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Effects of prenatal stress exposure on the reproductive behavior in mice

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Résumé

Le stress chronique est une source croissante de préoccupation dans la société moderne. Bien que le stress soit un terme difficile à définir, chaque personne vivante connait le stress et est y confronté à un moment donné de sa vie. Initialement, le stress est considéré comme un mécanisme évolutif permettant l'adaptation à des situations urgentes. Cependant, dans le mode de vie occidental, le stress est devenu un fardeau, qui est souvent associé à la maladie, y compris la perturbation mentale et comportementale. De plus, le stress semble avoir des effets très variés, affectant même la descendance des personnes stressées. Par exemple, la reproduction et le comportement sexuel, qui sont essentiels à la survie de l'espèce, ont été signalés comme étant altérés chez les enfants stressés avant la naissance. Sur la base de ces observations, nous avons étudié chez un modèle murin les effets permanents du stress prénatal sur le comportement sexuel de la progéniture féminine et masculine à l'âge adulte.

Pour induire un stress prénatal, des femelles gestantes ont été soumises à un stress par contention du deuxième jour de gestation jusqu'à mise bas. À l'âge adulte (8 semaines), l'ensemble des souris ont été soumis à différents test comportementaux. Les souris ont été soumises au elevated plus maze test (EPM) et à un prélèvement sanguin, afin d'évaluer les manifestations comportementales et physiologiques du stress. Des échantillons de sang ont été prélevés dans deux conditions, la cage d'hébergement et 30 minutes après l'EPM pour évaluer le fonctionnement de l'axe HPA par mesure de la corticostérone et la DHEA. Des frottis vaginaux ont été effectué sur des femelles pour étudier leur cycle œstral. La performance sexuelle a été testé chez les femelles et les mâles, puis un test de préférence de partenaire a été effectué sur les deux sexes pour évaluer la préférence sexuelle.

Nos résultats montrent que l'anxiété et les niveaux des hormones de stress (corticostérone et DHEA) n'étaient pas particulièrement altérées chez les femelles ayant subis un stress prénatal, tandis que les mâles semblaient avoir développé une résistance et une réduction de la réactivité au stress. En effet nous avons observé une faible réponse de corticostérone suite à l'exposition à l'EPM et un faible niveau basal de la DHEA. En ce qui concerne le comportement sexuel, les femelles et les males qui ont été exposé au stress prénatal ont montré des performances sexuelles similaires à celles du groupe témoin. En outre, nous avons noté que le stress prénatal avait un impact significatif sur la préférence sexuelle chez les femelles et les mâles. Ces observations montrent non seulement que le stress prénatal a eu un impact sur la progéniture mâle et femelle, mais aussi que certains effets étaient sexuellement dimorphes.

En conclusion, le stress prénatal est une question de plus en plus complexe qui entraîne des répercussions générationnelles. Dans notre étude, nous avons choisi d'évaluer l'impact du stress prénatal chez la progéniture féminine et masculine. Alors que le comportement anxieux et la performance sexuelle semblent être altérés uniquement chez les mâles, la préférence sexuelle semblait défectueuse dans les deux sexes.

Summary

Chronic stress is an increasing cause for concern in modern society. Stress is an evolutionary mechanism allowing adaptation to urgent situations. However, in western lifestyle, stress has become a burden, which is often associated to disease, including mental and behavioral disruption. Additionally, stress seems having a wide range of effects that could impact the offspring as well. For instance, impaired reproduction and sexual behavior have been reported to be the possible result of exposure to prenatal stress. Based on this, we aimed to investigate the permanent effects of prenatal stress on female and male offspring's sexual behavior.

To induce prenatal stress, pregnant dams were submitted to restrain stress from the second to delivery day. Thereafter, pups were not undisturbed until they reached adulthood (8 weeks) where they were finally tested. Mice were put through elevated plus maze test and blood sampling, to assess for the behavioral and physiological manifestations of stress. Blood samples were collected in two conditions, home cage and 30 min following the EPM test to evaluate the functioning of the HPA axis with assessment of corticosterone and DHEA. Vaginal smear assay was performed on female to investigate their estrous cycling. Sexual performance was tested on both virgin females and males, then partner preference test was performed on both sexes to assess for the sexual preference.

Our results show that anxiety and the level of stress hormones were not particularly altered in prenatally stressed females, while the males seemed to have developed resistance and reduced reactivity to stress. In fact, we observed low corticosterone response upon exposure to EPM test and low basal DHEA levels. Regarding sexual behavior, prenatally stressed females and males demonstrated similar sexual performance compared to control groups. Also, we noted that prenatal stress significantly impacted sexual preference in both female and male. These observations not only show that prenatal stress impacted male and female offspring but also that some effects were sexually dimorphic.

In conclusion, prenatal stress is an increasingly wording issue that has generational repercussions. In our study we chose to evaluate the impact of prenatal stress in female and male offspring. While anxious behavior and sexual performance were affected only in males, sexual preference seemed altered in both sexes.

Abbreviations

11-βHSD2	11β-Hydroxysteroid dehydrogenase enzymes
ACTH	Adreno- corticotropic hormone
AFP	Alpha-fetoprotein
AOB	Accessory olfactory bulb
AOB	Accessory olfactory bulb
AR	Androgen receptor
AVP	Arginine vasopressin
AVPV	Anteroventral periventricular nucleus
BNST	Bed nucleus of the stria terminalis
cAMP	Gαs/cyclic adenosine monophosphate
CORT	Corticosterone/cortisol
CRF	Corticotropin-releasing factor
CRFR1	Corticotropin-releasing factor type receptor 1
DHEA	Dehydroepiandrosterone
EPM	Elevated plus maze test
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptors
GREs	Glucocorticoid response elements
HPA	Hypothalamo-pituitary-adrenal axis
HSP	Heat shock in a protein
LH	Luteinizing hormone
MC2R	Melanocortin type 2 receptor

MeA	Medial amygdala
MeApd	Posterodorsal medial amygdala
MOB	Main olfactory bulb
MOE	Main olfactory epithelium
MOS	Main olfactory system
MOS	Olfactory system
MPOA	Medial preoptic area
MR	Mineralocorticoid receptors
Nac	Nucleus accumbens
NOS	Nitric oxide synthases
OA	Open arm
OC	Closed arm
POMC	Pre- proopiomelanocortin protein
PVN	The paraventricular nucleus
SON	Supraoptic nucleus
SRY	Testis-determining factor
Vb1	Vasopressin V1b (or V3)
VMH	Ventromedial hypothalamus
VMHvl	Ventromedial hypothalamus
VNO	Vomeronasal organ
VTA	Ventral tegmental area

Introduction

General introduction

Technological advances have brought many opportunities and undeniable comfort, thus the desire of increasingly more development. Ever since industrial booming in the 1920's, western modern lifestyle has evolved to become exceedingly dynamic leading to excessive consumerism. In 2022, the life of most people is characterized by hard labor and unhealthy lifestyle choices to sustain technological progress and high living standard. Even though, these lifestyle choices come with extensive ease and material abundance, the efforts that are necessary to maintain these standards trigger terrible psychological and even physical pressure and stress. Other factors, such as the ubiquitous presence of the media, the abusive use of social media, financial problems, family, and professional responsibilities and most recently the outbreak of the COVID-19 pandemic contribute to massive mental as well as physical overload, anxiety, and stress. To date, chronic stress has become a worldwide concerning problem and one of the main of factors associated with chronic diseases such as obesity, cardiovascular diseases, and cancer (Chida et al. 2008; Esch et al. 2002; Foss and Dyrstad 2011), it also known to be one of the primary triggers of mental health conditions such as burn-out and depression(Stephens and Wand 2012). Furthermore, what is even more worrying is that numerous studies suggested that the impact of stress might be transgenerational. Indeed, reports on descendance from parents that have suffered intense or chronic stress, show that their mental health and wellbeing are frequently compromised, as these children are more likely to be subjected to behavioral problems, anxiety and learning deficits along with health-related problems(Kinsella and Monk 2009). Another observed health problem in prenatally stressed children is that they are more likely to develop dysregulation of their stress response regulating system, the hypothalamo-pituitary-adrenal (HPA) axis (Charil et al. 2010). Thus, they seem to be less able to cope with stress. Interestingly, men and women suffering from sexual defective sexual behavior were also found expressing abnormal HPA axis function (Chatzittofis et al. 2016; Hamilton and Meston 2013), suggesting that there might be a link between HPA dysregulation induced by prenatal stress and sexual behavior.

Therefore, in mice, we aim in this study to investigate the effects of prenatal stress exposure on the sexual and anxiety behavior of both female and male subjects and whether the observed effects are associated with HPA dysregulation.

Introduction

Definition of stress

In 1932, a physiologist named Walter Bradford Cannon first introduced his model of flight-or-fight to describe stress. His model was largely influenced by Claude Bernard's concept of homeostasis, the body's internal balance that changes and adapts to the external demands. According to Cannon, when the organism is facing danger, threat, or a stress factor, it will react by adjusting its homeostatic systems to either fight or flee the stressors. Therefore, it will mobilize most of its energetic and physical resources to strengthen and optimize its abilities for better chances of survival before returning to its normal, homeostatic balance (McCarty 2016). In spite bringing undeniable findings to the field, especially because the theory linked the psychological dimension of stress to the physiological responses, a lot of Cannons work became obsolete with time.

Later, in 1975 stress was considered as a response reaction from the organism to its environment. In fact, Hans Selye, a pioneer of stress studies, defined stress as "the nonspecific response of the body to any demand made upon it,"(Martenson 1975). At that time, he also stated that despite the frequent association of stress with disease, stress was not necessarily a negative phenomenon, it was in fact a normal expression of the primary instincts that cannot be avoided. Moreover, to strengthen his postulate, he described as general adaptation syndrome the three successive stages undergone by the organism as a positive or negative reply to stressors (Rana, Gulati, and Veenu Wadhwa 2019):

The first stage is called, the alarm state and it consists in the activation of both the nervous and endocrine systems to optimize the body's reactions and maximize its abilities of detecting and overcoming the sources of stress.

In the second stage, stage of resistance, the organism returns to its homeostatic state, however some systems may remain on alert.

The third and final stage is the state of exhaustion, a deleterious stage where the body becomes exceeded by the constant activation eventually leading to organ failure and in the worst case even death. This type of negative stress is also referred to as distress as an opposed to eustress, which is the positive stress, just enough to excitement to overcome demands of the situations without damaging the organism (Le Fevre, Matheny, and Kolt 2003).

Comparable to Cannon's theory, Selye's stress model did bring interesting insight to the field, however it was not tough enough to stand the test of time.

Finally in 1984, Folkman and Lazarus developed the most robust model of stress(Rana, Gulati, and Veenu Wadhwa 2019), which has been at the base of most studies and research, describing stress from the psychological perspective which is also known as the transactional stress model. According to their hypothesis, a person starts experiencing stress only when facing a situation that is considered too challenging for his or her skills. Similarly, to the other animal models, stress is the result of the interaction between organism and environment except that Folkman and Lazarus introduced an additional cognitive aspect called cognitive

appraisal(Goodnite and Goodnite 2014). Appraisal involves a stressed individual to first evaluates the proprieties of the stressors and secondly deciding whether he or she has the ability of dealing with the situation. Furthermore, they suggested that not only stress had an impact on health but also the health status of an individual was a crucial factor that intervened in the coping with stress. In other words, they hypothesized that there is a reciprocal relationship between stress and health, one influencing the other and vice versa.

Causes of stress

There are many sources of stress, which can be classified as physical, chemical, or emotional stressors. In 1985, within these different types of stressors Wills and Schiffman were able to identify 3 main categories that they called (Rana, Gulati, and Veenu Wadhwa 2019):

> *The major life events,* short but intense stress exposure such as death of close person, facing an important academic evaluation or contracting a disease.

> *The everyday problems of life,* which are the common hardship that every individual encounter every day such as waiting for public transports, or dropping a drink. Although the exposure to these stressors might be daily, it generally for short period of time.

> *The enduring life strains,* meaning the stress that comes with lifestyle. Indeed, most people carry responsibilities and have duties linked to personal life, profession, or finance. These stressors trigger instant pressure or anxiety just by the thought of them. What's more is that these stressors have the tendency of having chronic and long duration effects.

Another important consideration about stressors are the effects they might bring to the body. As mentioned before, the organism will always express the same reaction when facing stress, independently to the type of stress. However, the amplitude of the response to the stress may vary depending on the properties of the stress factors so that different stress may have different outcomes (Guilliams and Edwards 2010).

Unsurprisingly, clinicians have been able to observe that the response to stress could be severe depending on if the patient is familiar or not with the stressor and if the stressing event happened unexpectedly or not. Besides, the individual's perception of the stress factor is also thought to account for the amplitude of the reaction. For instance, when considered as physical threat, humiliation, or the feeling of losing control, the source of stress tends to impact increase the response (Guilliams and Edwards 2010).

In the laboratories, researchers have developed standardized causes of stress. Different experiments are designed to induce and monitor stress in animal models. In the rodents for instance, there are different ways of inducing stress including model that need conditioning such as four-plate test, and some that only require natural animal reaction such as EPM test. Choosing between these different ways of inducing stress, depend on the aim of the study. Likewise different stress models may lead to different conclusions (Bourin et al. 2007).

The stress regulating system: HPA axis

To cope with all of the above mentioned stressors, the organism is equipped with the HPA system, that is designed to adapt and regulate metabolic, emotional, and physical balance when confronting a threat or homeostatic modifications (Walker, Terry, and Lightman 2010). HPA stands for hypothalamo-pituitary-adrenal axis, and as the name suggests, there are 3 main secretory structures involved in the stress response regulating system: The hypothalamus, the pituitary gland, and the adrenal glands (Figure 1).

Following stress, the organism will respond by the cascade activation of the HPA axis, which will successively lead to the secretion of different hormones by the different components of the HPA axis. This will eventually lead to the expression of a change within the body, which is intended to deal with the stressing circumstances. The first step of the response starts with the activation of the hypothalamus:

The Hypothalamus is a section of the forebrain that is located near the third ventricle. It is composed of different neuronal populations that produce different secretions. So the hypothalamus is considered to be a major component of the neuroendocrine system which has its implications in the function of various other systems such as musculoskeletal system, reproductive system, immune system and, of course, the stress-regulating system (Jones and Moller 2011; Shahid, Asuka, and Singh 2022).

After exposure to stress, a sub-population of neurons located in the paraventricular nucleus (PVN) of the hypothalamus are stimulated. As a response, these neurons will produce and secrete corticotropin-releasing factor (CRF) and arginine vasopressin (AVP). CRFs are a family of peptides composed of 41 amino acids, which have been shown to exert a variety of functions as hormone but also as a neurotransmitter. This neuropeptide is the main controlling agent of the HPA axis, as its release in the portal vessel of the pituitary, is the first reaction to stress, occurring only seconds after exposure to stressors (Claes 2004). Once in the blood, CRF travels to the anterior hypophysis, where it binds to its receptor, corticotropin-releasing factor type receptor 1 (CRFR1). As previously mentioned, in parallel to the CRF cascade, the AVP cascade is also activated. AVP is a nonapeptide that is produced in the hypothalamus in the supraoptic nucleus (SON) and the PVN. The peptide is known to be secreted in the periphery where it acts as a homeostatic agent involved in the osmotic water balance. The secretion of both AVP and CRF leads to the second step of the stress response, which takes place in the pituitary gland (or hypophysis). The hypophysis is a small organ located right below the hypothalamus. The pituitary gland can be divided into two distinct components, the anterior pituitary, which is an endocrine organ that produces and secretes hormones at hypothalamic demands, and the posterior hypophysis which is roughly the axonal prolongation of some neurons of the hypothalamus (Cole 2016). The anterior pituitary but more specifically the corticotropic cells (or basophilic cells) are the protagonist in the stress response as they respond to hypothalamic AVP and CRF secretion. The CRFR1 receptors bind to CRF and are coupled to G protein. Thus the binding to its ligand triggers the cascade reaction of the G protein, including the synthesis of Gαs/cyclic adenosine monophosphate (cAMP) by the adenylate cyclase resulting in the secretion of adreno- corticotropic hormone (ACTH) in the peripheric bloodstream (Smith and Vale 2006; Vasconcelos et al. 2020).

