HELLENIC REPUBLIC

National and Kapodistrian University of Athens

Department of Biology

Athens International Master's Programme in Neurosciences

Department of Dentistry and Department of Nursing , National and Kapodistrian University of Athens

RESEARCH THESIS PROJECT

Effects of prenatal exposure to endocrine disruptors on mice behavior and brain neurochemistry

Anastasia-Konstantina Papadopoulou

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2018-2019

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Three Member Evaluation Committee	**Signatures**

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Abstract:

Endocrine disrupting compounds (EDCs) are substances that can disturb the normal function of endogenous hormones in any manner. Recently, SELMA study of a pregnant women cohort showed that many of the known EDCs are present in the urine and the sera of these women and can potentially harm their offspring in multiple levels, including in their neuronal development. In the frame of EDC-MixRisk, the mixtures of EDCs associated with neurodevelopmental impairment in children of the SELMA study were defined. In the present study, we exposed pregnant mice dams to different doses of a mixture of endocrine disruptors during gestation. Then, in their adult male offspring we investigated the effects of this mixture on certain aspects of behavior and expression pattern of genes related to stress responses. We showed that exposure to high –relevant to human exposure-doses of this mixture of compounds induces hyperactivity and increases active coping in adult male mice, through deregulation of the Hypothalamus-Pituitary-Adrenals axis. These results indicate an association with equivalent phenotypes in patients with neurodevelopmental disorders and are influential for further investigation and prevention of the effects of EDCs.

Highlights:

- ✓ Prenatal exposure of mice to an epidemiologically-defined mixture of endocrine disruptors can modify their behavior and brain neurochemisty in adulthood
- ✓ Adult mice prenatally exposed to the N_1 mixture of endocrine disruptors display increased locomotion and active coping
- ✓ Adult mice prenatally exposed to the N_1 mixture of endocrine disruptors display increased levels in the relative expression of *Crh* in the hypothalamus and a reduction in the relative expression of *Nr3c1* in the hypothalamus as well as of *Crhr1* and *5htr1a* in the hippocampus, all of them being associated with deregulation of the HPA axis

Keywords: endocrine disruptors, neurodevelopment, hyperactivity, struggling, Nr3c1, Crhr1, 5htr1

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Introduction

Since 1962, when ***R.Carson*** published "Silent Spring", more and more people are being concerned about how some groups of chemical agents may interact and stall normal endocrine system responses and hormone-regulated events, such as behavior. These chemicals are characterized as Endocrine Disrupting Compounds (EDCs) and they include natural or synthetic exogenous substances or mixtures that affect the expression, biological actions and interactions of endogenous ligands by mimicking, blocking or displacing them, leading to adverse health effects in an organism or the following generations (*Zacharewski, 1998; Weiss, 2012; Bergman et al, 2013; Preciados et al, 2016*). Phytoestrogens, chemicals used as pesticides, such as DDT, fungicides, plastics, plasticizers, in pharmacy or industrial pollutants might contain phthalates, BPA, dioxins, alkylphenols or other chemical compounds that, either they or their metabolites, are found to emulate the structure and the action of hormones, such as estrogens or androgens, therefore preventing the natural performance of these molecules or aberrantly mimicking the result of their normal actions (*Roy et al, 1997; 2015; Ropero et al, 2006; Diamanti-Kandarakis et al, 2009; Aubert et al, 2012; Bergman et al, 2013; Zhang et al, 2013; Pinto et al, 2014; Xin et al, 2015; Preciados et al, 2016*).

Exposure to these compounds may occur through inhalation, digestion or dermal contact with contaminated air, water, food or items(*Birnbaum, 1994; Roy et al, 1997; Bergman et al, 2013; Preciados et al, 2016*). Moreover, most of these compounds cross both the placenta and the blood brain barrier, meaning that organisms can be exposed to them even prenatally, through an exposed mother (*Lopez-Espinoza et al, 2009; Masuo&Ishido, 2011; De Coster and van Larebeke, 2012; Bergman et al, 2013; Kajta andWojtowicz, 2013; Preciados et al, 2016*). Excretion of EDCs depends on whether they are non-persistent, meaning that they are metabolized in the liver, though in a low metabolic rate, and secreted through the feces and urine, or persistent, meaning that they are accumulated in the adipose deposits, including the breasts, from where through lactation they may pass to the offspring (*Lehmann et al, 2014; Derghal et al, 2016; Darbre, 2017; van der Berg et al, 2017*).

In fact, the most critical periods for long-term damages caused by environmental perturbations are the prenatal and early postnatal life periods, especially when examining the nervous system, while exposure to endocrine disruptors after maturity does not usually modulate permanently the hormone-related responses; the above consist the touchstone for the concept of "the fetal basis of adult diseases" (*Colborn et al, 1993; Barker et al, 2002; Gore, 2008; Mouritsen et al, 2010; Bao andSwaab, 2011; Walker & Gore, 2011; Weiss, 2012*).The transgenerational effects of EDCs eventuate from the alterations of epigenetic modifications that they may induce in a germline-dependent manner, when the epigenetic processes appertain to molecules of the sperm or ova, or in a germline-independent, context-dependent manner, when exposure to EDCs affects the maternal behavior leading to epigenetic alterations of offspring (*Crews et al, 2000; Crews, 2008; Wingfield, 2008; Sica et al, 2009; Walker and Gore, 2011; 2017*).

Endocrine disrupting compounds can bind on endogenous hormone receptors antagonizing or mimicking their actions, interfering with genes, proteins or enzymes important in hormone-regulated processes or they may act as mixtures, synergistically producing additive or complementary results and worsening adverse health effects (*Gilbert, 2005; Crain et al, 2008; Derghal et al, 2016; Darbre, 2017*). Based on the latter, the participants in the EDC-MixRisk project have explored, identified and examined some groups of chemicals that were found and analyzed in blood and urine of pregnant women that took part in the Swedish Environmental Longitudinal Mother and child, Asthma allergy (SELMA) cohort study and they associated them with three different aspects of health problems in their children regarding: 1) growth and metabolism, 2) neurodevelopment and 3) sexual differentiation

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(*http://edcmixrisk.ki.se; Bornehag et al, 2015, 2019; Repouskou et al, 2019*).The present study is geared toward the effects of the mixture of endocrine disrupting compounds that is associated with neurodevelopmental problems of the infants on the behavior and the neurochemistry of mouse brain.

