity, maturation levels and differentiation trajectories were characterized using scRNA-seq and scATAC-seq. The ventral telencephalic markers *EMX2* and *ASCL1* were found present from Day 13, whereas dorsal markers were absent, confirming the spatial identities of the cells. Integration of the two single-cell datasets were used to identify peak-to-gene links and putative cis-regulatory elements inferring epigenome regulation at Day 0 and Day 20. Moreover, single-cell data was made available in user-friendly webtools (**ShinyCell** and **ShinyArchR.UiO**) enabling the exploration of candidate genes or chromatin regions. This multi-omics approach showed that the neuronal differentiation protocol recapitulates stages of neuronal progenitor proliferation and specification.

4.3 Paper III: Multi-omics approach reveals dysregulated genes during hESCs neuronal differentiation exposure to paracetamol

In Paper III, we exposed hESCs undergoing neuronal differentiation (from Paper I) to therapeutic doses of paracetamol for 20 days to model the effect on early human brain development. The aim of this study was to identify the effect of 100 (P100) and 200 μM paracetamol (P200) on epigenetic and gene expression profiles of differentiating cells at Day 7 and Day 20 using a multi-omics approach. The results from this study are important to delineate the effect of paracetamol exposure during neurodevelopment and understand the mechanisms involved.

Bulk RNA-seq time-response analysis between Day 7 and Day 20 identified 121 and 1433 paracetamol-induced differentially expressed genes in P100 and P200 cells compared to control cells, respectively. These genes were associated with downregulation of biological processes involved in axogenesis, synaptic transmission, plasticity and organization. Transcriptional dysregulation of genes linked to neuronal development was identified, indicating a developmental delay in the paracetamol-exposed cells compared to control cells.

To study cell-type specific paracetamol-induced gene expression changes, scRNA-seq was performed. Analysis of 15201 cells revealed a shift in cluster annotations in P100 and P200 cells compared to control cells. Paracetamol induced dose-dependent changes in genes involved

in neuronal maturation, neurite outgrowth, cortical neurogenesis, expression of neurotransmitter transporters and WNT and FGF signalling. At Day 7 we observed downregulation of genes with the shared biological process (BP) *generation of neurons* and genes essential for neurogenesis, such as *FOXG1* and genes of the *HES* and *ID* families. At Day 20, expression of *PAX6* was increased in P200 cells compared to the control, indicating paracetamol-induced differentiation lag.

To assess whether paracetamol influenced chromatin state during differentiation, scATAC-seq was performed at Day 20. Integration of scATAC- and scRNA-seq at Day 20 identified 10 460 and 12304 unique putative cis regulatory elements in P100 and P200 cells respectively, indicating that paracetamol exposure during differentiation affects chromatin accessibility.

To study if paracetamol exposure induced changes in DNAm, EPIC array was performed at Day 7 and Day 20. DNAm time-response analysis of P200 cells identified significant changes in 3113 CpGs, involved in processes such as synaptic signalling. Of these CPGs, 180 differentially methylated genes (DMGs) were also differentially expressed. We observed a dose-dependent paracetamol-induced effect, showing increased DNAm in P100 and P200 cells compared to control cells at significant CpG sites at both Day 7 and 20. Comparing the differentially expressed genes (DEGs) and DMGs at Day 20 in the present model to our previous analysis in cord blood from children exposed to paracetamol during pregnancy, we identified overlapping genes involved in differentiation, Notch and Hedgehog signalling pathways, neuronal excitability, neuronal response to injury and toxic insult response.

Finally, scRNA-seq (**hescneuroparacet**) and integrative scATAC-seq/scRNA-seq datasets (**hescneurodiffparacet**) can be visualized using open-access webtools.

4.4 Paper IV: Citalopram exposure of hESCs during neuronal differentiation identifies differentially expressed genes involved in neuronal development and depression

In this study, we exposed hESCs undergoing neuronal differentiation (from Paper I) to therapeutic doses of citalopram for 13 Days to model the effect on early human brain development. The aim of this study was to identify the effect of 50, 100, 200 and 400 nM citalopram (Cit50-400) on epigenetic and gene expression profiles of differentiating cells at

Day 6, 10 and 13 using a multi-omics approach. The results from this study are important for assessing the effect of citalopram exposure during neurodevelopment and the potential mechanisms involved.

First, major changes in gene expression and DNAm during neuronal differentiation of control cells were observed, with many processes related to neuronal differentiation being affected. Loss of pluripotency markers (e.g. *POU5F1*, *NANOG*) and increased expression of key markers involved in neurogenesis (e.g. *PAX6*, *FOXG1*, *OTX2*), confirmed neuronal differentiation. The differentiation protocol used in this study showed good correspondence with data from paper II.

Linear time-response analysis of each concentration of citalopram from Day 6 to Day 13 compared to control cells identified affected gene expression but not DNA methylation (DNAm) levels over time. A dose-dependent effect of citalopram exposure on the number of DEGs was observed, with Cit400 showing the highest number of DEGs compared to controls. Some of the time-response DEGs are involved in neuronal development and brain function, including *CCL2*, associated with depression brain-immune system communication and suggested as a potential antidepressant target. The time-response analysis also revealed transcriptional changes in genes linked to ASD, depression, neurotransmission, and hippocampal neurogenesis. Gene set enrichment analysis showed enrichment of BPs related to related to amino acid metabolic and catabolic processes, in line with previous studies.

No specific dose- or time-dependent trend in cell-type composition was found using scRNA-seq analysis of 20217 cells. However, significant differences in pseudotime differentiation were identified between cells exposed to citalopram compared to control cells, driven by temporally expressed genes such as *TAGLN*, *HES5*, *FRZB, DDIT4* and *NNAT*. Citalopram-exposed cells exhibited higher mean pseudotime levels compared to controls, indicating enhanced neuronal maturation. Pairwise comparisons of each concentration of citalopram compared to controls cells revealed common BPs across all days involved in different catabolic and metabolic processes, protein targeting and viral gene expression. Some BPs related to neuronal development were also enriched. In contrast, only one CpG was differentially methylated in Cit400 cells compared to the controls, suggesting that the observed gene expression changes were regulated by mechanisms other than DNA methylation.

Finally, dose-response analysis of citalopram exposure revealed 685 DEGs at Day 6. We also identified 186 DEGs at Day 10 and 333 DEGs at Day 13, compared to baseline (Day 6). Differential methylation analysis revealed 27 DMCs in response to citalopram exposure at Day 13. Furthermore, BPs related to metabolic and catabolic processes were enriched at Day 13. The bulk RNA-seq dose-response analysis identified genes that were also found in the scRNA-seq pseudotime analysis. Notably, citalopram exposure led to dose-dependent downregulation of *DDIT4*, a gene associated with major depressive disorder and antidepressant effect. Additionally, fluctuations in *HES5* expression were observed, which is linked to cell state transitioning in neural progenitor cells. The scRNA dataset also revealed transcriptional changes in various other genes involved in brain function, synaptic transmission and plasticity, in response to citalopram exposure in a dose-dependent manner.

CHAPTER 5: DISCUSSION

5.1 What does the neuronal differentiation model reflect?

We developed a novel 2D differentiation protocol towards a heterogenous population of progenitors and neurons bearing telencephalic signatures. Combining RNA-seq, global DNA methylation, single-cell RNA-seq and ATAC-seq data and analysing the integration across timepoints (4D analysis), we constructed a molecular timeline and correlated TFs with time- and population-specific chromatin states in hESCs, early fate commitment and during differentiation. The in vitro neuronal differentiation protocol of hESCs mimics early human brain development. The hESCs HS360 used in our experiments represent the ICM of Day 6-preimplantation blastocysts[310], and differentiates towards neuronal cells up to 20 days. It is difficult to know the corresponding in vivo developmental timepoint, however it likely reflects early first trimester development. Neural rosette-structured derived cells at differentiation Day 6-7 correspond to the formation of the neural tube at embryonic week 3-4. The derived cells at Day 13 and 20 are similar to neuronal progenitor and neuroblast cells[309].

There are several advantages to this study. First, using this hESC neuronal differentiation model to study medication-exposure we gain valuable insight into a normally inaccessible developmental window of brain development. The cell-types derived from the neuronal differentiation protocol may resemble early stages of pregnancy, i.e., first trimester. Further, using human ESCs there is no need to account for interspecies translatability. Exposing differentiating cells to paracetamol and citalopram allows us to study the effect of these compounds without complicating the analysis and interpretation of results due to metabolites. Moreover, exposing cells to more than one medication dosage and sampling cells at several timepoints permits the elucidation of dose- and time-responses. In epidemiological studies, dose and time responses may be difficult to study due to power issues. Another major advantage of this study is the utilization of single-cell methods, which provide high resolution gene expression and chromatin opening data, and deconvolution of cell heterogeneity.

Using the HS360 neuronal differentiation model, we can study if exposure to medications induces changes to epigenetic modifications and gene expression. If so, which functional pathways do they affect and how does these translate to a clinical setting? The results may complement epidemiological studies where associations between medication exposure and

altered phenotype have been identified. Moreover, the results may be used to cautiously interpret potential mechanisms by which medications alter the phenotype.

5.2 What does the neuronal differentiation model not reflect?

5.2.1 Neurodevelopmental outcomes

We hypothesize that medications used during pregnancy, i.e., paracetamol and citalopram, may directly or indirectly affect epigenetic modifications, causing long-term adverse neurodevelopmental outcomes. The model used in the present study is a neuronal differentiation model, not a disease or phenotype model, thus we cannot answer the important scientific question if these medications cause adverse neurodevelopmental outcomes in vivo.

Human embryonic stem cells might seem like an unusual choice of starting material. However, the effects of drugs in reproductive biology are commonly studied using in vitro stem cell models including hESCs and hiPSCs[258]. Taking into consideration the 3R concept for lab animal use, and the limitations of animal models (as evidence shows that the human neocortex develops under the effect of additional mechanisms), we decided to use hESC during neuronal differentiation to gain insight into this inaccessible developmental window. We did not employ hESC differentiation claiming that we can recapitulate the effect of paracetamol during pregnancy, but as an alternative method to infer developmental epigenetic and transcriptome trajectories and the roles of specific genes in brain patterning.

To use the neuronal differentiation model in the context of a phenotype, iPSCs from a disease group and healthy controls should be used. For example, differentiating iPSCs from ADHD patients +/- paracetamol could be compared to iPSCs from controls +/- paracetamol. This way we account for the genetic vulnerability of ADHD patients and determine if paracetamol affects neurodevelopment differently in ADHD patients compared to controls.

5.2.2 Fetal-maternal interactions

The neuronal differentiation model uses a two-dimensional culturing protocol, which reflects early-stage neurodevelopment. However, the model does not account for in vivo tissue-tissue interactions within the fetus or fetal-maternal interactions, including hormonal signalling, placental function, maternal and fetal metabolism. For example, in vivo fetal exposure in the developing brain to the medication of interest and metabolites varies with time of exposure

during pregnancy. The ICM forms primitive ectoderm around Day 7.5 post fertilization. This leaves a window of 10-13 days before transport though the placental barrier from embryonal day 17-20[144]. Moreover, the fetal liver will start metabolism during the first half of the pregnancy, potentially resulting in the accumulation of metabolites on the fetal side of the placenta[311]. Thus, it is important to keep these limitations in mind to not overinterpret the in vitro findings.

5.2.3 Genetic and familial vulnerability

Paracetamol or antidepressant use during pregnancy may be associated with familial or genetic factors[169]. Thus, adverse neurodevelopmental outcomes observed after medication exposure could be confounded rather than caused by the medication (Fig. 10). For example, mothers who are genetically predisposed to ADHD and pass on this risk to the child may also be more inclined to take paracetamol during pregnancy due to inherent impulsive behaviour.

In this thesis, the effect of medication exposure during neuronal differentiation is studied using a single hESC line. An advantage of using only one genetic background is that the experiment is better controlled, we know that the differences we see after medication exposure is not due to differences in genetic or familial risk factors. A limiting factor in using one genetic background is that pharmacogenetic factors, how a persons' genotype affects the way they respond to medications, cannot be accounted for in this model.

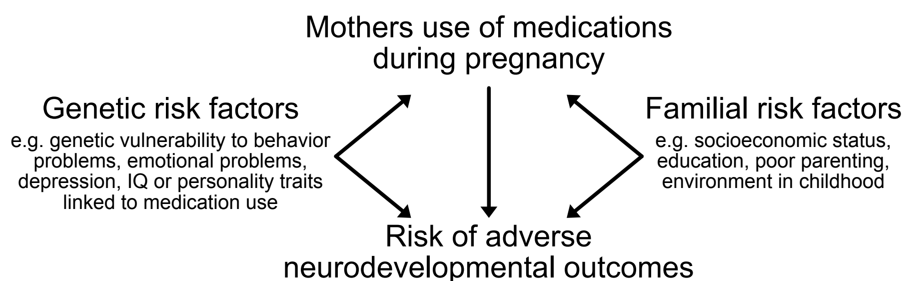

Figure 10. Confounding factors. Mothers use of medication during pregnancy is associated with an increased risk of adverse neurodevelopmental outcomes. The use of medication during pregnancy may be the cause of the adverse outcomes or may be confounded by genetic and/or familial factors.

