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Sex-specific differences in hippocampal development:

impact on stress and epileptogenesis

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Sex-specific differences in hippocampal development:

impact on stress and epileptogenesis

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RÉSUMÉ

Les différences sexuelles ne se limitent pas uniquement aux organes de reproduction, elles sont aussi très marquées dans plusieurs pathologies humaines. De ce fait, les études impliquant un seul sexe ne pourraient jamais permettre d'élucider les mécanismes qui sous-tendent ces pathologies. De plus, l'exclusion des femelles/filles/femmes des protocoles de recherche a des impacts négatifs sur la qualité de vie des patients, plus spécifiquement celle des filles et femmes.

Des études récentes ont suggéré que la testostérone et ses métabolites affectent le développement de l'hippocampe aux niveaux biochimique, morphologique et fonctionnel. En revanche, les données ne sont pas aussi extensives que celles de leurs rôles chez les adultes. Ainsi, une meilleure compréhension des mécanismes par lesquels l'hormone stéroïdienne influence le développement du cerveau facilitera l'identification des cibles thérapeutiques de plusieurs maladies neurodéveloppementales qui affectent le fonctionnement de l'hippocampe.

Afin de se développer adéquatement, le cerveau mâle requiert une exposition aux hormones sexuelles mâles pendant une période de temps donnée. En revanche, le cerveau femelle possède une phase critique peu après la naissance au cours de laquelle une exposition aux hormones sexuelles mâles le masculinise en produisant des caractéristiques comparables à celles rencontrées chez des mâles biologiques. Ainsi, la capacité de manipuler les cerveaux femelles dans le but de les masculiniser représente un outil expérimental important pour investiguer les différences sexuelles. Du fait que les hormones sexuelles telles que la testostérone et l'estradiol représentent respectivement l'élément caractéristique de chacun des sexes, cette thèse a pour objectif de disséquer l'implication de la testostérone dans le développement et le fonctionnement du cerveau en étudiant en plus des rats mâles et femelles, des femelles traitées avec la testostérone ainsi que des mâles rendus insensibles à la testostérone.

En premier lieu, nous avons investigué sur un système de neurotransmission spécifique, à savoir le système GABAergique, qui est important pour le contrôle des

convulsions communément observées dans l'épilepsie. Ce système possède des particularités notables en fonction du sexe, particularités qui pourraient être l'une des causes de la prédisposition des mâles à l'épilepsie. En effet, notre étude révèle qu'au niveau basal, les femelles ainsi que les mâles insensibles à la testostérone montrent très tôt au cours de leur développement une localisation à la membrane du co-transporteur KCC2 qui régule la force de la neurotransmission inhibitrice. Par ailleurs, nous avons aussi détecté des niveaux élevés du neurotrophine BDNF qui est un puissant modulateur du fonctionnement des cellules GABAergiques, ceci, au cours de la première semaine postnatale. Par ailleurs, chez les adultes, nous avons trouvé que les femelles ainsi que les mâles insensibles à la testostérone montrent une augmentation de la transmission GABergique spontanée comparativement aux mâles et aux femelles qui ont été exposées à la testostérone. En somme, ces données démontrent que le fonctionnement de la circuiterie GABAergique est modulé par le niveau de testostérone périnatal, ce qui suggère d'un rôle des hormones sexuelles dans la régulation de l'excitabilité cellulaire.

De plus, les différences sexuelles dans le cerveau sont largement déterminées par des facteurs extrinsèques. Parmi ces derniers, le stress du début de la vie est un facteur extrinsèque puissant qui altère l'habileté à contrôler la rétroaction négative des glucocorticoïdes sur l'axe hypothalamo-hypophyso-surrénalien (HHS). Le stress est également connu pour affecter différentiellement les rats mâles comparativement aux femelles. Nous démontrons alors que la corticostérone rend l'hippocampe vulnérable à une seconde insulte, telle que les épilepsies induites par l'hyperthermie. En effet, chez les rats traités à la corticostérone, la latence d'induction des épilepsies par hyperthermie est réduite, le temps de récupération plus long et le nombre d'évènements épileptiques plus nombreux. En outre, nous avons trouvé que tous ces effets sont plus proéminents chez les mâles que chez les femelles. Ces données confirment l'existence d'un lien entre le stress du début de la vie et la susceptibilité aux convulsions hyperthermiques chez les rats mâles et femelles. Une meilleure compréhension des conséquences des convulsions fébriles pourrait aider dans le pronostic et le traitement des patients souffrant d'épilepsie.

