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Analysis of epigenetic changes induced by exposure to a mixture of endocrine disrupting chemicals in the mouse brain and a hippocampus mouse cell model

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Abstract

Prenatal exposure to mixture N1, a chemical mixture consisting of four phthalate diesters, three pesticides and Bisphenol A, has been associated with behavioural changes as well as changes in gene expression in mice. In this study it was investigated whether the changes in gene expression could be explained by changes in DNA methylation. Mixture N1 was found to significantly change DNA methylation in three different genes (*Nr3c1*, *Nr3c2* and *Crhr1*) on totally eight different Cytosine Guanine dinucleotides (CpG) positions. To further investigate whether these changes could be induced already during differentiation of cells, a hippocampal cell model HT22 was exposed to mixture N1 during differentiation. In this mode, mixture N1 induced a statistically significant change in the promoter region of *Nr3c1*. Unfortunately, this change could however not be validated, and the experiments would need to be repeated. In conclusion, this study showed that exposure to mixture N1 can result in changes in DNA methylation.

Introduction

With the addition of new exogenous chemicals in our society in the past century and continuing to this day due to industrial activities, we are now facing problems we have not seen before. For a long time, our hormonal systems had been evolved together with our hormones without interruption from man-made chemicals with hormone-like properties (Combarnous 2017). Today, however, about 800-1000 chemicals are known or suspected Endocrine Disrupting chemicals (Gore *et al*. 2019, Engdahl & Rüegg 2020). They can be found all around us and are interfering with our hormonal systems in different ways. And since the brain is incredibly sensitive to small change in our hormonal system, especially during early development (Streifer & Gore 2021), exposure to these types of chemicals during development can have relevant consequences.

According to the World Health Organization (WHO), Endocrine Disruptors or Endocrine Disrupting Chemicals (EDCs) are "exogenous substances or mixtures that alter function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" (Damsta *et al*. 2002).

Even though some known EDCs, like Polychlorinated biphenyls (PCBS), are banned or on their way to be replaced, they still can be found both in our environment and in our own bodies (Patisaul 2021). This is mainly due to how widespread they once were, in addition to their physical properties which makes them very environmentally persistent (Patisaul 2021).

Effects of Endocrine Disrupting Chemicals

EDCs can interfere with all aspects of the hormonal system (Combarnous 2017, Combarnous & Nguyen 2019). Their mechanisms of action could be divided into direct and indirect actions. Direct actions would be where the EDC itself binds to the receptor, resulting in either stimulation or inhibition. The indirect actions would be when the EDCs interfere with synthesis, degradation, or availability of hormones (Combarnous

2017, Combarnous & Nguyen 2019, Lombó & Herráez 2021).

Endocrine Disrupting chemicals and their effects on neurodevelopment

Exposure to EDCs during early development can lead to lifelong and sometimes even transgenerational consequences (Streifer & Gore 2021). During development it is of importance that every process occurs correctly and on time (Engdahl & Rüegg 2020, Streifer & Gore 2021). The glucocorticoids are one type of hormones that play a vital role in healthy brain development (Davis & Sandman 2010). Moderate exposure to glucocorticoids during development has a positive effect on different important functions like stress response, memory and learning. However, an unbalanced exposure can instead have large negative consequences on stress response, memory and learning (Davis & Sandman 2010). The foetal gonads start to release a wide variety of hormones into circulation at the same time as many important processes occur, for example cell birth and differentiation (Streifer & Gore 2021). The level of hormones released from the gonads differ between sexes, with a higher release of hormones from the testes than from the ovaries. Due to this the developing male brain experience higher levels of hormones than females, resulting in a difference in vulnerability for changes in hormone levels in the brain caused by EDCs (Streifer & Gore 2021). This might however not be manifested until later in life (Streifer & Gore 2021) and could also explain why studies have shown that the effects of exposure to EDCs can differ between sexes.

Sex dimorphism in exposure to EDCs

A number of studies have shown that the effects of exposure to different EDCs can differ between sexes, and that their effects are more pronounced in males (Panagiotidou *et al*. 2014, Bornehag *et al*. 2018, Tanner *et al*. 2020, Curtis *et al*. 2020, Patisaul 2021). For example, in a study by Tanner *et al*. (2020) the authors have shown a stronger association between exposure to a mixture of EDCs and lower IQ at the age of seven in boys. Likewise in the study by Bornehag *et al*. (2018) the prevalence of language delay was higher in boys than in girls that were prenatally exposed to Phthalates. Sexual dimorphic effects have also been shown in the Michigan Polybrominated Biphenyl (PBB) Registry (Curtis *et al*. 2020), where female offspring to mothers with accidental exposure to PBB have an earlier onset of menstruation as well as increased risk for miscarriage, while male offspring reach puberty later and have a higher risk of genitourinary conditions (Curtis *et al*. 2020). Similar differences have also been seen in animal studies (Panagiotidou *et al*. 2014, Patisaul 2021). For example, Panagiotidou *et al*. (2014) showed that prenatal exposure to Bisphenol A (BPA) in rats resulted in reduced zona reticularis in both sexes, while it only resulted in increased basal corticosterone as well as decreased levels of the glucocorticoids in females. These findings, together with the knowledge that our hormonal pathways are different during development, suggests that it is of importance to study sex-dependent differences in the effect of exposure to EDCs.

Epigenetics

The long term effects of exposure to EDCs has been proposed to be a result of epigenetic mechanisms (Streifer & Gore 2021). The definition of epigenetics is widely

discussed; however, one way to explain the field is as the study of heritable changes in gene function which does not alter the DNA sequence (Weinhold 2006, Allis & Jenuwein 2016). There are several known epigenetic marks including DNA nucleotide and histone modifications. Out of these, the most studied epigenetic mark is DNA methylation (Weinhold 2006, Kundakovic & Jaric 2017). Epigenetic processes play an essential role during mammalian development (Bird 2002, Engdahl & Rüegg 2020) and any changes to it during early stages of development can result in permanently altered functions (Engdahl & Rüegg 2020). The epigenome has been shown to be responsive to environmental factors. Some known or suspected factors affecting the epigenome are heavy metals, pesticides, radioactivity, tobacco smoke (Weinhold 2006) and EDCs (Streifer & Gore 2021). Furthermore, accumulating evidence suggests that DNA methylation and histone marks can be programmed by hormones during the early organisational processes. This evidence suggests that the lifelong effects of EDCs could be due to the hormonal effects on the epigenome, e.g., in our brains (Streifer & Gore 2021).

DNA methylation

DNA methylation exists within all kingdoms (Petryk *et al*. 2021). DNA methylation is usually referred to as the addition of a methyl group (CH3) to a cytosine on carbon 5 of the pyrimidine ring (Weinhold 2006, Sant & Goodrich 2019). However, 5-methylcytosine is not the only methylation modification on cytosine (Sant & Goodrich 2019) (Fig.1). Other modifications are 5-Hydroxymethylcytosine (5-hmC), 5-Formylcytosine (5-fC) and 5-Carboxylcytosine (5caC). 5-hmC is the second most studied DNA modification and has been shown to be involved in both the regulation of gene expression in the brain and the differentiation of stem cells (Sant & Goodrich 2019).

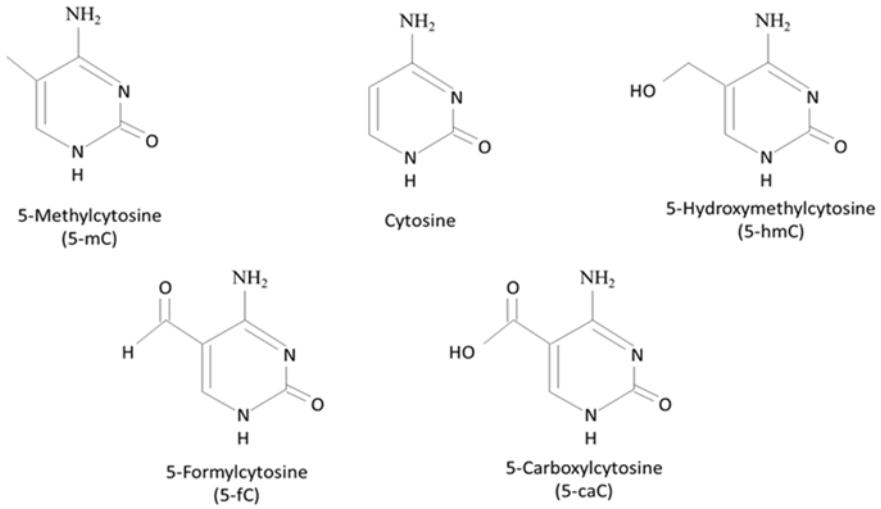

Figure 1. Structure of the different modifications of Cytosine compared to the structure of Cytosine.

In mammals DNA methylation is a crucial process, which plays an important role both in embryonic development as well as in cellular function (Petryk *et al*. 2021). In mammals DNA methylation is most commonly found at Cytosine guanine dinucleotides or so called CpGs (Sant & Goodrich 2019, Petryk *et al*. 2021). DNA methylation is often found in CpG-rich regions, so called CpG islands (CGIs) (Jones 2012, Sant &

Goodrich 2019). CpG islands are often located in the promoter regions of genes (Jones 2012, Sant & Goodrich 2019). Even though DNA methylation is most common in CGIs, most of them are non-methylated (Deaton & Bird 2011, Petryk *et al*. 2021). The maintenance of DNA methylation occurs both during and after DNA replication and is important for sustaining the marks (Petryk *et al*. 2021). To maintain methylation, it is necessary that the DNA methylation becomes symmetrical on each copy of the genome before the next S-phase, otherwise the methylation mark will disappear (Petryk *et al*. 2021).

When a CGI in a promoter region is methylated, transcription of the gene is usually silenced (Deaton & Bird 2011, Anastasiadi *et al*. 2018). Yet, it has also been shown that the methylation level differs between different exons in a gene, with the methylation of the first exon being suggested as a better indicator of gene expression than the methylation of the promoter (Anastasiadi *et al*. 2018). However, when it comes to the methylation of the gene body it has been shown that methylation of the gene body is positively correlated with gene expression (Anastasiadi *et al*. 2018, Petryk *et al*. 2021).