5.2.4 Later pregnancy stages

The neuronal differentiation model developed in this project reflects neuronal cells of varying maturity corresponding to development during first trimester pregnancies. It is currently

unknown which cell types in the brain or at which trimester medication exposure has the highest impact on neurodevelopment. The neuronal differentiation model does not relate to neurodevelopment at later pregnancy stages (2nd and 3rd trimesters). Given that brain growth accelerates in the 3rd trimester[312], medication exposure in cells corresponding to this stage may be relevant to the observed neurodevelopmental outcomes, including fully differentiated functional neurons and glial cells.

5.2.5 Metabolites

As described in chapter 3.3.1, paracetamol metabolites were not included in this experimental set-up. It is, however, possible that HS360 cells or HS360-derived NPCs from our protocol metabolize paracetamol to form the bioactive AM404. The genes encoding the enzymes that are required for deacetylation of paracetamol to p-aminophenol and further conjugated to form AM404 are expressed at all four sampling time points (Fig. 11A-B). Similarly, *CYP2E1*, coding for the enzyme that metabolize paracetamol to NAPQI (Fig. 11A), is expressed at increasing levels across differentiation albeit at low levels (Fig. 11C). Of note, *CYP2E1* is also expressed at low level in other cell types (e.g., blood) and tissues where paracetamol is not metabolised (https://www.gtexportal.org/home/gene/CYP2E1). The presence of the required enzymes AM404 or NAPQI have not been examined, thus these results must be interpreted with caution.

Furthermore, it is unlikely that HS360 cells or HS360-derived NPCs from our protocol metabolize citalopram, as *CYP2C19*, which codes for the enzyme responsible for the demethylation of citalopram, is not expressed (data not shown).

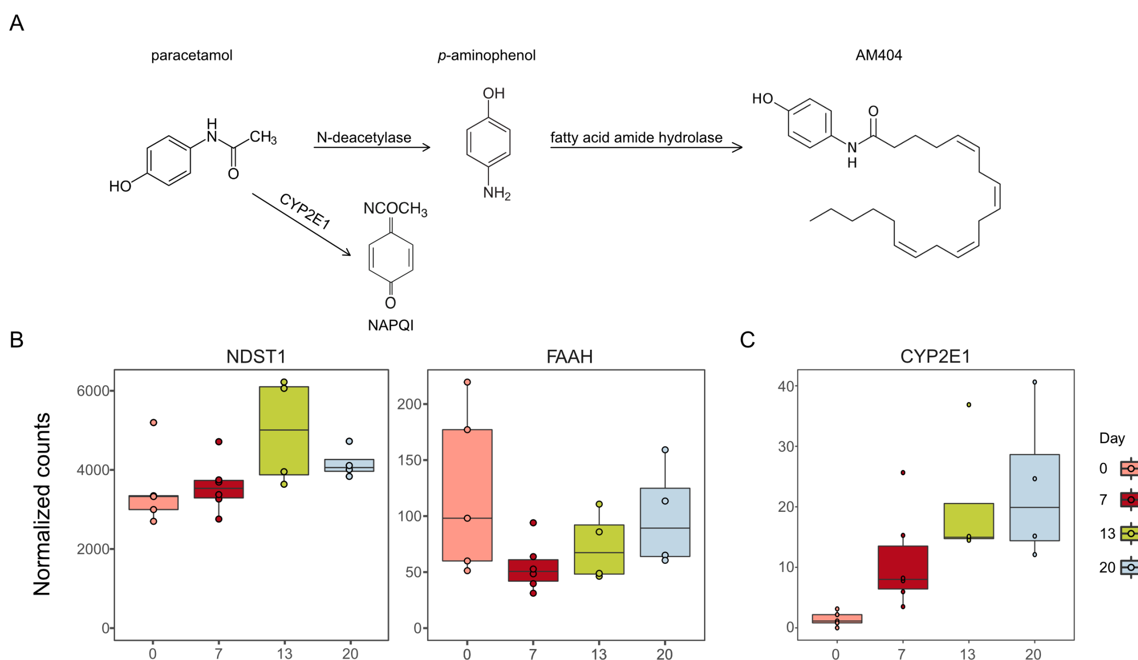

Figure 11. Metabolism of paracetamol to AM404 and NAPQI. A) Paracetamol is deacetylated by N-deacetylase to form *p*-aminophenol, which is further transported to the brain and conjugated with arachidonic acid by fatty acid amide hydrolase to form AM404. B) Normalized RNA-seq counts for genes *NDST1* and *FAAH* coding for the enzymes N-deacetylase and fatty acid amid hydrolase, respectively. C) Normalized RNA-seq counts for *CYP2E1*. B-C) Untreated cells (control) are shown at day 0, 7, 13 and 20.

5.3 Interpretation of findings

5.3.1 How to make sense of the large amount of acquired data?

Using a multi-omics approach to study the effect of medication exposure on neurodevelopment generates a very large quantity of data. For example, in Paper III we generated both bulk and single-cell data for gene expression analysis, bulk data for DNAm analysis and single-cell data for chromatin state. Thus, there were four large datasets from different timepoints and exposure concentrations. It is thus challenging to interpret the data and narrow it down to the biologically interesting genes or pathways or mechanisms.

If we have an idea of which mechanisms or biological pathways are involved, a hypothesis-driven approach can be used. However, as we do not know which biological pathways are involved in medication-induced adverse neurodevelopmental outcomes, an exploratory approach was used in this study. First, the datasets from each method were analyzed individually to find differential changes between conditions. Each comparison for each dataset

revealed everything between zero significant changes to hundreds of thousands of significant changes.

There are many ways of presenting significant findings. For example, we showed global gene expression or DNAm changes, changes in the top significant genes and top biological pathways related to the changes. We also integrated different datasets and compared significant genes/loci. Generally, an outcome is more convincing if more than one method identifies changes in the specific gene or pathway, or if it is consistent between different comparisons, for example across different exposure concentrations. Also, non-significant finding can be useful. For example, paracetamol did not induce significant changes in cell populations, suggesting that cell fate was not majorly changing upon exposure during differentiation. However, due to the abundance of data generated using the in vitro neuronal differentiation model, interpretation is complex even without trying to relate the findings to the clinical setting.

Finally, to adhere to the principles of Findability, Accessibility, Interoperability, and Reusability (FAIR), we provide access to all the single-cell datasets in intuitive, interactive web applications. By enabling diverse configurations of gene and cluster expression visualisation, including Uniform Manifold Approximation and Projections, heatmaps, violin plots, box plots, proportion plots, and bubble plots, these tools facilitate analysis during the neuronal differentiation protocol and during paracetamol and citalopram exposure. As such, they serve as valuable resources for future studies including, but not restricted to, the effect of specific disease variants or drugs in early brain physiology and development.

5.3.2 How to relate the results to the MoBa study?

Gervin et al.[118] studied the association between paracetamol exposure during pregnancy and DNAm changes in cord blood and clinical ADHD diagnosis. A similar EWAS looking at the connection between SSRI use during pregnancy and DNAm has recently been conducted[313]. To relate the results from the MoBa-study to the current study, we can compare DMCs and DMGs following paracetamol exposure as both studies have conducted DNAm analysis.

Crucial differences between the paracetamol studies, besides the obvious in vivo versus in vitro settings, are:

1. The neuronal differentiation model lacks a phenotype or disease outcome, whereas the MoBa study found associations with ADHD. In the MoBa study, no significant DMCs were identified

when comparing children without ADHD that had been exposed to paracetamol during pregnancy compared to controls (no ADHD, no paracetamol). In contrast, DNAm changes were found in children with ADHD that had been exposed to paracetamol during pregnancy compared to controls. Thus, paracetamol exposure during pregnancy only induced DNAm changes in individuals that were susceptible to ADHD. We were unable to account for this disease susceptibility in our neuronal differentiation model.

2. Different tissues were used in the current study compared to the MoBa study. The MoBa study used cord blood, whereas the present study used a model of early neuronal cells. We can speculate if common DMCs and DMGs between the two tissues suggests that paracetamol exposure modulates changes in early common ancestor cells. Thus, these CpGs could potentially be used as cord blood biomarkers for paracetamol-induced changes in brain cells. However, comparing in vivo and in vitro data we should expect to find many differences as well as some similarities.

In summary, the comparison of in vitro results to cohort studies must be conservative. When studying medication-induced outcomes, overlapping results between EWAS and the neuronal differentiation model is more convincing, but we should be careful not to over-interpret the results. Moreover, we expect large differences in the results between EWAS and the neuronal differentiation model due to the large differences in methodology.

CHAPTER 6: FUTURE DIRECTIONS

The safety of medications during pregnancy is a largely understudied field deserving of further study. For future studies related to the current project, it would be interesting to validate our findings in other birth cohorts to narrow down clinically relevant paracetamol- or citalopram-dysregulated genes. DNAm changes associated with citalopram-exposure have recently been studied in cord blood from MoBa[313]. It would be beneficial to compare our results to this study in a similar fashion to the paracetamol study.

In Papers III and IV, we study only the compound of interest, paracetamol and citalopram. For future studies, it would be of interest to conduct a time-course experiment to measure the concentrations of paracetamol/citalopram and metabolites in the culturing media using mass spectrometry. If cells are not capable of metabolizing the medications, it would be useful to study the effect of metabolites individually or in a cocktail with the mother compound.

We have investigated the effect of medications in differentiating cells originating from one cell type (HS360), and thus one genetic background. As DNAm and gene expression are established by the genotype, we could apply the model to other hESC lines to delineate if the effects we see are specific to HS360 or more widely applicable. Moreover, we could apply the neuronal differentiation model to iPSCs from patients diagnosed with NDDs and compare to controls to determine if exposure to medications have an effect on the epigenetic regulation of gene expression.

Our neuronal differentiation model reflects neuronal development during early pregnancy. It would be valuable to study the effects of paracetamol and citalopram in more mature neurons following a longer differentiation protocol reflecting second and third trimester development. Moreover, exposing cells using three-dimensional brain organoids in addition to the current two-dimensional protocol would allow us to study the effects on the development of other brain cells and the interactions between these cells.

Finally, it would be of interest to apply this neuronal differentiation model to study the effects of other potential environmental influences or medications used during pregnancy, such as NSAIDs, anticonvulsants, antihistamines, antidepressants and antianxiety medications, insect repellants, natural supplements or air pollutants.