Similarly, to CRF, AVP is also released in the hypophysial-portal circulation to finally reach its receptor, the vasopressin V1b (or V3) receptor on the basophilic cells. The binding activates the receptor which in its turn stimulates the G_q protein that initiates phospholipase C. Phospholipase C acts by switching on the protein kinase C, which is thought to enhances the release of ACTH (Maybauer, Maybauer, and Traber 2008; Smith and Vale 2006).

ACTH is a 39 amino acid peptide produced from the cleavage of the 6 times larger pre-proopiomelanocortin protein (POMC) in the pituitary. ACTH is considered to have a role in the stress reaction by acting on the adrenal glands. The secretion of ACTH in the systemic circulation initiates the third phase of stress response in the adrenal cortex:

The adrenal glands are small triangle shaped endocrine organs located above each kidney. These glands have a key role in the regulation of arterial pressure as well as in stress management through hormonal productions. Each adrenal cortex is histologically subdivided in three zones(Miller and Flück 2014). The outer most layer of the cortex is the glomerulosa that is located directly underneath the capsule. It makes 15% of the organ and is mostly responsible for the production of steroid hormone mineralocorticoid such as aldosterone. The fasciculata intermediate zone and the reticularis the inner most layer, constitute each 75% and 10% of the organ. Whereas, the zona reticularis is involved in the synthesis of androgen hormones, the zona fasciculata produces glucocorticoid hormones such as cortisol, which has a major role in the stress response.

Upon ACTH binding on its adrenal receptor, the melanocortin type 2 receptor (MC2-R) of the parenchymal cells of fasciculata, there is activation of MC2-R, which leads to the initiation of cAMP signaling pathway (Claes 2004; Smith and Vale 2006). This drives the accumulation of cholesterol in the mitochondrial membrane in the latter described cells. As a result of the cholesterol is converted to glucocorticoids by several mitochondrial cytochromes(Katsu and Baker 2021).

As mentioned in the previous paragraphs, glucocorticoids are a determinant component for stress management. In humans, the stimulation of the HPA axis leads to the final release of the main glucocorticoid, namely cortisol. Similarly, in rodents, exposure to stress will lead to the same successive activation of each organ in the HPA axis, except that in mice or rats, the principal glucocorticoid is corticosterone. Both cortisol and corticosterone are often referred to as CORT and they appear to have the similar functions (Oitzl et al. 2010). Corticosterone and cortisol are steroidal hormones that derive from cholesterol, so they are lipophilic agents. Once secreted in the blood stream, they passively travel through the vessels and cell membranes very easily to activate their intracellular receptors, the glucocorticoid receptors (GR) (Stephens and Wand 2012).

Before activation, the GR is engaged with the heat shock in a protein (HSP) complex which ensures the inactivation of the GR. After binding to its ligand, the receptor changes its conformation and frees itself from the HSP. Afterwards, the GR is translocated into the nucleus where it links to transcription factors. The new formed complex attaches on specific motifs of the DNA called glucocorticoid response elements (GREs) where it acts on the transcription of the genes that have GRE on their promotor by either enhancing or inhibiting the gene

expression (Smith and Vale 2006). Thereafter, the consequences of the glucocorticoid hormones are wide ranged. In fact, it has been shown that GR are ubiquitously expressed in the body. Consistent with this observation, most systems in the organism respond to corticosterone/cortisol release or in other words, apparently stress seems to affect the functioning of a vast majority of the body.

In healthy subjects, the HPA axis is highly regulated by negative feedback loops involving the glucocorticoid hormones (Smith and Vale 2006). In fact, it has been observed that along binding to GR, glucocorticoid hormones also bind to mineralocorticoid receptors (MR). In contrast to the ubiquitously expressed GR, MR are exclusively present in certain parts of the brain, including the hypothalamus. Interestingly, MR appears to have higher affinity for cortisol or corticosterone than GR. Therefore, they bind to the hormones at basal state when the circulating glucocorticoids are at low levels. This ensures the physiologic baseline functioning of the HPA axis (Smith and Vale 2006).

However, in stress conditions, there is imminent activation of the HPA axis, leading to increased cortisol/corticosterone secretion. In this case, as the levels of glucocorticoids are high, the GR finally bind to the hormones. The binding induces a series of changes in different tissues and systems including the HPA itself, as it triggers the negative feedback loop of the HPA axis that brings the organism back to basal state (Smith and Vale 2006).

Thus stress response is meant to be transient with rapid and reversible alterations in the behavior and metabolic processes but rapid return to homeostatic balance In other words, the stress response takes place in two distinct steps (Vasconcelos et al. 2020): the activation of HPA axis is as described above, leading to peak in cortisol levels within 30 minutes and progressive return to baseline after three hours (Elverson and Wilson 2005).

Besides being under control of hormonal feedback, the cortisol/ corticosterone levels are highly regulated by a circadian rhythm that aims at maintaining homeostasis in all the body parts. In humans, the cortisol levels are it reaches the highest levels during daytime before dropping to its lowest levels during the night (Spiga et al. 2014). In rodents, the rhythm of glucocorticoid release has a similar occurrence however, since mice and rats have active phases at night, their corticosterone levels rise during the night (when they are active) and drop during daytime (Spiga et al. 2014). Seemingly, in the context of stress however, homeostasis is disturbed so the body deploys tremendous efforts into bringing the normal balance back. Therefore, the levels of cortisol/corticosterone rise independently of circadian rhythm (Smith and Vale 2006).

CLASSICAL HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS
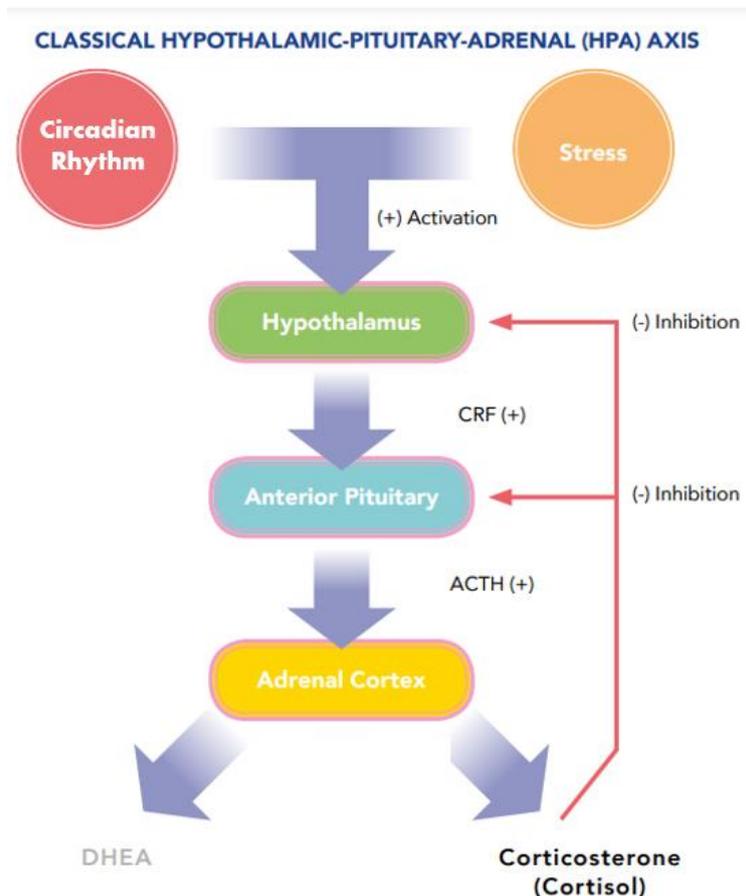
Figure 1: Schematic representation of the HPA axis activated by stress and by the circadian rhythm and stress. Adapted from https://researchfeatures.com/wp-content/uploads/2018/05/Doug-Vetter.pdf

The role of Dehydroepiandrosterone

Simultaneously to the cortisol secretion by the HPA axis following exposure to stress, there is a reduction of dehydroepiandrosterone (DHEA) secretion as a response to stress (Guilliams and Edwards 2010). DHEA is a steroidal molecule that, same as cortisol or corticosterone, derives from cholesterol processing in the adrenal cortex. However, its production takes place in the zona reticularis. In the plasma, DHEA is mostly found in its inactive sulfurized form DHEAS. Both DHEA and DHEAS are stable molecules with extended half-lives (A. K. Lennartsson et al. 2012). Interestingly, the secretion of these molecules during daily time frames is very stable and does not follow circadian rhythm unlike cortisol although its concentrations might fluctuate with HPA activation. Furthermore, it has been observed that DHEA concentrations vary with the age, as the levels rise during adolescence to peak at 20 to 30 years of age before dropping progressively with ageing (Leowattana 2004).

The exact roles of the DHEA(S) are not fully understood yet, but there are observations of its release with cortisol during stressing situations through ACTH stimulation. Research shows that DHEA antagonize the effects of cortisol and even inhibit cortisol secretion, perhaps indicating a protective function against potential detrimental effects of cortisol (A. Lennartsson et al. 2012). Besides, decreased DHEA has been observed in mice suffering from stress-related chronic diseases, such as diabetes (Coleman, Leiter, and Schwizer 1982). Thus many hypothesize that DHEA, aside from possibly providing protection to the organism and

the brain, also has an important role as anabolic agent that preserves against negative effects of stress or disease to a certain degree by inhibiting cell death, or reinforcing the immune system (A. Lennartsson et al. 2012; Maninger et al. 2009; Morgan et al. 2009).

HPA dysregulation

The primary causes for the hypothalamic-pituitary-adrenal axis dysfunction seem to be overactivation or repeated stimulation of the axis. In fact, prolonged or chronic stress are defined as risk factors of HPA dysregulation. Although the mechanisms of development of HPA dysregulation through stress, are not exactly known findings show that there might be an evolution from normal response to over-responsiveness and finally to silence of the axis due to overstimulation. As discussed in previous paragraphs, glucocorticoids, and DHEA, both released after activation of the HPA axis, have a precise pattern of expression in physiological conditions, so they are considered as major biomarkers that reflect the overall HPA functioning. Interestingly there are different ways to assess for HPA dysregulation. The classic way of screening for the condition is by measuring cortisol concentration. Hypercortisolism, supposedly the first phase that comes as a pathologic consequence of HPA overstimulation, shows of as exceedingly elevated levels of cortisol and impaired feedback loop. This stage of stress may last for extended period of time, but it is eventually followed by hypercortisolism, in other words the opposite state, characterized by extremely low levels of cortisol and low to no responsiveness of the axis even after stimulation. The mechanisms by which this phenomenon occurs have not been elucidated, however there is the thought that silencing the axis might be a way of protecting the organism against the effect of long term exposure to cortisol (Guilliams and Edwards 2010).

Another manner to determine HPA dysfunction is by the assessment of DHEA and its sulfureted derivates. Although this option has been less documented, there are various reports showing that lower levels of the hormones signal potential HPA damage (Danielle X. Morales, Sara E. Grineski 2016). Also, the disturbance in the circadian rhythm of HPA hormones release is also an alternative to detect the HPA malfunctions.

Lastly, increasingly more studies show that calculating the cortisol/DHEA ratio (CORT/DHEA) might also reflect the HPA function. Indeed, depressed people have higher cortisol/DHEA ratio whereas people presenting higher DHEA/cortisol seem to be more resistant against stress (Morgan et al. 2009). Hence, the measurements of the ratio of these hormones might, in combination with other clinical data and patients background, serve indicators for stress level in clinical medicine (Guilliams and Edwards 2010).

HPA axis dysregulation is associated to numerous other diseases such as diabetes, depression (Prestele, Aldenhoff, and Reiff 2003), obesity, metabolic disorders (Pasquali et al. 2006), neurodegenerative disorders and sexual dysfunctions in human and rodents. Considering the variation in hormone expression that comes with HPA dysregulation, some researchers mention the possibility that HPA dysfunction might be the origin and not the result of specific conditions such as the perturbations of the immune system (Guilliams and Edwards 2010).

Prenatal stress

A greater cause for concern is the fact that the impacts of stress range generations further than the individual that is directly submitted to the stressors (Glover, Connor, and Donnell 2010). In fact, multiple times it has been demonstrated that the sequels of stress affect the offspring of stressed subjects. Especially the impact of maternal stress during gestation, on their progeny has frequently been studied in animals as well as in humans. Although the findings among different studies are not unanimous, most of the results suggest that prenatal maternal stress interfere with the fetal development thereby permanently altering neurodevelopment, through interference with neuronal plasticity (María Eugenia Pallarés and Antonelli 2017), but also modifying physical and behavioral outcomes in the children (Yong Ping et al. 2015) as well as in rodent offspring (Van Den Hove et al. 2005). In the same manner, the offspring's HPA axis might also be affected, leading to enhanced or inappropriate responsiveness to stress and anxiety (Mueller and Bale 2008).

One theory to explain the mechanism underlying these observations, mentions that in stress conditions, the stress hormones shed by the pregnant women cross the placental barrier and reaches to the developing fetus (Figure 2). In normal conditions, the placenta serves as a natural selective barrier between mother and child. It is known to provide protection to the fetus by forming a solid barrier against harmful agents, including physiologically produced substances from the mother. The placenta is naturally equipped with 11β-Hydroxysteroid dehydrogenase enzymes (11- βHSD2), a protein that converts the active maternal cortisol, to harmless cortisone. This is believed to impede dangerous concentrations of glucocorticoids to interfere with fetal programming (Charil et al. 2010). However, stressed gestating mothers present reduced 11-βHSD2 enzymes combined with elevated glucocorticoid levels, so that the offspring are more likely to be overexposed to the circulating stress hormones, therefore they are at higher risk of developing the above-mentioned damages (O'Donnell, O'Connor, and Glover 2009).
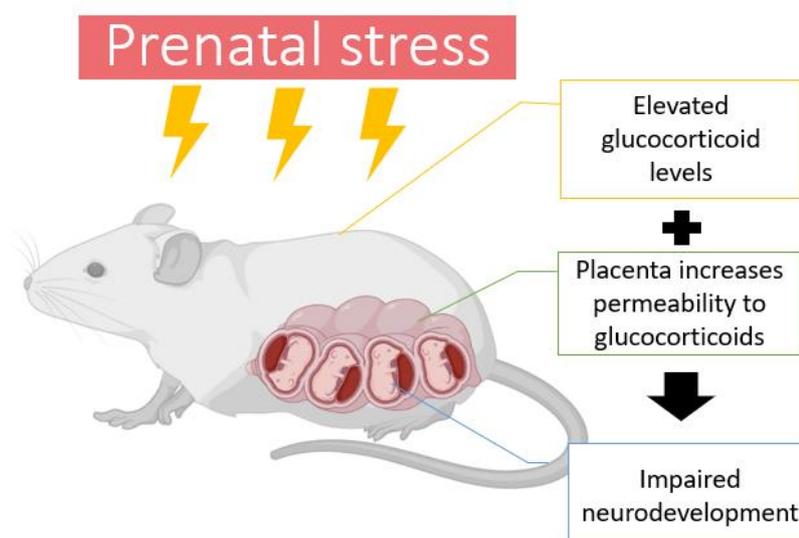
Figure 2: Self-made schematic illustration of potential mechanism of prenatal stress. Stress during gestation increases blood corticosterone of the dam and increases placental protection, thus the pubs are more likely to have alterations in their development.