Evidence for EDCs effects on DNA methylation

In numerous studies EDCs have been shown to affect epigenetic marks such as DNA methylation (Alworth *et al*. 2002, Bromer *et al*. 2010, Kitraki *et al*. 2015, Tian *et al*. 2016). For example, in a study by Kitraki *et al*. (2015) the authors found increased DNA methylation of the gene *Fkbp5* in the hippocampus of male rats perinatal exposed to BPA. Alworth *et al*. (2002) showed an increase in ribosomal DNA methylation in uterine samples from mice that were prenatally exposed to Diethylstilbestrol (DES). Similar findings have also been found in human cohorts. For example, in a study by Tian *et al*. (2016) the authors showed an association between exposure to benzo[a]pyrene and hypermethylation of the Glutathione-S-transferase Pi (*GSTP*) promoter region in hepatocellular carcinoma samples (Tian *et al*. 2016). In the study by Kundakovic *et al*. (2015) the authors saw an association between high BPA levels and alterations in DNA methylation at two CpG sites in human cord blood (Kundakovic *et al*. 2015). Further, in a study by Engdahl *et al*. (2021) the authors could show a positive association between prenatal Bisphenol F (BPF) exposure and DNA methylation at Glutamate Ionotropic Receptor NMDA Type Subunit 2B (*GRIN2B*) (Engdahl *et al*. 2021).

EDC-MixRisk

One of the large problems with EDCs is that they differ from other toxicants in several aspects, making it difficult to correctly assess their hazard and risk before they are released onto the market (Engdahl & Rüegg 2020). Firstly, EDCs often show non-monotonic dose responses meaning that a higher dose does not necessarily correspond to a higher effect (Combarnous 2017, Combarnous & Nguyen 2019) something which goes against the original idea that “the dose make the poison” (Patisaul 2021). Secondly, the effects of EDCs can be dependent on critical windows meaning that they can be more severe during certain periods of development (Engdahl & Rüegg 2020, Patisaul 2021). Thirdly, identification of EDCs is challenging since their effects can be seen later in life or even in later generations (Engdahl & Rüegg 2020, Patisaul 2021).

As today's methods used in chemical hazard assessment are not designed to pick up these specific features, the effects of EDCs risk are being missed during chemical risk assessments (Engdahl & Rüegg 2020). Additionally, research and risk assessment of chemicals are usually performed on one chemical at a time. However, exposure to chemicals is usually in the form of mixtures. Assessing the risks of chemicals one by one can hence create a peril that synergetic, additive, or opposing effects are missed and studying the exposure of one chemical at the time might therefore lead to a significant underestimation of their health risk (Repouskou *et al*. 2020, Patisaul 2021).

This difficulty in assessing the hazard and risk of EDCs in mixtures was addressed by the project EDC-MixRisk. This project was funded by the European Union as part of the union's Horizon 2020 research and innovation programme. The aim was to gain an understanding of the health effects of EDCs in mixtures, to be able to improve the risk management of these harmful chemicals.

In the EDC-MixRisk project critical EDC mixtures were identified using an epidemiological pregnancy cohort study, the Swedish Environmental Longitudinal, Mother and child, Asthma, and allergy (SELMA) study, in which prenatal EDC exposure was associated with different health outcomes regarding growth and metabolism, neurodevelopment and sexual development (Bergman *et al*. 2019).

Mixture N1

The mixture N1, which was used in this study, was identified based on 45 chemicals measured in urine and serum of pregnant SELMA mothers (Bornehag *et al*. 2012), and their association to language delay assessed when their children were 2.5 years old. It consists of four phthalate diesters (Di-ethyl phthalate, Diisodecylphthalate/ Di(2-propylheptyl) phthalate, Di-butyl Phthalate and Benzyl butyl phthalate), three pesticides (Dichloro diphenyl dichloro ethylene, Trichloropyridinol, 3-Phenoxybenzoic acid) and Bisphenol A (Repouskou *et al*. 2020). Phthalate diesters can be classified into high-molecular weight and low-molecular weight phthalates (Engdahl & Rüegg 2020). The high-molecular weight phthalates are commonly found as plasticizers in PVC plastics which can be found in for example toys, floors, and walls. The low-molecular weight phthalates are commonly found in cosmetic products, like deodorants, shampoos, and perfume. Further, Bisphenol A is commonly found in both polycarbonate plastics where it is used as a hardener as well as in epoxy resins used for coating of metal products and is a well-documented EDC (Engdahl & Rüegg 2020). As a part of the EDC-MixRisk project, Repouskou *et al*. (2020) performed a study on the mixture N1 in mice and identified long term transcriptional and behavioural effects.

Experimental findings with mixture N1

Repouskou *et al*. (2020) studied the long-term impacts of exposure to mixture N1 during development by prenatally exposing mice to mixture N1. The effects of exposure to mixture N1 on the progeny was investigated, both by examining behaviour and by analysing the gene expression of genes linked to stress response, anxiety, sociability, and learning (table 1). When the offspring reached adulthood, one to three mice per sex and litter from each exposure group were selected to undergo behavioural tests. The rest of the mice remained undisturbed and were referred to as basal animals. The behavioural tests that were performed were the elevated plus maze, open field, social

interaction, novel object location and, forced swimming stress test (Repouskou *et al*. 2020). The behavioural tests showed that exposure to mixture N1 increased active coping during swimming stress in both sexes, as well as increased locomotion and reduced social interaction in male offspring (Repouskou *et al*. 2020). Thirty minutes after the forced swimming stress test, the mice were sacrificed, and RNA was extracted for gene expression analysis (Repouskou *et al*. 2020).

The authors studied the gene expression of Corticotropin-releasing hormone receptor 1 (*Crhr1*), 5-hydroxytryptamine receptor 1A (*Hrt1a*), Nuclear receptor Subfamily 3 group C Member 1 (*Nr3c1*), Nuclear receptor Subfamily 3 group C Member 2 (*Nr3c2*), Estrogen receptor 2 (*Esr2*), Oxytocin receptor (*Oxtr*), Oxytocin (*Oxt*) and Corticotropin Releasing Hormone (*Crh*) in the hippocampus and found a statistically significant change in gene expression in males exposed to mixture N1 compared to the control group for *Nr3c1, Nr3c2, Crhr1* and *Hrt1a* (Table 2). The hippocampus is a part of the limbic system which is associated with memory function, stress response as well as spatial processing and navigation.

AIM

The aim of this study was to investigate

1. if the long term transcription effects found in prenatally exposed mice could be due to DNA methylation.
2. if exposure to mixture N1 could induce epigenetic changes during neuronal differentiation *i.e.*, during early development.

DNA methylation was analysed from the hippocampus of the mice from the study in the article by Repouskou *et al*. 2020 as well as from a hippocampal mouse cell model, HT22, exposed to mixture N1 during differentiation.

Table 1. Target genes and their respective functions. Entire table is based on information from Genecards - The Human Gene Database (genecards.org).

Gene name	Full name	Encode	Function
Crh	Corticotropin Releasing Hormone	The preproprotein Corticotropin Releasing Hormone.	A preproprotein which is proteolytically processed to generate the mature neuropeptide protein.
Crhr1	Corticotropin-releasing hormone receptor 1	A G-protein coupled receptor which binds the Corticotropin-releasing hormone family.	Major regulator of the hypothalamic-pituitary adrenal pathway. Regulates physiological processes like stress, reproduction, immune response, and obesity.
Esr2	Estrogen receptor 2	A nuclear hormone receptor.	Binds estrogens and activates expression of reporter genes containing estrogen response elements.
Fkbp5	FKBP Prolyl Isomerase 5	A protein within the family of immunophilin proteins	Encodes for a protein which is important for immunoregulation.
Htr1a	5-hydroxytryptamine receptor 1A	A G-protein coupled receptor for serotonin.	Is part of the regulation of release of 5-hydroxytryptamine as well as regulation of dopamine and 5-hydroxytryptamine metabolism.
Nr3c1	Nuclear receptor Subfamily 3 group C Member 1	A glucocorticoid receptor.	Binds to glucocorticoid response elements (GRE) and act as a transcription factor to glucocorticoid responsive genes.
Nr3c2	Nuclear receptor Subfamily 3 group C Member 2	A mineralocorticoid receptor.	Binds to mineralocorticoid response elements (MRE) and transactivate target genes.
Oxt	Oxytocin	A precursor protein that is processed to produce oxytocin and neurophysin I.	Oxytocin is a hormone and a neurotransmitter.
Oxtr	Oxytocin receptor	A G-protein coupled receptor for oxytocin.	Binds to oxytocin.

Table 2. Statistically significant expression changes upon Mixture N1 exposure for the genes tested by Repouskou *et al*. (2020). b stands for significance in basal tests and t stands for statistical significance in behaviourally tested animals.

Gene	*Male*					Female				
	0x	*0.5x*	*10x*	*100x*	*500x*	0x	0.5x	10x	100x	500x
Crh										
Crhr1				***b/t***	***b/t***					
Esr2										
Fkbp5										
Htr1a		***b***	***b***	***b/t***	***b/t***					
Nr3c1			***b/t***	***b/t***						
Nr3c2			***b***	***b***	***b***					
Oxt										
Oxtr										

Methods

Mouse DNA – Hippocampus

DNA was extracted from the hippocampus and sent to the Epitox group, Uppsala university from National and Kapodistrian University of Athens (NKUA), Athens, Greece. DNA was extracted from C57/BL6 mice that had been prenatally exposed to five different doses of the mixture N1. The doses of mixture N1 used were 0x, 0.5x, 10x, 100x and 500x of the geometric mean of the concentration found in the pregnant mothers in the mother-child cohort in the Swedish SELMA study, corresponding to a daily dose of 0, 0.001, 0.22, 2.2 or 11 mg/kg of the mixture. Before sacrifice, several behaviour tests were performed. For more details see the study by Repouskou *et al*. (2020).

