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A human-relevant mixture of endocrine disrupting chemicals induces changes in hippocampal DNA methylation correlating with hyperactive behavior in male mice

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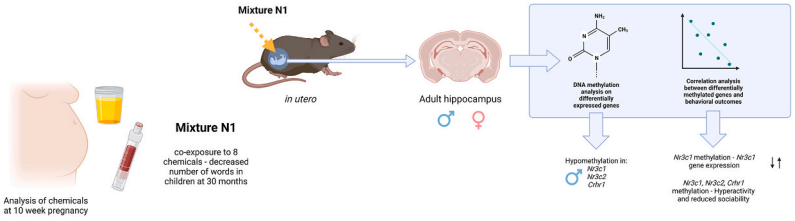
HIGHLIGHTS

- *In utero* exposure to a human-relevant EDC mixture affects hippocampal DNAm in males.
- DNAm changes at the *Nr3c1* gene correlate with altered expression.
- DNAm changes correlate with hyperactivity and decreased social behavior.

GRAPHICAL ABSTRACT



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ABSTRACT

Humans are ubiquitously exposed to endocrine disrupting chemicals (EDCs), substances that interfere with endogenous hormonal signaling. Exposure during early development is of particular concern due to the programming role of hormones during this period. A previous epidemiological study has shown association between prenatal co-exposure to 8 EDCs (Mixture N1) and language delay in children, suggesting an effect of this mixture on neurodevelopment. Furthermore, *in utero* exposure to Mixture N1 altered gene expression and behavior in adult mice. In this study, we investigated whether epigenetic mechanisms could underlie the long term effects of Mixture N1 on gene expression and behavior. To this end, we analyzed DNA methylation at regulatory regions of genes whose expression was affected by Mixture N1 in the hippocampus of *in utero* exposed mice using bisulfite-pyrosequencing. We show that Mixture N1 decreases DNA methylation in males at three genes that are part of the hypothalamus-pituitary-adrenal (HPA) axis: *Nr3c1, Nr3c2, and Crhr1,* coding for the glucocorticoid receptor, the mineralocorticoid receptor, and the corticotropin releasing hormone receptor 1, respectively. Furthermore, we show that the decrease in *Nr3c1* methylation correlates with increased gene expression, and that *Nr3c1*, *Nr3c2*, and *Crhr1* methylation correlates with hyperactivity and reduction in social behavior. These findings indicate

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that an EDC mixture corresponding to a human exposure scenario induces epigenetic changes, and thus programming effects, on the HPA axis that are reflected in the behavioral phenotypes of the adult male offspring.

1. Introduction

Humans are ubiquitously exposed to numerous man-made chemicals that are proven or suspected endocrine disrupting chemicals (EDCs), i.e. substances interfering with the hormonal system (Gore et al., 2015). EDCs include chemical classes such as bisphenols and phthalates, broadly used, e.g., in the production of plastic containers and can coating for food and drinks (Almeida et al., 2018; Serrano et al., 2014), personal care products (Nicolopoulou-Stamati et al., 2015), and building materials (Fucic et al., 2018), as well as pesticides, persistent organic pollutants (Mnif et al., 2011), and many more (Yilmaz et al., 2020). Chemical analyses of biofluids, such as blood, urine, as well as amniotic fluid, have shown persistent and ubiquitous human exposure to combinations of these chemicals (Zhang et al., 2013; Edlow et al., 2012; BornehagCarl-Gustaf et al., 2019; Kalloo et al., 2020).