REFERENCES

1. LB F, SK H. Disparities in rates of unintended pregnancy in the United States, 1994 and 2001. *Perspect Sex Reprod Health*. 2006;38(2). doi:10.1363/PSRH.38.090.06
2. Lo WY, Friedman JM. Teratogenicity of recently introduced medications in human pregnancy. *Obstet Gynecol*. 2002;100(3):465-473. doi:10.1016/S0029-7844(02)02122-1
3. Adam MP, Polifka JE, Friedman JM. Evolving knowledge of the teratogenicity of medications in human pregnancy. *Am J Med Genet Part C Semin Med Genet*. 2011;157(3):175-182. doi:10.1002/ajmg.c.30313
4. Little BB. Pharmacokinetics during pregnancy: Evidence-based maternal dose formulation. *Obstet Gynecol*. 1999;93(5 SUPPL.):858-868. doi:10.1016/S0029-7844(98)00444-X
5. Lupattelli A, Spigset O, Twigg MJ, et al. Medication use in pregnancy: A cross-sectional, multinational web-based study. *BMJ Open*. 2014;4(2):4365. doi:10.1136/bmjopen-2013-004365
6. Moore KL, Persaud TVN, Torchia MG. *The Developing Human-Clinically Oriented Embryology*. 10th ed. Saunders Elsevier; 2016. Accessed June 29, 2023. https://www.academia.edu/44502808/The_Developing_Human_Clinically_Oriented_Embryology_by_Keith_L_Moore
7. Shahbazi MN. Mechanisms of human embryo development: From cell fate to tissue shape and back. *Dev*. 2020;147(14). doi:10.1242/dev.190629
8. Muhr J, Ackerman KM. *Embryology, Gastrulation*. StatPearls Publishing; 2020. Accessed January 10, 2021. http://www.ncbi.nlm.nih.gov/pubmed/32119281
9. Yuskaitis CJ, Pomeroy SL. Development of the Nervous System. In: *Fetal and Neonatal Physiology*. Elsevier; 2017:1294-1313.e2. doi:10.1016/B978-0-323-35214-7.00131-1
10. Singh R, Munakomi S. *Embryology, Neural Tube*. StatPearls Publishing; 2019. Accessed January 13, 2021. http://www.ncbi.nlm.nih.gov/pubmed/31194425
11. Sadler TW. Embryology of Neural Tube Development. *Am J Med Genet Part C (Semin Med Genet*. 2005;135:2-8. doi:10.1002/ajmg.c.30049
12. Sauka-Spengler T, Bronner-Fraser M. A gene regulatory network orchestrates neural crest formation. *Nat Rev Mol Cell Biol*. 2008;9(7):557-568. doi:10.1038/nrm2428
13. Campbell, Neal A.; Reece JB. *Biology*. 7th ed. Pearson, Benjamin Cummings; 2005.
14. Stiles J, Jernigan TL. The basics of brain development. *Neuropsychol Rev*. 2010;20(4):327-348. doi:10.1007/s11065-010-9148-4
15. Tao Y, Zhang SC. Neural Subtype Specification from Human Pluripotent Stem Cells. *Cell Stem Cell*. 2016;19(5):573-586. doi:10.1016/j.stem.2016.10.015
16. Kirkeby A, Grealish S, Wolf DA, et al. Generation of Regionally Specified Neural Progenitors and Functional Neurons from Human Embryonic Stem Cells under Defined Conditions. *Cell Rep*. 2012;1(6):703-714. doi:10.1016/j.celrep.2012.04.009
17. Diez del Corral R, Morales A V. The multiple roles of FGF signaling in the developing spinal cord. *Front Cell Dev Biol*. 2017;5(JUN):58. doi:10.3389/fcell.2017.00058
18. Conti L, Cattaneo E. Neural stem cell systems: Physiological players or in vitro entities? *Nat Rev Neurosci*. 2010;11(3):176-187. doi:10.1038/nrn2761

19. Sarnat HB, Flores-Sarnat L. Nervous System, Neuroembryology of. In: *Encyclopedia of the Neurological Sciences*. Elsevier Inc.; 2014:350-359. doi:10.1016/B978-0-12-385157-4.01048-4
20. Johnson CA, Wright CE, Ghashghaei HT. Regulation of cytokinesis during corticogenesis: focus on the midbody. *FEBS Lett*. 2017;591(24):4009-4026. doi:10.1002/1873-3468.12676
21. Deans C, Maggert KA. What Do You Mean, "Epigenetic"? *Genetics*. 2015;199(4):887-896. doi:10.1534/GENETICS.114.173492
22. Greally JM. A user's guide to the ambiguous word "epigenetics." *Nat Rev Mol Cell Biol*. 2018;19(4):207-208. doi:10.1038/nrm.2017.135
23. Bird A. Perceptions of epigenetics. *Nat 2007 4477143*. 2007;447(7143):396-398. doi:10.1038/nature05913
24. Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. *Nat Rev Genet*. 2016;17(8):487-500. doi:10.1038/nrg.2016.59
25. Margueron R, Reinberg D. Chromatin structure and the inheritance of epigenetic information. *Nat Rev Genet*. 2010;11(4):285-296. doi:10.1038/nrg2752
26. Jurkowska RZ, Jurkowski TP, Jeltsch A. Structure and Function of Mammalian DNA Methyltransferases. *ChemBioChem*. 2011;12(2):206-222. doi:10.1002/cbic.201000195
27. Joosten SC, Smits KM, Aarts MJ, et al. Epigenetics in renal cell cancer: Mechanisms and clinical applications. *Nat Rev Urol*. 2018;15(7):430-451. doi:10.1038/s41585-018-0023-z
28. Ramazi S, Allahverdi A, Zahiri J. Evaluation of post-translational modifications in histone proteins: A review on histone modification defects in developmental and neurological disorders. *J Biosci*. 2020;45(1):1-29. doi:10.1007/s12038-020-00099-2
29. Bannister AJ, Kouzarides T. Regulation of chromatin by histone modifications. *Cell Res*. 2011;21(3):381-395. doi:10.1038/cr.2011.22
30. Ng SS, Yue WW, Oppermann U, Klose RJ. Dynamic protein methylation in chromatin biology. *Cell Mol Life Sci*. 2009;66(3):407-422. doi:10.1007/s00018-008-8303-z
31. Maher BA. The histone code. *Scientist*. 2003;17(2):13. doi:10.1007/978-981-10-7614-5_5
32. Pinney S. Mammalian Non-CpG Methylation: Stem Cells and Beyond. *Biology (Basel)*. 2014;3(4):739-751. doi:10.3390/biology3040739
33. Lister R, Pelizzola M, Dowen RH, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*. 2009;462(7271):315-322. doi:10.1038/nature08514
34. Greenberg MVC, Bourc'his D. The diverse roles of DNA methylation in mammalian development and disease. *Nat Rev Mol Cell Biol*. 2019;20(10):590-607. doi:10.1038/s41580-019-0159-6
35. Li E, Zhang Y. DNA methylation in mammals. *Cold Spring Harb Perspect Biol*. 2014;6(5):a019133. doi:10.1101/cshperspect.a019133
36. Unoki M. Recent Insights into the Mechanisms of De Novo and Maintenance of DNA Methylation in Mammals . In: *DNA Methylation Mechanism*. IntechOpen; 2020. doi:10.5772/intechopen.89238
37. Du J, Johnson LM, Jacobsen SE, Patel DJ. DNA methylation pathways and their crosstalk with histone methylation. *Nat Rev Mol Cell Biol*. 2015;16(9):519-532. doi:10.1038/nrm4043
38. Kim M, Costello J. DNA methylation: An epigenetic mark of cellular memory. *Exp Mol Med*. 2017;49(4):49. doi:10.1038/emm.2017.10

39. Spruijt CG, Vermeulen M. DNA methylation: Old dog, new tricks? *Nat Struct Mol Biol*. 2014;21(11):949-954. doi:10.1038/nsmb.2910
40. Jones PA. Functions of DNA methylation: Islands, start sites, gene bodies and beyond. *Nat Rev Genet*. 2012;13(7):484-492. doi:10.1038/nrg3230
41. Laurent L, Wong E, Li G, et al. Dynamic changes in the human methylome during differentiation. *Genome Res*. 2010;20(3):320-331. doi:10.1101/gr.101907.109
42. Gelfman S, Cohen N, Yearim A, Ast G. DNA-methylation effect on cotranscriptional splicing is dependent on GC architecture of the exon-intron structure. *Genome Res*. 2013;23(5):789-799. doi:10.1101/gr.143503.112
43. Shukla S, Kavak E, Gregory M, et al. CTCF-promoted RNA polymerase II pausing links DNA methylation to splicing. *Nature*. 2011;479(7371):74-79. doi:10.1038/nature10442
44. Ordoñez R, Martínez-Calle N, Agirre X, Prosper F. DNA methylation of enhancer elements in myeloid neoplasms: Think outside the promoters? *Cancers (Basel)*. 2019;11(10). doi:10.3390/cancers11101424
45. Klemm SL, Shipony Z, Greenleaf WJ. Chromatin accessibility and the regulatory epigenome. *Nat Rev Genet 2018 204*. 2019;20(4):207-220. doi:10.1038/S41576-018-0089-8
46. Markenscoff-Papadimitriou E, Binyameen F, Whalen S, et al. Autism risk gene POGZ promotes chromatin accessibility and expression of clustered synaptic genes. *Cell Rep*. 2021;37(10):110089. doi:10.1016/J.CELREP.2021.110089
47. Yoon SH, Choi J, Lee WJ, Do JT. Genetic and Epigenetic Etiology Underlying Autism Spectrum Disorder. *J Clin Med*. 2020;9(4). doi:10.3390/JCM9040966
48. Mossink B, Negwer M, Schubert D, Nadif Kasri N. The emerging role of chromatin remodelers in neurodevelopmental disorders: a developmental perspective. *Cell Mol Life Sci*. 2021;78(6):2517. doi:10.1007/S00018-020-03714-5
49. Zeng Y, Chen T. DNA methylation reprogramming during mammalian development. *Genes (Basel)*. 2019;10(4). doi:10.3390/genes10040257
50. Smith ZD, Sindhu C, Meissner A. Molecular features of cellular reprogramming and development. *Nat Rev Mol Cell Biol*. 2016;17(3):139-154. doi:10.1038/nrm.2016.6
51. Wang L, Zhang J, Duan J, et al. Programming and inheritance of parental DNA methylomes in mammals. *Cell*. 2014;157(4):979-991. doi:10.1016/j.cell.2014.04.017
52. Meissner A, Mikkelsen TS, Gu H, et al. Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature*. 2008;454(7205):766-770. doi:10.1038/nature07107
53. Hirabayashi Y, Gotoh Y. Epigenetic control of neural precursor cell fate during development. *Nat Rev Neurosci*. 2010;11(6):377-388. doi:10.1038/nrn2810
54. Xu R, Li C, Liu X, Gao S. Insights into epigenetic patterns in mammalian early embryos. *Protein Cell*. 2021;12(1):7-28. doi:10.1007/s13238-020-00757-z
55. Cantone I, Fisher AG. Epigenetic programming and reprogramming during development. *Nat Struct Mol Biol*. 2013;20(3):282-289. doi:10.1038/nsmb.2489
56. Burton A, Torres-Padilla ME. Chromatin dynamics in the regulation of cell fate allocation during early embryogenesis. *Nat Rev Mol Cell Biol*. 2014;15(11):722-734. doi:10.1038/nrm3885
57. Robertson KD. Epigenetic Mechanisms of Gene Regulation. In: *DNA Methylation and Cancer Therapy*.

Springer US; 2007:13-30. doi:10.1007/0-387-27443-x_2

58. Lewis EM, Kroll KL. Development and disease in a dish: the epigenetics of neurodevelopmental disorders. *Epigenomics*. 2018;10(2):219-231. doi:10.2217/epi-2017-0113

59. Fahrner JA, Bjornsson HT. Mendelian disorders of the epigenetic machinery: postnatal malleability and therapeutic prospects. *Hum Mol Genet*. 2019;28(R2):R254. doi:10.1093/HMG/DDZ174

60. Amir RE, Van Den Veyver IB, Wan M, Tran CQ, Francke U, Zoghbi HY. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl- CpG-binding protein 2. *Nat Genet*. 1999;23(2):185-188. doi:10.1038/13810

61. Shah RR, Bird AP. MeCP2 mutations: Progress towards understanding and treating Rett syndrome. *Genome Med*. 2017;9(1). doi:10.1186/s13073-017-0411-7

62. Cukier HN, Rabionet R, Konidari I, et al. Novel variants identified in methyl-CpG-binding domain genes in autistic individuals. *Neurogenetics*. 2010;11(3):291-303. doi:10.1007/s10048-009-0228-7

63. Satterstrom FK, Kosmicki JA, Wang J, et al. Large-Scale Exome Sequencing Study Implicates Both Developmental and Functional Changes in the Neurobiology of Autism. *Cell*. 2020;180(3):568-584.e23. doi:10.1016/j.cell.2019.12.036

64. Sanders SJ, He X, Willsey AJ, et al. Insights into Autism Spectrum Disorder Genomic Architecture and Biology from 71 Risk Loci. *Neuron*. 2015;87(6):1215-1233. doi:10.1016/j.neuron.2015.09.016

65. LaSalle JM, Yasui DH. Evolving role of MeCP2 in Rett syndrome and autism. *Epigenomics*. 2009;1(1):119-130. doi:10.2217/epi.09.13

66. Ward SP. Thalidomide and congenital abnormalities. *Br Med J*. 1962;2(5305):646-647. doi:10.1136/bmj.2.5305.646

67. Lammer EJ, Chen DT, Hoar RM, et al. Retinoic Acid Embryopathy. *N Engl J Med*. 1985;313(14):837-841. doi:10.1056/NEJM198510033131401

68. Macfarlane A, Greenhalgh T. Sodium valproate in pregnancy: What are the risks and should we use a shared decision-making approach? *BMC Pregnancy Childbirth*. 2018;18(1). doi:10.1186/s12884-018-1842-x

69. Bjørk MH, Zoega H, Leinonen MK, et al. Association of Prenatal Exposure to Antiseizure Medication With Risk of Autism and Intellectual Disability. *JAMA Neurol*. 2022;79(7):672-681. doi:10.1001/JAMANEUROL.2022.1269

70. Patorno E, Huybrechts KF, Bateman BT, et al. Lithium Use in Pregnancy and the Risk of Cardiac Malformations. *N Engl J Med*. 2017;376(23):2245-2254. doi:10.1056/nejmoa1612222