Bisulfite conversion

Upon arrival, the DNA was bisulfite converted at Uppsala University using the commercial kit EZ DNA methylation-gold kit (D5600) (Zymo). Low methylated (0%) and High methylated (100%) DNA from EpigenDx was also bisulfite converted according to the protocol. Thereafter different standards were prepared by mixing 0% and 100% methylated DNA in the respective proportions to generate three different standard curves: (1)0%, 5%, 10%, 15% and 20%, (2) 0%, 10%, 20%, 30% and 40% or (3): 0%, 25%, 50%, 75% and 100% methylation. Standards for the validation of the bisulfite pyrosequencing assay were used for each gene depending on the methylation levels of the test samples.

Assay design

Assays were designed at the Epitox group, Uppsala University, to cover the regions of interest in each target gene. The UCSC genome browser (genome.ucsc.edu) was used for annotations of regions of interest. The regions were selected in order to analyse CpG islands, Polycomb group proteins-binding sites, and/or histone-binding sites.

Primer optimisation

Prior to the analysis of the samples, 60 primer pairs were tested. First, the unmodified reverse and forward primers for each assay were optimised by testing three different annealing temperatures (54°C, 56°C and 58°C) and two different concentrations of MgCl2 (1.5mM and 2.5mM). In case of absence of amplification, two additional annealing temperatures (55°C and 57°C) were tested.

For the optimisation, random mouse DNA was used to verify amplification, check for the correct size of amplicons and optimal conditions of each primer pair. A SimpliAmp Thermal Cycler (Thermo Fisher) was used with the advanced setting "Veriflex" to obtain the three different temperatures in the three blocks of the instrument.

Since one primer in each assay needs to be biotinylated, one biotinylated primer was ordered for each assay with nonspecific products and strong bands of the correct size.

Establishment of CpG assays

A mixture of mouse DNA originated from mice exposed to different doses of mixture N1 was amplified with the established primer pairs and amplicons were checked by gel electrophoresis (1.5% agarose). Thereafter CpG assays for each sequence were made in the PyroMark Q24 Software v.2.0.8 (Qiagen). Then the amplified PCR product was sequenced using the PyroMark Q24 (Qiagen) according to the manufacturer's protocol. The assay results were analysed with the PyroMark Q24 Software v.2.0.8 (Qiagen).

If the first test was successful, the assays were tested using DNA standards (EpigenDX) with known percentage methylation. For each gene of interest at least one CpG assay was established. For *Nr3c2*, three assays were established, one for each CGI and for *Oxt* two assays were established. The results were analysed, and standard curves were made using Excel to verify the linearity by linear regression. Assays showing a R2 >0.90 were accepted as linear and used for analysis of samples.

DNA methylation analysis of the hippocampus samples

DNA methylation at regulatory regions of genes coding for *Crhr1*, Hrt1a, *Nr3c1*, *Nr3c2*, *Esr2*, *Oxtr*, *Oxt* and *Crh* was analysed.

For each gene, one 96 well plate with a total of 76 samples was prepared, 64 of which were bisulfite converted DNA, four were negative controls and eight replicates of control samples. The control samples, called CVs, were made by adding the same DNA mixture of unrelated samples to test for variability between runs. The negative controls were made by adding 1 µl of RNase free water to the master mix instead of mouse DNA. The same master mix was used for all the samples.

Amplification was initiated with an initial temperature of 95°C for 15 minutes, thereafter 45 cycles of denaturation at 94°C for 30 seconds, primer-specific annealing temperature (54°C for *Crh* and *Nr3c2* CGI2, 56°C for *Nr3c2* CGI1 and CGI6, *Esr2*, *Crhr1*, *Htr1a*,*Oxtr*, *Nr3c1* promoter and *Fkbp5*, 57°C for *Oxt* s2 and 58°C for *Oxt* s5) for 30 s, extension at 72°C for 30 seconds and lastly a final extension at 72°C for 10 minutes. Amplification and potential contamination were then checked using gel electrophoresis. Lastly, each 96 well plate was split into four PyroMark Q24 plates and analysed according to the protocol using the PyroMark Q24 (Qiagen) with the cartridge

method 0015. The layout of each plate can be seen in Fig. 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	8	16		24	32		40	48		56	64	
B	7	15		23	31		39	47		55	63	
C	6	14		22	30		38	46		54	62	
D	5	13		21	29		37	45		53	61	
E	4	12		20	28		36	44		52	60	
F	3	11	C water	19	27	C water	35	43	C water	51	59	C water
G	2	10	CV	18	26	CV	34	42	CV	50	58	CV
H	1	9	CV	17	25	CV	33	41	CV	49	57	CV

Figure 2. Layout of the 96 well plates used for each gene. The layout was chosen so that it could easily be transferable to a 24-well plate for pyrosequencing. Cell culture and treatments

Expanding the cells

The cell line HT22, which was derived from male mouse hippocampal cells, were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal bovine serum (FBS). The conditions used were 37°C and 5% CO2. A vial of passage 3 HT22 cells were retrieved from the liquid Nitrogen tank. The vial was thawed in a 37°C water bath for 1 minute and initially plated on a 60 mm plate. From passage 4 and on, the cells were instead plated on 100 mm plates with a seeding density of 2.2 x 106 cells. When the cells reached 70% confluency they were split according to the protocol (Appendix, see Protocol).

Since the cells were fast growing, a part of them were frozen down already when they reached confluency at passage 5, to be used later for the exposure experiments (Appendix, see Protocol). Cells from passage 6 and passage 8 were prepared for extraction of RNA and DNA.

Exposure of cells during differentiation

A vial of the cell line HT22, passage 5, was retrieved from the liquid nitrogen before the start of the experiments. The vials were thawed in a 37°C bead bath for 2 minutes. The cells were thereafter plated on a 100 mm plate and went through a number of passages before being passed into 6-well plates. Each biological replicate went through one more passage than the biological replicate before. The cells were cultured in phenol-free Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) (Sigma), 0.5 ml Sodium Pyruvate and 1 ml of L-Glutamine under standard conditions (37°C and 5% CO2).

After a few passages, the differentiation protocol was initiated by seeding the cells into the six well plates (0.5 x 105 cells/well) in phenol red-free DMEM containing 5% charcoal FBS, 0.5 ml Sodium Pyruvate and 0.5 ml L-Glutamine (Fig. 3).

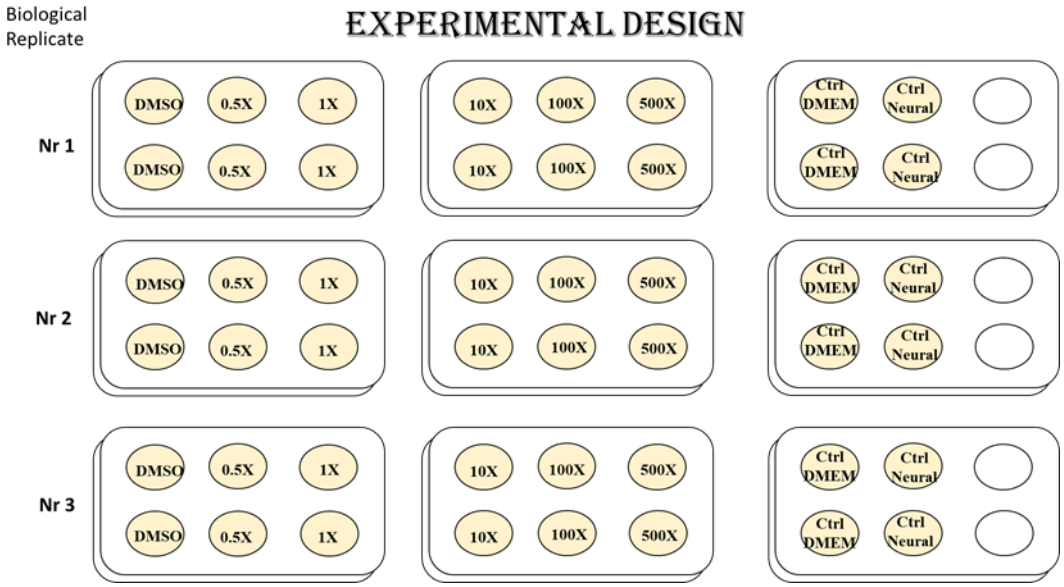

Figure 3. Experimental set up of the Mixture N1 exposure to HT22 cells.

After 24 hours the medium was changed to phenol-free Neurobasal medium supplemented with 1X N-2 supplement (Gibco), 5% charcoal-treated FBS (Gibco), 0.5 ml L-Glutamine, 0.5 ml Sodium Pyruvate and 100 uM dibutyrylcAMP. 4 ul of 0.5X, 1X, 10X, 100X or 500X Mixture N1 was also added to 4 ml of medium for each exposure. 4 ul of DMSO was added to the medium for the DMSO control. For the Neurobasal control, Neurobasal medium with its supplements were used, and DMEM with 10% FBS was used as a control for spontaneous differentiation.

After 48 hours of exposure to mixture N1 the cell medium was removed, and each well was washed twice with PBS before 350 µl of RLT plus buffer (Qiagen) from the All Prep Mini Kit (Qiagen) was added. The cell-buffer solution was then transferred to 1.5 ml Eppendorf tubes and thereafter put in -20°C until extraction. DNA and RNA was extracted with the All Prep Mini Kit (Qiagen) according to the protocol.

Efficiency tests of quantitative polymerase chain reaction assays

The quantitative polymerase chain reaction (qPCR) primers for the reference genes had already been established at the Epitox Group at Uppsala University. qPCR primers for analysis of *Nr3c1*, *Nr3c2* and *Crhr1* was derived from the Harvard Primer Bank(https://pga.mgh.harvard.edu/primerbank/). Before analysis of gene expression on the exposed cells, all qPCR primers were tested on cDNA converted from passage 6 and passage 8 HT22 RNA. Efficiency tests were performed for each assay by making technical triplicates of five point 1:2 serial dilutions of the converted cDNA starting on a concentration of 5 ng/ul. The cDNA from the serial dilution was mixed with 0.3uM primers and SsoAdvanced Universal SYBR® Green Supermix (Biorad) in a total volume of 10 µl according to manufacturer's protocol. All primers were tested with the same amplification protocol, which included a 2 minute incubation at 95°C, followed by 39 cycles of 96°C for 5 seconds (s) and 60°C for 30 seconds, and lastly a ramp from 65°C to 95°C with a ramp rate at

0.5°C/5 s. Assays with an efficiency between 90 and 110 were considered efficient.