Although EDCs can potentially affect the hormonal system during the whole lifespan (Rattan et al., 2017; Özen and Darcan, 2011), development represents a particularly sensitive window for EDC exposure due to the central role of the endocrine system in its control and coordination (Mathey, 2021). The developing brain is dependent on endocrine signaling for essential processes like neurogenesis, synaptogenesis, myelination, and migration of neurons (Benvenuti et al., 2008; Chen et al., 2012; Wang et al., 2003). Particularly sensitive to prenatal hormonal levels are the developing hypothalamic-pituitary-adrenal (HPA) axis and the limbic system, involved in stress and emotional response, learning, and memory (Dunn et al., 2010; Giesbrecht et al., 2017; Poimenova et al., 2010). The hippocampus, part of the limbic system and involved in HPA axis regulation (Xiong and Zhang, 2013), is characterized by higher expression of endocrine receptors than other brain regions, suggesting high sensitivity of this area to actions of EDCs. *In utero* and perinatal exposure to such chemicals, e.g. Bisphenol A (BPA) and Di (2-ethylhexyl) phthalate (DEHP), has been shown to disrupt HPA axis function and alter emotional and stress response (Panagiotidou et al., 2014; Palanza et al., 2008; Xu et al., 2015) as well as hippocampal functions in rodents (Wang et al., 2020; Liu et al., 2016). In humans, perturbation of hormonal levels during pregnancy (Miranda and Sousa, 2018; Davis and Sandman, 2010) and *in utero* EDCs has been associated with behavioral alteration and cognitive impairment later in life exposure (Erhardt et al., 2006; Day et al., 2021). The observed effects were often sex-specific, indicating a different susceptibility between males and females (Lim et al., 2017; Kamai et al., 2021; Philippat et al., 2018).

Increasing evidence supports epigenetic processes as a mechanism underlying long term effects of developmental endocrine system disruption (Streifer and Gore, 2021; Jacobs et al., 2017). Epigenetics is the study of "*the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states*" (Deichmann, 2016), and includes e.g. DNA methylation (DNAm) and histone modifications. DNAm is a dynamic epigenetic mark that, when established, can be preserved throughout development (Smith and Alexander, 2013) and regulates transcriptional activity (Anastasiadi et al., 2018). In the developing human brain, extensive DNAm changes take place and contribute to neurodevelopment (Spiers et al., 2015; Luo et al., 2016) presumably defining cell identities and activities (Bogdanović and Ryan, 2017). DNAm patterns have been found altered in connection to EDCs exposure in humans and have been linked to neurodevelopmental effects such as impaired cognitive functions (Engdahl et al., 2021) and thought problems (Mustieles et al., 2022). Moreover, experimental studies support the involvement of epigenetics on EDCs-related behavioral alterations, i.e. by altering brain DNAm in genes involved in cognition and stress modulation (Kitraki et al., 2015; Alavian-Ghavanini et al., 2018).

Most human studies have investigated associations between exposure for single EDCs and different health outcomes (Wolstenholme et al., 2011; Dutta et al., 2020). However, in real life, humans are always exposed to numerous chemicals in complicated mixtures. In previous studies, we have established such real life mixtures based on epidemiology data from the Swedish Environmental, Longitudinal, Mother and child, Asthma and allergy (SELMA) pregnancy cohort (BornehagCarl-Gustaf et al., 2012). Mixtures of chemicals in prenatal urine and serum have been associated with different outcomes in children, e.g., birthweight and growth (Svensson et al., 2021), reproduction (anogenital distance) (BornehagCarl-Gustaf et al., 2019), and neurodevelopment (cognitive function) (Tanner et al., 2020). In the case of neurodevelopment, a mixture of eight EDCs (Mixture N1) associated with language delay in children was established in a three step procedure: Firstly, we identified chemicals of concern measured in prenatal urine and serum of the SELMA mothers, that was associated with a language delay in their children at age 30 months, by the use of weighted quantile sum (WQS) regression. Secondly, we estimated the serum levels of the chemicals of concern. Urine compounds were converted into serum concentrations through the estimation of daily intake. Thirdly, the mixing proportions of the chemicals of concern were established using serum geometric mean levels from the SELMA mothers (additional details are reported in Supplemental material B – Table B1 and Supplemental material A – Table A.4). Mixture N1 was then tested in the experimental systems.

In mice, *in utero* exposure to Mixture N1 resulted in behavioral changes in adults, including an increase in distance moved in the Open field test, time of struggling in the Forced Swim Stress (FSS) test, and a decrease of discrimination index in the Social interaction test without alteration in the Elevated Plus-Maze scores or in the Novel object location ability. These results indicate that Mixture N1 induces a hyperactive phenotype and less interaction with co-specific, suggesting a decrease of stress coping and anxiety in *in utero* exposed mice (Repouskou et al., 2020). Behavioral outcomes were accompanied by significant expression changes of genes involved in the HPA axis, anxiety-related disorders, and neurodevelopment.