71. Baillie M, Allen ED, Elkington AR. The congenital warfarin syndrome: A case report. *Br J Ophthalmol*. 1980;64(8):633-635. doi:10.1136/bjo.64.8.633

72. Demers P, Fraser D, Goldbloom RB, et al. Effects of tetracyclines on skeletal growth and dentition. A report by the Nutrition Committee of the Canadian Paediatric Society. *Can Med Assoc J*. 1968;99(17):849-854. Accessed December 11, 2020. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1945410/

73. Conings S, Tseke F, Van Den Broeck A, et al. Transplacental transport of paracetamol and its phase II metabolites using the ex vivo placenta perfusion model. Published online 2019. doi:10.1016/j.taap.2019.03.004

74. Rodier PM. Developing brain as a target of toxicity. In: *Environmental Health Perspectives*. Vol 103. Public Health Services, US Dept of Health and Human Services; 1995:73-76. doi:10.1289/ehp.95103s673
75. Heyer DB, Meredith RM. Environmental toxicology: Sensitive periods of development and neurodevelopmental disorders. *Neurotoxicology*. 2017;58:23-41. doi:10.1016/j.neuro.2016.10.017
76. Giordano G, Costa LG. Developmental Neurotoxicity: Some Old and New Issues. *ISRN Toxicol*. 2012;2012:1-12. doi:10.5402/2012/814795
77. Costa LG. Toxic effects of pesticides. In: Klaassen CD, ed. *Casarett and Doull's Toxicology: The Basic Science of Poisons*. 7th editio. McGraw-Hill; 2008:883-930.
78. Kalkbrenner AE, Schmidt RJ, Penlesky AC. Environmental chemical exposures and autism spectrum disorders: a review of the epidemiological evidence. *Curr Probl Pediatr Adolesc Health Care*. 2014;44(10):277-318. doi:10.1016/j.cppeds.2014.06.001
79. Roze E, Meijer L, Bakker A, Van Braeckel KNJA, Sauer PJJ, Bos AF. Prenatal exposure to organohalogens, including brominated flame retardants, influences motor, cognitive, and behavioral performance at school age. *Environ Health Perspect*. 2009;117(12):1953-1958. doi:10.1289/ehp.0901015
80. Gascon M, Vrijheid M, Martínez D, et al. Effects of pre and postnatal exposure to low levels of polybromodiphenyl ethers on neurodevelopment and thyroid hormone levels at 4years of age. *Environ Int*. 2011;37(3):605-611. doi:10.1016/j.envint.2010.12.005
81. Clifford A, Lang L, Chen R, Anstey KJ, Seaton A. Exposure to air pollution and cognitive functioning across the life course - A systematic literature review. *Environ Res*. 2016;147:383-398. doi:10.1016/j.envres.2016.01.018
82. dos Santos MA, Vasconcelos GS, Soares de Sousa CN, Macedo D, Vasconcelos SMM. Consequences of ethanol exposure on neurodevelopment. In: *Neuroscience of Alcohol: Mechanisms and Treatment*. Elsevier; 2019:47-55. doi:10.1016/B978-0-12-813125-1.00006-4
83. Joelsson P, Chudal R, Talati A, Suominen A, Brown AS, Sourander A. Prenatal smoking exposure and neuropsychiatric comorbidity of ADHD: A finnish nationwide population-based cohort study. *BMC Psychiatry*. 2016;16(1):306. doi:10.1186/s12888-016-1007-2
84. Xu M-Q, Sun W-S, Liu B-X, et al. Prenatal Malnutrition and Adult Schizophrenia: Further Evidence From the 1959-1961 Chinese Famine. *Schizophr Bull*. 2009;35(3):568-576. doi:10.1093/schbul/sbn168
85. Sobral Da Silva PF, Eickmann SH, Arraes De Alencar Ximenes R, et al. Pediatric neurodevelopment by prenatal Zika virus exposure: A cross-sectional study of the Microcephaly Epidemic Research Group Cohort. *BMC Pediatr*. 2020;20(1):472. doi:10.1186/s12887-020-02331-2
86. Brown AS, Susser ES. In utero infection and adult schizophrenia. *Ment Retard Dev Disabil Res Rev*. 2002;8(1):51-57. doi:10.1002/mrdd.10004
87. Thapar A, Cooper M, Rutter M. Neurodevelopmental disorders. *The Lancet Psychiatry*. 2017;4(4):339-346. doi:10.1016/S2215-0366(16)30376-5
88. Zablotsky B, Black LI, Maenner MJ, et al. Prevalence and trends of developmental disabilities among children in the United States: 2009–2017. *Pediatrics*. 2019;144(4). doi:10.1542/peds.2019-0811
89. Allen DA. Autistic spectrum disorders: Clinical presentation in preschool children. *J Child Neurol*.

1988;3(SUPPL.). doi:10.1177/0883073888003001s10

90. Polanczyk G, De Lima MS, Horta BL, Biederman J, Rohde LA. The worldwide prevalence of ADHD: A systematic review and metaregression analysis. *Am J Psychiatry*. 2007;164(6):942-948. doi:10.1176/ajp.2007.164.6.942

91. Biederman J, Faraone S V. Attention-deficit hyperactivity disorder. In: *Lancet*. Vol 366. Elsevier; 2005:237-248. doi:10.1016/S0140-6736(05)66915-2

92. Sciberras E, Mulraney M, Silva D, Coghill D. Prenatal Risk Factors and the Etiology of ADHD—Review of Existing Evidence. *Curr Psychiatry Rep*. 2017;19(1). doi:10.1007/s11920-017-0753-2

93. Class QA, Abel KM, Khashan AS, et al. Offspring psychopathology following preconception, prenatal and postnatal maternal bereavement stress. *Psychol Med*. 2014;44(1):71-84. doi:10.1017/S0033291713000780

94. Oberlander TF, Reebye P, Misri S, Papsdorf M, Kim J, Grunau RE. Externalizing and attentional behaviors in children of depressed mothers treated with a selective serotonin reuptake inhibitor antidepressant during pregnancy. *Arch Pediatr Adolesc Med*. 2007;161(1):22-29. doi:10.1001/archpedi.161.1.22

95. Rommelse NNJ, Franke B, Geurts HM, Hartman CA, Buitelaar JK. Shared heritability of attention-deficit/hyperactivity disorder and autism spectrum disorder. *Eur Child Adolesc Psychiatry*. 2010;19(3):281-295. doi:10.1007/s00787-010-0092-x

96. Martens G, van Loo K. Genetic and Environmental Factors in Complex Neurodevelopmental Disorders. *Curr Genomics*. 2009;8(7):429-444. doi:10.2174/138920207783591717

97. Barker DJP. The origins of the developmental origins theory. *J Intern Med*. 2007;261(5):412-417. doi:10.1111/j.1365-2796.2007.01809.x

98. Mazahery H, Camargo CA, Conlon C, Beck KL, Kruger MC, von Hurst PR. Vitamin D and autism spectrum disorder: A literature review. *Nutrients*. 2016;8(4). doi:10.3390/nu8040236

99. Painter RC, Roseboom TJ, Bleker OP. Prenatal exposure to the Dutch famine and disease in later life: An overview. *Reprod Toxicol*. 2005;20(3):345-352. doi:10.1016/j.reprotox.2005.04.005

100. St Clair D, Xu M, Wang P, et al. Rates of adult schizophrenia following prenatal exposure to the Chinese famine of 1959-1961. *J Am Med Assoc*. 2005;294(5):557-562. doi:10.1001/jama.294.5.557

101. Park S, Cho SC, Kim JW, et al. Differential perinatal risk factors in children with attention-deficit/hyperactivity disorder by subtype. *Psychiatry Res*. 2014;219(3):609-616. doi:10.1016/j.psychres.2014.05.036

102. Weaver ICG, Cervoni N, Champagne FA, et al. Epigenetic programming by maternal behavior. *Nat Neurosci*. 2004;7(8):847-854. doi:10.1038/nn1276

103. Khan F, Momtaz S, Abdollahi M. The relationship between mercury exposure and epigenetic alterations regarding human health, risk assessment and diagnostic strategies. Published online 2018. doi:10.1016/j.jtemb.2018.11.006

104. Li S, Hansman R, Newbold R, Davis B, McLachlan JA, Barrett JC. Neonatal diethylstilbestrol exposure induces persistent elevation of c-fos expression and hypomethylation in its exon-4 in mouse uterus. *Mol Carcinog*. 2003;38(2):78-84. doi:10.1002/mc.10147

105. Ho SM, Tang WY, Belmonte De Frausto J, Prins GS. Developmental exposure to estradiol and

bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phosphodiesterase type 4 variant 4. *Cancer Res*. 2006;66(11):5624-5632. doi:10.1158/0008-5472.CAN-06-0516

106. Koturbash I, Baker M, Loree J, et al. Epigenetic dysregulation underlies radiation-induced transgenerational genome instability in vivo. *Int J Radiat Oncol Biol Phys*. 2006;66(2):327-330. doi:10.1016/j.ijrobp.2006.06.012

107. Onishchenko N, Karpova N, Sabri F, Castrn E, Ceccatelli S. Long-lasting depression-like behavior and epigenetic changes of BDNF gene expression induced by perinatal exposure to methylmercury. *J Neurochem*. 2008;106(3):1378-1387. doi:10.1111/j.1471-4159.2008.05484.x

108. Pilsner JR, Hall MN, Liu X, et al. Influence of prenatal arsenic exposure and newborn sex on global methylation of cord blood DNA. *PLoS One*. 2012;7(5). doi:10.1371/journal.pone.0037147

109. Bakulski KM, Lee H, Feinberg JI, et al. Prenatal mercury concentration is associated with changes in DNA methylation at *TCEANC2* in newborns. *Int J Epidemiol*. 2015;44(4):1249-1262. doi:10.1093/ije/dyv032

110. Pilsner JR, Hu H, Ettinger A, et al. Influence of prenatal lead exposure on genomic methylation of cord blood DNA. *Environ Health Perspect*. 2009;117(9):1466-1471. doi:10.1289/ehp.0800497

111. Miura R, Araki A, Minatoya M, et al. An epigenome-wide analysis of cord blood DNA methylation reveals sex-specific effect of exposure to bisphenol A. *Sci Rep*. 2019;9(1):1-13. doi:10.1038/s41598-019-48916-5

112. Feng F, Huang L, Zhou G, et al. *GPR61* methylation in cord blood: a potential target of prenatal exposure to air pollutants. *Int J Environ Health Res*. Published online June 1, 2020:1-10. doi:10.1080/09603123.2020.1773414

113. Miyake K, Kawaguchi A, Miura R, et al. Association between DNA methylation in cord blood and maternal smoking: The Hokkaido Study on Environment and Children's Health. *Sci Rep*. 2018;8(1):1-14. doi:10.1038/s41598-018-23772-x

114. Joubert BR, Felix JF, Yousefi P, et al. DNA Methylation in Newborns and Maternal Smoking in Pregnancy: Genome-wide Consortium Meta-analysis. *Am J Hum Genet*. 2016;98(4):680-696. doi:10.1016/j.ajhg.2016.02.019

115. Smith AK, Conneely KN, Newport DJ, et al. Prenatal antiepileptic exposure associates with neonatal DNA methylation differences. *Epigenetics*. 2012;7(5):458-463. doi:10.4161/epi.19617

116. Emes RD, Clifford H, Haworth KE, et al. Antiepileptic drugs and the fetal epigenome. *Epilepsia*. 2013;54(1):e16-e19. doi:10.1111/j.1528-1167.2012.03673.x

117. McLaughlin P, Mactier H, Gillis C, et al. Increased DNA Methylation of ABCB1, CYP2D6, and OPRM1 Genes in Newborn Infants of Methadone-Maintained Opioid-Dependent Mothers. *J Pediatr*. 2017;190:180-184.e1. doi:10.1016/j.jpeds.2017.07.026

118. Gervin K, Nordeng H, Ystrom E, Reichborn-Kjennerud T, Lyle R. Long-term prenatal exposure to paracetamol is associated with DNA methylation differences in children diagnosed with ADHD. *Clin Epigenetics*. 2017;9(1):77. doi:10.1186/s13148-017-0376-9

119. Fragou D, Pakkidi E, Aschner M, Samanidou V, Kovatsi L. Smoking and DNA methylation: Correlation of methylation with smoking behavior and association with diseases and fetus development following

prenatal exposure. Published online 2019. doi:10.1016/j.fct.2019.04.059

120. Cornelius MD, De Genna NM, Leech SL, Willford JA, Goldschmidt L, Day NL. Effects of prenatal cigarette smoke exposure on neurobehavioral outcomes in 10-year-old children of adolescent mothers. *Neurotoxicol Teratol*. 2011;33(1):137-144. doi:10.1016/j.ntt.2010.08.006