HPA axis and sex differences

Rodent studies:

In rodents, a large part of HPA programing takes place after birth (Kapoor et al. 2006). Nevertheless, prenatal development of the axis is also critical. This is illustrated by the various studies demonstrating deleterious impact of prenatal stress on HPA axis development (Weinstock 2005).

Strikingly, animals studies have reported that HPA axis programming might vary depending on the gender (Bale and Epperson 2015). This is demonstrated by the fact that prenatal stress seems particularly to impact male maturation and less female development. For instance, cognitive performances following prenatal stress, were found to be superior in the females compared to those in males (Mueller and Bale 2007). In contrast, female rats seem to be far more sensitive to stress exposure during pubertal development, than males (Barha et al. 2011). These observations indicate that maturation of the HPA axis in rodents not only is sexually dimorphic but also depend on specific timing depending on the sex.

Furthermore, sex-related differences in HPA responsiveness are also detected in adult wild-typed rodents (Goel et al. 2014). Indeed, female rodent respond with higher corticosterone secretion than males upon stress stimulation (Goel et al. 2014). Other studies showed that the decreased reaction to stress in post pubertal males is seemingly due to the interaction of testosterone with the stress system (Gomez, Manalo, and Dallman 2004). Besides, female anxiety and associated HPA function are reported vary depending on their cyclicity. In female animals and humans, that the intensity of the stress response varies according to their reproductive cycle, so that lower responsiveness to stress, much closer to what is measured in men, is detected in female individuals during the stage where estrogen secretions are the lowest, namely diestrus phase in rodents (Bale and Epperson 2015). Sexual dimorphic features of the HPA axis can be correlated with sexual differentiation and differential gonadal secretions. Reports show that HPA development is influenced by accurate steroidal hormone secretions (Goel et al. 2014).

Human studies:

In human, the responsiveness of the axis might vary depending on the gender (Bale and Epperson 2015). In addition, depending on the timing of the stress exposure, first, second or third trimester of gestation or childhood, adolescence, and adulthood the effects on HPA axis might differ and thus also the reaction to stress. For instance, in children born from mothers that have been stressed during pregnancy, boys are 3 times more at risk of presenting autism or attention deficit disorders than girls, suggesting that HPA axis impairment occurs differentially depending on the sex. Considering the proportion of behavior and learning disorders incidence, it seems that male children are more sensitive to stress exposure at fetal period compared to female individuals(Bale and Epperson 2015). In contrast, the HPA response to stress exposure during childhood seems to be quite similar in subjects regardless of their gender. Likewise, the number of anxiety and depression disorders cases found in stressed children are equally proportioned among male and female toddlers (Bale and Epperson 2015). There seems to be a sex dimorphic feature to HPA responsiveness, which

could be explained by the differential steroidal sex hormones expression in men and women. However, sex differences rapidly reemerge in adolescence and persist throughout adulthood. Indeed, young women appear to have higher odds of suffering from psychiatric and mood conditions than same aged men. Also, the levels of stress hormones found in both sexes significantly differ. For instance, cortisol secretions as a stress response in men is far less important than what is measured in women(Bale and Epperson 2015).

Undoubtedly, androgens and estrogens are both expressed during crucial development periods such as during fetal programming or teenage years. However, the pattern and amounts of expression vary substantially according to the individual's sex and life stage, which could be associated to the different HPA responses in relation to sex and developmental period (Bale and Epperson 2015; Heck and Handa 2019). The effects of estrogen and testosterone have been illustrated in numerous studies. For example, in 2006 Lund, Trent D. et. al demonstrated that androgens had inhibitory effect whereas estrogens had activation effects on neuronal population close to or within the PVN neurons of the HPA(Lund, Hinds, and Handa 2006). These observations associate the stress regulating system to the networks that govern steroidal hormone secretions.

HPA and HPG

Findings discussed in the previous paragraphs, clearly establish a link between HPA and the regulatory system of reproductive structures called the hypothalamic-pituitary-gonadal axis (HPG). Interestingly, animal studies have shown that not only sex hormones can regulate the HPA axis but also interfere in the development and maturation of the axis (Panagiotakopoulos and Neigh 2014). Reciprocally, the secretions mediated by the HPA axis influence the development and functions of the HPG system throughout life. Broadly speaking, the HPG is the orchestra of all events related to sexuality in male and females. Same as the HPA, the first structure of the systems is the hypothalamus, which secretes Gonadotropin-releasing hormone (GnRH) to stimulate Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release in the pituitary gland. LH and FSH activate sex steroidal hormones production by the gonads. Research has shown that sex hormones have a non-negligible role in the maturation of the brain as well as in the sex-specific development of multiple brain regions (Charil et al. 2010). Exposure to stress however may alter the proper functioning of the reproductive axis, thereby interfering with the cascade events leading to testosterone and estrogen secretion (Toufexis et al. 2014). These alterations were seen to ultimately block estrogen and testosterone secretion in both sexes with broad consequences on brain development (Charil et al. 2010), sex-specific brain differentiation(Anderson, Rhees, and Fleming 1985), fertility (Rooney and Domar 2018), female gonadal cycle (Toufexis et al. 2014) and reproduction including sexual behavior (Valsamakis, Chrousos, and Mastorakos 2019).

Brain development and sex specific differentiation

To date there is unambiguous evidence that in mammals, female, and male present sex specific features, including their sexual behavior. For instance, in wild-type rodents, male-typical mating behavior is expressed by mounting the opposite sex, whereas female mice or rats show typical lordosis posture (Yang and Shah 2014). Sexually dimorphic behaviors are suggested to be the result of a series of complex organizational and activational events that start before birth and may continue up to adulthood.

Fetal sexual differentiation starts during gestation, at a molecular level with either the presence or absence of testis-determining factor (SRY) gene. According to classic hypothesis, in humans and in rodents, male individuals have one X and one Y sexual chromosomes, in contrast female present two X chromosomes, which ultimately results in either the expression or the absence of a hormonal pool that drives sex typical development.

Male specific development

The presence of a functional Y chromosome is essential for male sexual differentiation, as it carries the SRY gene that drives transformation of undifferentiated bipotential gonads into male testes. From the moment that the testicles are formed, the developing fetus starts producing sexual hormones, namely testosterone (Yang and Shah 2014). The effects of testosterone are indispensable, especially in the brain. Testosterone binding to its receptor, the androgen receptor (AR), which is known to drive sexually dimorphic maturation of neuronal circuitries by inducing genetic and cellular phenomenon that either enhance expansion of cells, favoring certain neuronal connections over others, or inhibiting cell growth and even causing cell death in particular brain regions (Dulac and Kimchi 2007). These phenomena happen during prenatal development and are followed by testosterone surge during the 18th -19th day of gestation which consolidates male differentiation (J. Bakker and Brock 2010; Ward et al. 2003).

Female specific development

Contrary to male differentiation, prenatal development in females occurs in the absence of circulating sex hormones, which is why female gender is also referred to as the default sex. The feminine genitals and gonads are shaped into female organs without any hormonal stimulation (Yang and Shah 2014). Furthermore, studies showed that successful normal female brain development in mammals required protection against the hormonal secretions coming from the mother. According to various reports, inappropriate prenatal exposure to sex hormones in female rodents resulted in defeminized and partially masculinized females that were exhibiting low lordosis behavior and increased mounting behavior (Padmanabhan et al. 2006).

Alpha-fetoprotein (AFP) is a glycoprotein that is produced by the fetus. AFP is found in fetus and neonates, but its concentration diminished drastically after birth, so that it is only found in minimal amounts in adults. Importantly, AFP has a high affinity for estrogens and has thus been proposed as the protective agent against estrogens in female brains. Using an AFP null transgenic mouse model, Bakker et al. (Julie Bakker et al. 2006) was able not only to demonstrate the masculinizing and defeminizing role of estrogens at prenatal stages in both

sexes but also to show the implication of AFP in protecting feminine brain against hormonal exposure. However, the prenatal administration of aromatase inhibitors, which inhibit estrogen synthesis, to the transgenic females was able to rescue normal female features. In addition, the same study provided evidence that AFP was able to bind estrogen with high affinity thereby blocking the estrogen from interacting with its targets. In another study involving female guinea pigs, administration of high amounts of androgens, provoked similar behavioral disruption as observed in female AFP null mice (Dulac and Kimchi 2007).

All together these data suggest the role of estrogen as a masculinizing agent during prenatal and that the effect of AFP in female brains is to bind estrogens, thereby inhibiting its actions and allowing normal prenatal brain maturation (Figure 3).
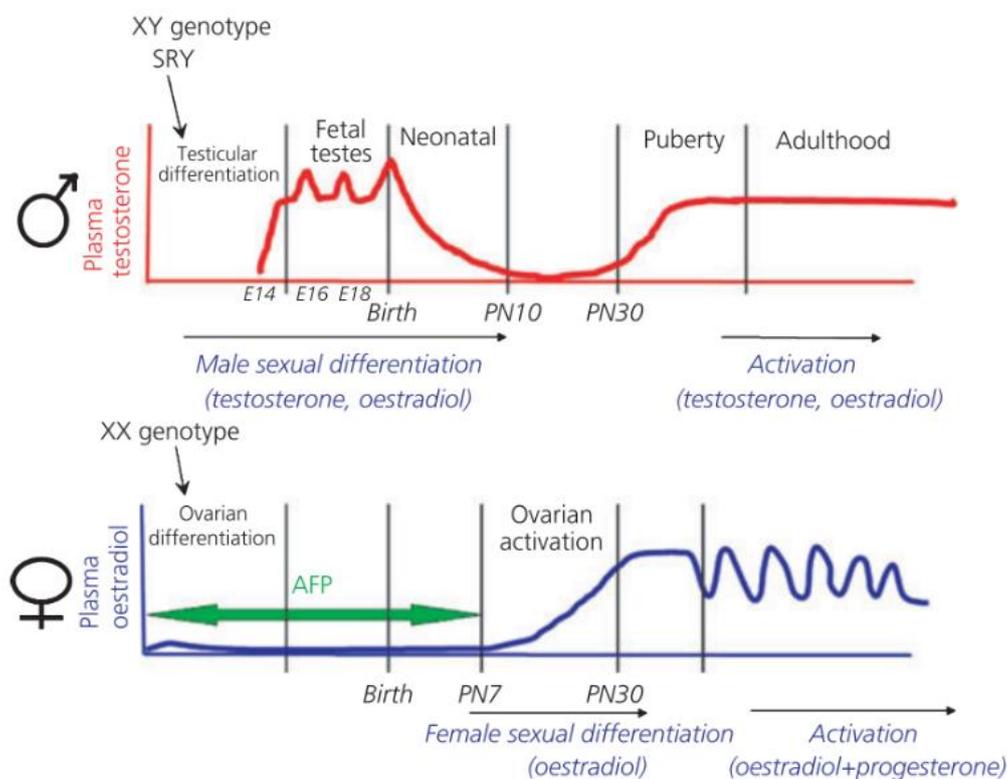
Figure 3: Illustration of gonadal hormone secretion in males and females from prenatal stages to adulthood. Differential hormone secretions are at the basis of the classical view of brain differentiation. While male require high doses of testosterone at prenatal stages, in females, high amounts of estradiol secretion start postnatally (J. Bakker and Brock 2010).

Neuronal input of sexual behavior

It is undeniable that among exhibiting biological differences, male and female animals, express sex-specific behavioral differences. One of the most robust sexually dimorphic behaviors seen in mammals is the sexual behavior(Ishii and Touhara 2019).

In most vertebrates including human, sexual behavior can be split in two distinct components (Jennings and Lecea 2020): appetitive and consummatory behavior. In rodents, for instance the two behaviors are very characteristic. Before even engaging in sexual intercourse, mice start by investigating and showing interest for the opposite sex, which also termed as appetitive behavior. This behavior includes snuffling and touching before expressing clear preference and attraction for the potential mate partner. Very rapidly, these first interactions will lead to the expression of the consummatory behavior, in which the animals take the correct postures that will allow the actual act of sex. Male mice mount the female in attempt to intromit, whereas female express the lordosis posture to facilitates the intromission.

In normal conditions, lordosis posture is only observed in female subjects (Sakamoto 2012), while mounting and intromission behavior are almost exclusively seen in males (Manoli et al. 2013). These behaviors are strongly sex specific, which is why there are thought to be controlled by sexually dimorphic neuronal pathways in males and females (Figure 4).

Sexual behavior in both sexes starts with the perception of olfactory cues emitted by the opposite sex (Ishii and Touhara 2019). To draw a big picture of the complex neuronal circuits of sexual behavior (appetitive or consummatory), there are two types of olfactory cues that can be detected by two distinct parts of the olfactory system. **(I) The** Volatile molecules like pheromones for instance, are recognized by the main olfactory epithelium (MOE), the primary sensory organ of the main olfactory system (MOS). From the MOE the information is processed to the main olfactory bulb (MOB) to pass through the olfactory cortex and finally reach the lateral amygdala (Ishii and Touhara 2019). The MOB has also been shown to sends information to GnRH neurons of the hypothalamus, which thought to be also capable to regulate reproduction and fertility to some extent (Dulac and Kimchi 2007). **(II) The** Non-volatile molecules such as urinary proteins and steroids are probably detected by the vomeronasal organ (VNO) which send the information to the accessory olfactory bulb (AOB). The AOB transmits the information to further nuclei of the amygdala such as the medial amygdala (MeA), the basolateral amygdala and bed nucleus of the stria terminalis (BNST), which in turn activate the hypothalamic nuclei, including medial preoptic area (MPOA) and ventromedial hypothalamus (VMH) (Dulac and Kimchi 2007; Ishii and Touhara 2019).

Interestingly, the above-described circuits seem to be necessary to express normal sexual behavior in both sexes and only a minority of nuclei within the above listed structures have been identified to present sexual dimorphism and sex specific activation patterns (Dulac and Kimchi 2007):

Appetitive sexual behavior

In male mice, female cues activate the VNO, which in turn stimulates the ventral tegmental area (VTA), a hypothalamic nucleus. The VTA enhances dopamine release in the nucleus accumbens (NAc), thereby triggering the reward system, thus inducing sex motivation. In parallel, the VNO activates the MPOA which inhibits specific neurons in the VTA, resulting in a disinhibiting signal from the VTA to dopaminergic neurons of the NAc, hence reinforcing the motivational aspect of sexual behavior (Ishii and Touhara 2019).