Somme toute, cette thèse met en lumière le rôle complexe des hormones sexuelles dans la régulation des circuits GABAergiques, des réponses au stress et de l'hyperexcitabilité

du cerveau en développement. Une meilleure compréhension des mécanismes pathologiques propres aux modèles animaux mâles et femelles résulterait en de meilleures interventions et thérapies aussi bien chez les hommes que les chez les femmes.

MOTS-CLÉS: Différences sexuelles, Testostérone, Circuits GABAergiques, Hippocampe, KCC2, BDNF, Corticostérone, Stress chronique, Convulsions fébriles, HHS

ABSTRACT

Sex differences extend far beyond reproductive health — there is a widespread prevalence of sex differences in many human diseases and conditions. Therefore, studies limited to a single-sex cannot fully give a comprehensive picture of the underlying disease mechanisms, and the neglect of females/girls/women in biological research negatively impacts patients' quality of life, especially women.

Recent data suggest that testosterone and its metabolites affect the hippocampus during development at the biochemical, morphological, and functional levels, although the data are not nearly as extensive as what is known in adults. Therefore, a better understanding of these effects will elucidate steroid hormone-dependent mechanisms of brain development and, possibly, help identifying ways to mitigate the burden of the many neurodevelopmental disorders that involve hippocampal function.

The male brain is unique in that it must be exposed to male sex hormones for a fixed period of time, which is so-called critical period. This is deemed a critical period because if androgens levels do not rise at this time in males, the brain will fail to be masculinized. The female brain, on the other hand, has a sensitive period shortly after birth, during which exposure to male sex hormones may masculinize the brain and produce features comparable to those seen in biological males. This capacity to manipulate females toward more masculinized brains represent an important experimental tool to investigate sex differences. Because sexual hormones, such as testosterone and estradiol, are a distinct point of divergence between sexes, my thesis proposes to study the implication of testosterone by using, in addition to male and female animals, females treated with testosterone as well as testosterone-insensitive male rats.

First, we investigated a specific neurotransmitter system, the GABAergic system, which contributes to the control of seizures commonly observed in epilepsies. This system shows robust differences between males and females, which may be involved with the predisposition to epilepsy observed in males. Our study revealed that at baseline conditions female and testosterone-insensitive male rats show an earlier localization at

the membrane of the chloride co-transporter KCC2, which regulates the strengths of inhibitory neurotransmission, and higher levels of the neurotrophin BDNF, which is a powerful modulator of GABAergic cell function, during the first postnatal week. In addition, we found that female and testosterone-insensitive male rats show enhanced spontaneous GABA synaptic transmission when compared to males and testosterone-exposed females in adults. Overall, these data show that perinatal testosterone levels modulate GABAergic circuit function, suggesting a role of sex hormones in regulating cell excitability.

Second, sex differences in the brain are largely determined by extrinsic factors. Early-life stress is one such powerful extrinsic factor that impairs the ability to control glucocorticoid negative feedback on the HPA axis. Stress is also known to differentially affect male and female rats. Here, we show that corticosterone alone renders the hippocampus vulnerable to a second insult, namely hyperthermia-induced seizures, in fact in corticosterone-treated rats the latency to hyperthermia-induced seizures was shorter, the recovery time longer, and the number of hyperthermia-induced seizures larger. Further, these effects were a lot more prominent in males than in females. These findings support a link between early-life stress and hyperthermic seizure susceptibility in both male and female rats. A better understanding of the consequences of febrile seizures could help improve the prognosis and treatment of patients with epilepsy.