121. Tran NQV, Miyake K. Neurodevelopmental Disorders and Environmental Toxicants: Epigenetics as an Underlying Mechanism. *Int J Genomics*. 2017;2017. doi:10.1155/2017/7526592

122. Milutinovic S, D'Alessio AC, Detich N, Szyf M. Valproate induces widespread epigenetic reprogramming which involves demethylation of specific genes. *Carcinogenesis*. 2007;28(3):560-571. doi:10.1093/carcin/bgl167

123. Smith A, Kaufman F, Sandy MS, Cardenas A. Cannabis Exposure During Critical Windows of Development: Epigenetic and Molecular Pathways Implicated in Neuropsychiatric Disease. *Curr Environ Heal Reports*. 2020;7(3):325-342. doi:10.1007/s40572-020-00275-4

124. Hansen HH, Mikkelsen JD. Long-term effects on serotonin transporter mRNA expression of chronic neonatal exposure to a serotonin reuptake inhibitor. *Eur J Pharmacol*. 1998;352(2-3):307-315. doi:10.1016/S0014-2999(98)00349-5

125. Csoka AB, Szyf M. Epigenetic side-effects of common pharmaceuticals: A potential new field in medicine and pharmacology. *Med Hypotheses*. 2009;73(5):770-780. doi:10.1016/J.MEHY.2008.10.039

126. Graham GG, Davies MJ, Day RO, Mohamudally A, Scott KF. The modern pharmacology of paracetamol: Therapeutic actions, mechanism of action, metabolism, toxicity and recent pharmacological findings. *Inflammopharmacology*. 2013;21(3):201-232. doi:10.1007/s10787-013-0172-x

127. Mazaleuskaya LL, Sangkuhl K, Thorn CF, Fitzgerald GA, Altman RB, Klein TE. PharmGKB summary: Pathways of acetaminophen metabolism at the therapeutic versus toxic doses. *Pharmacogenet Genomics*. 2015;25(8):416-426. doi:10.1097/FPC.0000000000000150

128. Högestätt ED, Jönsson BAG, Ermund A, et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. *J Biol Chem*. 2005;280(36):31405-31412. doi:10.1074/jbc.M501489200

129. Sharma C V., Long JH, Shah S, et al. First evidence of the conversion of paracetamol to AM404 in human cerebrospinal fluid. *J Pain Res*. 2017;10:2703-2709. doi:10.2147/JPR.S143500

130. Miners J, Robson R, Birkett D. Paracetamol metabolism in pregnancy. *Br J Clin Pharmacol*. 1986;22(3):359-362. doi:10.1111/j.1365-2125.1986.tb02901.x

131. Jozwiak-Bebenista M, Nowak JZ. Paracetamol: Mechanism of action, applications and safety concern. *Acta Pol Pharm - Drug Res*. 2014;71(1):11-23. Accessed March 17, 2021. https://europepmc.org/article/med/24779190?utm_medium=email&utm_source=transaction&client=bot&client=bot&client=bot

132. Bertolini A, Ferrari A, Ottani A, Guerzoni S, Tacchi R, Leone S. Paracetamol: New vistas of an old drug. *CNS Drug Rev*. 2006;12(3-4):250-275. doi:10.1111/j.1527-3458.2006.00250.x

133. Raffa RB, Pawasauskas J, Pergolizzi J V., et al. Pharmacokinetics of Oral and Intravenous Paracetamol (Acetaminophen) When Co-Administered with Intravenous Morphine in Healthy Adult Subjects. *Clin Drug Investig*. 2018;38(3):259-268. doi:10.1007/s40261-017-0610-4

134. Zafeiri A, Mitchell RT, Hay DC, Fowler PA. Over-the-counter analgesics during pregnancy: A

comprehensive review of global prevalence and offspring safety. *Hum Reprod Update*. 2021;27(1):67-95. doi:10.1093/humupd/dmaa042

135. Werler MM, Mitchell AA, Hernandez-Diaz S, Honein MA. Use of over-the-counter medications during pregnancy. *Am J Obstet Gynecol*. 2005;193(3):771-777. doi:10.1016/j.ajog.2005.02.100
136. Nordeng H, Ystrøm E, Einarson A. Perception of risk regarding the use of medications and other exposures during pregnancy. *Eur J Clin Pharmacol*. 2010;66(2):207-214. doi:10.1007/s00228-009-0744-2
137. Castillo Barrio B, Bravo Laguna MC, de la Calle Fernández M, Pellicer Martínez A. Consumption of paracetamol during pregnancy. *An Pediatría (English Ed*. 2020;93(3):196-197. doi:10.1016/j.anpede.2019.12.007
138. Bandoli G, Palmsten K, Chambers C. Acetaminophen use in pregnancy: Examining prevalence, timing, and indication of use in a prospective birth cohort. *Paediatr Perinat Epidemiol*. 2020;34(3):237-246. doi:10.1111/ppe.12595
139. Navaro M, Vezzosi L, Santagati G, Angelillo IF. Knowledge, attitudes, and practice regarding medication use in pregnant women in Southern Italy. Ciccozzi M, ed. *PLoS One*. 2018;13(6):e0198618. doi:10.1371/journal.pone.0198618
140. Nitsche J, Patil A, Langman L, et al. Transplacental Passage of Acetaminophen in Term Pregnancy. *Am J Perinatol*. 2017;34(06):541-543. doi:10.1055/s-0036-1593845
141. Van Der Marel CD, Anderson BJ, Pluim MAL, De Jong THR, Gonzalez A, Tibboel D. Acetaminophen in cerebrospinal fluid in children. *Eur J Clin Pharmacol*. 2003;59(4):297-302. doi:10.1007/s00228-003-0622-2
142. Anderson BJ, Holford NHG, Woollard GA, Chan PLS. Paracetamol plasma and cerebrospinal fluid pharmacokinetics in children. *Br J Clin Pharmacol*. 1998;46(3):237-243. doi:10.1046/j.1365-2125.1998.00780.x
143. Kumpulainen E, Kokki H, Halonen T, Heikkinen M, Savolainen J, Laisalmi M. Paracetamol (acetaminophen) penetrates readily into the cerebrospinal fluid of children after intravenous administration. *Pediatrics*. 2007;119(4):766-771. doi:10.1542/peds.2006-3378
144. Ahokas RA, McKinney ET. *Development and Physiology of the Placenta and Membranes*. Sapiens Publishing, LTD; 2009. doi:10.3843/GLOWM.10101
145. Reuter I, Knaup S, Romanos M, Lesch K-P, Drepper C, Lillesaar C. Developmental exposure to acetaminophen does not induce hyperactivity in zebrafish larvae. *J Neural Transm*. 2016;123(8):841-848. doi:10.1007/s00702-016-1556-z
146. Viberg H, Eriksson P, Gordh T, Fredriksson A. Paracetamol (Acetaminophen) Administration During Neonatal Brain Development Affects Cognitive Function and Alters Its Analgesic and Anxiolytic Response in Adult Male Mice. *Toxicol Sci*. 2014;138(1):139-147. doi:10.1093/toxsci/kft329
147. Philippot G, Gordh T, Fredriksson A, Viberg H. Adult neurobehavioral alterations in male and female mice following developmental exposure to paracetamol (acetaminophen): characterization of a critical period. *J Appl Toxicol*. 2017;37(10):1174-1181. doi:10.1002/jat.3473
148. Hay-Schmidt A, Finkielman OTE, Jensen BAH, et al. Prenatal exposure to paracetamol/acetaminophen and precursor aniline impairs masculinisation of male brain and behaviour. *Reproduction*.

2017;154(2):145-152. doi:10.1530/REP-17-0165

149. Dean A, van den Driesche S, Wang Y, et al. Analgesic exposure in pregnant rats affects fetal germ cell development with inter-generational reproductive consequences. *Sci Rep*. 2016;6(1):19789. doi:10.1038/srep19789

150. Blecharz-Klin K, Joniec-Maciejak I, Jawna-Zboińska K, et al. Cerebellar level of neurotransmitters in rats exposed to paracetamol during development. *Pharmacol Reports*. 2016;68(6):1159-1164. doi:10.1016/j.pharep.2016.06.005

151. Blecharz-Klin K, Joniec-Maciejak I, Jawna K, et al. Developmental exposure to paracetamol causes biochemical alterations in medulla oblongata. *Environ Toxicol Pharmacol*. 2015;40(2):369-374. doi:10.1016/J.ETAP.2015.07.001

152. Blecharz-Klin K, Piechal A, Jawna-Zboińska K, et al. Paracetamol − Effect of early exposure on neurotransmission, spatial memory and motor performance in rats. *Behav Brain Res*. 2017;323:162-171. doi:10.1016/J.BBR.2017.01.051

153. Koehn LM, Huang Y, Habgood MD, et al. Effects of paracetamol (acetaminophen) on gene expression and permeability properties of the rat placenta and fetal brain. *F1000Research*. 2020;9. doi:10.12688/F1000RESEARCH.24119.2

154. Petersen TG, Liew Z, Andersen A-MN, et al. Use of paracetamol, ibuprofen or aspirin in pregnancy and risk of cerebral palsy in the child. *Int J Epidemiol*. 2018;47(1):121-130. doi:10.1093/ije/dyx235

155. Bornehag C-G, Reichenberg A, Hallerback MU, et al. Prenatal exposure to acetaminophen and children's language development at 30 months. *Eur Psychiatry*. 2018;51:98-103. doi:10.1016/j.eurpsy.2017.10.007

156. Fan G, Wang B, Liu C, Li D. Prenatal paracetamol use and asthma in childhood: A systematic review and meta-analysis. *Allergol Immunopathol (Madr)*. 2017;45(6):528-533. doi:10.1016/j.aller.2016.10.014

157. Jensen MS, Rebordosa C, Thulstrup AM, et al. Maternal Use of Acetaminophen, Ibuprofen, and Acetylsalicylic Acid During Pregnancy and Risk of Cryptorchidism. *Epidemiology*. 2010;21(6):779-785. doi:10.1097/EDE.0b013e3181f20bed

158. Brandlistuen RE, Ystrom E, Nulman I, Koren G, Nordeng H. Prenatal paracetamol exposure and child neurodevelopment: a sibling-controlled cohort study. *Int J Epidemiol*. 2013;42(6):1702-1713. doi:10.1093/ije/dyt183

159. Vlenterie R, Wood ME, Brandlistuen RE, Roeleveld N, van Gelder MMHJ, Nordeng H. Neurodevelopmental problems at 18 months among children exposed to paracetamol in utero : a propensity score matched cohort study. *Int J Epidemiol*. 2016;45(6):dyw192. doi:10.1093/ije/dyw192

160. Trønnes JN, Wood M, Lupattelli A, Ystrom E, Nordeng H. Prenatal paracetamol exposure and neurodevelopmental outcomes in preschool-aged children. *Paediatr Perinat Epidemiol*. 2020;34(3):247-256. doi:10.1111/ppe.12568

161. Liew Z, Ritz B, Rebordosa C, Lee P-C, Olsen J. Acetaminophen Use During Pregnancy, Behavioral Problems, and Hyperkinetic Disorders. *JAMA Pediatr*. 2014;168(4):313. doi:10.1001/jamapediatrics.2013.4914

162. Liew Z, Ritz B, Virk J, Olsen J. Maternal use of acetaminophen during pregnancy and risk of autism spectrum disorders in childhood: A Danish national birth cohort study. *Autism Res*. 2016;9(9):951-958.

doi:10.1002/aur.1591

163. Thompson JMD, Waldie KE, Wall CR, Murphy R, Mitchell EA, ABC study group. Associations between Acetaminophen Use during Pregnancy and ADHD Symptoms Measured at Ages 7 and 11 Years. Hashimoto K, ed. *PLoS One*. 2014;9(9):e108210. doi:10.1371/journal.pone.0108210

164. Sznajder KK, Teti DM, Kjerulff KH. Maternal use of acetaminophen during pregnancy and neurobehavioral problems in offspring at 3 years: A prospective cohort study. *PLoS One*. 2022;17(9):e0272593. doi:10.1371/JOURNAL.PONE.0272593

165. Stergiakouli E, Thapar A, Davey Smith G. Association of Acetaminophen Use During Pregnancy With Behavioral Problems in Childhood. *JAMA Pediatr*. 2016;170(10):964. doi:10.1001/jamapediatrics.2016.1775

166. Avella-Garcia CB, Julvez J, Fortuny J, et al. Acetaminophen use in pregnancy and neurodevelopment: attention function and autism spectrum symptoms. *Int J Epidemiol*. 2016;45(6):dyw115. doi:10.1093/ije/dyw115