In this study, we addressed whether DNAm alterations could underlie the observed long term changes in hippocampal gene expression and behavior induced by Mixture N1. To this end, we analyzed DNAm levels in hippocampi of mice that were prenatally exposed to Mixture N1, and subsequently, we performed correlation analysis to investigate the relationship between differentially methylated *loci* and gene expression as well as behavioral alterations.

2. Methods

2.1. Hippocampal DNA

This study made use of tissue and data generated in Repouskou et al., (2020). Briefly, C57/BL6 mice from the Hellenic Pasteur Institute (Athens, Greece) were fed with BPA-Phthalate-free food and water. Pregnant mice were daily exposed during gestation to 0.001, 0.22, 2.2, or 11 mg/kg body weight of Mixture N1. These exposures correspond to 0.5 × , 10 × , 100 × , and 500 × the geometric mean of the serum concentrations of the pregnant women included in the SELMA study. DMSO was used as control (0×).

At postnatal day 90 (PND90) one to three animals per sex and litter from each exposure group were randomly selected for the behavioral testing. This resulted in 14 males and 12 females from the control group (0×), 10 males and 12 females from the 0.5x group, 12 males and 12 females from the 10× group, 10 males and 13 females from the 100× group, 12 males, 11 females from the 500× group. 30 min after the last

test, animals were sacrificed and hippocampi were isolated. Hippocampi from siblings having same sex and treatment were pooled and DNA was extracted as described in our previous study. The experimental protocol was approved by the Ethical Licensing Committee of the Prefecture of Attica-Veterinary department (#4783) and performed in accordance with relevant guidelines and regulations (European Communities Council Directive of September 22, 2010; 2010/63/EU).

2.2. Assay design

In order to analyze DNAm changes induced by *in utero* exposure to Mixture N1, we selected putative epigenetic regulatory regions using annotation in UCSC. Target regions included CpG islands, Polycomb group (PcG) proteins-binding sites, or/and histone-binding sites.

Assays were designed using the PyroMark Assay Design 2.0 software (Qiagen). Assay linearity was verified using bisulfite-converted low and high methylated mouse standard (EpigenDx) and performing linear regression. Best assays were then selected for methylation analyses (supplemental material A - Table A.1).

2.3. Bisulfite-pyrosequencing

DNAm in the hippocampus was assessed by bisulfite-pyrosequencing. DNA bisulfite conversion was performed using EZ DNA Methylation-Gold Kit D5006 (Zymo). The PCR was performed in SimpliAmp™ Thermal Cycler using PyroMark PCR Kit (Qiagen), biotinylated primers (Biomers), primers (IDT), and approximately 10 ng of bisulfite-converted template DNA, following the manufacturer's instructions. Amplification, absence of non-specific amplification, and absence of DNA contamination were confirmed by electrophoresis on a 1,5% agarose gel. 10 μl of amplification products were immobilized on Streptavidin Sepharose® High Performance (Sigma) in PyroMark Binding Buffer (Qiagen) on plate-shaker for 20 min and Pyrosequencing was performed on a PyroMark Q24 using PyroMark Gold Q24 Reagents, PyroMark Denaturation Solution, PyroMark Wash Buffer, Annealing Buffer (Qiagen) according to manufacturer's instructions. Assays were run including a DNA control sample in each pyrosequencing run, in order to detect intra- and interplate variation in pyrosequencing analysis. The methylation was analyzed on Pyromark Q24 2.0.8 software (Qiagen).

2.4. Statistical analysis

Kruskal Wallis test was performed for methylation and doses, analyzing CpG sites, as well as the average of methylation per region, stratifying sexes. The analysis was followed by the non-parametric Dunn's test. Significant results were identified using a p-value <0.05 and validated according to inter- and intraplate variation (variation higher than inter-group differences were considered not significant). Additionally, a Spearman correlation analysis was performed between exposure and DNA methylation at CpG sites and regions identified as affected by Mixture N1, whereby p-values <0.05 were considered significant.

For significantly changed CpG sites and regions, we also performed Spearman correlation analyses between methylation and relative gene expression as well as methylation and behavioral data. We analyzed the correlation between methylation and Open field, Social interaction and, Forced swimming stress. p-values <0.05 were considered significant. Kruskal Wallis and Spearman correlation analyses were performed in GraphPad Prism version 5.01. Statistical evaluation of gene expression and behavioral data used in the present study has been reported in our previous study (Repouskou et al., 2020).