Chemicals that can interfere with the proper function of the endocrine system, especially during development, may also affect the nervous system, since many hormones play a crucial role in brain development. Thyroid hormones' levels are essential for the survival, proliferation and synaptogenesis of hippocampal cells (*Rami and Rabie, 1990; Uchida et al, 2005; Zhang et al, 2009; Gong et al, 2010; Parent et al, 2011*). Gonadal hormones are required for the sexual differentiation of brain areas, such as the hypothalamus or the hippocampus, leading also to sexually dimorphic behaviors, for example different approach styles in social interaction or in spatial learning, respectively (*Williams and Meck, 1991; Roof and Havens, 1992; Schanz and Widholm, 2002*).Adrenal steroids, the glucocorticosteroids, also, regulate spatial learning and memory and the responsiveness of the hypothalamus-pituitary-adrenal axis to stress, being implicated in cell proliferation, degeneration, myelination and synaptogenesis (*de Kloet et al, 1988; Gould and Cameron, 1996; Schanz and Widholm, 2002*). Signalling of neurotransmitters, such as acetylcholine, dopamine, or serotonin, as well as their synthesis and metabolism, transport, uptake and reuptake, also attunes brain development and defects in these processes might lead to disorders, such as Attention-Deficit Hyperactivity Disorder (ADHD) or Autism's Spectrum Disorders (ASDs) (*Heyer and Meredith, 2017; Rock and Patisaul, 2018*). In order to examine how the aforementioned group of endocrine disruptors, involved in neurodevelopmental adversities in offspring of SELMA mothers, affects brain neurochemistry and sequentially behavior, we exposed pregnant mice dams throughout the gestation period to different doses of a refined mixture of these compounds (N_1 mix) and studied the behavior of their adult male offspring in a series of tests, including Elevated Plus Maze, Open Field, Novel Object Location and Forced Swim Stress. The outcomes of these experiments were that prenatally exposed male mice display increased mobility that is also reflected in the increased adoption of active coping strategies. Based on these observations, we sought for the transcriptional profile of these mice that could be associated to these behavioral alterations and we found that exposure to N_1 mix prenatally is involved in the deregulation of genes involved in the Hypothalamus-Pituitary-Adrenals (HPA)-mediated response to stress and the serotonin signaling.

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Methods

1. EXPERIMENTAL MODEL & SUBJECT DETAILS:

- **Animals:**

Drug- and test-naïve 2-months old male and female C57BL/6 mice, purchased from the Hellenic Pasteur Institute (*Athens, Greece*) and allowed to acclimate for 2 weeks, were used as breeders. They were maintained in polypropylene cages, in controlled temperature (22±1^{o}C) and humidity (40-60%) conditions under a 12-hour light-dark cycle (0700-1900: light period). Food (4% fat, phytoestrogen-free, *Altromin 1324 P, Lage, Germany*) and tap water were provided *ad libitum*. One adult female and one adult male C57BL/6J mouse were housed together for 5 days in order for them to mate. On the 5th day, the pair was separated and the male mouse was housed again with sibling male mice, while the female remained in single-housing.

N_1 Mixture of endocrine disruptors:

Based on the analysis of serum and urine of pregnant women of the SELMA cohort, a group of molecules was identified to be associated with neurodevelopmental problems in their male progeny, presented as language delay and cognitive difficulties, and a refined mixture of bisphenols, phthalates and pesticides was composed for use in experimental animal models (*http://edcmixrisk.ki.se*). The initial mixture was diluted in DMSO, aliquoted and stored at -20^{o}C.

From the first morning after the beginning of the breeding period to the end of the gestation period, meaning the day the dam gave birth to the litter of pups (PostNatal Day 0- PND0), the dams were given daily an organic cornflake saturated either with vehicle, (0.2% DMSO in PBS) or the N_1 mixture of endocrine disruptors diluted in vehicle (0.2% DMSO in PBS)in different concentrations, corresponding to multiples of the geometric mean of these chemicals found in women sera and urine.

A total of 5 groups of treatment have been employed:

1) DMSO
2) 0.5x SELMA mother levels
3) 10x SELMA mother levels
4) 100x SELMA mother levels
5) 500x SELMA mother levels.

Each dam was randomly allocated to a treatment group. The body weight of the dams was recorded every three days in order to verify the pregnancy and to adjust the volume of the mixture provided.

All animal handling and experiments were conducted in accordance to the European Communities Council Directive of 22 September 2010 (2010/63/EU) and the experimental protocol was approved by the Ethical Committee of the Prefecture of Attica-Veterinary department (#4784-11/9/2017)

2. METHOD DETAILS:

- **Behavioral tasks:**

90-days old male C57BL/6 mice, progenies of dams exposed to either DMSO or to different concentrations of the N_1 mixture of endocrine disruptors, were used for the behavioral tests, that included elevated plus maze (EPM), open field (OF), novel object location (NOL) and forced swim stress(FSS). All behavioral tests were performed during the light period in a specific equipped room in which the temperature and the humidity were adjusted to the housing conditions of the mice. The behavioral tests were performed in the aforementioned order progressing from the mildest to the most stressful event. Behaviors were recorded using an Everio GZ-MS110 Memory Camera (*JVC*) and the recorded videos were analyzed by three different observers, blind to the treatment of each subject and also by using NOLDUS software to track the locomotion of the subjects in OF.

The ***elevated plus maze test*** takes place on a cross-shaped platform 60 centimeters above the ground that is composed by two opposing "open" and two opposing "enclosed" arms. The "enclosed" arms are surrounded by 40 cm tall mirroring walls, while the "open" ones do not have walls. Mice are placed on the intersection of the four arms and are free to move to whichever direction for five minutes. Based on their instinct, mice are more prone to move to the enclosed corridors where they would be more protected, but some may also dare to explore the "open" ones. The number of entries in either the "enclosed" or the "open" arms and the time, counted in seconds, that they spend in each type of arms are indicative of the level of anxiety of the mice. Less anxious mice tend to attempt more entries and spend more time in the "open" arms, while the more anxious ones remain longer in the "enclosed" arms.

The ***open field test*** is another experimental procedure that deals with the opposing drives of mice to explore new environments and to assure their protection. This test takes place in a square box open at the top in which the mouse is placed and left to move freely for five minutes. We focus our interest on the motility of the mouse mirroring the locomotor condition of the subject and on the time that it spends either at the central space or near the edges, assessing the anxiety levels, as less anxious mice would spend more time in an open space where they would be more vulnerable to predators. Another behavior that is being studied in this test is the eagerness of mice to stand on their hind legs, called rearing. There are two types of rearing; the supported one, otherwise called wall-leaning that is related to locomotion and activity studies and the unsupported rearing, where the animal instantly stands on its hind legs without any support and that is negatively related to anxiety (*Whimbey & Denenberg, 1967; Crusio et al, 1989; Lever et al, 2006; Sturman et al, 2018*)

Another assessment of mice innate novelty preference, but also of their spatial learning and memory is the ***Novel Object Location*** (NOL) test. As the abovementioned ones, this test is performed in a square box open at the top where two identical objects (tea strainers in our experiment) are placed one on the upper left quadrant (as viewed in the video image) and one on the upper right quadrant of the floor, in equal distances from the walls, and the mouse is left to freely explore them for five minutes. The mouse is rehoused in its cage and ninety minutes later, it is returned in the test area, where one of the two objects is "misplaced", and left again to explore the two objects for five minutes. This test examines the ability of the mouse to comprehend that one of the two objects is placed in

a novel location compared to the previous trial and whether this novel location would be more interesting for the mouse to explore. To assist the navigation of the mouse, a sign is placed on the wall that is next to the object that is being "misplaced" (black tape). The observer records the time that the mouse spends exploring the object that does not change location or the one that does and a preference index is calculated according to the following formula: (Time exploring the misplaced object)/(Time exploring the misplaced object + Time exploring the non-misplaced object).