167. Liew Z, Bach CC, Asarnow RF, Ritz B, Olsen J. Paracetamol use during pregnancy and attention and executive function in offspring at age 5 years. *Int J Epidemiol*. 2016;45(6):dyw296. doi:10.1093/ije/dyw296

168. Liew Z, Ritz B, Virk J, Arah OA, Olsen J. Prenatal Use of Acetaminophen and Child IQ: A Danish Cohort Study. *Epidemiology*. 2016;27(6):912-918. doi:10.1097/EDE.0000000000000540

169. Ystrom E, Vollrath ME, Nordeng H. Effects of personality on use of medications, alcohol, and cigarettes during pregnancy. *Eur J Clin Pharmacol*. 2012;68(5):845-851. doi:10.1007/s00228-011-1197-y

170. Ystrom E, Gustavson K, Brandlistuen RE, et al. Prenatal exposure to acetaminophen and risk of ADHD. *Pediatrics*. 2017;140(5):20163840. doi:10.1542/peds.2016-3840

171. Masarwa R, Levine H, Gorelik E, Reif S, Perlman A, Matok I. Prenatal exposure to acetaminophen and risk for attention deficit hyperactivity disorder and autistic spectrum disorder: A systematic review,meta-analysis, and meta-regression analysis of cohort studies. *Am J Epidemiol*. 2018;187(8):1817-1827. doi:10.1093/aje/kwy086

172. Gou X, Wang Y, Tang Y, et al. Association of maternal prenatal acetaminophen use with the risk of attention deficit/hyperactivity disorder in offspring: A meta-analysis. *Aust N Z J Psychiatry*. 2019;53(3):195-206. doi:10.1177/0004867418823276

173. Masarwa R, Platt RW, Filion KB. Acetaminophen use during pregnancy and the risk of attention deficit hyperactivity disorder: A causal association or bias? *Paediatr Perinat Epidemiol*. 2020;34(3):309-317. doi:10.1111/ppe.12615

174. Addo KA, Bulka C, Dhingra R, et al. Acetaminophen use during pregnancy and DNA methylation in the placenta of the extremely low gestational age newborn (ELGAN) cohort. *Environ Epigenetics*. 2019;5(2):1-10. doi:10.1093/eep/dvz010

175. Addo KA, Bulka C, Dhingra R, et al. Acetaminophen use during pregnancy and DNA methylation in the placenta of the extremely low gestational age newborn (ELGAN) cohort. *Environ Epigenetics*. 2020;6(1). doi:10.1093/eep/dvaa006

176. Eslamimehr S, Jones AD, Anthony TM, et al. Association of prenatal acetaminophen use and acetaminophen metabolites with DNA methylation of newborns: analysis of two consecutive generations

of the Isle of Wight birth cohort. *Environ Epigenetics*. 2022;8(1):1-10. doi:10.1093/EEP/DVAC002

177. Parker W, Hornik CD, Bilbo S, et al. The role of oxidative stress, inflammation and acetaminophen exposure from birth to early childhood in the induction of autism. *J Int Med Res*. 2017;45(2):407-438. doi:10.1177/0300060517693423

178. Bauer AZ, Kriebel D, Herbert MR, Bornehag C-G, Swan SH. Prenatal paracetamol exposure and child neurodevelopment: A review. *Horm Behav*. 2018;101:125-147. doi:10.1016/J.YHBEH.2018.01.003

179. Garcia KLP, Yu G, Nicolini C, et al. Altered balance of proteolytic isoforms of pro-brain-derived neurotrophic factor in autism. *J Neuropathol Exp Neurol*. 2012;71(4):289-297. doi:10.1097/NEN.0b013e31824b27e4

180. Ferreira FF, Ribeiro FF, Rodrigues RS, Sebastião AM, Xapelli S. Brain-derived neurotrophic factor (BDNF) role in cannabinoid-mediated neurogenesis. *Front Cell Neurosci*. 2018;12. doi:10.3389/fncel.2018.00441

181. Sangkuhl K, Klein TE, Altman RB. PharmGKB summary: Citalopram pharmacokinetics pathway. *Pharmacogenet Genomics*. 2011;21(11):769-772. doi:10.1097/FPC.0b013e328346063f

182. Pollock BG. Citalopram: A comprehensive review. *Expert Opin Pharmacother*. 2001;2(4):681-698. doi:10.1517/14656566.2.4.681

183. Brummelte S, Mc Glanaghy E, Bonnin A, Oberlander TF. Developmental changes in serotonin signaling: Implications for early brain function, behavior and adaptation. *Neuroscience*. 2017;342:212-231. doi:10.1016/j.neuroscience.2016.02.037

184. Feighner JP. *Mechanism of Action of Antidepressant Medications*. Vol 60. Physicians Postgraduate Press, Inc.; 1999. Accessed April 6, 2021. https://www-psychiatrist-com.ezproxy.uio.no/jcp/depression/mechanism-action-antidepressant-medications

185. Cipriani A, Furukawa TA, Salanti G, et al. Comparative efficacy and acceptability of 21 antidepressant drugs for the acute treatment of adults with major depressive disorder: a systematic review and network meta-analysis. *Lancet*. 2018;391(10128):1357-1366. doi:10.1016/S0140-6736(17)32802-7

186. Bezchlibnyk-Butler K, Aleksic I, Kennedy SH. Citalopram - A review of pharmacological and clinical effects. *J Psychiatry Neurosci*. 2000;25(3):241-254. Accessed April 15, 2021. /pmc/articles/PMC1407724/?report=abstract

187. Hyttel J, Bøgesø KP, Perregaard J, Sánchez C. The pharmacological effect of citalopram resides in the (S)-(+)-enantiomer. *J Neural Transm*. 1992;88(2):157-160. doi:10.1007/BF01244820

188. Evans J, Heron J, Francomb H, Oke S, Golding J. Cohort study of depressed mood during pregnancy and after childbirth. *Br Med J*. 2001;323(7307):257-260. doi:10.1136/bmj.323.7307.257

189. Dubovicky M, Belovicova K, Csatlosova K, Bogi E. Risks of using SSRI / SNRI antidepressants during pregnancy and lactation. *Interdiscip Toxicol*. 2017;10(1):30-34. doi:10.1515/intox-2017-0004

190. Jimenez-Solem E, Andersen JT, Petersen M, et al. Prevalence of Antidepressant Use during Pregnancy in Denmark, a Nation-Wide Cohort Study. Alessi-Severini S, ed. *PLoS One*. 2013;8(4):e63034. doi:10.1371/journal.pone.0063034

191. Mitchell AA, Gilboa SM, Werler MM, Kelley KE, Louik C, Hernández-Díaz S. Medication use during pregnancy, with particular focus on prescription drugs: 1976-2008. *Am J Obstet Gynecol*. 2011;205(1):51.e1-51.e8. doi:10.1016/j.ajog.2011.02.029

192. Cooper WO, Willy ME, Pont SJ, Ray WA. Increasing use of antidepressants in pregnancy. *Am J Obstet Gynecol*. 2007;196(6):544.e1-544.e5. doi:10.1016/j.ajog.2007.01.033
193. Zoega H, Kieler H, Nørgaard M, et al. Use of SSRI and SNRI Antidepressants during Pregnancy: A Population-Based Study from Denmark, Iceland, Norway and Sweden. Branchi I, ed. *PLoS One*. 2015;10(12):e0144474. doi:10.1371/journal.pone.0144474
194. Charlton R, Jordan S, Pierini A, et al. Selective serotonin reuptake inhibitor prescribing before, during and after pregnancy: a population-based study in six European regions. *BJOG An Int J Obstet Gynaecol*. 2015;122(7):1010-1020. doi:10.1111/1471-0528.13143
195. Bakker MK, Kölling P, Van Den Berg PB, De Walle HEK, De Jong Van Den Berg LTW. Increase in use of selective serotonin reuptake inhibitors in pregnancy during the last decade, a population-based cohort study from the Netherlands. *Br J Clin Pharmacol*. 2008;65(4):600-606. doi:10.1111/j.1365-2125.2007.03048.x
196. Andrade SE, Raebel MA, Brown J, et al. Use of antidepressant medications during pregnancy: a multisite study. *Am J Obstet Gynecol*. 2008;198(2):194.e1-194.e5. doi:10.1016/j.ajog.2007.07.036
197. Nordeng H, Van Gelder MMHJ, Spigset O, Koren G, Einarson A, Eberhard-Gran M. Pregnancy outcome after exposure to antidepressants and the role of maternal depression: Results from the Norwegian mother and child cohort study. *J Clin Psychopharmacol*. 2012;32(2):186-194. doi:10.1097/JCP.0b013e3182490eaf
198. Pariente G, Leibson T, Carls A, Adams-Webber T, Ito S, Koren G. Pregnancy-Associated Changes in Pharmacokinetics: A Systematic Review. *PLoS Med*. 2016;13(11):e1002160. doi:10.1371/journal.pmed.1002160
199. Hendrick V, Stowe ZN, Altshuler LL, Hwang S, Lee E, Haynes D. Placental Passage of Antidepressant Medications. *Am J Psychiatry*. 2003;160(5):993-996. doi:10.1176/appi.ajp.160.5.993
200. Rampono J, Simmer K, Ilett KF, et al. Placental Transfer of SSRI and SNRI Antidepressants and Effects on the Neonate. *Pharmacopsychiatry*. 2009;42(03):95-100. doi:10.1055/s-0028-1103296
201. Paulzen M, Goecke TW, Stingl JC, et al. Pregnancy exposure to citalopram – Therapeutic drug monitoring in maternal blood, amniotic fluid and cord blood. *Prog Neuro-Psychopharmacology Biol Psychiatry*. 2017;79:213-219. doi:10.1016/j.pnpbp.2017.06.030
202. Paulzen M, Lammertz SE, Gründer G, Veselinovic T, Hiemke C, Tauber SC. Measuring citalopram in blood and central nervous system: Revealing a distribution pattern that differs from other antidepressants. *Int Clin Psychopharmacol*. 2016;31(3):119-126. doi:10.1097/YIC.0000000000000114
203. Christensen HD, Rayburn WF, Gonzalez CL. Chronic prenatal exposure to paroxetine (Paxil) and cognitive development of mice offspring. *Neurotoxicol Teratol*. 2000;22(5):733-739. doi:10.1016/S0892-0362(00)00099-4
204. Vorhees C. A Developmental Neurotoxicity Evaluation of the Effects of Prenatal Exposure to Fluoxetine in Rats. *Fundam Appl Toxicol*. 1994;23(2):194-205. doi:10.1006/faat.1994.1098
205. Lee LJ. Neonatal Fluoxetine Exposure Affects the Neuronal Structure in the Somatosensory Cortex and Somatosensory-Related Behaviors in Adolescent Rats. *Neurotox Res*. 2009;15(3):212-223. doi:10.1007/s12640-009-9022-4
206. Rodriguez-Porcel F, Green D, Khatri N, et al. Neonatal exposure of rats to antidepressants affects

behavioral reactions to novelty and social interactions in a manner analogous to autistic spectrum disorders. *Anat Rec*. 2011;294(10):1726-1735. doi:10.1002/ar.21402

207. Ansorge MS, Morelli E, Gingrich JA. Inhibition of serotonin but not norepinephrine transport during development produces delayed, persistent perturbations of emotional behaviors in mice. *J Neurosci*. 2008;28(1):199-207. doi:10.1523/JNEUROSCI.3973-07.2008

208. Weaver KJ, Paul IA, Lin RCS, Simpson KL. Neonatal Exposure to Citalopram Selectively Alters the Expression of the Serotonin Transporter in the Hippocampus: Dose-Dependent Effects. *Anat Rec*. 2010;293(11):1920-1932. doi:10.1002/ar.21245

209. Simpson KL, Weaver KJ, De Villers-Sidani E, et al. Perinatal antidepressant exposure alters cortical network function in rodents. *Proc Natl Acad Sci U S A*. 2011;108(45):18465-18470. doi:10.1073/pnas.1109353108

210. Sprowles JLN, Hufgard JR, Gutierrez A, et al. Perinatal exposure to the selective serotonin reuptake inhibitor citalopram alters spatial learning and memory, anxiety, depression, and startle in Sprague-Dawley rats. *Int J Dev Neurosci*. 2016;54(1):39-52. doi:10.1016/j.ijdevneu.2016.08.007

211. Schaefer TL, Grace CE, Braun AA, et al. Cognitive impairments from developmental exposure to serotonergic drugs: Citalopram and MDMA. *Int J Neuropsychopharmacol*. 2013;16(6):1383-1394. doi:10.1017/S1461145712001447

212. Zohar I, Shoham S, Weinstock M. Perinatal citalopram does not prevent the effect of prenatal stress on anxiety, depressive-like behaviour and serotonergic transmission in adult rat offspring. Herman J, ed. *Eur J Neurosci*. 2016;43(4):590-600. doi:10.1111/ejn.13150