Altogether, these findings shed light on the complex roles of sex hormones in regulating GABAergic circuits, stress responses and circuit hyper-excitability in the developing brain. A better understanding of disease-mechanisms underlying male and female animal models could lead to better interventions and therapeutics in both men and women.

KEYWORDS: Sex differences, Testosterone, GABAergic circuits, Hippocampus, KCC2, BDNF, Corticosterone, Chronic stress, Febrile seizures, HPA

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ABBREVIATIONS

[Cl-]i: Intracellular chloride concentration

ACTH: adrenocorticotropic hormone

AD: androgen receptor

ASD: autism spectrum disorder

AVP: arginine vasopressin

BDNF: brain-derived neurotrophic factor

CA1: cornus ammonis

CBG: corticosteroid-binding globulin

CIHR: Canadian Institutes of Health Research

CNS: central nervous system

CORT: corticosterone

CRH: corticotropin-releasing hormone

EC: European Commission

EEG: clectroencephalogram

E_{GABA}: reversal potential of GABA

ER: estrogen receptor

FDC: focal cortical dysplasia

FS: febrile seizures

FSH: follicle stimulating hormone

GABA: γ-aminobutyric acid

GAD: glutamic acid decarboxylase enzyme

GAT: GABA transporter

GnRH: gonadotropin-releasing hormone

GR: glucocorticoid receptor

HPA: hypothalamic-pituitary-adrenal

HPG: hypothalamic-pituitary-gonadal

HS: hyperthermia-induced seizures

KCC2: K–Cl cotransporter

LH: luteinizing hormone

MAPK: mitogen-activated protein kinase

mIPSC: miniature inhibitory postsynaptic current

MR: mineralocorticoid receptor

MRI: magnetic resonance imaging

mTLE: mesial temporal lobe epilepsy

NIH: National Institutes of Health

NKCC1: Na–K–2Cl cotransporter

P: postnatal day

PR: progesterone receptor

PVN: paraventricular nucleus of the hypothalamus

SABV: sex as a biological variable

SAGER: sex and gender equity in research

sEPSC: spontaneous excitatory post-synaptic currents

SGBA: sex- and gender-based analysis

SHRP: stress hypo-responsive period

sIPSC: spontaneouss inhibitory postsynaptic current

siRNA: small interfering RNA

SRS: spontaneous recurrent seizures

SRY: sex-determining region of the Y

TDF: testis-determining factor protein

Tfm: testicular feminization mutant

To my mother, father, sister, and nephew,

for their unlimited love and support.

‘Come for the sex, stay for the science

Come for the science, stay for the sex’

— Dr. Jennifer Gunter,

Canadian OB/GYN and women's health advocate.

‘When we exclude half of humanity [women] from the production of knowledge

we lose out on potentially transformative insights.’

— Caroline Criado Pérez,

Invisible Women: Data Bias in a World Designed for Men.

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My Ph.D. journey was not the easiest one, but in between moving to a different country, change of supervisors and a pandemic, I am proud of what I have accomplished because even with all the up and downs, because I met wonderful people that helped me through this process.

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I also would like to acknowledge being part of the IGH Trainee Network from CIHR. Being a Trainee Leader at Université de Montréal brought me great understanding on the importance of studying sex and gender in health research.

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– CHAPTER I –

GENERAL INTRODUCTION

1.1. Overview

This doctoral thesis' project focuses on understanding how developmental sex differences underlie sexual dimorphism in brain inhibitory network, the stress response and, ultimately, seizure susceptibility. The following introduction will provide relevant background on five important topics: 1) sex and gender in health research; 2) sex differences in the brain; 3) sex differences in the GABA circuit; 4) neurobiology of stress; and 5) sex hormones and seizures. Finally, I will outline the main objectives as well as the hypotheses of the experimental work presented in chapters II and III.