The last behavioral test that was conducted with these mice was the ***Forced Swim Stress***(FSS). Each mouse is placed in a large glass transparent beaker that contains enough tap water in order for the mouse not to reach the bottom and is left to act freely for ten minutes. The behavior of the mouse during forced swim stress is sorted into three types: "floating", when it only moves one leg in order to remain on the surface, "swimming", when it moves more than one leg and it gets around having its body mostly in a horizontal position and "struggling", when it swims against the glass, having its body mostly in a vertical position and rippling the water with its front legs. The observer records the duration of each one of the aforementioned behaviors. The mouse may adopt an active or a passive coping strategy, depending on whether it spends more time struggling or floating respectively.

Sample collection:

Thirty minutes after the initiation of the FSS test, the mice were euthanized, using isoflurane for inducing deep anesthesia and then decapitated. Trunk blood was collected for hormone level measurements, the adrenal glands and the pituitaries were carefully removed and kept frozen for gene expression analyses and the brains were carefully removed from the skull; the two hemispheres were separated and the right one was snap-frozen in 2-methyl butane, in order to be used for immunostaining assays whereas the left one was further dissected into subregions (prefrontal cortex, hippocampus, amygdala, hypothalamus) and used for gene expression analyses.

- **Biological assays:**

For the present study the pituitaries and the brain regions of the left hemisphere were used for relative gene expression analyses. Total DNA, RNA and proteins were extracted using the NucleoSpin®TriPrep kit (*MACHEREY-NAGEL*). The concentration and the purity of the nucleic acids were measured using spectrophotometry (*BioSpec-nano Life Science, Shimadzu*). First strand cDNA was synthetized using FIREScript RT cDNA Synthesis KIT (*Solis BioDyne*) in order for the final cDNA concentration to be 0.5-0.7μg. cDNA products were diluted and used for quantitative real-time polymerase chain reaction (RT-qPCR). 96-well plates containing duplicates of each diluted sample were prepared using SYBR Select Master Mix (*Applied Biosystems*) and were performed in an Mx3005P Real-Time PCR System (*Agilent*) using primers for each gene of interest purchased from PrimerBank (Table 1). We applied the protocol of polymerase chain reaction recommended in SYBR Select Master Mix manual, but we adjusted the temperature of the annealing step to the melting temperature (T_m)of each set of primers. Melting curve analysis was used to assess the purity of the PCR amplicon. The results were taken as raw data, the level of expression

of each gene of interest was normalized using b-actin as an internal reference gene. The relative expression of the gene of interest to b-actin in the different experimental groups in comparison to the control group was calculated using the $2^{-\Delta\Delta CT}$ method (*Livak and Schmittgen, 2001*).

Gene	***Forward primer (5'-3')***	***Reverse primer (5'-3')***	***T annealing***	***Primer Bank ID / Reference***
Crhr1	GGAACCTCATCTCGGCTTTCA	GTTACGTGGAAGTAGTTGTAGGC	59°C	6681013a1
Crh	GAGGCATCCTGAGAGAAGTCC	GTTAGGGGCGCTCTCTTCTC	60°C	This study
Nr3c1	GGACCACCTCCCAAACTCTG	GCTGTCCTTCCACTGCTCTT	60°C	This study
Htr1a	GGATGTTTTCCTGTCCTGGT	CACAAGGCCTTTCCAGAACT	59°C	*Chiavegatto et al, 2010*
Htr2a	AGAACCCCATTCACCATAGC	ATCCTGTAGCCCGAAGACTG	59°C	*Chiavegatto et al, 2010*
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT	59°C	6671509a1

Table 1: List of primers used in the present study

3. QUANTIFICATION & STATISTICAL ANALYSIS:

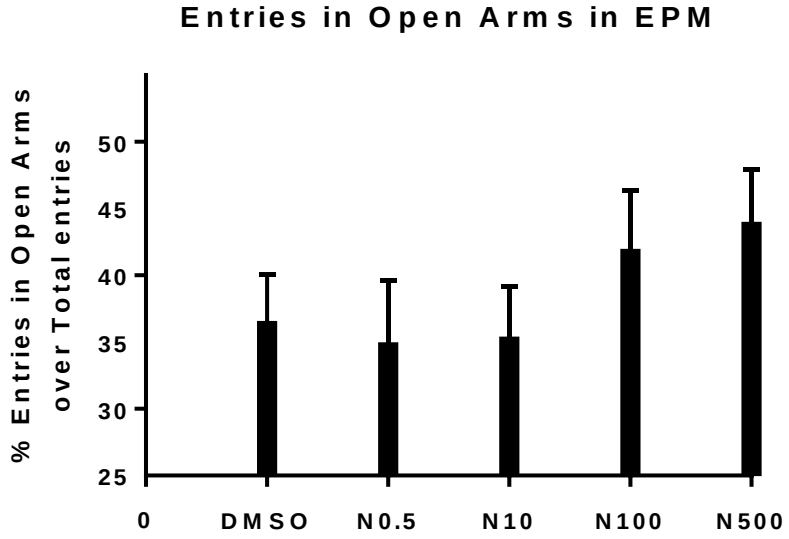
Statistical analysis was performed using the SPSS software "IBM Statistics version 21". We statistically analyzed animal behaviors with Generalized linear estimate models in which the litter was nested within the treatment followed by post-hoc Sequential Bonferroni pairwise comparisons. The statistical analysis of relative gene expression was assessed with One-Way ANOVA followed by 2-sided Dunnet's post-hoc multiple comparisons. Statistical details can be found in the "Results" section and in the legends of figures.