Gene expression analysis

RNA extracted from the cells were converted to 10 ng/µl of cDNA with the Iscript ™ cDNA synthesis kit 100 reactions (Biorad). Gene expression was analysed for the DMSO control and 0.5X, 1X, 10X, 100X and 500X of mixture N1 of the first two biological replicates. The third biological replicate was excluded due to issues with reagents. Each sample was prepared by mixing 5 ng of cDNA with 0.3uM primers and SsoAdvanced Universal SYBR® Green Supermix (Biorad) in a total volume of 10 µl according to manufacturer's protocol and analysed in triplicates for gene *Nr3c1*, Rp1p and in duplicates for gene Tbp. The quantitative amplification was measured with the same amplification protocol as described above. A relative gene expression was calculated using the 2-ΔΔCT method.

Statistical analysis

Since the sample size for the mouse dataset was small, the non-parametric Kruskal Wallis test was considered more suitable than a parametric test. The test was performed in R v.4.0.5 (R Core Team 2021) and used to identify statistically significant changes in methylation between doses at each CpG position for male and female mice respectively. In case of statistical significance ($p<0.05$), the non-parametric Dunn's test with Bonferroni p-value adjustment was used to identify specific experimental groups within the dataset, responsible for the statistical significance.

For analysis of the interaction between sex and doses the non-parametric Friedman's two-way ANOVA was used. This type of test is hypothesis driven and needs a null hypothesis and an alternative hypothesis:

Null hypothesis: There are no differences in doses between male and female.

Alternative hypothesis: There is a difference between at least one dose and DMSO between male and females.

Several packages exist for performing Friedman's test in R. However, all these packages require that the data is already ranked. Therefore, the data were ranked beforehand. The package "stats" and the function "friedman.test()" were chosen to perform the test.

Table 3. Shows the system of the ranking. The lowest value received the lowest rank.

	DMSO	0.5X	10X	100X	500X
Male	98	93,5	93	97	93
RANK	4	2	1	3	1
Female	95	93,5	92,5	95	95
RANK	3	2	1	3	3

To rank the data, the median of percentage methylation was calculated at each dose in males and females (table 3). Each dose was then ranked from lowest to highest median value, with the lowest median getting the lowest rank. Subsequently, a matrix consisting of the ranking of the male and female doses was made in excel and saved as a csv file. The file was then loaded into R v.4.0.5 (R Core Team 2021) using "read.csv2(file.choose())" and thereafter converted into a matrix with the function "data.matrix()". Thereafter the function "friedman.test()" was used. If two doses had the same median value, they were given the same rank.

Both the methylation data and the gene expression data for HT22 cells were analysed in R v.4.0.5 (R Core Team 2021) with one-way ANOVA.

Table 4. Information about the final assays, including primer information, amplicon length (bp), chromosomal localisation, gene region, number of CpGs analysed and condition used. Results

Gene	Primer	Sequence	Amplicon length (bp)	Chromosomal localisation (bp) **	Gene region	No. Of CpGs	Condition
Nr3c1 Promoter	Forward Reverse Sequencing	GGGAGAAGTTGTAAAGTAGAATT AAATACAAAAAAACCCAACTC GAGTTTGAGAGGAGG	242	chr18:39,490,688–39,490,930	Promoter	7	56°C with $MgCl_2$
Nr3c2 CGI 1	Forward Reverse Sequencing	GTTAGTAGGGAGATTGTTAGGATTGTTA TACTACCCACAACCCACCTCCTTAA TGTAGATTAGTAGGTATTGGATTT	288	chr8:76,900,175–76,900,363	Exon 1 and intron 1	3	56°C with $MgCl_2$
Nr3c2 CGI 2	Forward Reverse Sequencing	GGGGTTATTTTAGGGAATTAGGAGAA CCCCCTAAAACCATTTCCATTAACCA GGAAGTTGAGGAGTTTAGG	205	chr8:76,902,028–76,902,137	Intron 1	3	54°C with $MgCl_2$
Nr3c2 CGI 3	Forward Reverse Sequencing	AAGTGGGTTAAGGTATTTTTAGGTA ATCTTCCATACTCATACCAAAATACTATAC AGGTATTTTTAGGTAGGAT	107	chr8:77,186,122–77,186,164	Intron 5 or Intron 4	4	56°C with $MgCl_2$
Htr1a	Forward Reverse Sequencing	GTTTTGGATGTGTTGTGTTGTA TCCCTTCTTTTCCACCTTCT GTATTTAGTTTATTTTGTATTTGTG	372	chr13:105444637–105445143	Exon 1	3	56°C with $MgCl_2$
Esr2	Forward Reverse Sequencing	TTTATTTTGGTAGGGGTAGGTT AATATAAAAATAAACATTAACTCCAATCC GTTGTTATTATTATA	261	chr12:76,152,555–76,152,587	Intron 4	4	56°C with $MgCl_2$
Crhr1	Forward Reverse Sequencing	AGAATGGGTGATGGTGAAAT ACCATCCCCTCTCAAAAATACTAAAT GTAGAAAGTTTTAGA	291	chr11:104,133,345–104,133,554	Intron 1	6	56°C with $MgCl_2$
Crh	Forward Reverse Sequencing	TGGAAGGTGAGATTTAGAGAGAT TACTCCACTATCCCCCAACTCCAC GTATTTGTAGTTGTTGTAGTAA	246	chr3:19,694,033–19,694,513	Exon 2	2	54 °C without $MgCl_2$
Oxt s2	Forward Reverse Sequencing	GTTTTGGGAAGTGTTAGGTTTT CTCCCCCAAAACTTCTAACCAAACTAAC GGATTAGGATTGTGT	292	chr2:130,576,489–130,576,970	Intron 1	3	57 °C with $MgCl_2$
Oxt s5	Forward Reverse Sequencing	TGTTTTAGTTTTGTTAGTTTGGTTAGAAGT ACAACCATCTACAAAAAAAAATAAATCAAT GGGTGATTAGGTAAGGAG	173	chr2:130,576,489–130,576,970	Missing info	2	58 °C with $MgCl_2$
Oxtr	Forward Reverse Sequencing	AGAGTTAGGAATAGTATGAGATATAGGA CCCCACCTACCACACTTTAAAAAACCT CTCAAAACCCAATACTCCCT	235	chr6:112,489,025–112,489,883	Exon 1	7	56°C without $MgCl_2$
Fkbp5	Forward Reverse Sequencing	GTTGTGTATATAGGGAGGTTAGAG AACACCCCCCTCACCTACC AGTATTTTAGAGATTTTT	236	chr17:28,485,510–28,485,520	Intron 1	3	56 °C with $MgCl_2$

Results

Primer optimisation for pyrosequencing

Twelve assays out of approximately 60 assays tested during optimisation were successfully optimised with an R2 value over 0.9. The final assays consisted of three assays for *Nr3c2*, two for *Oxt*, one for *Htr1a*, *Esr2*, *Crh*, *Crhr1*, *Oxtr*, *Nr3c1* and *Fkbp5*. Information about the final assays can be seen in table 4.

Analysis of methylation changes in mice

To analyse epigenetic changes, 64 samples of DNA which had been extracted from mice that had been prenatally exposed to different doses of mixture N1 were analysed with PyroMark Q24 (Qiagen). Two samples in each PyroMark Q24 plate were identical and worked as a quality validation. Of all the genes that were analysed, a statistically

significant difference was found in *Nr3c2* CGI 1 and CGI 3, *Nr3c1* promoter region and in *Crhr1* (table 5). DNA methylation analysis of *Nr3c2* showed that exposure to mixture N1 resulted in hypomethylation on several positions. In CGI 1 (Fig. 4A), a statistically significant hypomethylation was seen in position 2 between DMSO and 500X (p-value:0.03, Dunn's test with Bonferroni correction). In CGI 3 (Fig. 4B), a statistically significant hypomethylation was seen at position 3 between DMSO and 500X (p-value:0.04, Dunn's test with Bonferroni p-value adjustment). Interestingly, in CGI 1, the methylation was already low, and was decreased further by the exposure to mixture N1. However, in CGI 3, methylation was high, but decreased after exposure. Further, no significant change in methylation could be seen in CGI 2 (see Appendix, DNA methylation mice). Hypomethylation of the gene was also seen on several positions in the promoter region of *Nr3c1* (Fig. 5A). Here, significant changes in methylation were seen between DMSO and 100X at position 1 (p-value: 0.03, Dunn's test with Bonferroni p-value adjustment), position 3 (p-value :0.003, Dunn's test with Bonferroni p-value adjustment) and position 4 (p-value :0.01, Dunn's test with Bonferroni correction), as well as between DMSO and 500X at position 1 (p-value: 0.006, Dunn's test with Bonferroni p-value adjustment), position 2 (p-value: 0.04, Dunn's test with Bonferroni p-value adjustment), position 3 (p-value: 0.003, Dunn's test with Bonferroni p-value adjustment), position 4(p-value: 0.001, Dunn's test with Bonferroni correction), position 5 (p-value: 0.0009, Dunn's test with Bonferroni correction) and position 7 (p-value: 0.001 Dunn's test with Bonferroni correction). Similarly, hypomethylation (p-value:0.005, Dunn's test with Bonferroni p-value adjustment) between DMSO and 500X was also seen in the *Crhr1* gene at position 1 in males (Fig. 5B). No further statistically significant changes could be seen in the other genes: *Esr2*, *Htr1a*, *Oxtr* and *Oxt* (see Appendix, DNA methylation).