Similarly, it has been observed that in female rodents, male cues also lead to dopamine release in the NAc through the activation of the MPOA followed by the stimulation of the VTA. The induction of this circuit seems to also increase female to male interaction (Ishii and Touhara 2019).

Consummatory sexual behavior

As previously described mounting and intromitting are major constituents of male consummatory behavior. The main olfactory system (MOS) is essential to sexual behavior in male mice as suggested by diverse studies, in which alteration of normal MOE function resulted in significantly reduced mating (Dulac and Kimchi 2007). However, the consummatory component of mating behavior largely depends on the circuits initiated by the accessory olfactory bulb (AOB) that lead to the activation of posterodorsal medial amygdala (MeApd), followed by the MPAO and the ventrolateral part of the ventromedial hypothalamus (VMHvl) stimulation, which in turn end in the enhancement of the consummatory behavior seen as increased mounting behavior (Ishii and Touhara 2019).

In female mice, consummatory behavior translates as the expression of the characteristic lordosis posture. Studies have shown that the activation of the VMHvl is not only necessary but also completely sufficient to the induction of lordosis. In other words, the VMHvl plays a major role in the display of female consummatory behavior.

The expression of lordosis behavior is mediated through the activation of kisspeptin neurons in the anteroventral periventricular nucleus (AVPV) by olfactory cues. The Kisspeptin neurons directly stimulate the nitric oxide synthases (NOS) neurons of the VMHvl and eventually trigger the expression of lordosis (Ishii and Touhara 2019).

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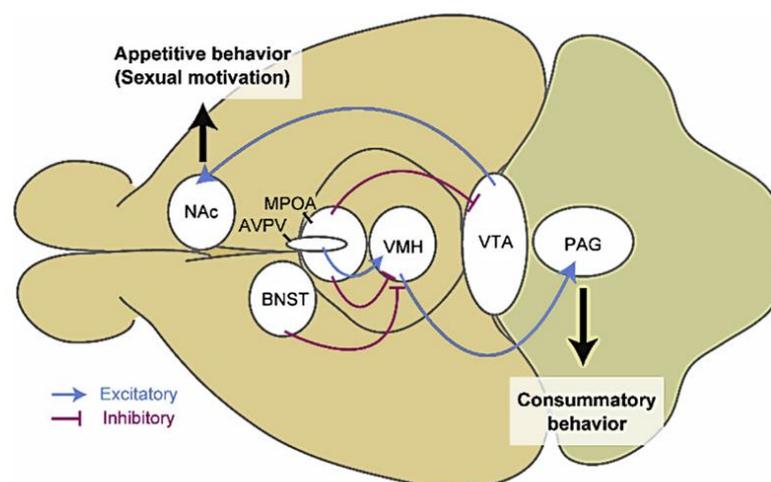
Figure 4: Graphic representation of the neuronal circuitry that regulates sexual behavior (Ishii and Touhara 2019)

Objective and Hypothesis

Objective and Hypothesis

The scientific literature about stress highlight how maternal stress during gestation can have devastating effect on behavior, cognitive development, mental health, and different body functions of the offspring.

Here, we hypothesized that prenatal stress might permanently impact reproductive behavior of the offspring. Thus, the goal of the present study is to investigate the effects of developmental stress exposure on the sexual behavior of both adult female and male CB57/Bl6 mice.

Anxiety and HPA functioning were tested with the EPM test and ELISA assays for corticosterone and DHEA in males and females. Vaginal smear assay was performed to assess the estrous cyclicity of the females. Consummatory and appetitive behaviors were assessed with lordosis and partner preference test respectively in females. Similarly, male mice were submitted to copulation test then partner preference.

Material & Methods

Material & Methods

The exhaustive list of material and tools can be found in annex 1.

Animals

Female and male wild type C57BL/6JRj mice were purchased from Janvier Labs. Couples of both sexes were housed together for mating. Following vaginal plugs observation, dams were randomly split into two groups; control and prenatal stress. The control females were left undisturbed, while the ones in the prenatal group were exposed to daily restrain stress as described below. Following birth, all animals were kept in the same housing conditions, weaned at the age of P21 and housed in groups of four animals per cage.

Animal testing started when subjects reached adulthood (2 months). Mice were housed under reversed light/dark cycle with food and water available *ad libitum*. All behavioral tests were performed in the dark phase of the diurnal cycle. For this study, ethical approval was obtained from the University of Liège ethics committee.

Maternal stress induction

To induce prenatal stress in pups, pregnant dams were put under chronic stress during gestation. The first day of pregnancy (E1) was determined early in the morning when the vaginal plug was observed in females. Stress exposure was performed on the pregnant dams from the second day of gestation (E2) until delivery (E20/21). Restraint stress was applied on the gestating mice by placing them in a plastic tube (4,3cm diameter, 10,8cm length). This procedure was applied for 45 minutes and repeated three times a day. The females from the control group were not handled, and left in their home cage during all the gestation period.

Vaginal smear assay

There are different technics to identify the stages of the estrous cycle, however sampling of vaginal lining cells is the most reliable (Byers et al. 2012) and therefore the method that was executed in this research. Smear assay was performed using metallic swab with circular tip. To collect vaginal cells, the swab was dipped into saline buffer then directly inserted into the vaginal cavity of the mouse. The samples were immediately deposited on a super frost microscopic slide. The slides were dried at 60°C for 20 minutes and stained with Thionine blue solution (1%). The slides were dried again at room temperature for 10 minutes before microscope observation. The stages of the estrous cycle were determined by evaluating the proportion of three different cell types as follow:

- Proestrus phase is characterized by the appearance of a high proportion of round nucleated epithelial cells and low to no lymphocyte cells.
- In the estrus stage, the epithelial cells are high in proportion with little to no visible lymphocytes. However, in contrast to round shaped, nucleated cells found in proestrus, the vaginal cells appear to have an irregular shape without nucleus, they are also called cornified cells.
- During metestrus, all three cell types can be found. Whereas cornified cells and lymphocytes are found in large quantities, the round shaped epithelial cells maybe visible but at low proportions.

- Finally, diestrus is characterized by the absence of cornified cells and presence of a high number of lymphocytes.

Ambiguous transitioning stages were sometimes visible, so to minimize the odds of sampling during these confusing in-between phases, the vaginal cells were collected in the morning from 10 a.m. to 11 a.m. (lights off at 9 am).

Elevated plus maze test

The aim of the elevated plus maze (EPM) test is to induce a dilemmatic situation, in which the mice are conflicted between their natural curiosity, explorative instinct and their strong dislike of heights. In principle, the more the mice explore the open spaces, the less they are considered anxious and more they spend time in the closed arms, the more they are anxious (Bourin et al. 2007).

The EPM is a cross-shaped apparatus composed of 4 arms that cross to form a central section. The opposite facing arms are either closed arms or open arms. All the arms are 38cm long but the difference between open arms and closed arms are the delimiting walls. The walls of the closed arms are 15cm high and those from the open arms are only 2,5cm high. The apparatus is elevated 80cm above the floor (Figure 5).

Mice were placed at the middle section at the beginning of the test, then left to freely explore the apparatus for 5 minutes. The test was performed between 2 p.m. and 4 p.m. The behavior was videotaped, and manually scored later for the time and frequency of entries in the open (OA) and the closed arms (CA) using a custom-made JavaScript program.

Finally, anxiety was assessed by calculating the anxiety index. Anxiety index = 1- [((Time OA/(Time OA +Time CA))+(Entries OA/(Entries OA + Entries CA))]/2 as suggested in previous studies (Lguensat et al. 2019; Rao and Sadananda 2016). A value close to 1 reflect a high anxiety.
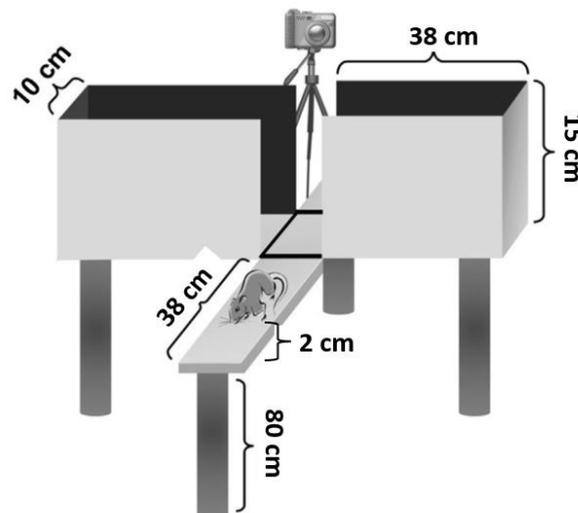

Figure 5: Schematic representation of the three-compartment box used to test sexual preference. Adapted from: (Sweis, Brian M. et al. 2016).

Blood sampling

To evaluate the functioning of the HPA axis, blood samples were collected in two conditions; home cage and 30 mins after the end of EPM test with at least 7-day time gap between the two sampling days. In both conditions, blood sampling was performed in females, only in proestrus or estrus stage of the estrous cycle. Also, to avoid diurnal fluctuation of the CORT and DHEA levels, samples from all animals were strictly obtained between 2 p.m. and 4:30 p.m.

The blood was collected from the tail in a heparinized capillaries (1.1 -1.2 mm internal and 1.5-1.6 mm external diameter; Carl Roth GmbH). Samples were centrifuged at 4°C for 15 mins at 5000 rpm. The supernatant plasma was carefully collected by pipetting and stored at -80°C until the ELISA test was performed.

Ovariectomy and hormones supplementation

Estradiol implants

Handmade hormone implants were manufactured using 1cm long silicone tubes (inner and outer diameters 0,062 - 0,094 mm; Degania Silicone Silclear Tubing). Each tube was filled with 1:1 ratio of cholesterol and estradiol (Estradiol: 101565, ICN Biomedicals Inc.; Cholesterol: C8667, Sigma). Both ends of filled tubes were sealed with a medical adhesive paste (Dow Corning Corporation; Silastic medical adhesive silicone type A).

Bilateral ovariectomy

Bilateral ovariectomy was performed on both experimental and stimulus adult mice. The females were put under general anesthesia: 80mg/kg ketamine (Nimatek) and 0,9mg/kg medetomidine (Domitor). Ovaries were removed through direct surgical incision on the flank. Fallopian tubes were ligated to prevent hemorrhagic bleeding after gonad ablation, then the ovaries were removed. Muscle and skin were sutured separately, and the estradiol implant was placed subcutaneously, in the back of each mouse. To assist the wakening up process, mice were injected 7,5mg/kg atipamezole (Antisedan) right after hormonal implantation and before putting them in a clean home cage. The females were kept on heating pad until they recovered. All subjects were given 7 to 10 days of convalescence before being involved in any testing. Hormonal priming was performed on the testing days by injection of 50ul progesterone (500 ug; Sigma life science) 2 to 3 hours prior to testing for the sexual behavior.

Testing for female mice sexual performance: Lordosis test

Lordosis behavior was tested during four sessions, with three to four days between each testing. Stimulus male mouse was habituated for 15 min in a 33cmx19cmx18cm Plexiglas aquarium containing clean sawdust bedding. Following that, a stimulus female was introduced to the aquarium and once the male started showing sexual behavior, the stimulus female was replaced with the experimental female. Lordosis behavior was evaluated in response to 10 mounts attempt by the male or during 10 minutes, whatever comes first. Lordosis, which is the typical posture adopted by receptive females, was considered when the females lowered their four limbs, arched their backs, tilted their head to the back and raised their tail, to facilitate the intromission.

The lordosis behavior was scored as a percentage of the number of lordosis responses to male mounting divided by the total number of mounts × 100%

Testing for Male mice sexual performance: Copulation test

Three testing sessions were performed at the frequency of one session per week. Animals were tested in the afternoon from 1p.m. to 5 p.m. Two to three hours prior to testing, stimulus females were injected with progesterone as indicated above to induce sexual receptivity. Experimental male mice were habituated in a 33cmx19cmx18cm Plexiglas aquarium containing clean sawdust bedding for 15mins before introducing a sexually receptive female. Each test lasted 30 minutes. The complete sessions were videotaped, and manually video-scored. To evaluate the sexual behavior, the latency to the first mount and the first intromission as well as the latency to ejaculation were measured. In addition, the number of intromissions and the number of mounts were counted.

Partner preference test

Partner preference test was performed to evaluate the sexual preference of both male and female subjects. A 60 cm x 30 cm x 30 cm three compartments box, connected by small doors, was used for this test. The two lateral compartments contained a section for the stimuli animals with the following dimensions (10 × 20 cm). Two opaque 30m x30 cm separating walls were installed on each side of the box to block visual cues coming from the stimulus animals. These separations contained wholes to diffuse the smell of the stimulus animals.

Before performing the test, a 10 minutes habituation session was held the day before without the presence of the stimuli animals. The next day, an intact male and hormonally primed female were placed into the small sections of the lateral compartments with their bedding to boost the olfactory cues. The experimental mice were placed in the central compartment and left to explore for 10 minutes. The test was scored live using the above-mentioned script.

Sexual preference was evaluated by quantifying the time spent near each stimulus mouse, and a preference index was calculated with the following formula: (time spent with the opposite sex – time spent with the same sex) / (total time spent with both the stimulus animals). A positive value indicates preference for the opposite sex, whereas a negative value indicates preference towards the same sex.
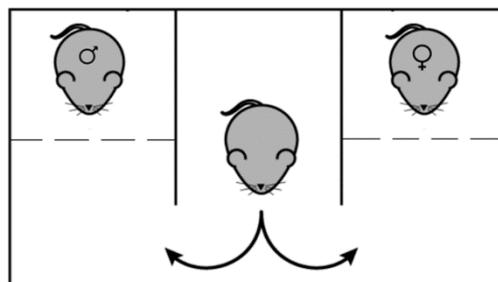

Figure 6: Schematic representation of the three-compartment box used to test sexual preference. Adapted from:
https://openbooks.lib.msu.edu/neuroscience/chapter/social-bonding/

Corticosterone and DHEA ELISA Assays

Corticosterone and DHEA were measured in two conditions to evaluate the baseline and the response levels. As indicated above, blood samples were collected in the home cage of the mice and 30 minutes following exposure to the elevated plus maze. The ELISA assays were performed using 96-well plates purchased for Enzo Life science (Corticosterone: ADI-901-097; LOT :05252110B ; DHEA: ADI-900-093; LOT NO :06222113). The assays were performed according to the manufacture instructions and the plates were read by a the Multiskan Ascent V1.24 spectrophotometer. Concentrations were calculated following the instructions of the product manual.