Results

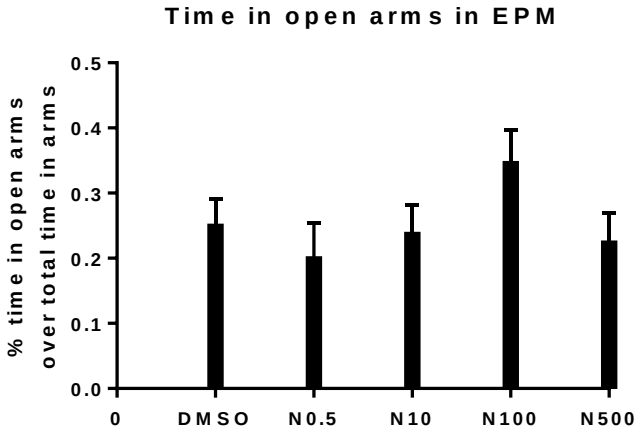
1. Lack of effect of prenatal exposure to N_1 EDC mix on the performance of mice in the Elevated Plus Maze

Mice are nocturnal animals that are often a prey for larger predators. Therefore, they have the innate tendency to prefer darker and more protected sites to open, elevated and illuminated ones. In this regard, we assessed the performance of adult offspring of dams exposed to N_1 EDC mix during pregnancy, in the Elevated Plus Maze test. Using this test, we intended to investigate whether the neuroendocrine disruptors we administered to the mothers have enhanced or decreased the preference of their offspring for the "safer" closed arms over the more "dangerous" open ones; to this end we evaluated the number of entries and the time spent in each type of arm, and their eagerness to perform risky behaviors, as assessed by the number of head dippings. However, there was no statistically significant difference among these parameters between prenatally exposed and control mice (Fig. 1A,B,C)

A



B



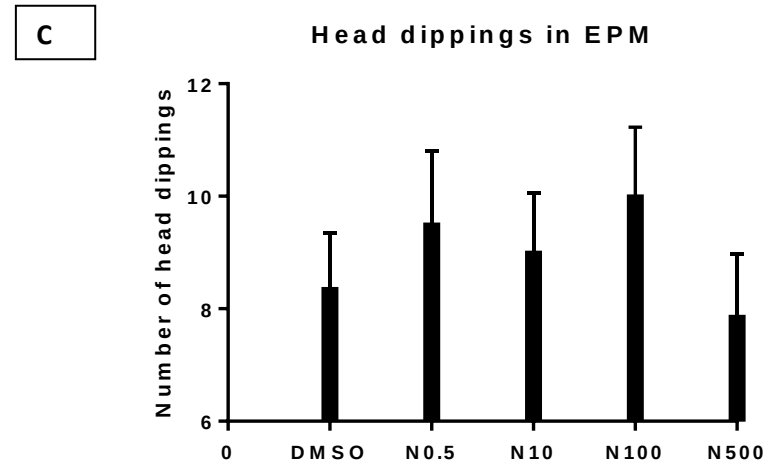


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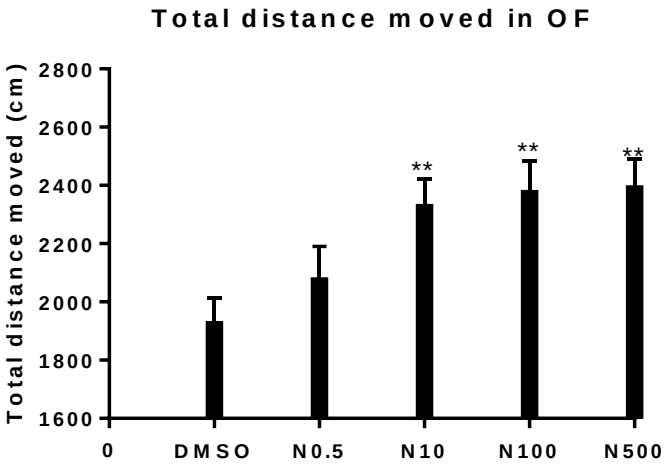
Figure 1:Lack of effect of endocrine disruptors on the performance of EDC-prenatally exposed mice in the Elevated Plus Maze. Prenatal exposure to N_1 mix did not affect mice performance in EPM as estimated by the percentage of entries (A, W=71.477, p=0.000) and time spent (B, W=47.662, p=0.016) in the open arms and the number of head dippings (C, W=2.185, p=0.702). Bars represent the estimated marginal means ± SEM of each of the aforementioned parameters. Generalized linear estimate models in which the litter was nested within the treatment followed by post-hoc Sequential Bonferroni pairwise comparisons. Number of animals used: 10-14 per group.

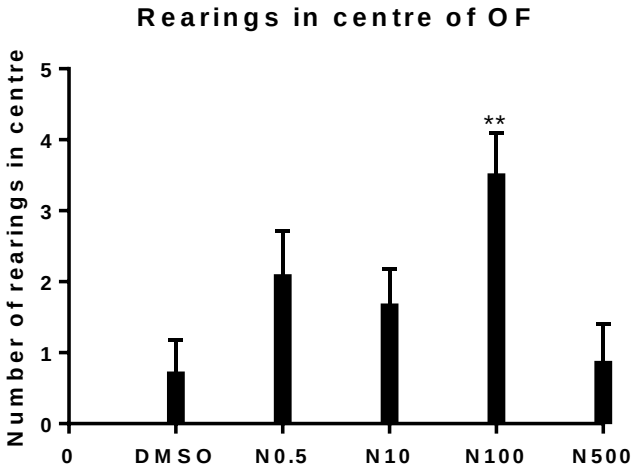
2. Increased mobility observed in mice prenatally exposed to N_1 EDC mix in the Open Field

On the second day of the battery of behavioral tests, mice were freely left to explore an Open Field. Not only does this test assess the tendency of mice to become vulnerable to potential predators and explore unfamiliar environments, but also their locomotor activity, depending on the distance they traverse during the test session. Interestingly, mice prenatally exposed to 10x, 100x and 500x SELMA levels of N1 mix showed a statistically significant increase in mobility ($p<0,01$), approximately 20% above the mean distance travelled by the control mice. Moreover, the 100x group of prenatally exposed mice displayed increased number of rearings in the centre of the arena compared to the control group ($p<0,01$), though the total number of rearings was not statistically different than that of the control group, indicating that mice prenatally exposed to 100x SELMA levels of N1 mix might be less afraid to be exposed. (Fig. 2A, B, C)

A



B



C

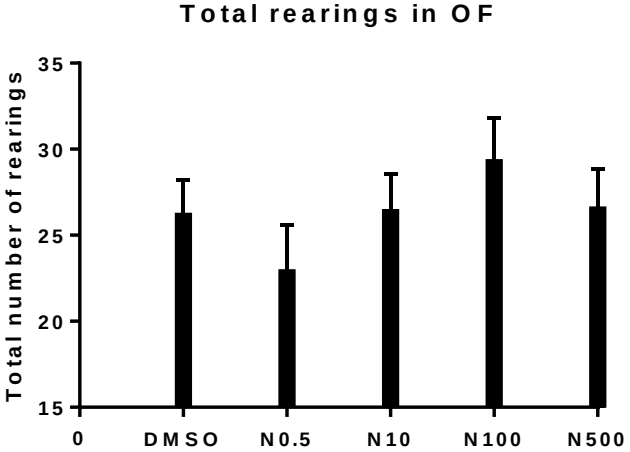


Figure 2:Increased mobility observed in mice prenatally exposed to N_1 mix. Hyperactivity of mice prenatally exposed to N_1 mix of endocrine disruptors shown by increased total distance travelled (A, W=20.485, p=0.000) and increased number of rearings in the centre (B, W=16.106, p=0.003), not reflected to the total number of rearings (C, W=3.126, p=0.537) in the Open Field. Bars represent the estimated marginal means ± SEM of each of the aforementioned parameters. Generalized linear estimate models in which the litter was nested within the treatment followed by post-hoc Sequential Bonferroni pairwise comparisons. Number of animals used: 10-14 per group.