3. Results

3.1. In utero exposure to mixture N1 alters DNAm at Nr3c1, Nr3c2, and Crhr1 in males

To analyze DNAm changes induced by *in utero* exposure to Mixture N1 (composition and description shown in Table B.1 Supplemental material), we performed bisulfite-pyrosequencing analysis at putative regulatory regions of 4 genes in the hippocampi of mice from our previous study, including Glucocorticoid receptor (*Nr3c1*), Mineralocorticoid receptor (*Nr3c2*), Corticotropin-Releasing Hormone Receptor 1 (*Crhr1*), and Serotonin receptor 1a (*Htr1a*). These genes were selected as they were the only ones showing altered expression in adult hippocampus upon exposure to Mixture N1 under basal and/or stress conditions (Repouskou et al., 2020).

Our results showed a significant decrease in DNAm in HPA axis genes *Nr3c1*, *Nr3c2,* and *Crhr1* in *in utero* exposed males, but not in females (supplemental material A - Table A.2), compared to control. For *Nr3c1*, hypomethylation of 6 CpG sites out of the 7 analyzed positions was observed at the 500X dose, while the 100X dose induced significantly decreased methylation at 3 CpG sites (Fig. 1A, C, 1D, supplemental material A - Table A.2). Moreover, significantly decreased DNAm was observed in the average methylation of the entire *Nr3c1* target region at 500X (Fig. 1H). For *Nr3c2* and *Crhr1* only one position was found differentially methylated at the highest dose (Fig. 1I and J, supplemental material A - Table A.2). No changes in DNAm upon Mixture N1 exposure were found in the *Htr1a* (supplemental material A - Table A.2).

To validate these results, a correlation analysis between prenatal exposure levels and DNAm at significant sites and regions was performed. Our results showed a strong or moderate negative correlation between DNAm and Mixture N1 exposure for all the significant CpG sites identified in *Nr3c1*, *Nr3c2* and *Crhr1* as well as *Crhr1* and *Nr3c1* regions (Fig. 2, supplemental material B – Table B.2).

3.2. DNAm correlates with gene expression and behavioral outcomes

We have previously shown that Mixture N1 induces changes in hippocampal gene expression of *Nr3c1*, *Nr3c2,* and *Crhr1* as well as behavioral alterations in *in utero* exposed males (Repouskou et al., 2020). Behavioral changes included distance moved in the Open field test, duration of struggling in the Forced Swim Stress (FSS) and discrimination index in the Social interaction test.

To investigate the functional implications of the DNAm changes, in this study, we addressed their correlation with gene regulation on one hand, and with behavioral outcomes on the other hand.

Spearman correlation analysis showed a significant negative correlation for all 6 differentially methylated CpGs in *Nr3c1* and gene expression (supplemental material B - Figure B.1). A similar result was found in the analysis of *Nr3c1* average DNAm (Table 1, Fig. 3). No correlation was found between *Nr3c2* and *Crhr1* expression and methylation at the investigated CpG sites (supplemental material B - Table B.3).

Correlation analysis with performance in behavioral tests showed a significant negative correlation between *Nr3c1* and locomotion in the Open field test for CpG1, 2, 3, 4, and average methylation, as well as struggling in the FSS test for CpG2 and 4. In both cases, a decrease in methylation was correlated with hyperactivity (Table 2, supplemental material B - Figure B.2).

Nr3c2 CGI3 CpG3 and *Nr3c2* CGI3 average methylation were negatively correlated with duration of struggling in the FSS test (Table 2, supplemental material B - Figure B.3), however, no correlation was found with activity in the Open field test (supplemental material B -