Experimental procedure

The timeline of the experimental procedure is shown in the figure (7). Below. To induce prenatal stress, pregnant dams were stressed by restrain stress during the entire gestation period (from E2 until delivery). Two months after delivery and once the offspring were adults, smear assay was performed for 3 weeks, on the females to evaluate the estrous cycle. Both male and female subjects were submitted to the EMP test. Blood samples were collected from the animals before and after EPM test to assess for HPA axis and anxiety. Female mice were ovariectomized and allowed to recover for 7 to 10 days. To evaluate for their sexual performances, females underwent 4 sessions of lordosis test, one session every 3 to 4 days, while males underwent 3 sessions of copulations that were separated by one week. Finally, sexual preference in both sexes was evaluated with the partner preference test.
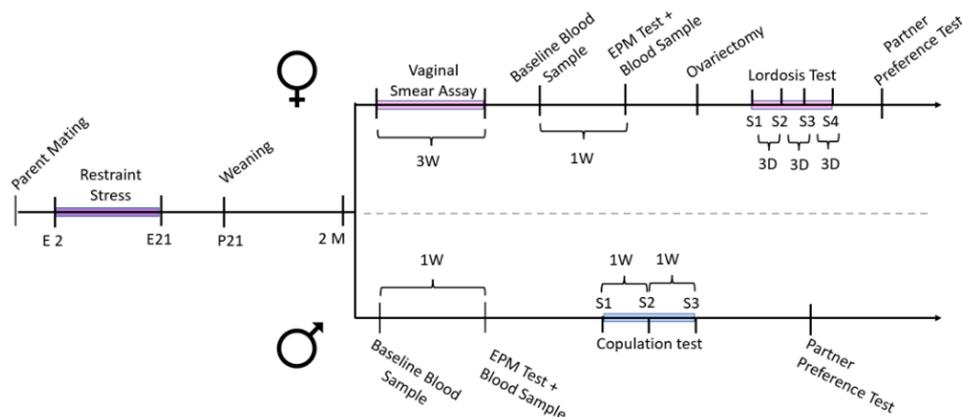
Figure 7: Schema representing the experimental timeline. The precise time is illustrated in day (D), weeks (W) and month (M). Upper part shows experiments performed on females and bottom part present male testing.

Statistics

Statistical analyses and graphs were obtained using GraphPad Prism 8. Normal data distribution was determined using D'Agostino & Pearson test. Depending on the data, we performed a t-test or repeated measure two-way ANOVA. Parametric Student's t-test was applied when the data presented a normal distribution and non-parametric Mann–Whitney U test was computed in the case of non-normal data distribution. All sessions average of lordosis, number and latency of mounts and intromissions were analyzed using a t-test. We performed the same test to analyze all sessions average of the ejaculation latency, the anxiety index and the sexual preference index as well. One sample t-test was applied to compare the sexual preference index to hypothetical value of 0. Repeated measure two-way ANOVA was applied to compare the number of days spent in each stage of the estrous cycle, stress hormone levels, lordosis behavior, sexual preference and the males copulation data. When appropriate, ANOVA was followed by Sidak's post hoc testing. Values were considered significant when $p < 0.05$.

Results

Results

The effect of prenatal stress on the murine estrous cycle.

The estrous cycle of the mice was investigated to look for eventual differences within the cycle and to check for the normal progression of cyclicity (Fig. 8A and Fig 8B). Two-way ANOVA analysis of the number of days spent in each stage of the estrous cycle revealed that prenatal stress had not affected the estrous cycling. No group effect was detected ($F_{(1, 25)}$ = 3,852, p=0,0609). However, a significant estrous stage effect was observed ($F_{(2,536, 63,40)}$ = 31,98, p<0,0001). Finally, no interaction effect between the stage and group factor was found ($F_{(3, 75)}$ = 1,286, p=0,2855).
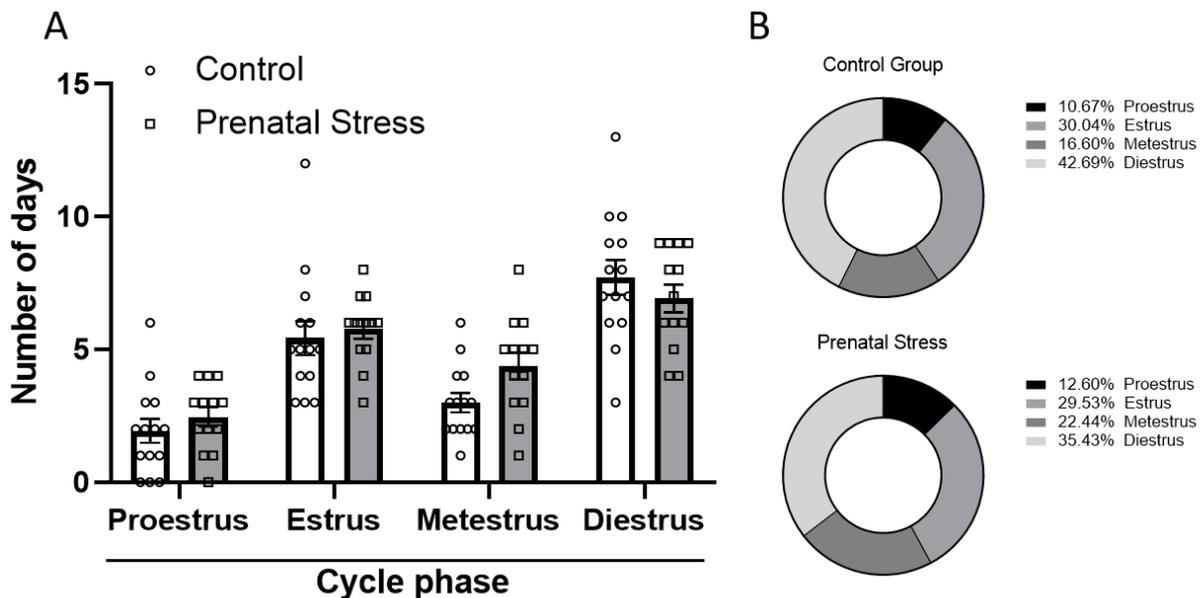
Figure 8: Effect of prenatal stress exposure on the estrous cycle. (A) The mean ± standard error of the mean (SEM) of the number of days spent in each of the 4 stages of the estrous cycle during the period of vaginal smears monitoring. (B) The number of days spent in every stage of the cycle expressed in percentages in a pie chart for both groups.

Prenatal stress did not alter the number of cycles undergone by the females during cycle monitoring period. Indeed, the statistical analysis indicated no significant difference between the animals exposed parental stress and the control (*p* = 0,8929, t = 0,7321; Fig. 9).

Figure 9: Number of cycles that the females went through during the period of the estrous cycle monitoring.

Determining the impact of prenatal stress on anxiety and the HPA axis.

Anxiety was evaluated with the elevated plus maze test (EPM test) whilst biological functioning of the HPA axis was determined through the assessment of stress hormones (corticosterone and DHEA) levels in plasma and calculating the CORT/DHEA ratio at baseline and after exposure to the EPM test.

Anxiety behavior

No clear effect of prenatal stress was observed on anxiety behavior of the females (Figure 10). This was confirmed by statistical analysis. For instance, according to the unpaired parametric t-Student test, when comparing both groups, the time spent in the open arms, the frequency of entries in the open arms and the time in the closed arms did not significantly differ. The same test demonstrated a significantly increased number of entries in the closed arms was observed in the prenatally stressed group although Mann-Whitney test did not show any effect of prenatal stress on the time spent in the closed arms (see values in Fig. 10A). Unpaired parametric t-test, comparison revealed no significant difference in the anxiety index between the two groups (p = 0,2508; t=1,176; Figure 10B). In addition, the statistical test did not reveal any significant difference in the total number of entries in all arms (p = 0,0778; t=1,839; Fig. 10C).

A

		Control	Prenatal stress	t-Test
		Mean ± SEM	Mean ± SEM	p-value
Open arms	Time (sec)	92,96 ± 6,861	114,8 ± 14,84	0,1831
	Number of entries	10 ± 0,9778	9,308± 0,97	0,6202
Closed arms	Time (sec)	114,7 ± 9,038	104 ± 13,93	0,2388
	Number of entries	10,15 ± 0,8110	13,14 ± 1,061	0,033
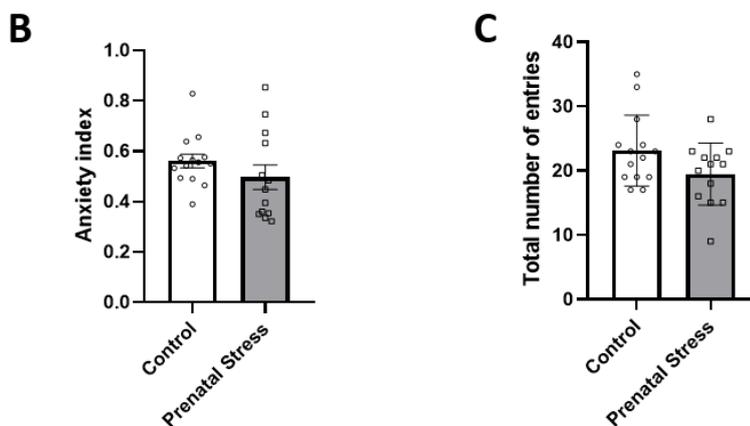
Figure 10: Anxiety assessment in female mice trough elevated plus maze test. A) Summary of the data and the statistical analyses comparing the frequency and time spent in the open and closed arms in both experimental groups. The values are expressed as mean ± SEM. B) Anxiety index: The bar plot illustrates the mean anxiety index ± SEM of the control and prenatal stress groups. C) Total number of entries in all arms during the EPM test.

Corticosterone levels

Prenatal stress did not affect corticosterone secretion in the females (Fig. 11). Repeated measure two-way ANOVA analysis showed a significant effect of the blood sampling condition ($F_{(1, 25)} = 162,3$; $<0,0001$), indicating a significant increase in the levels of corticosterone following exposure to the EPM. However, no significant group (p =0,0482; $F_{(1, 25)} = 4,314$) or interaction between the two factors were observed ($F_{(1, 25)} = 0,08546$; p = 0,7724).
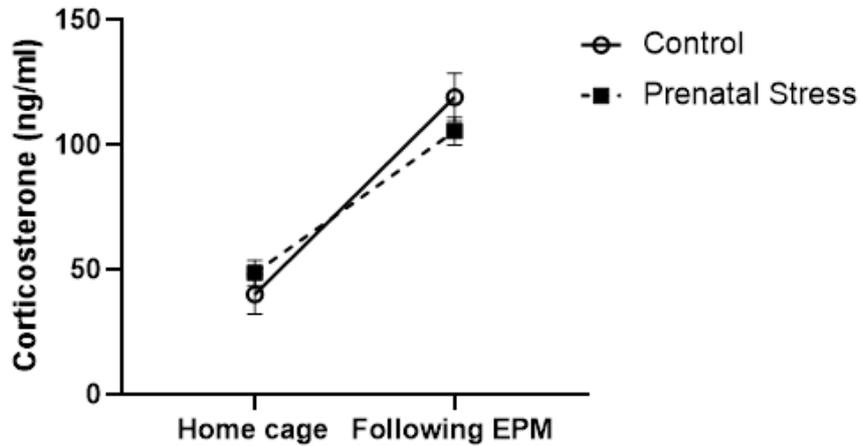
Figure 11: The graph represents the plasma concentrations of the corticosterone in both female groups measured in the home cage and following the EPM test.

DHEA Levels

Similarly, to corticosterone, DHEA secretion does not seem to be impacted by the prenatal stress exposure (Fig. 12). No significant group effect was detected by the repeated measure two-way ANOVA ($F(1, 48) = 2,269$; $p = 0,1385$). Similarly, no significant effect of the blood sampling condition (home cage vs following the EPM) ($F (1, 48) = 0,1529$; $p = 0,6975$) or the interaction between the two factors ($F (1, 48) = 0,8913$; $p = 0,3499$) were found. Interestingly, prenatal stressed females showed a tendency to lower DHEA levels after EPM test in comparison to the control animals.
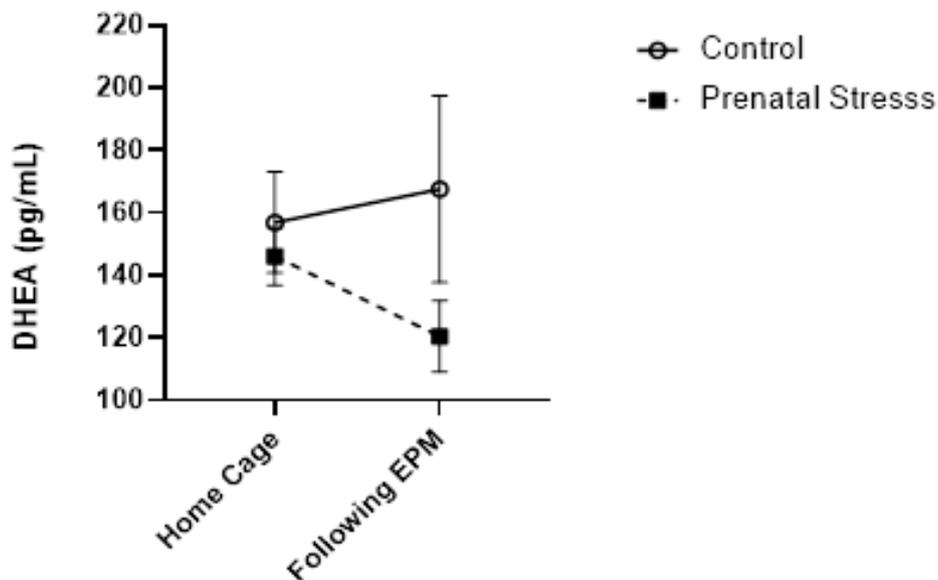

Figure 12: The graph represents the plasma concentrations of the DHEA in both female groups measured in the home cage and following the EPM test.

CORT/DHEA ratio

Consistent with the independent measurements of DHEA and corticosterone levels, the effect of developmental stress during prenatal period did not lead to the modifications of the general HPA axis functioning as demonstrated by repeated measure Two-way ANOVA analysis of CORT/DHEA ratios (Fig. 13). In fact, statistics did not show any interaction effect between the group and performing EPM test ($F_{(1, 19)} = 0,5792$); p=0,4560 > 0,05), nor was there a groups effect ($F_{(1, 25)} = 0,03177$ p=0,8600 > 0,05). However, highly significant increase was observed in the mean value of the CORT/DHEA ratio, upon submission to the EPM test ($F_{(1, 19)} = 52,69$; p < 0,0001 < 0,05).
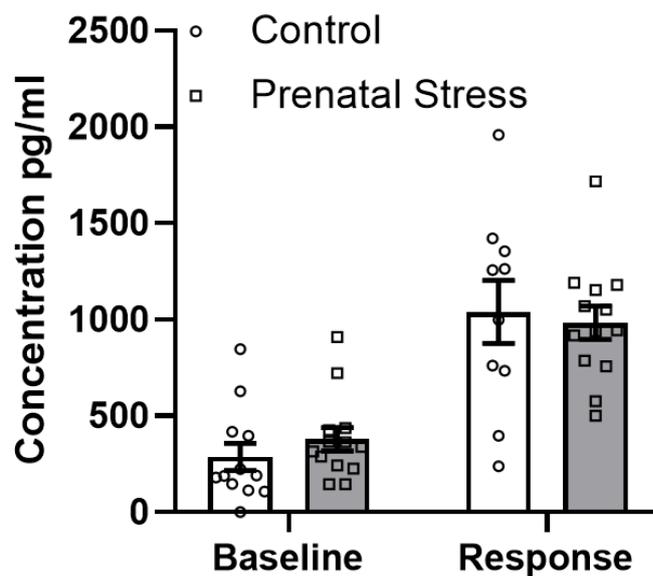

Figure 13: Assessment of the HPA axis in female mice. The mean corticosterone/DHEA ratio ± SEM before (Baseline) and after EPM test (Response).

Investigation of the impact of prenatal stress on the lordosis behavior.