3. No difference in discrimination of a relocated and a stationary object between N1 mix-exposed and unexposed mice

In an attempt to examine mice's spatial learning and memory as well as novelty preference, we tested them in a Novel Object Location paradigm. They were left to explore a steady object and an object that after a familiarization trial will be relocated in another place of the arena. Mice are novelty seekers and we would expect that they would spend more time exploring the misplaced object perceiving it as a novel one, as long as they can comprehend the change of position and discriminate it from the stationary one. For that reason, we calculated the ratio of the time mice spent with the misplaced object to the total time they spent exploring both objects after the familiarization trial and we refer to it as "discrimination index". The comparison of the discrimination indices of the prenatally exposed and unexposed mice showed that there was no statistically significant difference between them (Fig. 3). This means that either the exposure to N_1 mix of EDCs prenatally does not alter the novelty preference and spatial memory of the offspring or that the object relocation was not salient enough to interest them.

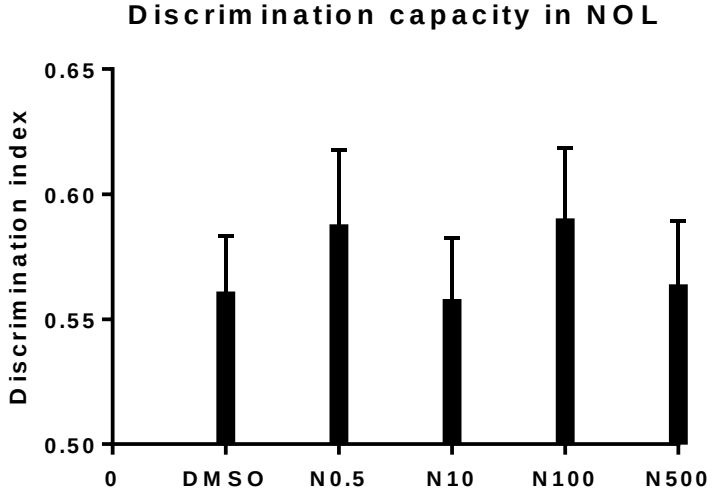


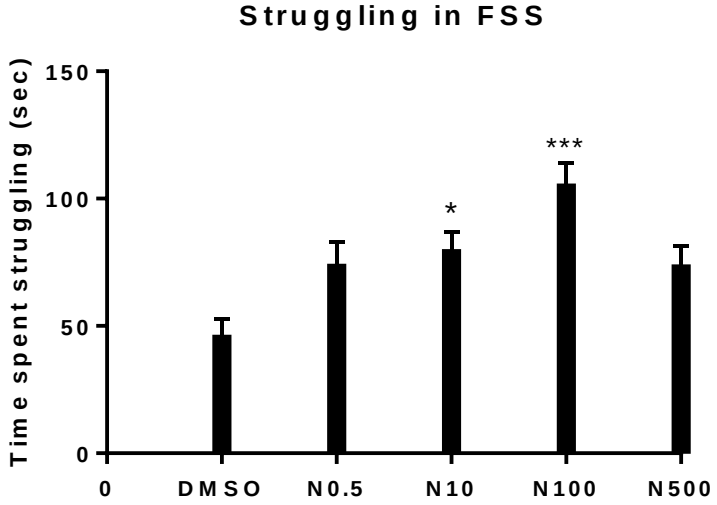
Figure 3:No difference in discrimination of a relocated and a stationary object between mixture N1-exposed and unexposed mice. No statistical difference in the ability to discriminate and the willingness to explore a misplaced object rather than a steady one between exposed and control mice. Bars represent the estimated marginal means ± SEM of the discrimination index. W=1.264, p=0.868 Generalized linear estimate model in which the litter was nested within the treatment followed by post-hoc Sequential Bonferroni pairwise comparisons. Number of animals used: 10-14 per group.

4. Increased active coping in FSS for mice prenatally exposed to N_1 mix

The final behavioral test for the adult offspring was the Forced Swim stress in order to investigate the coping style of each group during an inescapable stress i.e. swimming stress. Mice that spend more time struggling during the FSS seem to adopt a more active coping strategy, while the ones that prefer floating seem to have a more passive coping style. In our experiments, the 10x and 100x SELMA levels

treated groups displayed a statistically significant higher degree of active coping (struggling) compared to the control group, while there was no statistically significant difference in passive coping (floating) between the treatment groups and the control group (Fig. 4A, B)

A



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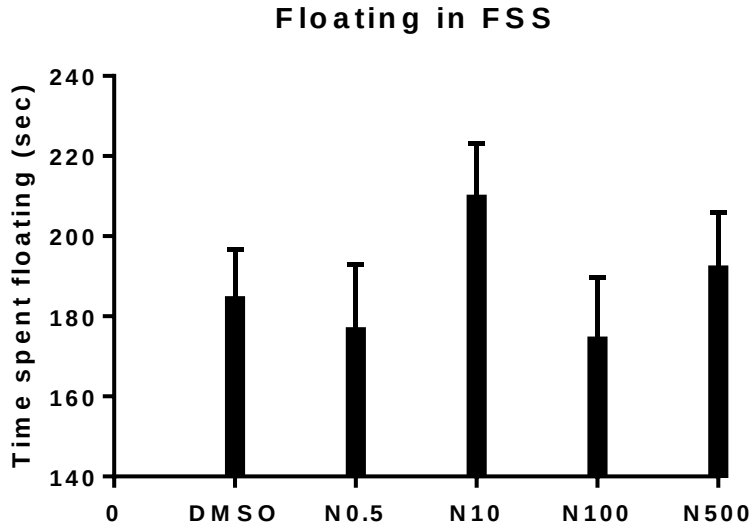
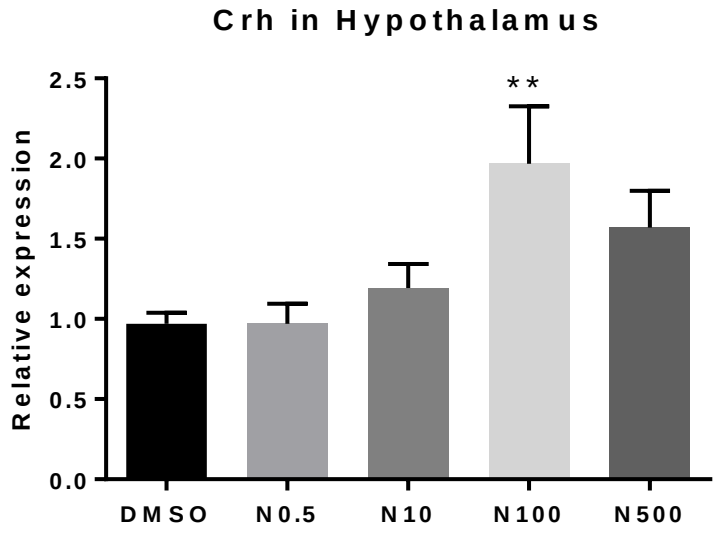