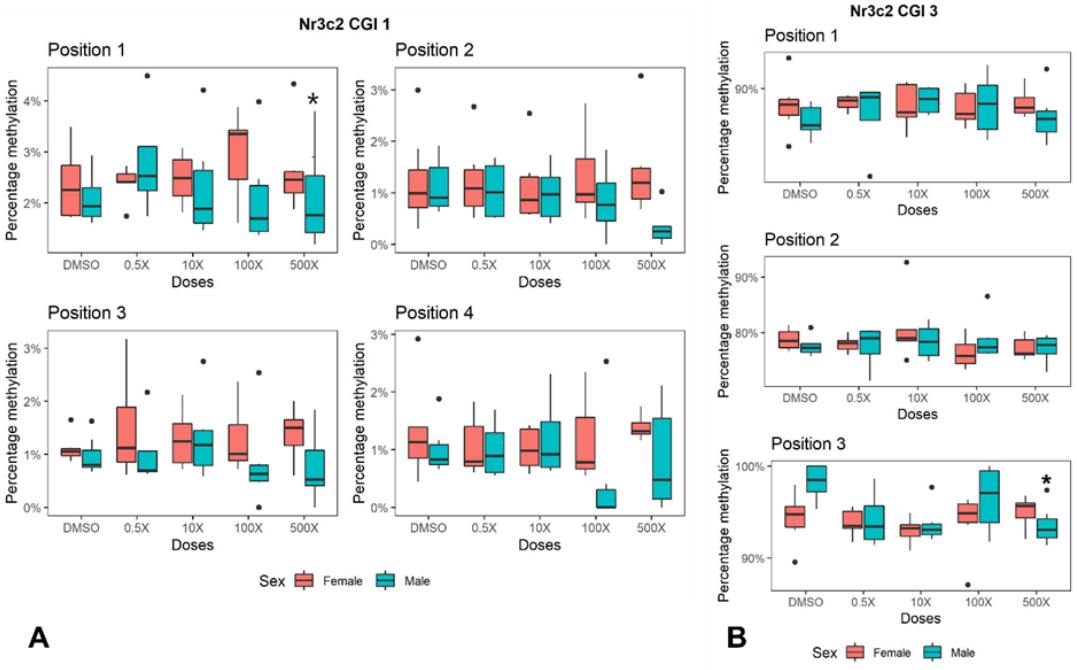

Figure. 4 A DNA methylation at each CpG position of *Nr3c2* CGI 1 at different doses of exposure. Male data is shown in blue and female in pink. Statistically significant results with a p-value <0.05 is shown with *. B DNA

methylation at the different doses of exposure at each CpG position of *Nr3c2* CGI 3. Male data is shown in blue and female in pink. Statistically significant results with a p-value <0.05 is shown with *.

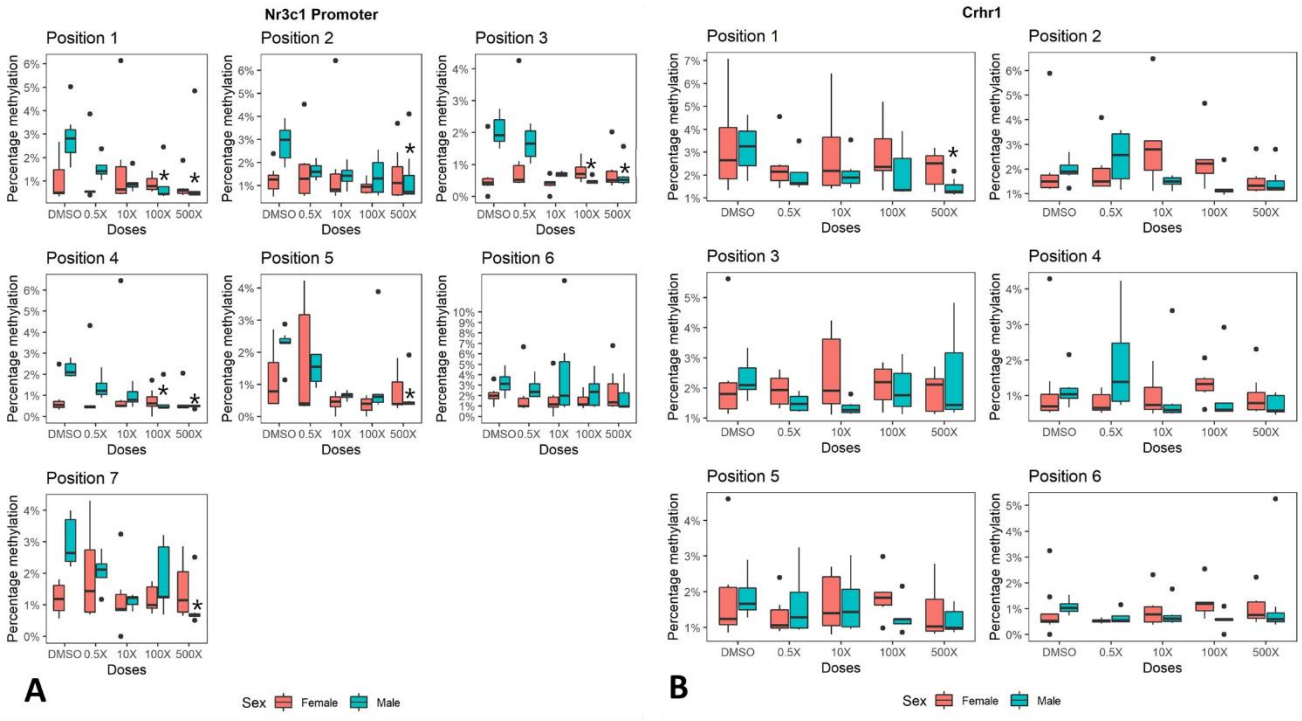

Figure. 5 A DNA methylation at each CpG position of *Nr3c1* Promoter region at different doses of exposure. Male data is shown in blue and female in pink. Statistically significant results with a p-value <0.05 is shown with *. B DNA methylation at each CpG position of *Crhr1* at different doses of exposure. Male data is shown in blue and female in pink. Statistically significant results with a p-value <0.05 is shown with *

Table 5. The table shows the statistically significant changes in DNA methylation at each position in the target genes and which p-value that were found with Dunn's test with Bonferroni correction.

Assay	Dose	CpG position	Significant p-values
Crhr1	500X	1	0.005
Nr3c1	100X	1	0.03
Nr3c1	500X	1	0.006
Nr3c1	500X	2	0.04
Nr3c1	100X	3	0.003
Nr3c1	500X	3	0.003
Nr3c1	100X	4	0.01
Nr3c1	500X	4	0.001
Nr3c1	500X	5	0.0009
Nr3c1	500X	7	0.001
Nr3c2 CGI 1	500X	2	0.03
Nr3c2 CGI 3	500X	3	0.04

Primer optimisation for qPCR

After the DNA and RNA had been extracted, the expression of the target genes in our cell model was checked both before and after differentiation. Unfortunately, HT22 cells only expressed *Nr3c1*. Therefore, only *Nr3c1* was analysed with qPCR in the cell model.

Morphological effects of Mixture N1 in the HT22 cell model

After both 24 and 48 hours (Fig. 6) of exposure to mixture N1, morphology of the cells was checked. The DMEM control showed that the cells reached 100% confluency in the 6-well plate within 48 hours and that no differentiation happened in the DMEM medium. The neurobasal control showed that the medium itself resulted in differentiation of the cells within 48 hours. More cell death appeared in the Neurobasal medium than in the DMEM medium. In figure 6 this cell death is seen as white cell clumps. Cell death could be seen in all wells with neurobasal medium, and it was increased in the medium with mixture N1 compared to the wells for the neurobasal control and the DMSO control. Further, more cell death was seen in the lower doses of mixture N1. Due to problems with the reagents used in the third biological replicate, it was excluded from the study.

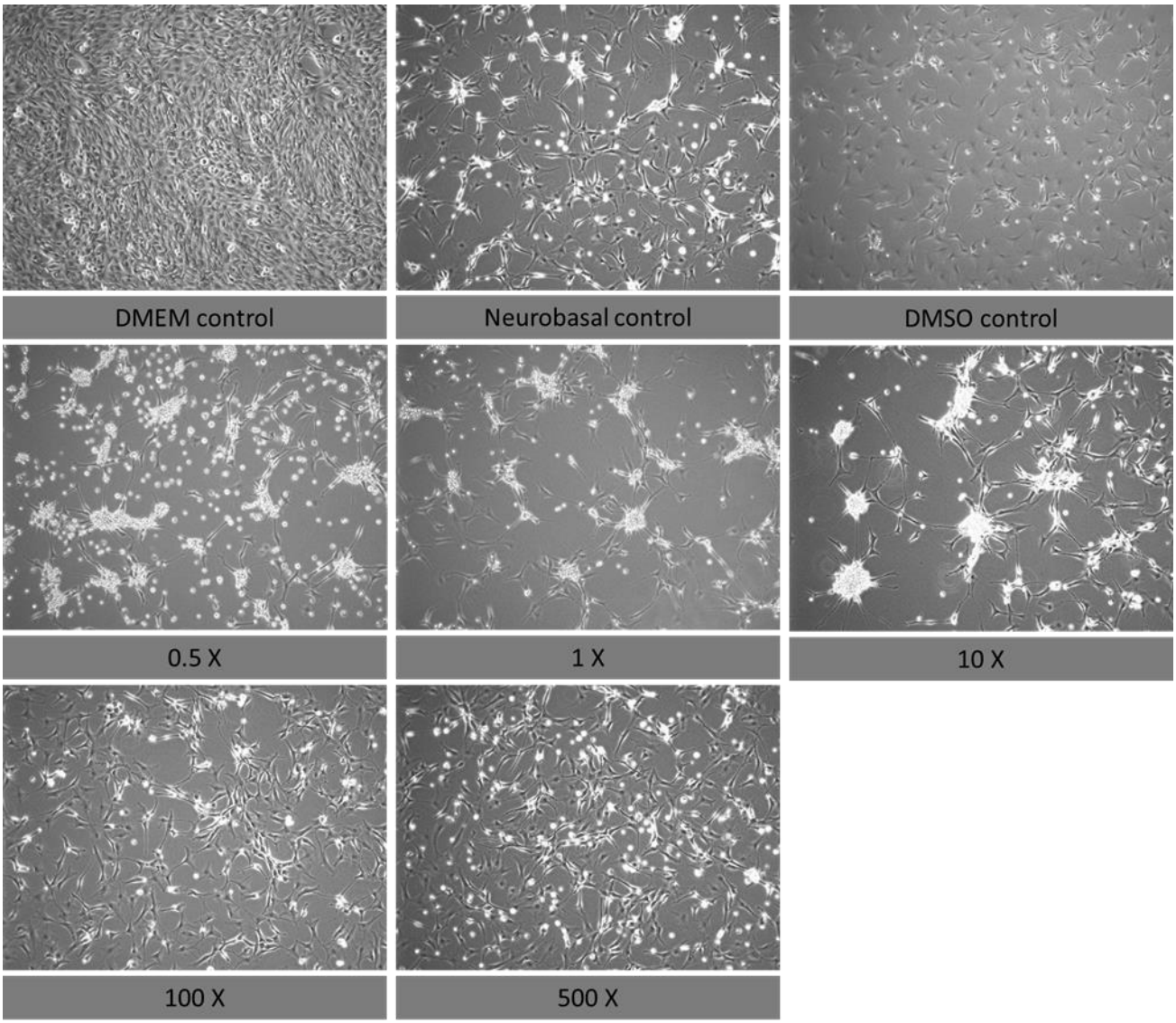

Figure 6. Representative images of the morphology of the HT22 cells in respective exposure.