Lordosis behavior in the female mice was assessed during four sessions. Statistical analysis confirms the absence of any alteration in the lordosis behavior upon prenatal stress exposure (Figure 14). The repeated two-way measures ANOVA revealed no group ($F_{(1, 25)} = 0,01177$; p = 0,9145) or interaction effect of group × session factors ($F_{(3, 75)} = 1,46$; p = 0,2324) on the lordosis behavior. Interestingly the analysis detected highly significant impact of the session on lordosis quotient ($F_{(2,856, 71,40)} = 41,04$; p<0,0001). Indeed, the percentages of lordosis increased over time until reaching a plateau (Fig. 14A).

Furthermore, unpaired parametric t-test of all session average lordosis percentages confirmed that there was indeed no difference in lordosis behavior when comparing prenatally stressed animals to controls (p =0,9144; t=0,1085; Figure 14B).
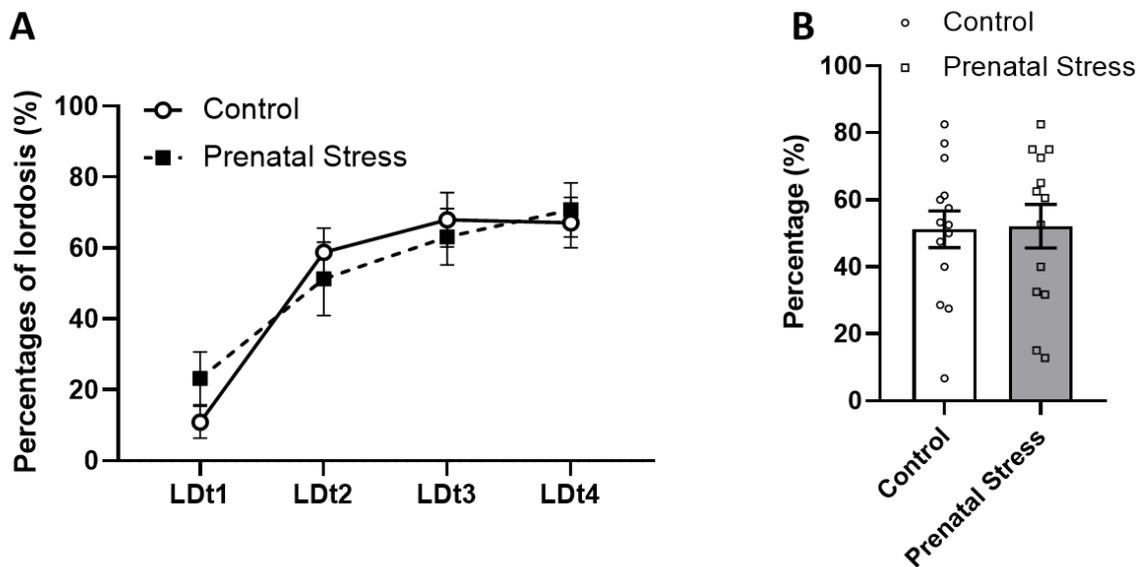
*Figure 14: Lordosis behavior in female mice. Four sessions of lordosis test (LDt) were performed on the adult female mice to assess for the consummatory behavior. A) Graphical illustration of the mean lordosis quotient (%) ± SEM of the four sessions, B) All sessions lordosis average ± SEM represented by bar plot.*

The effect of prenatal stress on the sexual preference in females.

Female sexual preference was evaluated with the partner preference test (Fig. 15). The results indicated that prenatal stress reduces sexual preference in female mice.

Unpaired parametric t test analysis did not detect significant difference in the comparison of sexual preference of both groups (p = 0,4345; t=0,7944; Fig 15A). However statistical analysis of the sexual preference index with a one sample t-test indicated that sexual preference is significantly different than the hypothetical value of 0 in the control group (p=0.021, t=2.643) while the prenatally stressed animals was not (p=0.298, t=1.085) (Fig. 15A).

Also, unpaired parametric t-test did reveal that the total number of entries performed by both groups did not significantly differ from each other (p = 0,5526; t = 0,6019; Fig. 15B), indicating that the observed effect in the sexual preference is not associated with locomotion.
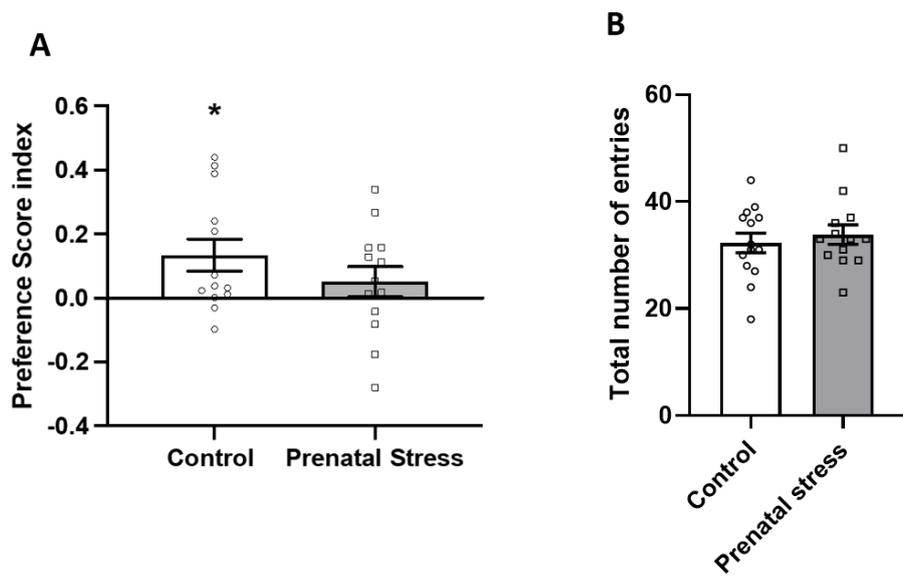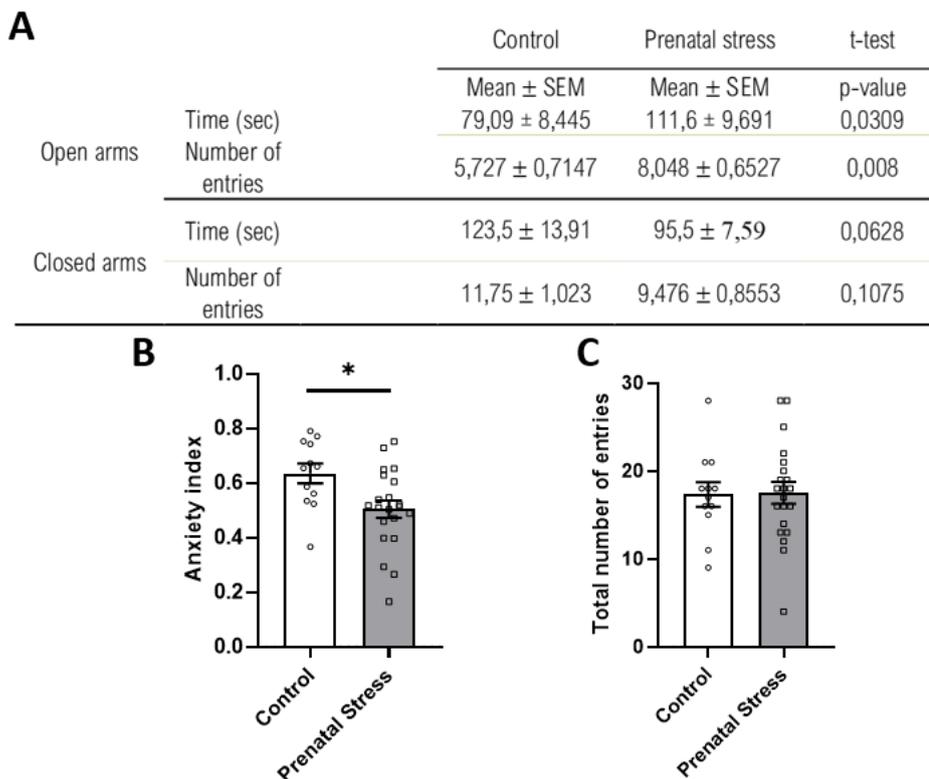
Figure 15: Application of partner preference test to explore female appetitive behavior. A) Female sexual preference index ±SEM for each group represented in a bar plot illustration. B) Indicators for mobility: The mean average counts ±SEM of the total number of entries in both compartments illustrated in a bar plot.

Determining the impact of prenatal stress on anxiety and the HPA, the innate stress management system in male adult mice.

Similarly to females, the EPM behavioral test and the measurement of the stress hormones were performed on the males to assess for behavioral and biological components of anxiety.

Anxiety behavior

According to the statistical analysis, prenatal stress induced a significant reduction of anxiety. Unpaired t-test showed that prenatal stress significantly increased the time and the number of entries in the open arms. In contrast, the analysis did not find any difference between the groups for the time or the number of entries in the closed arms (Fig. 16A). Similarly, the total number of entries in both arms did not differ between the groups (p = 0,9226; t=0,09791; Fig 16C). Also, the anxiety index in the prenatally stressed males was significantly lower in comparison to the control as shown by the unpaired parametric t-test (p = 0,0148; t=2,582; Fig. 16B).

		Control	Prenatal stress	t-test
		Mean ± SEM	Mean ± SEM	p-value
Open arms	Time (sec)	79,09 ± 8,445	111,6 ± 9,691	0,0309
	Number of entries	5,727 ± 0,7147	8,048 ± 0,6527	0,008
Closed arms	Time (sec)	123,5 ± 13,91	95,5 ± 7,59	0,0628
	Number of entries	11,75 ± 1,023	9,476 ± 0,8553	0,1075

Figure 16: Anxiety assessment in male mice trough elevated plus maze (EPM) test. A) Data comparing the EPM test result expressed in mean (M) ± SEM summarized in a table. B) Anxiety index: The bar plot illustrates the mean anxiety index ± SEM of the control and prenatal stress groups. C) Indicators for mobility: The mean ± SEM average counts of the total entries in the arms of the maze per group by of a bar plot.

Corticosteroid levels

ELISA assay revealed a significantly reduced levels of corticosterone in male mice exposed to prenatal stress (Figure 17). The repeated measure two-way ANOVA analysis indicated a significant effect of group, significant effect of the blood sampling condition (Home cage vs EPM) and a significant interaction between the two factors (Group: p = 0,0023; F (1, 31) = 11,06; sampling condition: p < 0,0001; F (1, 31) = 293,0; Interaction: p <0,0001; F (1, 31) = 22,82). Post hoc comparisons demonstrated that the baseline corticosterone in both groups were not significantly different (p = 0,9836). However, after exposure to the EPM test, prenatally stressed males presented a significantly less increase in corticosterone release compared to the controls (p < 0,0001). Nevertheless, post hoc analysis also showed that within both groups the application of EPM test treatment resulted in increased corticosterone release (C: p < 0,0001 PN: p < 0,0001).
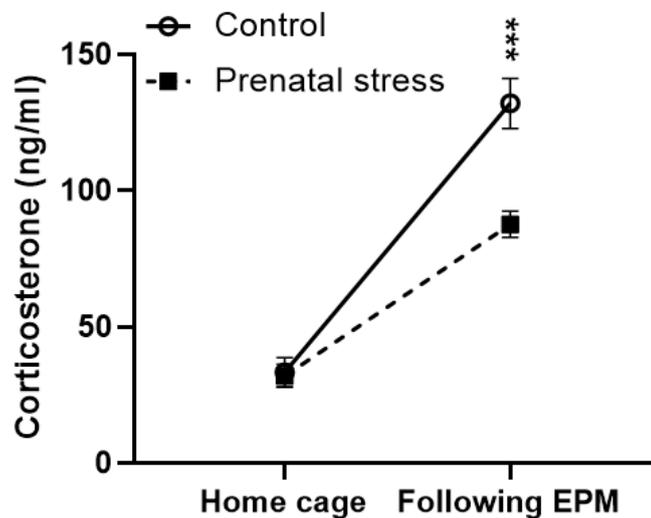
Figure 17: Investigating the HPA axis functioning in male mice trough measurements of the plasma concentration of corticosterone at base line and after EPM test. The graph represents the mean corticosterone levels ±SEM in the blood of the animals of both groups.

DHEA levels

Likewise, to corticosterone levels, prenatal stress impacted the levels of DHEA (Figure 18). Statistical analysis using two-way ANOVA revealed a significant effect of the group, significant effect of the blood sampling condition and a significant interaction (Group: F (1, 31) = 9,313; p=0,0046; EPM test: F (1, 31) = 16,15; p = 0,0003; Interaction: F (1, 31) = 5,366; p = 0,0273).

While control males had significantly higher basal DHEA levels in comparison to the prenatally stressed animals (p = 0,0008), the difference between the groups disappeared after performing EPM test (p = 0,1456) as revealed by Sidak's post hoc test. This was further illustrated in the post hoc test when comparing the DHEA values within the groups. In fact, the control males had significantly higher DHEA secretions at baseline than after EMP test

(p=0,0008), but the prenatally stressed animals presented no significant change in DHEA secretion before and after EPM test (p = 0,3078) (Figure 18).
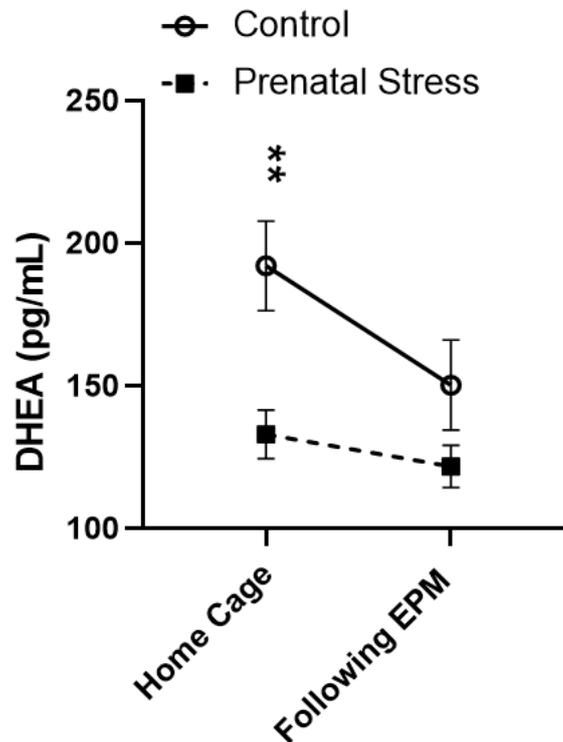

Figure 18: Investigating HPA axis in male mice trough measurements of the plasma DHEA of corticosterone, at base line, and after EPM test. The graph represents the mean DHEA levels $\pm$SEM of the animals in their home cage and after stress.

CORT/DHEA ratio

The effect of prenatal stress on the HPA axis is further confirmed by statistical analysis of CORT/DHEA ratio (Figure 19). Repeated measure two-way ANOVA showed no significant effect of the group (F (1, 31) = 0,6502; p = 0,4262) but significant effect of the EPM exposure (F (1, 31) = 189,7; p < 0,0001) and a significant interaction of both factors (F (1, 31) = 8,896; p = 0,0055).