Figure 4:Increased active coping in FSS for mice prenatally exposed to N_1 EDC mix. Mice prenatally exposed to N_1 mix assume more active coping strategy, reflected in struggling in FSS (A, W=28.810, p=0.000), but do not show any difference in passive coping, i.e. floating, compared to the control group (B, W=4.161, p=0.385). Bars represent the estimated marginal means ± SEM of each of the aforementioned parameters. Generalized linear estimate models in which the litter was nested within the treatment followed by post-hoc Sequential Bonferroni pairwise comparisons. Number of animals used: 10-14 per group.*p < 0.05, ***p<0.001

5. Altered expression of genes related to stress response in the hypothalamus of male mice prenatally exposed to N_1 mix

After the euthanization of the mice, their brains were collected, separated into two hemispheres and one of them was further dissected into subregions in order to examine whether the differences observed in behavioral tests were reflected on alterations –at the transcriptional level- of some genes of interest. One of the regions of concern was the hypothalamus, a unit of the tripartite axis, Hypothalamus-Pituitary-Adrenals (HPA), since most of the behavioral tests that were included in the abovementioned battery dealt with the response of the animals to stress and their coping strategy. The genes of interest examined in this region were the *Crh*, encoding the corticotropin-releasing hormone (CRH) and the *Nr3c1*, encoding the glucocorticoid receptor (GR). The expression of these genes was altered in the hypothalamus of mice exposed prenatally to the higher doses of the N_1 mixused. Specifically, the relative expression of *Crh* was significantly increased in the 100x group and that of *Nr3c1* showed a statistically significant decrease in the 500x group. (Fig. 5A, B)

A



B

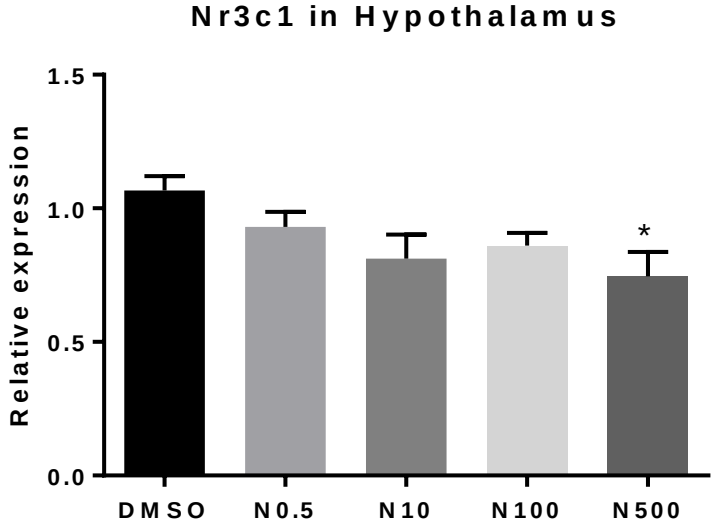
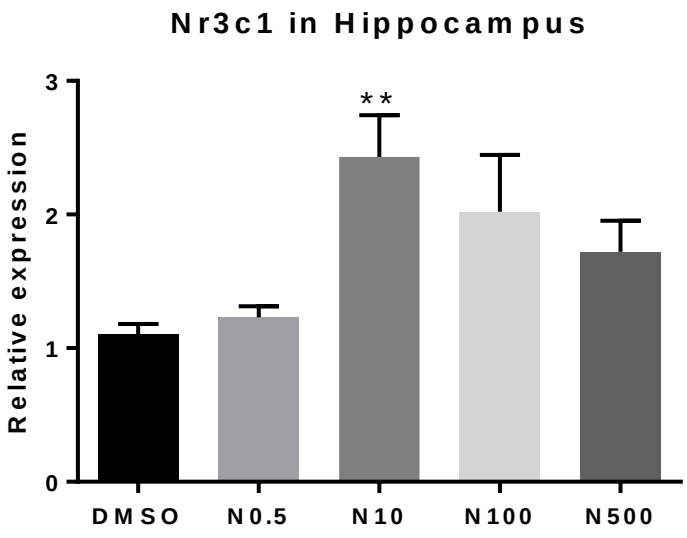


Figure 5:Alteration in relative expression of genes related to stress response in the hypothalamus of mice prenatally exposed to N_1 mix. (A) *Crh* levels were increased in the hypothalamus of mice prenatally exposed to 100x SELMA levels of the EDC mix, increased by 203% (One-way ANOVA, $F_{4,29}$=3.83, p=0.015, 2-sided Dunnett's post-hoc multiple comparisons, p=0.009). (B) *Nr3c1* levels were decreased in the hypothalami of mice prenatally exposed to 500x SELMA levels of N_1 mix, 30% reduction (One-way ANOVA, $F_{4,29}$=3.121, p=0.033, 2-sided Dunnett's post-hoc multiple comparisons, p=0.010). The expression of each gene of interest has been normalized to the expression of b-actin, used as a reference gene. Bars represent the means ± SEM of each of the relative expression of each of the aforementioned genes . Number of animals used: 10-14. *p<0.05, **p<0.01

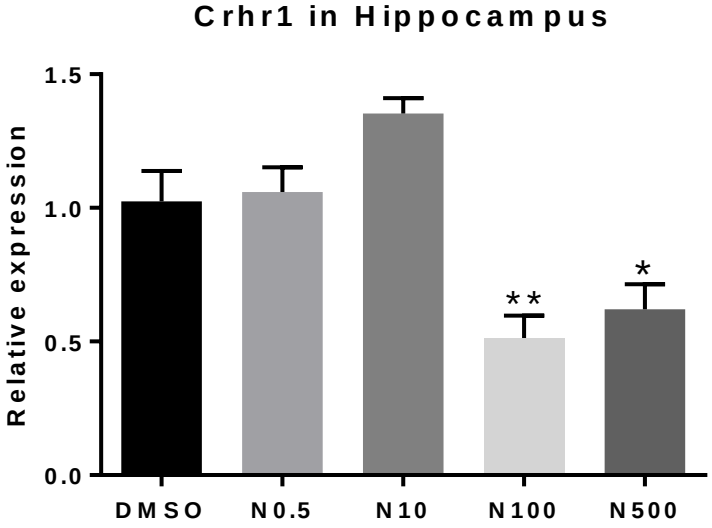
6. Prenatal exposure toN_1 mix modulates the expression of stress response related genes in adult offspring hippocampus