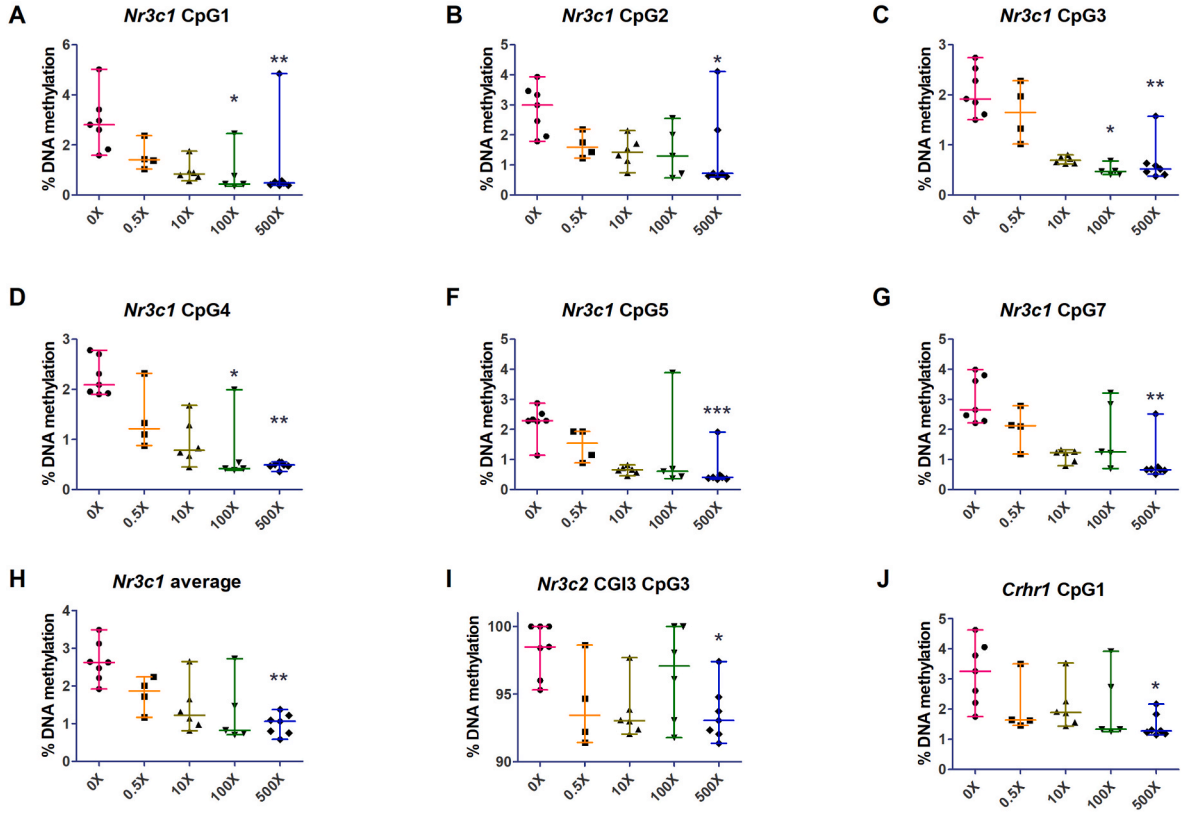

Fig. 1. Changes induced by *in utero* Mixture N1 exposure in the male adult hippocampus. DMSO in pink (Control), 0.5X in orange, 10X in light green, 100X in dark green, and 500X in blue. Bar plots show median and range. Exposure refers to SELMA mothers' levels. (A, B, C, D, E, F, G) *Nr3c1* methylation in CpG sites, (H) *Nr3c1* average, (I) *Nr3c2* CGI3 CpG3 and (J) *Chrh1* CpG1. p-value <0.05*, p-value <0.01**, p-value*** <0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

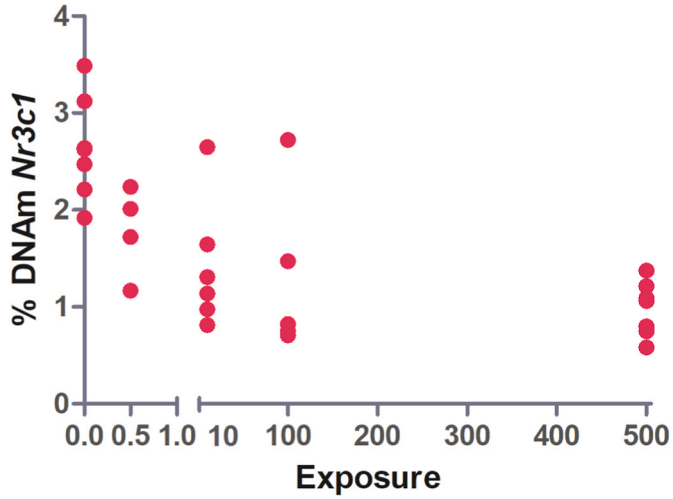

Fig. 2. Correlation between *Nr3c1* average methylation and prenatal exposure to Mixture N1 using Spearman analysis. *r* −0,70, p-value <0,0001.