Post hoc test indicated that comparison of the ratio values at baseline where not significantly different (p = 0,3462), but it reached statistical significance, with the prenatally stressed males having lower ratio values in comparison to controls when exposed to the EPM test (p = 0,0265). Nevertheless, Sidak's comparison test demonstrated that in both groups, the effect of EPM test was significantly inducing an increase of the CORT/DHEA ratio (C: p < 0,0001; PN: p <0,0001).
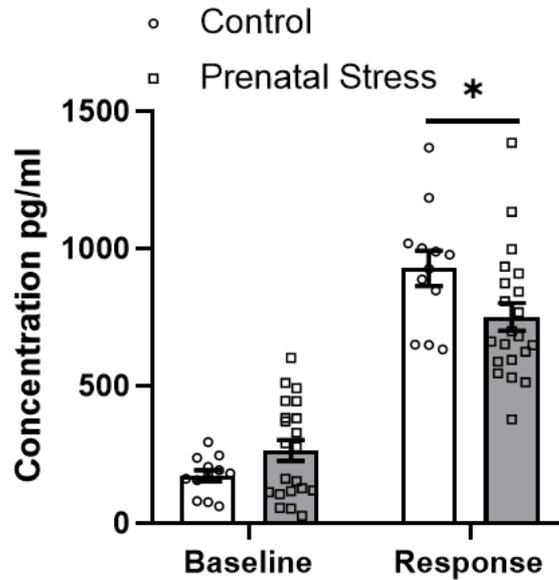
Figure 19: Assessment of the HPA axis in male mice. The mean corticosterone/DHEA ratio ± SEM before (Baseline) and after EPM test (Response).

## The effect of prenatal stress on the consummatory sexual behavior of male mice.

Copulatory test was performed on the adult male mice to evaluate their consummatory sexual behavior. In total, five parameters were evaluated, latencies to ejaculation, to the first intromission and to first mount, plus the number of mounts and intromissions.

Latencies

The results show that prenatal stress did not impact the males' sexual performance as demonstrated by the statistical analysis (Figure 20). Repeated measure two-way ANOVA was applied to compare each dataset. For the mounts, latencies, the analysis detected significant trial effect ($F_{(2, 62)} = 23,05$; $p < 0,0001$; Fig. 20A) and interaction effect of trial and group ($F_{(2, 62)} = 3,379$; $p = 0,0405$; Fig. 20A) but no effect of the group ($F_{(1, 31)} = 0,7430$; $p = 0,3953$; Fig. 20A) on the latency to the first mount. Post hoc analysis showed no difference in the latency to the first mount between the groups in neither of the trials (Trail 1: $p = 0,9479$; Trial 2: $p = 0,1328$; Trial 3: $p = 0,9027$; Fig. 20 A).

Regarding the latencies to the first intromission, no effect of the group ($F_{(1, 31)} = 0,3336$; $p = 0,5677$; Fig. 20B) or interaction effect of group and trial ($F_{(2, 62)} = 0,6419$; $p = 0,5298$; Fig. 20B) could be determined on the latency to the first intromission by data analysis, even though there was a significant trail effect on the variable ($F_{(2, 62)} = 13,21$ $p < 0,0001$; Fig. 20B). Likewise, comparison of the latency to the first ejaculation indicated that group and interaction (group x trial) effect were not significant (Group: $F_{(1, 31)} = 0,5576$; $p = 0,4609$;

Interaction: F (2, 62) = 0,07985; p = 0,9234; Fig. 20C), whereas significant trial effect was detected (F (2, 62) = 11,20; p = 0,0001).
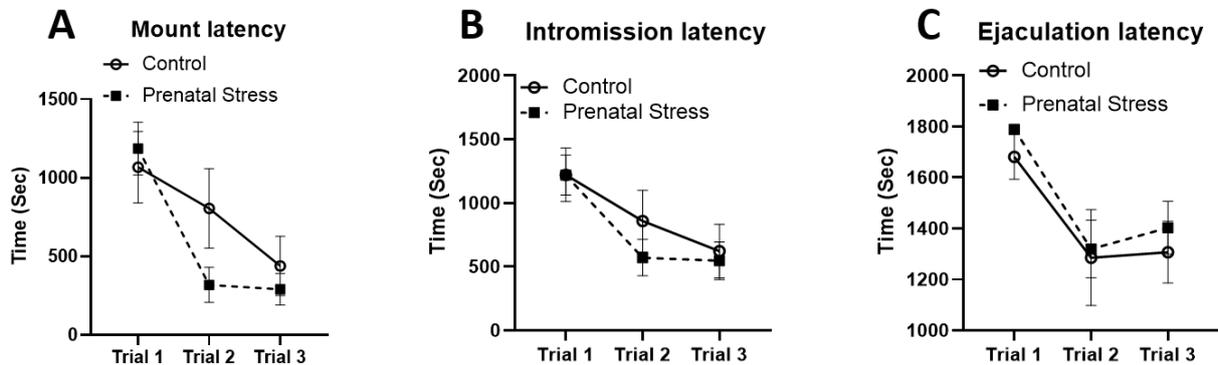
Figure 20: Latency to sexual behavior in adult male mice. The latency to 1st mounting (A), 1st intromission (B) and ejaculation (C) was measured in every copulation test session. The graph mean latency ±SEM for each test session.

Unpaired t-test analysis did not show significant effect of prenatal stress on all session averages latency to 1st mount or latency to 1st intromission nor latency to ejaculation (mount: p=0,5422; U = 109; Fig. 21A; intromission: p=0,6708; U = 114; Fig. 21B; ejaculation: p = 0,4609; t=0,7467; Fig. 21C).
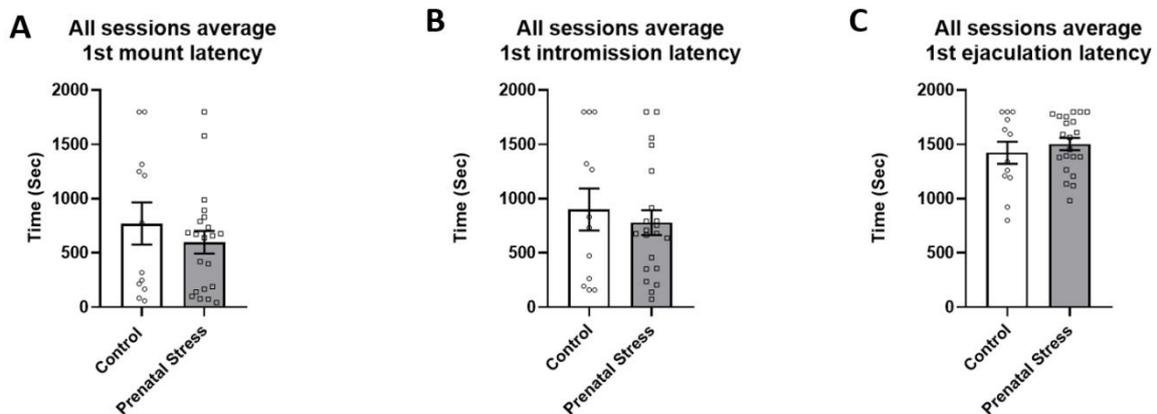
Figure 21: Latencies to multiple components of sexual behavior in male mice. The all sessions average latency to 1st mounting (A), 1st intromission (B) and ejaculation (C) ±SEM represented in the bar graph.

Frequency of mounting or intromission

Data presented in figure 22 show no clear effect of exposure to parental stress on the frequency of mounting or intromission behaviors. Repeated measure two-way ANOVA on the number of mounts per testing trial revealed no group effect, or trial effect nor interaction effect of both factors (group: $F_{(1, 31)} = 0,6046$; $p = 0,4427$; trial: $F_{(2, 62)} = 1,333$; $p = 0,2711$; interaction: $F_{(2, 62)} = 0,7392$; $p = 0,4817$; Fig. 22A).

For the frequency of intromission, a significant trial effect was detected but group effect and interaction effect were statistically not significant (group: $F_{(1, 31)} = 1,338$; $p = 0,2562$; trial: $F_{(2, 62)} = 6,007$; $p = 0,0041$; interaction: $F_{(2, 62)} = 0,6370$; $p = 0,5323$; Fig. 22B)

Figure 22: Sexual behavior of male mice. The number of mounts and intromissions were count in every copulation trial. The graphs represent the evolution of the number of mounts and intromissions during the sessions.

Analysis of all sessions average corroborate the observations of the individual session data (Figure 23). Prenatal stress males did not express any significant changes in the number of mounts or intromission. Group comparison by unpaired parametric t-test analysis indicated no significant differences in neither Mounts ($p = 0,4427$; $t=0,7776$; Fig. 23A) or Intromissions ($p = 0,2563$; $t=1,157$ Fig. 23B).
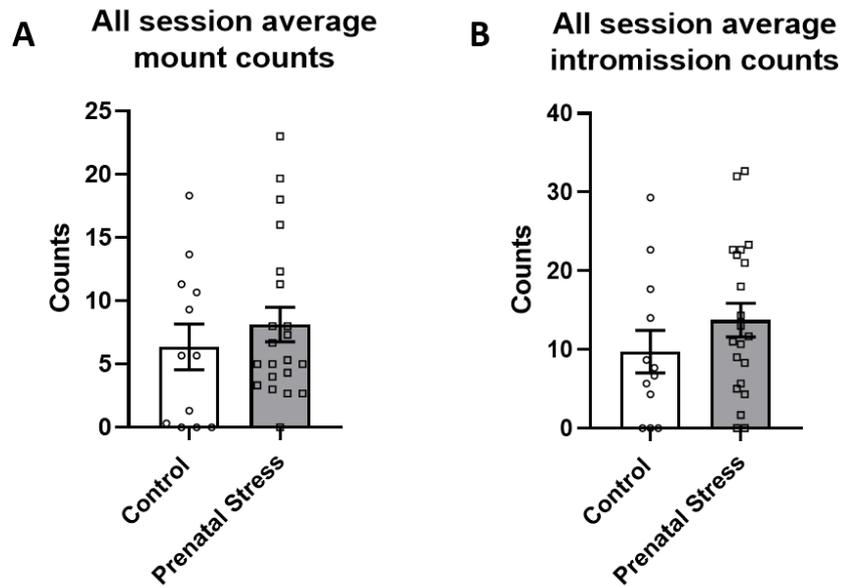
Figure 23: Sexual behavior of male mice. All sessions average number of mounts and intromissions± SEM graph representation.

The effect of prenatal stress on appetitive sexual behavior of male mice.

Exploration of male sexual preference was performed with the partner preference test. Interestingly application of stress during prenatal development attenuated sexual preference in the male offspring.

Statistical analysis using unpaired parametric t-test revealed a lower preference index toward the female stimulus compared to the control group (p = 0,0497; t=2,042; Fig. 24A). Similarly, one sample t-test showed that, compared to a hypothetical value of 0 (no preference), the control males presented highly significant preference for the females (p = 0,0002; t=5,578), whereas the prenatally stressed males also expressed a significant preference for the females (p = 0,0461; t=2,126), but not strong as the control animals.

Finally, unpaired parametric t-test did not reveal a difference in the total number of entries when comparing both groups (p = 0,2710; t=1,121; Fig. 24B).
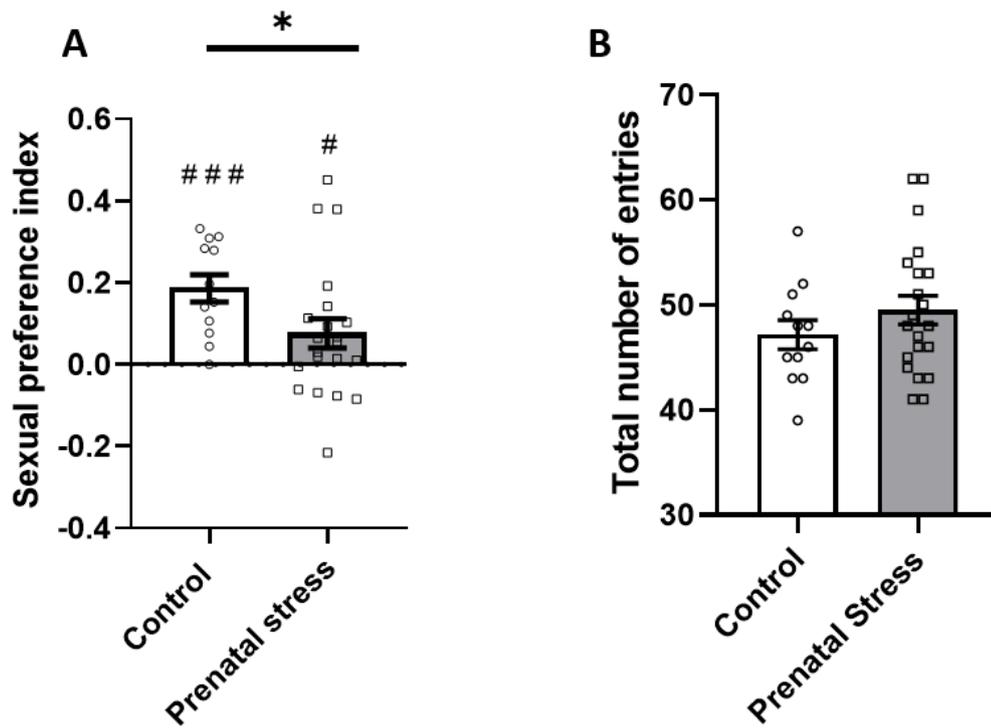
*Figure 24: Male appetitive behavior assessed with the partner preference test. A) Male sexual preference index ± SEM represented in a bar plot illustration. B) Indicators for mobility: The mean ± SEM average counts of the total entries in compartments per group illustrated in a bar plot. ***p < 0,0001; *p < 0,05 group comparison; ###: p < 0,0001; #: p < 0,05 comparison to hypothetical value of 0 (no preference).*

Discussion

Discussion

Effect of prenatal stress on the estrous cycle.

The examination of the estrous cycle showed no difference in the cyclicity, nor in the duration of each stage, when comparing the prenatally stressed animals to the controls. Prenatally stressed females presented every stage of the estrous cycle with the same amount of time as the control subjects. Additionally, the number of cycles observed in the prenatally stressed mice was similar to the control group. These results indicate that prenatal stress did not impact the estrous cycle , which is in contrasts with studies evaluating the effects of stress during adulthood (Casillas et al. 2021).

The exact mechanism by which the estrus cycling seems to be preserved of the deleterious prenatal stress effects are not quite known yet. We propose the hypothesis that this phenomenon may be linked to the timing of ovarian maturation, because ovarian secretions are among the primary factors that regulate estrous cyclicity (OECD 2008).

According to previous reports, ovarian maturation in rodents takes place most likely after birth. So females are born with quiescent ovaries, which is supported by histological investigations showing immature ovaries right after birth (Luis and Moncayo 2007). In our experiment the chronic stress was applied to the females before they were even born. So, we hypothesized that because ovarian maturation and activity onset takes place postnatally, prenatal stress could not have altered ovarian functioning, simply because they are not even functioning anyways at that stage. Considering this, logically prenatal stress has no impact on ovarian maturation thus resulting in normal ovarian activity that eventually yielding proper estrous cycling.