Another brain region that is implicated in the response of mice to stressful stimuli is the hippocampus. In this area, we addressed our interest to a gene that we previously discussed regarding the hypothalamus, *Nr3c1*, but also to some other genes, namely the genes encoding for the receptor of CRH, corticotropin releasing hormone receptor 1 (*Crhr1*) and the receptors of serotonin, 5-hydroxytryptophan receptors 1A and 2A (*5htr1a* and *5htr2a*). Among the aforementioned genes, the ones that showed statistically significant difference in relative expression between some of the prenatally exposed groups and the control group were the *Nr3c1*, the *Crhr1* and the *5htr1a* genes. The *Nr3c1* was elevated in the 10x treatment group and the *Crhr1* and the *5htr1a* were reduced in both the 100x and the 500x groups. The levels of *5htr2a* were not statistically significantly different between the prenatally exposed animals and the control animals. (Fig. 6A, B, C, D)

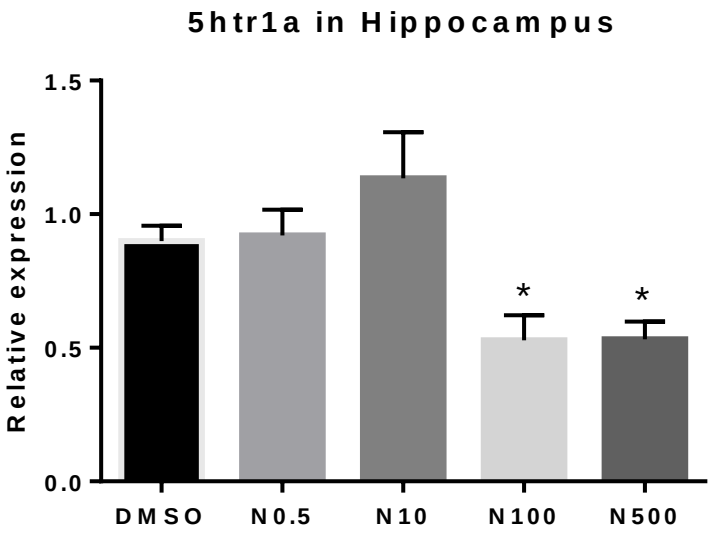
A



B



C



D

Figure 6: Prenatal exposure to N_1 mix modulates the expression of stress response related genes in the hippocampus of mice prenatally exposed to N_1 mix. (A) *Nr3c1* levels were increased in the hippocampus of mice prenatally exposed to 10x SELMA levels of the EDC mix, 220% increase (One-way ANOVA, $F_{4,29}$=4.113, p=0.011, 2-sided Dunnett's post-hoc multiple comparisons, p=0.005). (B) *Crhr1*levels were decreased in the hippocampus of mice prenatally exposed to 100x and 500x SELMA levels of the EDC mix, 50% and 40% reduction respectively (One-way ANOVA, $F_{4,29}$=13.551, p<0.001, 2-sided Dunnett's post-hoc multiple comparisons, p=0.002, p=0.011). (C) *5htr1a* expression was reduced in the hippocampus of mice prenatally exposed to 100x and 500x SELMA levels of N_1 mix, 37% decrease in both cases (One-way ANOVA, $F_{4,29}$=6.904, p=0.001, 2-sided Dunnett's post-hoc multiple comparisons, p=0.046, p=0.038). The expression of each gene of interest has been normalized to the expression of b-actin, used as a reference gene. Bars represent the means ± SEM of each of the relative expression of each of the aforementioned genes. Number of animals used: 10-14. *p<0.05, **p<0.01

Title: Effects of prenatal exposure to endocrine disruptors in mice behavior and brain neurochemistry
Student's name: Anastasia-Konstantina Papadopoulou

Parameters	**N0.5**	**N10**	**N100**	**N500**
EPM	⟺	⟺	⟺	⟺
OF	⟺	Increased locomotion	➢ Increased locomotion ➢ Increased rearings in the centre	Increased locomotion
NOL	⟺	⟺	⟺	⟺
FSS	⟺	Increased struggling (active coping)	Increased struggling (active coping)	⟺
Hypothalamus	⟺	⟺	↑ *Crh*	↓ *Nr3c1*
Hippocampus	⟺	↑ *Nr3c1*	↓ *Crhr1, 5htr1a*	↓ *Crhr1, 5htr1a*

Discussion

Endocrine disrupting chemicals (EDCs) are being found in progressively more products and are being associated with an increasing number of health adversities, such as cancers or neurological disorders. Among the latter, most of them concern the development of the nervous system, for instance autism spectrum disorders (ASD), attention deficit and hyperactivity disorder (ADHD) or learning disabilities (*Bergman et al, 2013; Kajta and Wojtowicz, 2013; Preciados et al, 2016*). In the SELMA pregnancy cohort study, a group of endocrine disruptors detected in the sera and urine of pregnant women, including bisphenols, phthalates and pesticides, was associated with neurodevelopmental problems of the offspring (*http://edcmixrisk.ki.se*). In the present study, the effects of a refined mixture(N_1 mixture) of the abovementioned EDCs in the ratio detected in SELMA pregnant women, were tested in mice. It appears that N_1 mixture affects mice neurodevelopment as reflected in their behavior and neuronal gene expression observed in adulthood.

Increased locomotion and active stress coping strategy in mice prenatally exposed to N_1 mixture

Results collected from the battery of behavioral tests that prenatally exposed to N_1 mix adult male mice went through, showed that the exposure to EDCs at levels equal or higher than 10-fold the geometric mean levels found in SELMA study led to increased mobility, indicative of hyperactivity. Hyperactivity is by definition a trait of ADHD, but it is also apparent in other neurodevelopmental disorder models of rodents, such as mania, schizophrenia and ASD (*Yen et al, 2013; Degroote et al, 2014*). In fact, Degroote et al (*2014*) showed that perinatal exposure to another mixture of endocrine disruptors containing Polybrominated Diphenyl Ethers (PBDEs), used as flame retardants, and phthalates can induce in rats the hyperactive phenotype that is evident in valproic acid-induced ASD-like model in rodents. Moreover, prenatal and perinatal exposure to bisphenol A is positively associated with hyperactivity both in childhood and in adulthood in rodents and in humans (*Rochester et al, 2018*).