213. Maciag D, Williams L, Coppinger D, Paul IA. Neonatal citalopram exposure produces lasting changes in behavior which are reversed by adult imipramine treatment. *Eur J Pharmacol*. 2006;532(3):265-269. doi:10.1016/j.ejphar.2005.12.081

214. Bourke CH, Stowe ZN, Neigh GN, Olson DE, Owens MJ. Prenatal exposure to escitalopram and/or stress in rats produces limited effects on endocrine, behavioral, or gene expression measures in adult male rats. *Neurotoxicol Teratol*. 2013;39:100-109. doi:10.1016/j.ntt.2013.07.008

215. Ehrlich DE, Neigh GN, Bourke CH, et al. Prenatal stress, regardless of concurrent escitalopram treatment, alters behavior and amygdala gene expression of adolescent female rats. Published online 2015. doi:10.1016/j.neuropharm.2015.05.012

216. Popa D, Léna C, Alexandre C, Adrien J. Lasting syndrome of depression produced by reduction in serotonin uptake during postnatal development: Evidence from sleep, stress, and behavior. *J Neurosci*. 2008;28(14):3546-3554. doi:10.1523/JNEUROSCI.4006-07.2008

217. Ramsteijn AS, Van de Wijer L, Rando J, van Luijk J, Homberg JR, Olivier JDA. Perinatal selective serotonin reuptake inhibitor exposure and behavioral outcomes: A systematic review and meta-analyses of animal studies. *Neurosci Biobehav Rev*. 2020;114:53-69. doi:10.1016/j.neubiorev.2020.04.010

218. Rogers A, Obst S, Teague SJ, et al. Association Between Maternal Perinatal Depression and Anxiety and Child and Adolescent Development: A Meta-analysis. *JAMA Pediatr*. 2020;174(11):1. doi:10.1001/JAMAPEDIATRICS.2020.2910

219. Gjerde LC, Eilertsen EM, Hannigan LJ, et al. Associations between maternal depressive symptoms and risk for offspring early-life psychopathology: the role of genetic and non-genetic mechanisms. *Psychol*

Med. 2021;51(3):441-449. doi:10.1017/S0033291719003301
220. Hanley GE, Brain U, Oberlander TF. Prenatal exposure to serotonin reuptake inhibitor antidepressants and childhood behavior. *Pediatr Res*. 2015;78(2):174-180. doi:10.1038/pr.2015.77
221. Malm H, Brown AS, Gissler M, et al. Gestational Exposure to Selective Serotonin Reuptake Inhibitors and Offspring Psychiatric Disorders: A National Register-Based Study. *J Am Acad Child Adolesc Psychiatry*. 2016;55(5):359-366. doi:10.1016/j.jaac.2016.02.013
222. Lupattelli A, Wood M, Ystrom E, Skurtveit S, Handal M, Nordeng H. Effect of Time-Dependent Selective Serotonin Reuptake Inhibitor Antidepressants During Pregnancy on Behavioral, Emotional, and Social Development in Preschool-Aged Children. *J Am Acad Child Adolesc Psychiatry*. 2018;57(3):200-208. doi:10.1016/J.JAAC.2017.12.010
223. Cohen JM, Wood ME, Hernández-Díaz S, Ystrom E, Nordeng H. Paternal antidepressant use as a negative control for maternal use: assessing familial confounding on gestational length and anxiety traits in offspring. *Int J Epidemiol*. 2019;48(5):1665-1672. doi:10.1093/ije/dyz170
224. Klinger G, Frankenthal D, Merlob P, et al. Long-term outcome following selective serotonin reuptake inhibitor induced neonatal abstinence syndrome. *J Perinatol*. 2011;31(9):615-620. doi:10.1038/jp.2010.211
225. Brandlistuen RE, Ystrom E, Eberhard-Gran M, Nulman I, Koren G, Nordeng H. Behavioural effects of fetal antidepressant exposure in a Norwegian cohort of discordant siblings. *Int J Epidemiol*. 2015;44(4):1397-1407. doi:10.1093/ije/dyv030
226. Morales DR, Slattery J, Evans S, Kurz X. Antidepressant use during pregnancy and risk of autism spectrum disorder and attention deficit hyperactivity disorder: Systematic review of observational studies and methodological considerations. *BMC Med*. 2018;16(1):1-14. doi:10.1186/s12916-017-0993-3
227. Andalib S, Emamhadi MR, Yousefzadeh-Chabok S, et al. Maternal SSRI exposure increases the risk of autistic offspring: A meta-analysis and systematic review. *Eur Psychiatry*. 2017;45:161-166. doi:10.1016/j.eurpsy.2017.06.001
228. Jiang HY, Peng CT, Zhang X, Ruan B. Antidepressant use during pregnancy and the risk of attention-deficit/hyperactivity disorder in the children: a meta-analysis of cohort studies. *BJOG An Int J Obstet Gynaecol*. 2018;125(9):1077-1084. doi:10.1111/1471-0528.15059
229. Kobayashi T, Matsuyama T, Takeuchi M. Autism spectrum disorder and prenatal exposure to selective serotonin reuptake inhibitors: A systematic review and meta-analysis. *Reprod Toxicol*. 2016;65:170-178. doi:10.1016/j.reprotox.2016.07.016
230. Sujan AC, Rickert ME, Öberg AS, et al. Associations of Maternal Antidepressant Use During the First Trimester of Pregnancy With Preterm Birth, Small for Gestational Age, Autism Spectrum Disorder, and Attention-Deficit/Hyperactivity Disorder in Offspring. *JAMA*. 2017;317(15):1553-1562. doi:10.1001/jama.2017.3413
231. Rai D, Lee BK, Dalman C, Newschaffer C, Lewis G, Magnusson C. Antidepressants during pregnancy and autism in offspring: Population based cohort study. *BMJ*. 2017;358. doi:10.1136/bmj.j2811
232. Zhou XH, Li YJ, Ou JJ, Li YM. Association between maternal antidepressant use during pregnancy and autism spectrum disorder: An updated meta-analysis. *Mol Autism*. 2018;9(1):1-7. doi:10.1186/s13229-018-0207-7

233. Lupattelli A, Mahic M, Handal M, Ystrom E, Reichborn-Kjennerud T, Nordeng H. Attention-Deficit/Hyperactivity Disorder in Children Following Prenatal Exposure to Antidepressants: results from the Norwegian Mother, Father and Child Cohort Study. *BJOG An Int J Obstet Gynaecol*. Published online May 13, 2021:1471-0528.16743. doi:10.1111/1471-0528.16743
234. Heikkinen T, Ekblad U, Kero P, Ekblad S, Laine K. Citalopram in pregnancy and lactation. *Clin Pharmacol Ther*. 2002;72(2):184-191. doi:10.1067/mcp.2002.126181
235. Sivojelezova A, Shuhaiber S, Sarkissian L, Einarson A, Koren G. Citalopram use in pregnancy: Prospective comparative evaluation of pregnancy and fetal outcome. *Am J Obstet Gynecol*. 2005;193(6):2004-2009. doi:10.1016/j.ajog.2005.05.012
236. Klieger-Grossmann C, Weitzner B, Panchaud A, et al. Pregnancy outcomes following use of escitalopram: A prospective comparative cohort study. *J Clin Pharmacol*. 2012;52(5):766-770. doi:10.1177/0091270011405524
237. Cardenas A, Faleschini S, Cortes Hidalgo A, et al. Prenatal maternal antidepressants, anxiety, and depression and offspring DNA methylation: Epigenome-wide associations at birth and persistence into early childhood. *Clin Epigenetics*. 2019;11(1):1-11. doi:10.1186/s13148-019-0653-x
238. Non AL, Binder AM, Kubzansky LD, Michels KB. Genome-wide DNA methylation in neonates exposed to maternal depression, anxiety, or SSRI medication during pregnancy. *Epigenetics*. 2014;9(7):964-972. doi:10.4161/epi.28853
239. Schroeder JW, Smith AK, Brennan PA, et al. DNA methylation in neonates born to women receiving psychiatric care. *Epigenetics*. 2012;7(4):409-414. doi:10.4161/epi.19551
240. Gurnot C, Martin-Subero I, Mah SM, et al. Prenatal antidepressant exposure associated with CYP2E1 DNA methylation change in neonates. *Epigenetics*. 2015;10(5):361-372. doi:10.1080/15592294.2015.1026031
241. Gartstein MA, Hookenson K V., Brain U, Devlin AM, Grunau RE, Oberlander TF. Sculpting infant soothability: the role of prenatal SSRI antidepressant exposure and neonatal SLC6A4 methylation status. *Dev Psychobiol*. 2016;58(6):745-758. doi:10.1002/dev.21414
242. Devlin AM, Brain U, Austin J, Oberlander TF. Prenatal exposure to maternal depressed mood and the MTHFR C677T variant affect SLC6A4 methylation in infants at birth. *PLoS One*. 2010;5(8):e12201. doi:10.1371/journal.pone.0012201
243. Galbally M, Watson SJ, Van Ijzendoorn M, et al. The role of glucocorticoid and mineralocorticoid receptor DNA methylation in antenatal depression and infant stress regulation. Published online 2020. doi:10.1016/j.psyneuen.2020.104611
244. Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. *Epigenetics*. 2008;3(2):97-106. doi:10.4161/epi.3.2.6034
245. Mansell T, Vuillermin P, Ponsonby AL, Collier F, Saffery R, Ryan J. Maternal mental well-being during pregnancy and glucocorticoid receptor gene promoter methylation in the neonate. In: *Development and Psychopathology*. Vol 28. Cambridge University Press; 2016:1421-1430. doi:10.1017/S0954579416000183
246. Ciesielski TH, Marsit CJ, Williams SM. Maternal psychiatric disease and epigenetic evidence suggest a

common biology for poor fetal growth. *BMC Pregnancy Childbirth*. 2015;15(1):1-9. doi:10.1186/s12884-015-0627-8

247. Ju C, Fiori LM, Belzeaux R, et al. Integrated genome-wide methylation and expression analyses reveal functional predictors of response to antidepressants. *Transl Psychiatry*. 2019;9(1):1-12. doi:10.1038/s41398-019-0589-0
248. Kanherkar RR, Getachew B, Ben-Sheetrit J, et al. The Effect of Citalopram on Genome-Wide DNA Methylation of Human Cells. *Int J Genomics*. 2018;2018. doi:10.1155/2018/8929057
249. Nissen T, Wynn R. The clinical case report: A review of its merits and limitations. *BMC Res Notes*. 2014;7(1):1-7. doi:10.1186/1756-0500-7-264
250. Lao KSJ, Chui CSL, Man KKC, Lau WCY, Chan EW, Wong ICK. Medication safety research by observational study design. *Int J Clin Pharm*. 2016;38(3):676-684. doi:10.1007/s11096-016-0285-6
251. Polifka JE, Friedman J. Clinical teratology: identifying teratogenic risks in humans. *Clin Genet*. 1999;56(6):409-420. doi:10.1034/j.1399-0004.1999.560601.x
252. Campagna MP, Xavier A, Lechner-Scott J, et al. Epigenome-wide association studies: current knowledge, strategies and recommendations. *Clin Epigenetics*. 2021;13(1):1-24. doi:10.1186/S13148-021-01200-8/FIGURES/12
253. Wei S, Tao J, Xu J, et al. Ten Years of EWAS. *Adv Sci*. 2021;8(20):2100727. doi:10.1002/ADVS.202100727
254. Lin JH. Species similarities and differences in pharmacokinetics. *Drug Metab Dispos*. 1995;23(10):1008-1021.
255. Nestler EJ, Hyman SE. Animal models of neuropsychiatric disorders. *Nat Neurosci*. 2010;13(10):1161-1169. doi:10.1038/nn.2647
256. Bailey J, Knight A, Balcombe J. The future of teratology research is in vitro. *Biog Amin*. 2005;19(2):97-145. doi:10.1163/1569391053722755
257. OECD. *Test No. 416: Two-Generation Reproduction Toxicity*. OECD Publishing; 2001. doi:10.1787/9789264070868-en
258. Schmidt BZ, Lehmann M, Gutbier S, et al. In vitro acute and developmental neurotoxicity screening: an overview of cellular platforms and high-throughput technical possibilities. *Arch Toxicol 2016 911*. 2016;91(1):1-33. doi:10.1007/S00204-016-1805-9
259. Genschow E, Spielmann H, Scholz G, et al. The ECVAM international validation study on in vitro embryotoxicity tests: Results of the definitive phase and evaluation of prediction models. *ATLA Altern to Lab Anim*. 2002;30(2):151-176. doi:10.1177/026119290203000204
260. Luz AL, Tokar EJ. Pluripotent Stem Cells in Developmental Toxicity Testing: A Review of Methodological Advances. *Toxicol Sci*. 2018;165(1):31-39. doi:10.1093/toxsci/kfy174
261. van Dartel DAM, Piersma AH. The embryonic stem cell test combined with toxicogenomics as an alternative testing model for the assessment of developmental toxicity. *Reprod Toxicol*. 2011;32(2):235-244. doi:10.1016/j.reprotox.2011.04.008
262. Pennings JLA, Theunissen PT, Piersma AH. An optimized gene set for transcriptomics based neurodevelopmental toxicity prediction in the neural embryonic stem cell test. *Toxicology*. 2012;300(3):158-167. doi:10.1016/j.tox.2012.06.016