Table 1
Significant results in Spearman correlation analyses on DNAm and relative gene expression. Spearman *r* 0.2–0.39 (weak), 0.4–0.59 (§ moderate), 0.6–0.79 (§§ strong).

Target	CpG	Spearman *r*	p-value
Nr3c1	1	−0.47 §	0.010
Nr3c1	2	−0.65 §§	<0.001
Nr3c1	3	−0.55 §	0.002
Nr3c1	4	−0.48 §	0.008
Nr3c1	5	−0.44 §	0.018
Nr3c1	7	−0.52 §	0.004
Nr3c1	average	−0.65 §§	<0.001

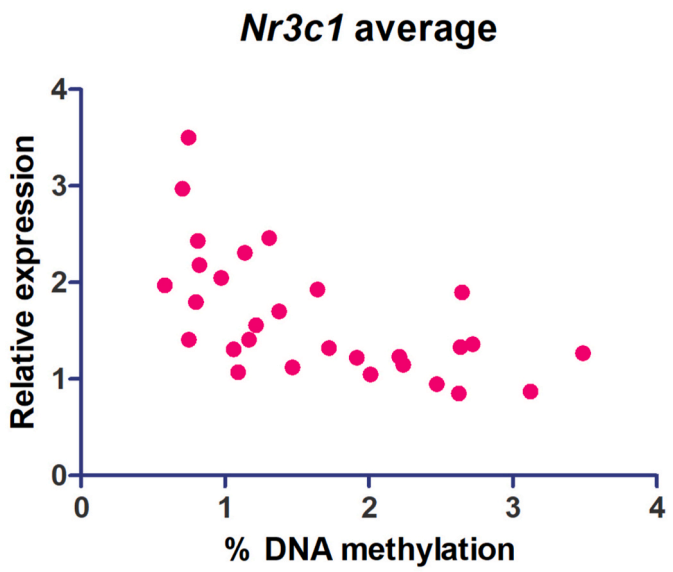

Fig. 3. Correlation between *Nr3c1* average methylation and relative gene expression. Spearman *r* is −0,65, p-value 0,0002.

Table 2
Significant results in Spearman correlation analyses on DNAm and behavioral measurement for Open field, Struggling in FSS and Social interaction. For Open field, Struggling (FSS) and Social interaction we consider respectively distance moved (cm), seconds of struggling and discrimination index. Spearman *r* 0.2–0.39 (weak), 0.4–0.59 (§ moderate), 0.6–0.79 (§§ strong).

Target	CpG	Behavioral outcome	Spearman *r*	p-value
Nr3c1	1	Open field	−0.46 §	0.013
Nr3c1	2	Open field	−0.53 §	0.003
		Struggling (FSS)	−0.62 §§	<0.001
Nr3c1	3	Open field	−0.50 §	0.006
Nr3c1	4	Open field	−0.42 §	0.024
		Struggling (FSS)	−0.38	0.044
Nr3c1	average	Open field	−0.39	0.038
Nr3c2 CGI3	3	Struggling (FSS)	−0.39	0.034
Nr3c2 CGI3	average	Struggling (FSS)	−0.37	0.044
Crhr1	1	Social interaction	0.52 §	0.004
		Open field	−0.46 §	0.013
Crhr1	average	Social interaction	0.44 §	0.016

Table B.4), suggesting that the observed decrease in *Nr3c2* methylation is linked to hyperactivity and decrease in the handling of intense stress but not of moderate stress as addressed in the Open field test.

For *Crhr1* CpG1, results showed a significant negative correlation with locomotion in the Open field test and an additional significant positive correlation between the *Crhr1* CpG1 and the corresponding average methylation with the discrimination index in Social interaction (Table 2, supplemental material B - Figure B.4). This indicates that hypomethylation at *Crhr1* is linked to hyperactivity in moderate, but not intense, stress conditions as well as to less time in the proximity of a conspecific (Table 2).