Another behavioral task that provided evidence of differences between the prenatally exposed to N_1 mix mice and the control ones was the Forced Swim Stress. FSS is not a test that indicates a depression-like behavior as claimed in the past, but rather a mean to measure the coping strategies of rodents to an acute and inescapable stressor (Commons et al, 2017).We tend to classify movement of rodents in the water during FSS into three types: (a) floating, when they remain immobile, but barely move one hind leg in order not to drown, (b) swimming, when they move more than one legs vividly and (c) struggling, when they swim against the walls of the container on a vertical position and ripple the water with their front legs. Among them, floating is considered as a passive stress coping strategy and struggling is accounted as an active stress coping style. Normally, when the mice are exposed to this task, they initially perform swimming and struggling and progressively become more passive (*Commons et al, 2017*). In the present study, it is shown that mice prenatally exposed to 10-fold and 100-fold SELMA levels of the N_1 mix of endocrine disruptors adopt a more active coping strategy, reflected in increased duration of struggling, that seems to be moderated in the group exposed to the highest dose, though in a statistically non-significant manner. Active coppers seem to be more rigid, than passive coppers and persistent struggling can be associated to the repetitive behaviors of ASD, while increased active coping is also a common trait of mania and ADHD (*Hawley et al, 2010*; *Yen et al, 2013; Commons et al, 2017*). In effect, we could conclude that increased struggling results from a generalized hyperactivity, as it was also suggested for other analogous findings in the literature, for example in the research of Warden et al (*2012*) where stimulation of the dorsal raphe nucleus led to the increase of both locomotor activity in the OF and active coping in the FST at a serotonergic-dependent manner

Increased mobility, but also increased active coping style preference is observed in low anxiety-related behavior (LAB) CD1 mouse line, that is considered as "non-anxious" (*Ohl et al, 2002; Krömer et al, 2005; Yen et al, 2013*). In our study, low levels of anxiety can be supported by the lack of effects of mixture N_1 in the EPM and could be also related with the observation that the 100x group of mice are more eager to stand on their hind legs (rearing) in the centre of an open field, despite being "unprotected", in our study. However, this increase in rearing attempts might be a corollary of the hyperactivity phenotype of these mice. Overall, based on the outcomes of our research study, we can assume that prenatal exposure to the N1 mixture of endocrine disruptors can interfere with the control of the mobility during the development of the male organism leading to excessive motion and alertness in adulthood, reflecting the equivalent symptoms of patients with neurodevelopmental disorders. Therefore, further investigation of other phenotypes related to these disorders could lead in a more well-supported causal effect of neuroendocrine disruptors on hyperactivity.

Prenatal exposure to the N_1 mix of endocrine disruptors garbles HPA axis responses

One of the two principle systems that induces and controls stress responses is the Hypothalamus-Pituitary-Adrenals (HPA) axis. In the presence of a threatening or stressful stimulus, the HPA axis is activated and certain hypothalamic neurons synthesize and secrete the corticotropin-releasing hormone (CRH), that sequentially binds on specific receptors, especially the type 1 corticotropin-releasing hormone receptors (CRHR1) that are located on several regions throughout the brain, including the pituitary from where a signal is transmitted to the adrenal cortex inducing the synthesis and secretion of glucocorticoids (*Uht, 2012*). Glucocorticoids occupy two types of receptors depending on their levels; melanocorticoid receptors (MRs), even at low hormone levels and glucocorticoid receptors (GRs), at high levels, for example during stress (*de Kloet et al, 2005; Scharf et al, 2011*). GRs are found in hypothalamic neurons expressing CRH and act by negatively modulating the transcription of the corresponding gene (*Evans et al, 2013*).

Our finding of increased *Crh* expression in the hypothalamus of adult offspring of the 100x group imply an innate hyperactivity of the HPA axis, which is reflected on a generalized locomotor hyperactivity, shown by increased mobility, rearing attempts and active coping strategy during FSS. Another observation regarding HPA axis-related gene expression in the hypothalamus is that *Nr3c1*, the GR encoding gene. This gene expression is downregulated in the hypothalamus of mice prenatally exposed to the 500x dose of mixture N1. A decrease in GR levels could explain the increase in CRH expression, since the negative feedback loop between them seems to be corrupted. In addition, we could deem the downregulation of the target receptors of CRH, CRHR1, in the hippocampus a compensatory mechanism of the brain in order to mitigate the effects of the elevated expression of CRH in the hypothalamus. These findings are in accordance with a recent study by Lopez-Rodriguez et al (*2019*), who showed that perinatal exposure to various endocrine disruptors, including bisphenols, phthalates and pesticides led to similar deregulations of the expression of the aforementioned genes accompanied by corresponding alterations in the methylation patterns of these genes in the hypothalamus of the F1 and F3 generations of female rats. Thus, it would be of interest to future study the epigenetic modification profile of the deregulated genes in order to define the effects of this mix of endocrine disruptors both at a genetic and at an epigenetic level.

Another potential compensatory mechanism for the hyperactivity of HPA axis through the hippocampus might be the increase of Nr3c1 expression in mice prenatally exposed to 10x SELMA levels, since GR activity in the hippocampus acts as a negative feedback for HPA axis response (*Goel and Bale, 2009*). Also, by comparing the graphs of the duration of struggling in FSS (Fig. 4A) and that of the relative expression of

Nr3c1 in the hippocampus (Fig.6A), we can observe that both of them have an inverted-U shape in their dose-response relation. It has been shown that glucocorticoids can have biphasic effects, leading to an inverted-U shape dose-response curves of their receptors and potentially of other factors regulated by them (*Ebner and Singerwald, 2017);* although, there are no previous findings in the literature for an association of GR upregulation in the hippocampus and increased active coping in FSS.

The shortfall in *5htr1a* expression might contribute to active coping selection

It is hypothesized that 5HTR1A activation favors passive coping strategies, while 5HTR2A is involved in active stress coping (*Puglisi-Allegra and Andolina, 2015; Carhart-Harris and Nutt, 2017*). Based our findings, *5htr1a* expression is significantly decreased in the hippocampus of mice prenatally exposed to 100x and 500x SELMA levels of the N1 mix, while 5htr2a expression does not show any statistically significant difference between the prenatally exposed animals and the control ones. These results should be further investigated through protein analyses in the future.

All the aforementioned findings coalesce to the need of increase in awareness of the adverse effects of the exposure to EDCs, found in products of everyday use, especially during the critical period of pregnancy and might lead to the emergence of neurodevelopmental disorders. Moreover, it is important for governments and councils to establish stricter legislature on the permitted limits of industrial use and exposure to EDCs. Otherwise, "the chemical war is never won and all life is caught in its crossfire" as R. Carson quoted.

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