Also, these results seem to be consistent with what has been observed in our HPA axis investigation, considering that stress exposure in adulthood induces reversible problems by mediating HPA-HPG interaction. This has also been observed in humans. For instance, functional amenorrhea in women is believed to be the result of the exposure to highly stressful environments. Interestingly, the condition is resolved with time when the stressors disappear (Podfigurna and Meczekalski 2021). According to the classic HPA-HPG interaction theory, stress promotes glucocorticoid secretion, which in turn directly act on the hypothalamus to block the GnRH neurons activity. Probably that once the stress factor dissolve, stress hormones return to the homeostatic levels, thus no more interfering with hypothalamic functioning. In our case, the absence of prenatal stress exposure effect on the estrous cycle is probably associated with the normal functioning of the HPA axis.

Impacts of the prenatal stress on anxiety and the functioning of the HPA axis.

The main utility of the elevated plus maze test in our case was to trigger the HPA axis but also to assess for the anxiety levels. In the present study, the EPM test showed no difference between the control and the prenatally stressed females, as both spent equal amounts of time in the open arms of the elevated plus maze. This suggests that anxiety was not impacted by

chronic stress exposure to prenatal stress. In addition, corticosterone and DHEA levels measured in both groups before and after the EPM test were statistically not different, showing normal secretion even in prenatally stressed females. Surprisingly, for the male mice, the results show that prenatally stressed animals spent significantly more time in the open arms than the control animals. Similarly, the anxiety index of the prenatally stressed males was significantly lower compared to the control group. Both results indicate a reduced anxiety in the offspring that were exposed to prenatal stress. Note that the mobility, which was assessed via the total number of entries, indicated that in both groups of mice were investigating the at the same rate the maze arms, thus the observed anxiety findings are not due abnormal locomotion.

Also, the overall HPA functioning was assessed by the calculation of the CORT/DHEA ratio. In the female mice, there was no difference between the stressed and the control groups when comparing the ratio at baseline or after EPM test. The corticosterone levels of the prenatally stressed males were similar to the control's levels at baseline but were significantly lower after exposure to the EPM test. No differences were found for the DHEA levels, however the CORT/DHEA ratio after EPM test was lower in the prenatally stressed males. These data suggest that prenatal stress induced alterations of the HPA axis in males. One could argue that prenatally stressed females did not show any signs of HPA dysfunction is due to ineffective prenatal stress induction. However, prenatal stress exposure was performed through 3 restraint sessions per day (45 min each) on the pregnant dams. This method was successfully used in different studies and has been shown to effectively induce prenatal stress in the offspring (Baier et al. 2012; Bustamante et al. 2010, 2013). Besides, the male littermates of the female mice did show altered HPA function in our study (discussed below), which indicates that the protocol we used was effective. Other potential explanations for the competent HPA axis in our female lies in the fact that female offspring were housed in groups during the whole experience, which could have constituted a positively enriched environment. Reports show that exposure to enriched environment, has the capacity to reverse the negative effects of prenatal stress (McCreary and Metz 2016). Also, it is important to consider that the estrous cycle of the mice may also have played a role in the anxiety behavior of the females. Our females were tested for anxiety when they were on proestrus-estrus stage of the estrous cycle. Several reports have demonstrated that these stages interfere with the anxious behavior by lowering the expression of anxiety in rodents (D'Souza and Sadananda 2017). Furthermore this effect is thought to be the result of hormonal and chemical changes that happen during the stage (D'Souza and Sadananda 2017; Mora, Dussaubat, and Díaz-Véliz 1996). In our case, the effect of the proestrus-estrus stage might have also influenced the mechanism that regulate anxious behavior thus might have masked potentially HPA dysfunction in our prenatally stressed females.

Regarding our results for the male subjects, there are multiples hypothesis to explain our observations. In fact, studies in non-human primates found that prenatally stressed offspring were less likely to present depression and anxiety-like behavior. Instead, they often showed signs of adaptation and resilience (Ceniceros, Capitanio, and Kinnally 2021). Similarly, children of women that suffered partner violence during pregnancy, where found to be much more robust against developing psychiatric and emotional disorders when exposed to adverse

events later in life (Serpeloni et al. 2019). In the same paper, the authors established an association between epigenetic modifications that have previously been linked to resilience, and those that were also found in the prenatally stressed children (Serpeloni et al. 2019). However, the authors point out that the sampling method they used was not adequate, so further investigations are required. Nevertheless, the paper provided interesting hypothesis that might explain our findings. Likewise, prenatally stressed rat presented better performances in forces swimming test with lower immobility time as compared to control animals (Montes et al. 2016). Again, this effect of prenatal stress was associated to epigenetic changes leading to differential gene expression in the brain eventually resulting with improved capacities of coping with stress exposure during adulthood.

Another important consideration is that the results of prenatal stress depend on two major factors, which are the parameters of the stressor and individual sensitivity to stress. These factors might account for the different observations made on stress studies in literature. For instance, stress is determined by multiple features such as the timing of stress exposure, the magnitude of the stressor, the duration and frequency of stress. So the final effect of stress will probably depend on how these parameters are set (Rao and Sadananda 2016). As an example, performing twice the same stress experiment but varying the duration of stress exposure might yield different results. Then, more and more studies indicate individual sensitivity to stress that might derive from genetic or epigenetic inputs, influence the outcome of stress (Rodgers et al. 2015; Yao et al. 2014; Yehuda et al. 2005). Therefore, the fact that our male data contrast with other studies that show augmented anxiety in prenatally stressed male rodents (Fride and Weinstock 1988; Van Den Hove et al. 2005) , might not only rely on different methodology concerning prenatal stress and species but also on epigenetic and genetic signature of our experimental animals. Likewise, undisrupted anxiety behavior observed in our prenatally stressed female, which is contradictory to other reports (Fride and Weinstock 1988; María E. Pallarés et al. 2007) could be due to the same reasons as earlier explained for male data.

Effects of prenatal stress exposure on sexual behavior.

Sexual behavior can be categorized to consummatory behavior (sexual performance) and appetitive behavior (sexual preference). In our experiment both components were tested in female and male animals separately.

A. Sexual performance

Lordosis test, which assesses the consummatory component of the sexual behavior in females showed that the prenatally stressed females were as receptive to male mice as the control mice. Additionally, the proportions of non-receptive females were similar to what is observed in the controls. These observations suggest proper copulatory behavior, thus prenatal stress seemed not to affect the consummatory component of sexual behavior in the females.

Regarding the copulation test, the prenatally stressed males performed equally to the control animals. So, at first sight the data show that prenatal stress did not affect sexual performance of the male mice. However, we hypothesize that these

observations are probably due to the poor performance of the control animals. Indeed, according to unpublished data in our lab and a previous study using the same parameters of testing as our study (Honda et al. 1998), the control animals are supposed to express much higher number of mounts and intromissions then what we observed in here. The origin of this discrepancy is not exactly known. However, it is probable that an issue during the video scoring might account for this observation.

Our initial hypothesis was that the sexual behavior can be altered in males but not females following exposure to prenatal stress. This idea was based on the sexually dimorphic fetal development of the males and the females. Indeed, it has been shown that the sexual differentiation of the males happens mainly during the prenatal period under the effect of steroid hormones, while females brain differentiate during the prepubertal period (Bakker and Brock 2010). Thus, contrary to males, females' typical sexual behavior is less likely to be altered by parental stress.

B. Sexual preference

Sexual preference was evaluated in both males and females using a partner preference test. In the females, we found that, compared to the control group, subjects exposed to prenatal stress express low preference toward the male. As shown in the results, the lack of preference was not associated with a locomotion problem.

The understanding of female neuronal circuits involved in the regulation of female sexual behavior is required to explain how prenatal stress can probably impair the preference but not the lordosis. Partner preference, which reflects sexual motivation, and lordosis, which reflects the sexual performance are controlled by close, yet divergent neuronal circuits. Both circuits start with the olfactory inputs that innervate the amygdala. The neuronal information is then transmitted to the AVPV kisspeptin neurons. From there, the circuits diverge, as the AVPV neurons send projections to the GnRH neurons to modulate mate preference (Hellier et al. 2018) while the AVPV neurons project to the nNOS neurons of the ventrolateral part of the ventromedial nucleus (VMHvl) to regulate lordosis behavior (Bentefour and Bakker 2021).

Intact lordosis behavior in prenatally stressed females indicated proper functioning of the neurons implicated in both appetitive and consummatory behavior such as the AVPV neurons. Thus, only the neuronal structures involved in the expression of sexual preference downstream the AVPV neurons are potentially disrupted leading to reduced partner preference. The dysfunction can be associated with various problems such as defective GnRH neurons, impaired kisspeptin receptors on the GnRH neurons and thus incapacity of responding to AVPV stimuli or damaged neuronal transmission between and the two nuclei. A counterargument to this explanation would lie in the normal estrous cycling observed in these mice,

which points to a rather proper functioning of the GnRH system. However, we cannot exclude the possible existence of specialized subpopulations of the GnRH neurons controlling the estrous cycle and sexual preference that are differently impacted by the prenatal stress.

Another possible explanation of the reduced sexual preference involves the serotonin system. Different animal studies have demonstrated that prenatal stress, including restraint stress, is able to alter and even suppress the functioning of the serotoninergic system of the offspring with permanent effects (St-Pierre et al. 2016). Indeed, the serotonin system has previously been shown to participate to the modulation of sexual preference of the female mice (Zhang, Liu, and Rao 2013). The same study found that two separate mice models with a genetic serotonin depletion (model 1: absence of serotonin neurons; model 2: absence of serotonin release) that females expressed a sexual preference for females. Interestingly these animal models presented a normal lordosis behavior (Zhang, Liu, and Rao 2013).

Males exposed to prenatal stress also expressed a reduced time spent next to the female stimulus compared to the control group. The reasons for the impairment of male sexual behavior may be traced back to fetal development. As explained earlier, sexual hormones are necessary for the normal development of male typical behavior. Their primitive testis produce testosterone already in utero, which contribute to the sexual differentiation and the development of the neuronal circuits involved in sexual behavior (J. Bakker and Brock 2010). Actually, it was found that prenatal stress exposure in rats interferes with these hormone levels thereby completely altering the fetal development in male rats. This resulted in an impaired sexual behavior (Ward and Weisz 1980). Other reports show that glucocorticoids can suppress testosterone release directly by inhibiting the action of the Leydig cells of the testis in adult rats (Welsh, Bambino, and Hsueh 1982). Based on these finding it is possible that excess maternal corticosterone of stressed dams interferes with the normal functioning of the fetal testis and blocking the normal development of male typical behavior. This is supported by studies investigating the effects of prenatal stress on brain development. For instance, in rat Anderson et al. found a reduced size of the preoptic area in male following exposure to prenatal stress (Anderson, Rhees, and Fleming 1985).

Equally to female preference, disruption in serotonin system might also be implicated in the partner preference of the male mice, as supposedly, genetically serotonin depleted mice have lost sexual preference, which could be rescued with pharmaceutical injections of serotonin precursor (Liu et al. 2011). However according to a following study using the same serotonin depleted mice, serotonin is not associated with mate preference in males (Angoa-Pérez et al. 2015). They propose that differences linked to different methods used to investigate preference might be one of the factors that explain the contrasting results. In addition, they argue that the pharmaceutical agent used for rescue experiments performed in the first study, has a wide range of effects, including interaction with

neuronal system other than serotonin circuit, so it is not specific enough to associate the effect of rescued partner preference exclusively to serotoninergic system.

For our study, additional testing using GnRH and serotonin precursor are necessary, first, to understand the mechanisms behind lacking sexual preference in the prenatally stressed females and males and secondly, to confirm or refute the theory of serotonin involvement in male mate preference.

Conclusions and Perspectives

Conclusions and Perspectives

In both sexes, the expression of normal sexual behavior needs prior organizational and activational inputs of sex hormones at different time points, and stress exposure during a specific time frame, can have a major impact on the normal development of the neuronal circuits regulating sexual behavior. In our study, we demonstrated that stress exposure during a critical period of brain development can lead to a permanent disruption of some aspects of reproduction.

Consistent with prior reports, we provide evidence not only that prenatal stress affects male and female offspring's behaviors, but also that these changes are sexually dimorphic. Indeed, our study confirmed our hypothesis by showing altered sexual preference in both female and male subjects exposed to prenatal stress. Nevertheless, the experiments that we conducted are mainly behavioral observations. So, the next step is to identify the exact neuronal circuits that were impacted by prenatal stress by performing rescue experiments for instance. As discussed above, female lost their sexual preference and according to the literature there is two potential candidates that could explain the observed effects; serotonin and GnRH neurons. Thus, rescue experiments using serotonin precursor or GnRH injections might allow us to confirm or not their implication. Similarly, male subject also showed lower preference toward the females. Literature on the implication of serotonin in male preference is rather conflictual, so serotonin rescue experiment might clarify the question and potentially show the origin of altered mate preference in males.

In addition, histological investigations are necessary. For instance, immunostaining of the brain tissue following sexual stimulation might also give us important information, such as a reduced number of neurons or reduced neuronal activation of the brain areas implicated in the regulation of sexual preference.

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Annexe 1

Exhaustive list of used materials

Material	Reference
Antisedan	KELA Nacro stop Atipamezole HCL 5mg/ml LOT: 17I211
Audio & video Software	Scion Visicapture 1.1
Bedding	Osafe bedding – premium scientific bedding rettenmaier & schöne GMBH + co KGworldwide headquarters 7394 Roseberg Germany
Camera	Color digital camera Scion corporation Model CFW-1612C
Capilaires	Marienfeld microheamatocrit capillary tubes, Sodium heparinized 80iu/ml lot: 2405424
ELISA Kit (Corticosterone)	Enzo corticosterone ELISA kit CAT. No ADI-901-097; LOT NO :05252110B EXP DATE 30 apr 2022
ELISA Kit (DHEA)	Enzo DHEA ELISA kit CAT. No ADI-900-093 LOT NO :06222113 EXP DATE 30 apr 2022
Estradiol	SIGMA Life Science, beta-estradiol LOT NO: 060M0149V
Eye ointment	PFIZER Terramycine + polymyxine B 5 mg/g +10000IU/g
Ketamin	Nimatek Ketamin 100mg/ml lot 115632
Magnetic plate/stirrer	Cenco instrumenten b.v. ETS VANGELE hotplate magnetic stirrer CAT no 34532
Material	Reference
Medetomidine hydrochlochride	Orionpharma Vetoquinol Domitor 1mg/ml 10 ml 134737-5 lot: 2049108
Medical adhesive paste	Dow Corning Corporation Midland, MI 48686-0994 USA Silastic medical adhesive scilicone type A not sterile
Microscope	BH2 Olympuus BHTU Trinocular Microscope
Microscope slide	Epredia Superfrost plus adhesion microscope slides REF J1800AMNZ 25x75x1mm Gerhard Menzel GmbH Saarbrückener St 248 Germany
Microscope slide	Epredia Super frost® Plus; Erie Scientific LLC; USA; Ref: J1800AMNZ
Progesterone	Sigma life science; Progesterone 99% 4-pregnene-3-20-dione lot #SLBK5683V
Scale	Scale: Kern & Sohn GmbH Ser No: WB14AJ0064 D-72336 Balingen
Seasame Oil	Sigma life science sesame oil; LOT 3 noMKBQ0543V
Serynge and needles	Terumo Syringe with needle 26Gx1/2 1ml (0,45x13mm) Terumo (Philippines) Corporation LAGUNA Technopark, BBinan, Laguna Philippines, Terumo europe N.V, Leuven Belgium
Statistic software	Graphpad Prism 8
Toluidin blue	Vel n.v. s.a; Toluidin blue 0 lot 97022143