4. Discussion

4.1. Mixture N1 induces DNAm changes in HPA-axis related genes

Humans are continuously exposed to a combination of environmental chemicals. In previous studies using the SELMA cohort, we have associated prenatal exposure to a mixture of 8 EDCs (Mixture N1) with delayed language development in children, and demonstrated that *in utero* exposure to Mixture N1 induces behavioral changes in adult mice and concomitant gene expression alteration. Here we provide evidence that some of these long term changes could be brought about by alterations in epigenetic regulation in the hippocampus. Specifically, we found hypomethylation in *Nr3c1* promoter in exposed mice at 100X and 500X times SELMA concentrations. Additionally, we found hypomethylation at *Nr3c2* intron 5 and *Crhr1* intron 1 at the highest tested dose.

While this is one of the first studies addressing epigenetic effects of EDCs in mixtures, single compound studies on chemicals contained in Mixture N1 have previously shown effects of *in utero* exposure on specific DNAm in adult brain, concomitant with behavioral changes (e.g. (Dolinoy et al., 2007; Kundakovic et al., 2013; Wolstenholme et al., 2011; Nadeem et al., 2021). In a previous study, where developmental effects of BPA at comparable doses to that in Mixture N1 (40 μg BPA/kg bw/day compared to 47 μg BPA/kg bw/day at 100X Mixture N1) were investigated in rats, we found that BPA increased DNAm at *Fkbp5*, an important regulator of the HPA axis, but no changes were observed in hippocampal *Nr3c1* methylation (Kitraki et al., 2015). This suggests that the combination of chemicals may have different epigenetic effects from single substance exposure.

4.2. Mixture N1-induced DNAm changes were sex-specific

We found DNAm alterations exclusively in males, in accordance with emerging evidence of sexually dimorphic effects of EDCs on DNAm (Kundakovic et al., 2013; Cheong et al., 2018; Alavian-Ghavanini et al., 2018; Palanza et al., 2021). While we cannot exclude effects on the female hippocampi in genes and *loci* not investigated in this study, the results are in line with the fact that Mixture N1 had a stronger effect on gene expression in males than in females (Repouskou et al., 2020). This suggests that the mixture had sex-specific effects on brain development and function, which could be due to biological differences (e.g. different hormonal and gene expression levels) or due to differences in placenta protective response in males and females, and hence in local chemical concentrations (Björvang and Mamsen, 2022).

4.3. Relationship between DNAm changes and gene expression

While for *Nr3c1,* DNAm showed a significant correlation with gene expression, no such relationship was found for *Nr3c2* and *Crhr1*. This might implicate that for the latter genes, DNAm at the investigated regions has no functional role for gene expression in the hippocampus. Indeed, the *locus* analyzed in *Nr3c2* is a binding site for a component of the Silencing transcriptional factor, which is specifically expressed during neuroendocrine differentiation (Supplemental material A - Table A.3) (Monaghan et al. 2017). Thus, it is possible that the observed DNAm changes induced by Mixture N1 at these genes are mainly important during neurodevelopment but are nevertheless maintained into adulthood. On the other hand, it is important to underline that DNAm and expression analyses were performed after behavioral tests, thus it may be possible that the differentially methylated *loci* at *Nr3c2* and *Crhr1* do not have a role in transcriptional regulation under stress but rather under basal conditions. This is corroborated by the fact that Mixture N1 had a stronger impact on *Nr3c2* transcription under basal conditions than under stress (Repouskou et al., 2020). Further studies with different sampling points are needed to clarify this question.

4.4. Relationship between DNAm changes and behavioral outcomes

Dysregulation in the HPA axis and epigenetic changes in HPA axis-related genes has been associated with psychiatric and developmental disorders such as depression, anxiety (Erhardt et al., 2006), and ADHD (Kaneko et al., 1993). For *NR3C1,* hypermethylation in exon 1F has been found in the salivary DNA of maltreated children and linked with higher emotional lability, depressive symptoms, and more externalizing behavior (Cicchetti and Handley, 2017). Hypermethylation at 1B, 1C, 1F, and 1H promoters has also been found to correlate with a decrease in