1.2. Sex and gender in health research

1.2.1. Introduction to sex and gender

Health and disease are influenced by both sex and gender. Therefore, it is critical to incorporate sex and gender considerations over the course of the research in order to generate the best possible outcomes. However, sex and gender are frequently used interchangeably and erroneously which can cause misunderstandings across different fields of health research [1,2]. It is important to understand these concepts, and therefore sex and gender are defined by the Canadian Institutes of Health Research (CIHR) [3] as follows:

a. Sex is a biological variable in animals and humans and differentiates males from females from intersex, which represents 1.7% of the population [4,5]. Sex is related to chromosomes, gene expression and hormone levels, which in turn affect phenotype and biological functions such as physiology, neuroendocrinology, metabolic systems, and ultimately behavior.

b. Gender is a social construct. It represents social, cultural, and psychological external influences that shape identity, behaviors, stereotypes of girls, boys, women, men, and gender diverse people (non-binary). Despite gender being conceptualized as a binary (girl/woman and boy/man), gender exists in a spectrum that varies across time and cultures [6].

Sex and gender are interrelated and intersect. They both affect symptoms and manifestations of disease, as well as responses to treatments. Only humans have a gender; therefore, animal models allow us to separate sex and gender considerations. Given the nature of this project, sex will be the only variable considered throughout this doctoral thesis.

1.2.2. International funding agencies initiatives

As it has become increasingly clear that sex differences exist beyond the reproductive system and in order to address negligence of sex and gender considerations in health research, the US National Institutes of Health (NIH) [7], the CIHR [3], and the European Commission (EC) [8], began requesting that preclinical and clinical researchers must address and integrate sex and gender considerations while designing experiments, as well as while analyzing and reporting outcomes [8].

The NIH focused on sex as a biological variable (SABV) as a policy [9], CIHR established a policy on sex- and gender-based analysis (SGBA) [10]; and the EC mandated the inclusion of the "gender dimension", which integrates sex, gender, and intersectional analysis [11]. The purpose of these policies and/or mandates is to promote rigorous science, improve reproducibility via rigor and transparency and therefore increase our understanding of health determinants for girls, boys, women, men, and gender diverse people. As a tool, guidelines (such as the Sex And Gender Equity in Research (SAGER))

were designed to aid researchers and journal editors in disclosing sex and gender considerations in publications when applicable [12].

1.2.3. Sex bias and sex omission in Neuroscience

The existence of sex differences in Neuroscience, from biochemistry to physiology to structure and function, is well documented [13–16]. Still, for decades, in most basic and clinical studies, single-sex – usually male – animals and participants dominated research while excluding female animals and participants [17,18]. Sex bias and sex omission are the two most common types of negligence in Neuroscience research [19,20]. The first is characterized by a preference for one sex over another. The second is the failure to report animal or participant sex. Both common practices can mask sex differences and lead to false interpretation [21–23].

According to Beery and Zucker [20], neuroscience, among other biological domains such as pharmacology, physiology, and endocrinology, has the highest sex biases, with male animal studies outnumbering female animal studies by a ratio of 5.5 to 1. Failure to report the sex of the animals accounted for ~22% of neuroscience articles [20]. In another study, when sex is taken into consideration based on neuroscience research published in the most prominent journals in the field, such as Nature, Science, Nature Neuroscience, Neuron, and the Journal of Neuroscience, the results showed that ~32% studied exclusively males, while in ~28% sex was omitted and ~7% studied exclusively females [21]. A more recent study indicated that although significant progress has been made in the reduction of sex omission, in which a decrease of 28% was observed from 2010-2014, sex bias towards male remains a persistent phenomenon in the neuroscience literature, with an increase of 9% in the same period, even after policies were put in place [19]. It is important to emphasize that even if no difference is found, it is still paramount to finally identify similarities and differences among sexes (**Figure 1**).