DNA methylation analysis in HT22

Even though only *Nr3c1* was expressed in the cell model, all target genes (*Nr3c1*, *Nr3c2* and *Crhr1*) were analysed with pyrosequencing.

Only one assay, *Nr3c1* promoter region, showed statistically significant changes. Compared to the hypomethylation seen in the mouse DNA, the changes found in the promoter region of the HT22 cells were hypermethylation at position 1 between DMSO and 10X (Fig.7). However, the results could not be validated with the CVs since one of them had a high deviation compared to the others. The rest of the data can be seen in Appendix – DNA methylation in HT22 cells.

Gene expression analysis of the exposed HT22 cells

Gene expression analysis of *Nr3c1* was normalised to the two house-keeping genes Tbp and Rp1p previously established in the Epitox group. No significant changes in gene expression between the different doses of mixture N1 was seen. The results from the gene expression analysis can be seen in figure 8.

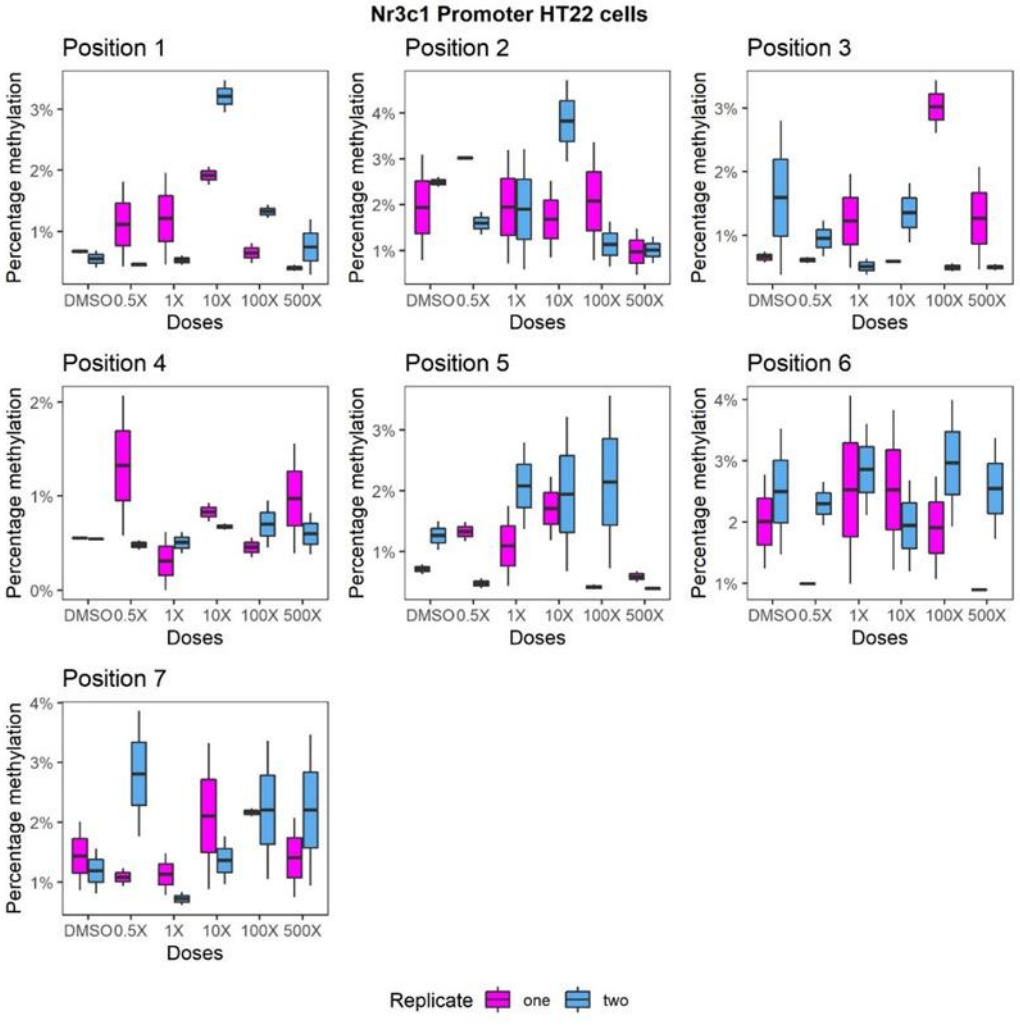

Figure 7. DNA methylation at the different doses of exposure of the two different biological replicates. Replicate 1 is shown in magenta while replicate 2 can be seen in blue.

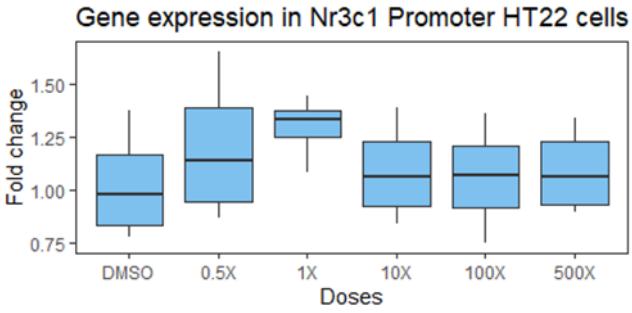

Figure 8. Gene expression of *Nr3c1* normalised to the housekeeping genes and DMSO control after exposure to different concentrations of mixture N

Discussion

The aim of this study was to investigate if the long term transcriptional effects found in prenatally exposed mice could be due to epigenetic changes. DNA methylation changes were therefore investigated with the use of bisulfite-pyrosequencing for nine genes: *Crh*, *Crhr1*, *Esr2*, *Fkbp5*, *Htr1a*, *Nr3c1*, *Nr3c2*, *Oxtr* and *Oxt*. Statistically significant changes were found between DMSO and 500X at several positions in *Nr3c1*, and at one position at *Crhr1* and *Nr3c2*. Interestingly, these findings corresponded to most of the statistically significant findings in the previous study by Repouskou *et al*. (2021). However, the authors have shown a significant change between DMSO and almost all the exposures at *Htr1a*. They also saw statistically significant changes between other exposure doses at *Nr3c1* and *Nr3c2* than us.

The changes that were seen in this study were mainly hypomethylation between the control DMSO and the dose 500X. The biological relevance of these findings can be argued since the significant doses are 100 and 500 times higher than the geometric mean found in the urine and plasma samples from the SELMA mothers. These results may however be more relevant than initially thought due to several reasons. First, the mice were orally exposed to Mixture N1, with the given doses 0.5X, 10X, 100X and 500X. This does not necessarily mean that it is the same high dosage that reaches the offspring. Second, the levels of chemicals found in the urine and the plasma of the SELMA mothers does not necessarily correspond to the prenatal exposure of the foetus. In a study by Björvang *et al*. (2021) the authors concluded that maternal serum and placenta may underestimate actual foetal exposure (Björvang *et al*. 2021). There are however not many studies conducted on the differences of chemical levels in tissue, and further research is needed. Third, the dose 1x of the geometric mean of the SELMA mothers is just an average of what is found in the mothers. Some mothers will have both higher and lower concentrations, which makes it important to also know the consequences of both higher and lower concentrations. Fourth, it is also meaningful to remember that there are biological differences between humans and mice, and that findings in mice cannot entirely be transferred to humans. However, it can be used as an indicator of potentially harmful chemicals. Nevertheless, these types of studies can still provide us with very important information about the mechanisms of EDCs, which we cannot receive from humans due to ethical reasons.

Statistically significant changes were found in different regions of the genes. For *Nr3c1*,

hypomethylation was found in all positions except for position 6 in the promoter region. The general consensus is that high methylation of the promoter region corresponds to silencing of the gene, while low methylation of it corresponds to high gene expression (Deaton & Bird 2011, Anastasiadi *et al*. 2018). In our data the DMSO control was low methylated, but the DNA that came from mice that had been exposed to 500X of mixture N1 was even less methylated. This should accordingly correspond to higher gene expression. Indeed, comparing these results to the gene expression data from Repouskou *et al*. (2020), higher gene expression at 500X exposure compared to the DMSO control was found. For *Nr3c2*, hypomethylation was seen on three different positions, two of them in exon 1 and one at intron 5. According to Anastasiadi *et al*. (2018) there is a strong negative correlation between DNA methylation in the intron and gene expression, meaning that if DNA methylation in an intron decrease, gene expression will increase. They also found low negative correlation between DNA methylation of the first exon and gene expression. Further, they suggested that the first exon is a better indicator of gene expression than methylation of the promoter (Anastasiadi *et al*. 2018).