gene expression in the hippocampi of suicide victims with a history of childhood abuse (McGowan, 2009; Labonte et al., 2012). Most studies identified *NR3C1* hypermethylation linked with a decrease in gene expression and psychiatric disorders. Yet, *NR3C1* DNAm hypomethylation at 1C and 1B promoters has been found in blood cells of post-traumatic stress disorder (PTSD) patients as well as individuals with adverse lifestyles and has been associated with lower cortisol levels and higher perceived stress (Labonté et al., 2014; de Rooij et al., 2012). Moreover, in armed forces personnel, overexpression of *NR3C1* has been found as a vulnerability factor for PTSD symptoms (van Zuiden et al., 2011). In this study, we found a decrease in hippocampal *Nr3c1* DNAm that correlates with increased expression and hyperactivity under stress conditions. As reported in our previous work, exposed mice did not show depressive symptoms i.e. increased floating time in the FSS test, or changes in corticosterone levels compared to the control group. Therefore, *Nr3c1* hypomethylation, if assumed as an early epigenetic effect, seems not to be related to depressive symptoms or linked to hyperactivation of the HPA axis. Most likely, *Nr3c1* hypomethylation and increased gene expression could lead to prolonged perceived stress and fear in the open field and in FSS. This hypothesis is supported by evidence that *Nr3c1* overexpression can affect BDNF signaling involved in fear extinction in mice (Lu et al., 2021).

Much fewer studies have linked DNAm at *CRHR1* or *NR3C2* to behavioral outcomes in rodents or humans. One study describes the association between hypermethylation of *NR3C2* in peripheral blood and aggressive behavior in humans (Qing et al., 2021). Yet, in this study, we found a decrease in methylation linked with hyperactivity under intense stress while no correlation with social interaction was observed. This could be due to species differences or due to the fact that DNAm was measured in different tissues. Mice lacking *Crhr1* have been observed to have impaired endocrine response to stress and decreased anxiety-like behavior (Timpl et al., 1998; Smith et al., 1998). Furthermore, a decrease in promoter CpG island DNAm in the hypothalamus was observed upon *in utero* hypoxic stress, which was suggested to play a role in programming of anxiety-like behaviors in male rats (Wang et al., 2003). Consistent with the previous findings, we found that a decrease of *Crhr1* DNAm at the same CpG island by Mixture N1 was associated with hyperactivity in stress conditions. Additionally, we showed that the decrease of *Crhr1* DNAm was associated with lower duration of interaction with a con-specific. This last finding could be related to social anxiety/phobia, linked with HPA axis functionality (Condren et al., 2002).

4.5. Human relevance of the reported findings

Although significant DNAm changes were only found at higher doses corresponding to 100–500× human mean concentrations, effects reported here could still be relevant for humans. Firstly, it should be emphasized that some of the women in the SELMA cohort had far higher exposure levels, corresponding to at least 10× the geometric mean. Secondly, species differences could render mice less sensitive to the exposure, and inter-individual differences could render vulnerable individuals more sensitive. Indeed, these uncertainty factors are accounted for when safety thresholds are established, usually with a factor of 100 (Naidenko, 2020). Therefore, *Nr3c1* DNAm alterations are in the exposure range of interest. Additionally, the correlation analyses showed a decrease in DNAm even at the lower doses although they did not reach statistical significance with the Kruskal Wallis test due to inter-individual variations.

As discussed in our previous publication (Repouskou et al., 2020), identified hyperactivity and abnormal social behaviors are traits in animal models for ADHD and autism spectrum disorder in humans. Thus, while our results might not easily be linked to language delay (which was used to establish Mixture N1) as trait *per se*, they indicate, just as language delay, neurodevelopmental changes that can manifest in cognitive deficits and/or behavioral alterations. Future analyses should address DNAm levels at the same genes in humans as a function of exposure and the behavioral/cognitive outcomes.

4.6. Limitations

There are certain limitations in this study. Firstly, the sample size was limited, which compromised the power of our analyses due to inter-individual differences. Secondly, due to limited material we could not investigate the impact of Mixture N1 on DNAm under basal conditions - and, consequently, its effect on gene expression - as well as the impact on methylation in other areas and effect on behavioral outcomes. Lastly, we performed a hypothesis-driven approach based on looking at putative regulatory regions of candidate genes based on the literature. We can thus not exclude that Mixture N1 affects DNAm in other regions at the investigated genes and in additional genes, also in females.

5. Conclusion

This study shows that *in utero* exposure of mice to a human-relevant EDC mixture decreases hippocampal DNAm in the HPA axis-related genes *Nr3c1*, *Nr3c2*, and *Crhr1*, which provides a potential mechanism by which the mixture induces hyperactivity under stress conditions and reduced social behavior in adult male offspring.

Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.137633.

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