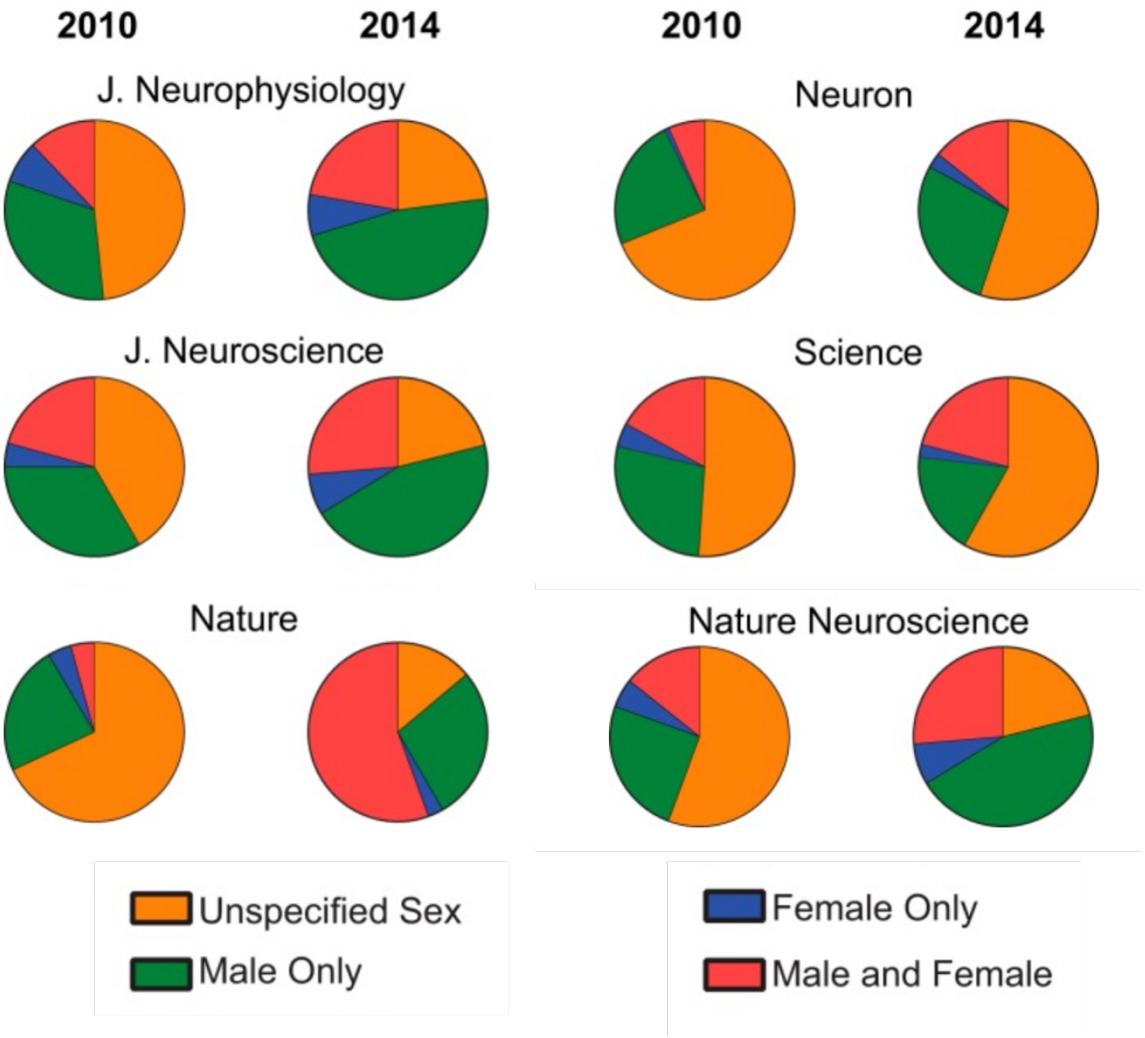

Figure 1. Sex bias and sex omission by journal in 2010 and 2014. Articles were analyzed from the following journals: Journal of Neurophysiology, Journal of Neuroscience, Nature, Nature Neuroscience, Science and Neuron. A decrease of 28% was observed related to sex omission. On the other hand, sex bias towards male remained a persistent phenomenon in the Neuroscience literature, with an increase of 9% in the same period. Charts adapted from Will *et al*., 2017 [19].