This does not seem to be the case for either *Nr3c2* or *Crhr1*. In regard to *Nr3c2*, where hypomethylation is seen at three CpG sites, an already low DNA methylation in exon 1 became lower when the mice were exposed to the 500X dose. Meanwhile at intron 5, the quite high methylation levels became lower when exposed to the 500X dose. In disregard of the data found by Anastadiadi *et al*. (2018) the findings in gene expression from Repouskou *et al*. (2020) suggests that hypomethylation at these positions decreases the gene expression. Still, it is important to bear in mind that the changes seen on each CGI are few and might not be enough to impact the gene expression. Further, there could be other, more important CpG positions located in other places of the gene, like for example in the promoter region that plays a more relevant role on the gene expression. However, correlation tests should be performed to validate the relevance of regions analysed and the gene expression levels. Additionally, in this study we do not distinguish between 5-hmC and 5-mC and therefore cannot be certain of whether the decrease in methylation levels is due to 5-hmC or 5-mC. This would however be interesting to study, mainly since recent studies have suggested that 5-hmC play a more regulatory role than previously believed (Luo *et al.* 2018).

Hypomethylation is also seen at CpG position 1 on intron 1 in *Crhr1*. Once again, the hypomethylation can be seen at an already low methylated CpG position after exposure to the dose 500X. When we compare this to the data from Repouskou *et al*. (2020), we can again see that the decreased methylation in our case is related to a decreased gene expression compared to the DMSO treated mice. Nonetheless, we can suggest that the exposure to 500X of mixture N1 is associated with the changes observed in the DNA methylation.

Sex specificity

In our analysis clear differences between exposure and DNA methylation are only seen in males. Statistical significance is seen between DMSO and 500X on several positions in the non-parametric Kruskal-Wallis test, indicating that there are significant changes in DNA methylation. However, when the data is analysed with a non-parametric Friedman's test no significance

in interaction between sexes can be seen, indicating that the difference seen is not due to sex specificity. The question is then, why do we see these differences within males? Could it be due to how exposure seems to differ between males and females? Björvang *et al* (2021) saw that a generally higher chemical exposure in males than in females was observed when comparing chemical exposure in tissues of stillborn human foetuses. It would therefore be of interest to validate whether the sexual differences seen after prenatal exposure to mixture N1 are correlated to different chemicals levels in the tissue between sexes for example with the use of mass spectrometry. Further, it would also be interesting to investigate if the genotype of female/male could affect the observed changes in gene methylation by repeating the experiments in two different cell models, one originating from a female and another from a male.

Behaviours associated with methylation of *Nr3c1*, *Nr3c2* and *Crhr1*.

All of the genes found to be significant play a role in the stress regulation of the hypothalamic-pituitary-adrenal (HPA) axis (Nicolaides *et al*. 2015, ter Heegde *et al*. 2015, NCBI gene data bank 2021 p. 1). The mineralocorticoid receptor *Nr3c2* is more expressed in the hippocampus while the glucocorticoid receptor *Nr3c1* is more widely expressed with higher expression in regions like the PFC-hippocampal- amygdala circuitry (de Kloet *et al*. 2018). However, during the first weeks of life the glucocorticoid receptor is more actively expressed in the hippocampus than it is later on in life (de Kloet *et al*. 2018). Still, in this study and the previous study by Repouskou *et al*. (2020) differences in both methylation status and gene expression in the glucocorticoid receptor was seen.

Several studies have been performed on how DNA methylation of *Nr3c1* is associated to different social and stress related outcomes (van der Knaap *et al*. 2015, Niknazar *et al*. 2017, Conradt *et al*. 2019, Folger *et al*. 2019, Mattern *et al*. 2019, Gatta *et al*. 2021). Since these studies have been focusing on different aspects of social and stress outcomes as well as different regions of the gene, it is difficult to draw a conclusion of what role hyper- or hypomethylation plays in the CGIs of *Nr3c1*. However, it seems like environmental stressors like paternal stress (Niknazar *et al*. 2017) and chronical alcohol use (Gatta *et al*. 2021) is associated with hypermethylation of the gene.

Although the mineralocorticoid *Nr3c2* is more expressed in hippocampus than the glucocorticoid receptor *Nr3c1* there seem to be less studies focusing on associations between DNA methylation and potential health outcomes in *Nr3c2*. Nonetheless, a few studies have investigated the outcomes between social-behavioural outcomes like schizophrenia (Qing *et al*. 2020), aggressive behaviour (Qing *et al*. 2021) and early pregnancy depression (Galbally *et al*. 2020) and their association with *Nr3c2* methylation. Once again, it is difficult to draw any conclusions from this. However, a sex dependency seems to exist between hyper- and hypomethylation.

The corticotropin releasing hormone receptor 1 (*Crhr1*) also plays an important role by releasing the corticotropin releasing hormone which has a regulatory role in the HPA axis (NCBI gene data bank 2021 p. 1). In a few studies (Wang *et al*. 2013, Schartner *et al*. 2017) hypomethylation of the *Crhr1* has been associated with anxiety.

Exposure of mixture N1 during differentiation in HT22 cells

Our secondary goal was to investigate if these findings also could be seen during the brain development. To do this we exposed a male hippocampal cell line called HT22 to mixture N1. Unfortunately, HT22 was not the optimal model, since it did not express all our target genes: *Crhr1*, *Nr3c1* and *Nr3c2*. However, it did express one of them: *Nr3c1*. Due to this we only checked *Nr3c1* with qPCR, while *Nr3c1*,*Nr3c2* and *Crhr1* were also tested with pyrosequencing. With the PyroMark Q24 (Qiagen) we could detect statistically significant changes only in one of the genes, *Nr3c1*. The changes seen in methylation levels of *Nr3c1* in the HT22 cells were different from the ones found in the mice DNA. While we observed a hypomethylation in 100X and 500X compared to DMSO in the prenatally exposed mice, we found a hypermethylation at position 1 at 10X compared to the DMSO control in the promoter region in the differentiated cells. It is unclear why we see these differences between the prenatally exposed mice DNA and the cell model, and it would be necessary to both repeat the experiment and test with another cell model to be able to draw any conclusions. However, one explanation could be that we are studying two different time points, one during brain development and the second one in the adult brain. Hence, our findings could show that the changes observed during brain development are not permanent. Further, since it has been shown that the concentrations of chemicals in the mothers can be misleading and not necessarily corresponds to the concentration of chemicals found in foetus (Björvang *et al*. 2021) it is uncertain whether the doses that the mice and the cells were exposed to is the same. Either way, since the control samples (the CVs) could not validate our findings, it would be necessary to repeat the experiments.

When exposing the cells during the differentiation we also saw changes in morphology of the cells. When comparing the undifferentiated cells in the DMEM control and the non-exposed differentiated cells in the Neurobasal medium control with the exposed cells, it was evident that all the cells with neurobasal medium had a lot of cell death compared to the DMEM control. We also saw that in, especially the lower exposure doses, the cell death was higher and structures in the form of cell clumps were formed to a higher degree than in the other wells. Unfortunately, there was no time for testing what this was due to. However, some of these changes are a natural consequence of cell differentiation of these type of cells (Saxena *et al*. 2020). To the best of my knowledge no studies have been done on why this happens during differentiation and investigating it could therefore be of interest. It would also be of interest to perform a cytotoxicity test on mixture N1 to see if the mixture itself induces cell death, since we could see higher density of cell death in the lower doses of mixture N1 than in the DMSO control.

Limitations of the study

The main limitation of this study was the number of assays to perform compared to the amount of DNA available. Unfortunately, this made it impossible to run the PCR at least twice for each assay which otherwise would have been preferable due to the limitations of the PyroMark Q24. To bypass this all pyrosequencing runs were validated with two control samples in each plate.

Conclusions

An association between exposure to mixture N1 and changes in DNA methylation was seen in three different genes on totally eight different CpG positions. The main findings were found in the promoter region of *Nr3c1*. Some were also found at CpG-sites on *Crhr1* and *Nr3c2*. In conclusion, the results suggest that exposure to mixture N1 can result in changes in DNA methylation levels.