263. De Los Angeles A, Ferrari F, Xi R, et al. Hallmarks of pluripotency. *Nature*. 2015;525(7570):469-478. doi:10.1038/nature15515
264. Sylvester KG, Longaker MT. Stem Cells: Review and Update. *Arch Surg*. 2004;139(1):93-99. doi:10.1001/archsurg.139.1.93
265. Ma DK, Bonaguidi MA, Ming GL, Song H. Adult neural stem cells in the mammalian central nervous system. *Cell Res*. 2009;19(6):672-682. doi:10.1038/cr.2009.56
266. Zeineddine D, Hammoud AA, Mortada M, Boeuf H. The Oct4 protein: More than a magic stemness marker. *Am J Stem Cells*. 2014;3(2):74-82. Accessed January 14, 2021. www.AJSC.us/ISSN:2160-4150/AJSC0001509
267. Li M, Belmonte JCI. Ground rules of the pluripotency gene regulatory network. *Nat Rev Genet*. 2017;18(3):180-191. doi:10.1038/nrg.2016.156
268. James D, Levine AJ, Besser D, Hemmati-Brivanlou A. TGFβ/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development*. 2005;132(6):1273-1282. doi:10.1242/dev.01706
269. Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med*. 2004;10(1):55-63. doi:10.1038/nm979
270. Mossahebi-Mohammadi M, Quan M, Zhang JS, Li X. FGF Signaling Pathway: A Key Regulator of Stem Cell Pluripotency. *Front Cell Dev Biol*. 2020;8:79. doi:10.3389/fcell.2020.00079
271. Pauklin S, Vallier L. Activin/nodal signalling in stem cells. *Dev*. 2015;142(4):607-619. doi:10.1242/dev.091769
272. Sokol SY. Maintaining embryonic stem cell pluripotency with Wnt signaling. *Development*. 2011;138(20):4341-4350. doi:10.1242/dev.066209
273. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell*. 2007;131(5):861-872. doi:10.1016/j.cell.2007.11.019
274. Höpfl G, Gassmann M, Desbaillets I. Differentiating embryonic stem cells into embryoid bodies. *Methods Mol Biol*. 2004;254:79-98. doi:10.1385/1-59259-741-6:079
275. Jagtap S, Meganathan K, Gaspar J, et al. Cytosine arabinoside induces ectoderm and inhibits mesoderm expression in human embryonic stem cells during multilineage differentiation. *Br J Pharmacol*. 2011;162(8):1743-1756. doi:10.1111/j.1476-5381.2010.01197.x
276. Stummann TC, Hareng L, Bremer S. Hazard assessment of methylmercury toxicity to neuronal induction in embryogenesis using human embryonic stem cells. *Toxicology*. 2009;257(3):117-126. doi:10.1016/j.tox.2008.12.018
277. Colleoni S, Galli C, Gaspar JA, et al. Development of a neural teratogenicity test based on human embryonic stem cells: Response to retinoic acid exposure. *Toxicol Sci*. 2011;124(2):370-377. doi:10.1093/toxsci/kfr245
278. Colleoni S, Galli C, A. Gaspar J, et al. Characterisation of a Neural Teratogenicity Assay Based on Human ESCs Differentiation Following Exposure to Valproic Acid. *Curr Med Chem*. 2012;19(35):6065-6071. doi:10.2174/092986712804485926
279. Meganathan K, Jagtap S, Wagh V, et al. Identification of Thalidomide-Specific Transcriptomics and

Proteomics Signatures during Differentiation of Human Embryonic Stem Cells. *PLoS One*. 2012;7(8). doi:10.1371/journal.pone.0044228

280. Dhara SK, Stice SL. Neural differentiation of human embryonic stem cells. *J Cell Biochem*. 2008;105(3):633-640. doi:10.1002/jcb.21891
281. Tropepe V, Hitoshi S, Sirard C, Mak TW, Rossant J, Van Der Kooy D. Direct neural fate specification from embryonic stem cells: A primitive mammalian neural stem cell stage acquired through a default mechanism. *Neuron*. 2001;30(1):65-78. doi:10.1016/S0896-6273(01)00263-X
282. Perrier AL, Tabar V, Barberi T, et al. *Derivation of Midbrain Dopamine Neurons from Human Embryonic Stem Cells*.; 2004. Accessed January 19, 2021. www.pnas.orgcgidoi10.1073pnas.0404700101
283. Schulpen SHW, de Jong E, de la Fonteyne LJJ, de Klerk A, Piersma AH. Distinct gene expression responses of two anticonvulsant drugs in a novel human embryonic stem cell based neural differentiation assay protocol. *Toxicol Vitr*. 2015;29(3):449-457. doi:10.1016/j.tiv.2014.12.001
284. Ehashi T, Suzuki N, Ando S, Sumida K, Saito K. Effects of valproic acid on gene expression during human embryonic stem cell differentiation into neurons. *J Toxicol Sci*. 2014;39(3):383-390. doi:10.2131/jts.39.383
285. Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol*. 2009;27(3):275-280. doi:10.1038/nbt.1529
286. Wong KA, Trembley M, Wahab SA, Viczian AS. Efficient retina formation requires suppression of both Activin and BMP signaling pathways in pluripotent cells. *Biol Open*. 2015;4(4):573-583. doi:10.1242/bio.20149977
287. Inman GJ, Nicolás FJ, Callahan JF, et al. SB-431542 is a potent and specific inhibitor of transforming growth factor-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol*. 2002;62(1):65-74. doi:10.1124/mol.62.1.65
288. Galiakberova AA, Dashinimaev EB. Neural Stem Cells and Methods for Their Generation From Induced Pluripotent Stem Cells in vitro. *Front Cell Dev Biol*. 2020;8:815. doi:10.3389/fcell.2020.00815
289. Du ZW, Chen H, Liu H, et al. Generation and expansion of highly pure motor neuron progenitors from human pluripotent stem cells. *Nat Commun*. 2015;6(1):1-9. doi:10.1038/ncomms7626
290. Arber C, Precious S V., Cambray S, et al. Activin a directs striatal projection neuron differentiation of human pluripotent stem cells. *Dev*. 2015;142(7):1375-1386. doi:10.1242/dev.117093
291. Mahajani S, Raina A, Fokken C, Kügler S, Bähr M. Homogenous generation of dopaminergic neurons from multiple hiPSC lines by transient expression of transcription factors. *Cell Death Dis*. 2019;10(12):1-15. doi:10.1038/s41419-019-2133-9
292. Balmer N V., Weng MK, Zimmer B, et al. Epigenetic changes and disturbed neural development in a human embryonic stem cell-based model relating to the fetal valproate syndrome. *Hum Mol Genet*. 2012;21(18):4104-4114. doi:10.1093/hmg/dds239
293. Kamata S, Hashiyama R, Hana-ika H, et al. Cytotoxicity comparison of 35 developmental neurotoxicants in human induced pluripotent stem cells (iPSC), iPSC-derived neural progenitor cells, and transformed cell lines. *Toxicol Vitr*. 2020;69:104999. doi:10.1016/j.tiv.2020.104999

294. Yamada S, Yamazaki D, Kanda Y. Silver nanoparticles inhibit neural induction in human induced pluripotent stem cells. *Nanotoxicology*. 2018;12(8):836-846. doi:10.1080/17435390.2018.1481238
295. Chan HYA, Stanton LW. A pharmacogenomic profile of human neural progenitors undergoing differentiation in the presence of the traditional Chinese medicine NeuroAiD. *Pharmacogenomics J*. 2016;16(5):461-471. doi:10.1038/tpj.2016.21
296. Kugler J, Huhse B, Tralau T, Luch A. Embryonic stem cells and the next generation of developmental toxicity testing. *Expert Opin Drug Metab Toxicol*. 2017;13(8):833-841. doi:10.1080/17425255.2017.1351548
297. Elkabetz Y, Panagiotakos G, Al Shamy G, Socci ND, Tabar V, Studer L. Human ES cell-derived neural rosettes reveal a functionally distinct early neural stem cell stage. *Genes Dev*. 2008;22(2):152-165. doi:10.1101/gad.1616208
298. Koch P, Opitz T, Steinbeck JA, Ladewig J, Brüstle O. A rosette-type, self-renewing human ES cell-derived neural stem cell with potential for in vitro instruction and synaptic integration. *Proc Natl Acad Sci U S A*. 2009;106(9):3225-3230. doi:10.1073/pnas.0808387106
299. Ostermann L, Ladewig J, Müller F-J, et al. In Vitro Recapitulation of Developmental Transitions in Human Neural Stem Cells. *Stem Cells*. 2019;37(11):1429-1440. doi:10.1002/stem.3065
300. Samara A, Falck M, Spildrejorde M, et al. Robust neuronal differentiation of human embryonic stem cells for neurotoxicology. *STAR Protoc*. 2022;3(3):101533. doi:10.1016/j.xpro.2022.101533
301. Falk A, Koch P, Kesavan J, et al. Capture of neuroepithelial-like stem cells from pluripotent stem cells provides a versatile system for in vitro production of human neurons. *PLoS One*. 2012;7(1). doi:10.1371/journal.pone.0029597
302. Osete JR, Akkouh IA, de Assis DR, et al. Lithium increases mitochondrial respiration in iPSC-derived neural precursor cells from lithium responders. *Mol Psychiatry*. Published online June 1, 2021:1-17. doi:10.1038/s41380-021-01164-4
303. Löser P, Schirm J, Guhr A, Wobus AM, Kurtz A. Human embryonic stem cell lines and their use in international research. *Stem Cells*. 2010;28(2):240-246. doi:10.1002/stem.286
304. Main H, Hedenskog M, Acharya G, Hovatta O, Lanner F. Karolinska Institutet Human Embryonic Stem Cell Bank. *Stem Cell Res*. 2020;45:101810. doi:10.1016/j.scr.2020.101810
305. Sundberg M, Jansson L, Ketolainen J, et al. CD marker expression profiles of human embryonic stem cells and their neural derivatives, determined using flow-cytometric analysis, reveal a novel CD marker for exclusion of pluripotent stem cells. *Stem Cell Res*. 2009;2(2):113-124. doi:10.1016/j.scr.2008.08.001
306. Hiemke C, Bergemann N, Clement HW, et al. Consensus Guidelines for Therapeutic Drug Monitoring in Neuropsychopharmacology: Update 2017. *Pharmacopsychiatry*. 2018;51(1-2):9-62. doi:10.1055/s-0043-116492
307. Pidsley R, Zotenko E, Peters TJ, et al. Critical evaluation of the Illumina MethylationEPIC BeadChip microarray for whole-genome DNA methylation profiling. *Genome Biol*. 2016;17(1). doi:10.1186/S13059-016-1066-1
308. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet*. 2009;10(1):57. doi:10.1038/NRG2484
309. Samara A, Spildrejorde M, Sharma A, Gervin K, Lyle R, Eskeland R. A multi-omics approach to

visualize early neuronal differentiation from hESCs in 4D. *iScience*. 2022;25(11):105279. doi:10.1016/j.isci.2022.105279

310. Ström S, Holm F, Bergström R, Strömberg AM, Hovatta O. Derivation of 30 human embryonic stem cell lines—improving the quality. *In Vitro Cell Dev Biol Anim*. 2010;46(3):337. doi:10.1007/S11626-010-9308-0

311. Pelkonen O, Kaltiala EH, Larmi TKI, Kärki NT. Comparison of activities of drug-metabolizing enzymes in human fetal and adult livers. *Clin Pharmacol Ther*. 1973;14(5):840-846. doi:10.1002/CPT1973145840

312. Falk D. Evolution of brain and culture: the neurological and cognitive journey from Australopithecus to Albert Einstein. 2016;94:99-111. doi:10.4436/jass.94027

313. Olstad EW, Marie H, Nordeng E, Sandve GK, Lyle R, Gervin K. Effects of prenatal exposure to (es)citalopram and maternal depression during pregnancy on DNA methylation and child neurodevelopment. *Transl Psychiatry 2023 131*. 2023;13(1):1-11. doi:10.1038/S41398-023-02441-2