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Appendix

DNA Methylation data Mice

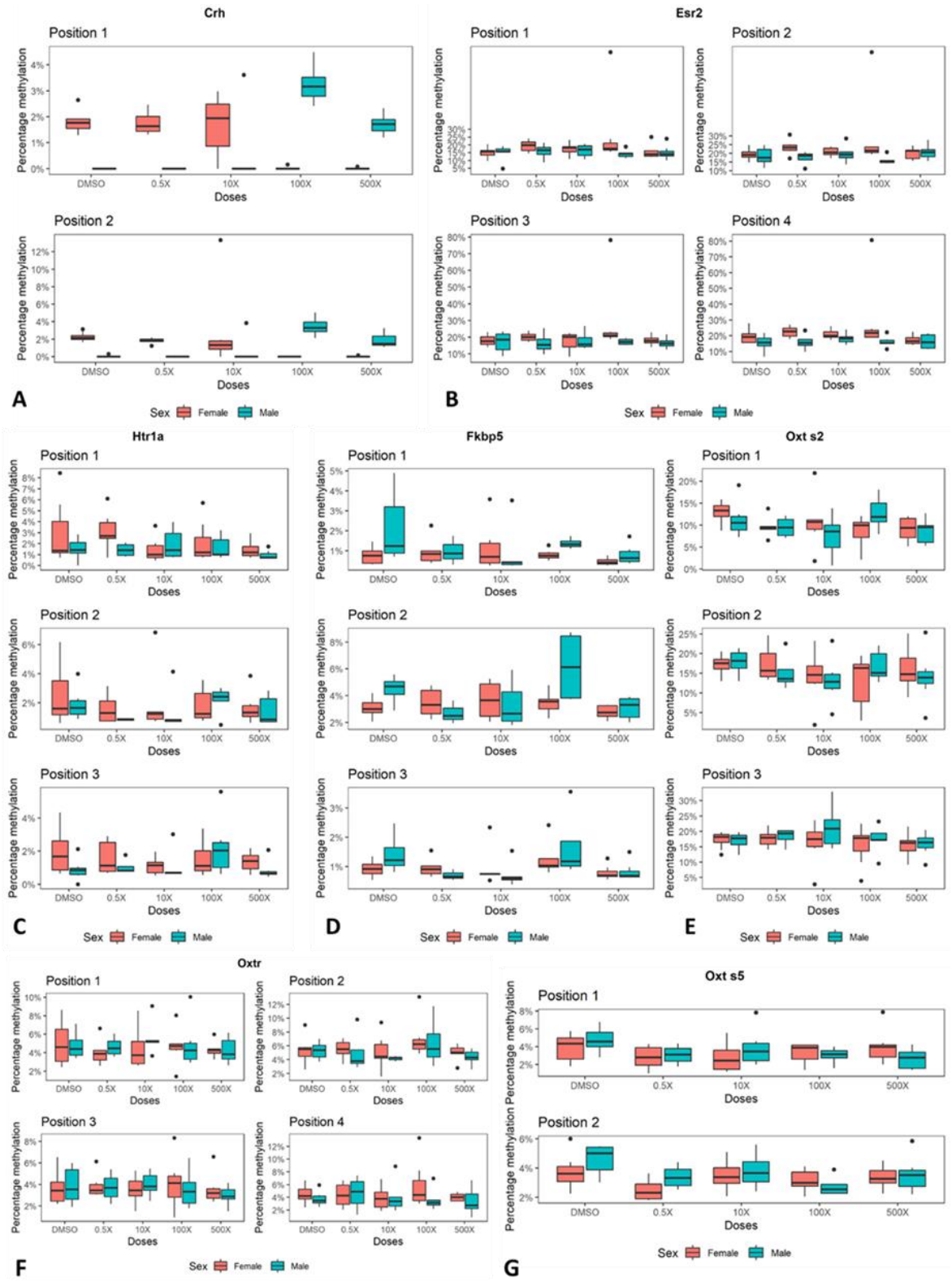

Figure 1. Shows the DNA methylation for each dose of exposure to mixture N1 at each CpG position in A gene *Crh*. B gene *Esr2*. C gene *Htr1a*. D gene *Fkbp5*. E. gene *Oxt* assay S2. F gene *Oxtr*. G gene *Oxt* assay 5.

DNA methylation in HT22 cells

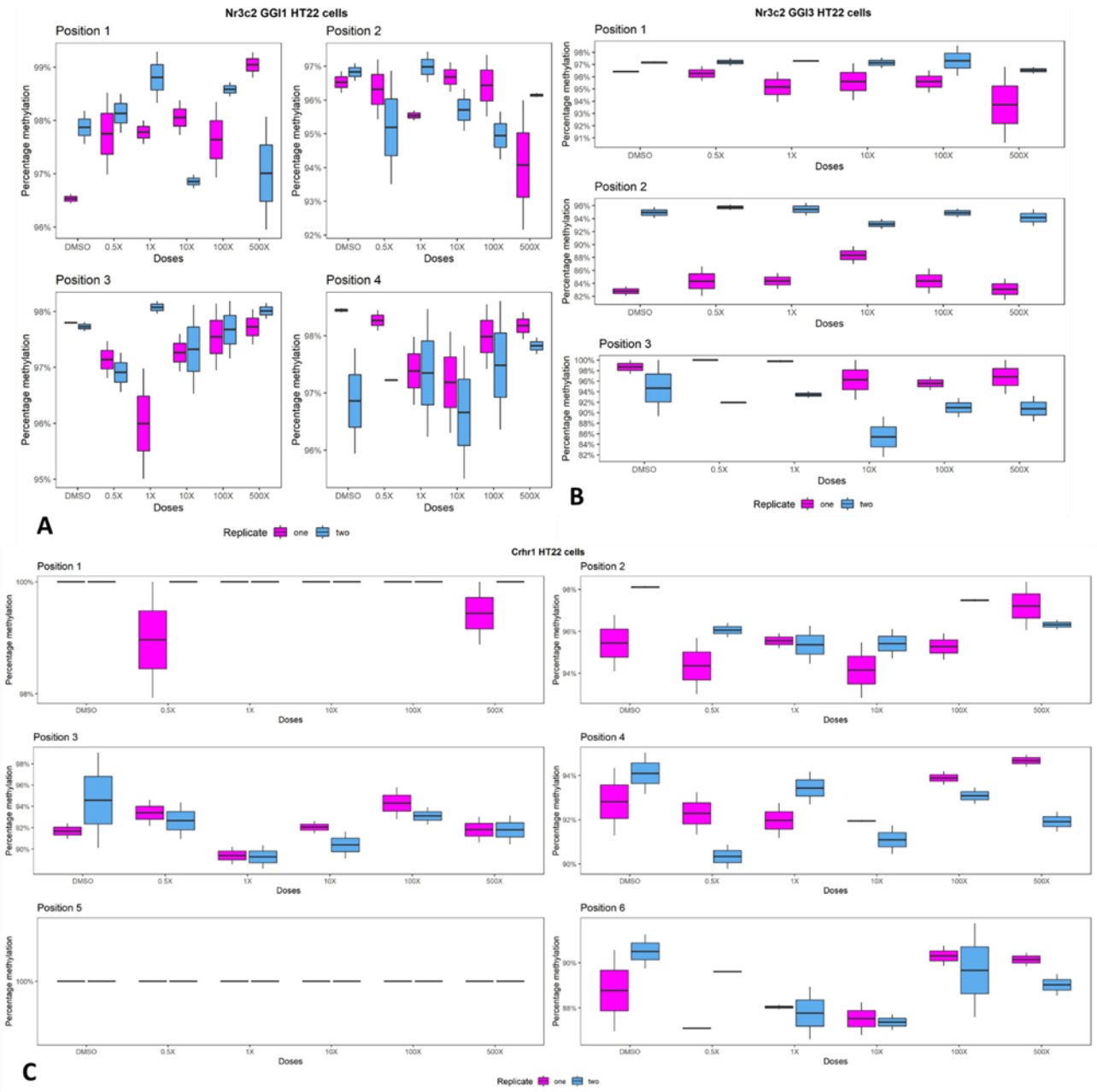

Figure 1. Shows the DNA methylation for each dose of exposure to mixture N1 at each CpG position in A gene *Nr3c2* CGI1 B gene *Nr3c2* CGI3. C gene *Crhr1*. Replicate 1 is shown in the R colour magenta while replicate 2 can be seen in the R colour Steelblue2.

Protocol

Splitting cells

Material needed:

DMEM with 10% FBS

DPBS (-calcium , -)

Tryple select (or other trypsin reagent)

Falcon tubes 5 ml

Falcon tubes 2 ml

Pipetboy

1 ml pipette

10 ul pipette

Pipette tips (10 ul and 1 ml)

10 and 25 ml pipetboy tips

100 mm plates

Steps:

1. Remove the medium from the plate
2. Gently wash the plate with 5 ml DPBS twice.
3. Add 5 ml of Tryple Select
4. Incubate at least 2 minutes in standard conditions
5. Check the plate under the microscope to check that the cells have detached
6. Add 6 ml of medium
7. Transfer the medium/tryple/cell solution to a falcon tube
8. Centrifuge at 1000 rpm for 5 minutes
9. Remove the supernatant without disrupting the cell pellet
10. Resuspend in 1 ml of medium and mix gently with the pipette
11. Transfer 10 ul of medium to a 1 ml Eppendorf tube
12. Mix the 10 ul of medium with 10 ul of Trypan blue and then add 10 ul of this to the chamber.
13. Count the cells in the EVE automated cell counter
14. Calculate the volume needed to seed a plate by taking (Cells needed)/(Cells counted (/ml))*1000
15. Take the ul needed and add to the medium required to plate the cells (RULE: 12 ml of medium for each 100 mm plate)
16. Add the medium required to each plate and make sure the medium covers the entire plate by gently moving the plate in a crosswise manner
17. Incubate in standard conditions.

Freezing of cells

Material needed:

FBS

DMSO

DPBS (-calcium and - magnesium)

Tryple select (or other trypsin reagent)

Falcon tubes 5 ml

Falcon tubes 2 ml

Freezing tubes

Mr cool

Pipetboy

1 ml pipette

10 ul pipette

Pipette tips (10 ul and 1 ml)

10 and 25 ml pipetboy tips

100 mm plates

DMEM with 10% FBS

Steps:

1. Prepare the freezing medium by mixing 9 ml of FBS with 1 ml of DMSO to achieve a 10% DMSO freezing medium.
2. Remove the medium from the plate
3. Gently wash the plate with 5 ml DPBS twice.
4. Add 5 ml of Tryple Select
5. Incubate at least 2 minutes in standard conditions
6. Check the plate under the microscope to check that the cells have detached
7. Add 6 ml of medium
8. Transfer the medium/tryple/cell solution to a falcon tube
9. Centrifuge at 1000 rpm for 5 minutes
10. Remove the supernatant without disrupting the cell pellet
11. Resuspend in 1 ml of medium and mix gently with the pipette
12. Transfer 10 ul of medium to a 1 ml Eppendorf tube
13. Mix the 10 ul of medium with 10 ul of … and then add 10 ul of this to the chamber. Count the cells in the EVE automated cell counter
14. Calculate the volume needed to seed a plate by taking 1000000/(Cells counted (/ml))*1000
15. Add 1 ml of freezing medium to each vial.
16. Take the ul needed and put in the vials needed
17. Put the vials in mr Cool and put in the -80 °C freezer

DNA AND RNA preparation with Allprep DNA/RNA mini kit

1. Prepare the RLT plus Buffer (Qiagen) by adding 10 µl of β-Mercaptoethanol to each millilitre of RLT plus Buffer. Note: when the β-Mercaptoethanol has been added, the buffer can be stored in the fridge for 1 month.
2. Aspirate the cell culture medium
3. Wash the cells with DPBS
4. Disrupt the cells by adding Buffer RLT plus. If the diameter of the plate is <6 cm, add 350 µl of buffer, and if it has a diameter of 6 to 10 cm, add 600 µl.
5. Transfer the cells to the correct amount of Eppendorf tubes, one for each plate or well, and store in -80 °C until extraction.