1.2.4. Sex as a risk factor for nervous system disorders

The importance of SABV is due to sex bias in the prevalence, incidence, age of onset, progression, manifestation and/or responses to treatment of many nervous system disorders [24]. For example, autism spectrum disorder (ASD) is diagnosed in boys four to

five times more often than girls [25,26]. Schizophrenia has consistently been shown to develop at an earlier age in men than women and symptoms differ between both sexes [27–29]. On the other hand, affective disorders such as major depressive disorder, post-traumatic stress disorder, and anxiety disorders are up to twice as frequent in women and girls[30,31]. Women are also more likely to develop numerous clinical pain conditions [32] and show faster progression in Alzheimer's disease [33] (**Figure 2**).

In particular for this thesis project, neurodevelopmental disorders are either more frequently diagnosed or more severe in symptomology in males, both clinically and when modeled in rodents [26,27,34–36]. Also, the hippocampus is implicated in a variety of the aforementioned neurological disorders. This includes epilepsy, which varies in frequency and/or presentation between boys/men and girls/women [37–39] and will be discussed in the following sections.

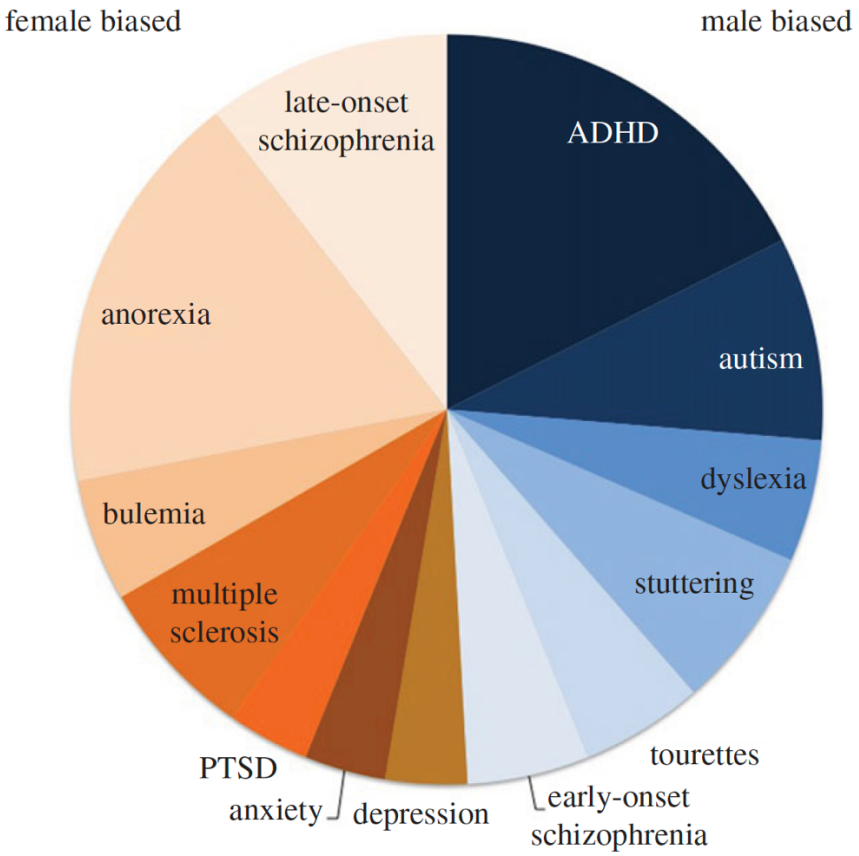

Figure 2. Sex-bias in some neurologic and neuropsychiatric disorders. Many factors influence the diagnosis of different disorders, however identifying biological variables

emphasises those influences that are not biological in nature (e.g., diagnosis bias, differing symptomology). This diagram depicts only a subset of the numerous neurologic and neuropsychiatric disorders known to differ in incidence between boys and girls, men and women. The size for each disorder shows the relative degree of bias toward females (left) versus males (right). ADHD, attention deficit hyperactivity disorder; PTSD, post-traumatic stress disorder. Diagram adapted from McCarthy, 2016 [40].

1.3. Sex differences in the brain

1.3.1. Contribution of chromosomal repertoire to sex differences in the brain

Sex chromosomes (XX for female and XY for male, for most mammals) are one of the most important factors that lead to sex differences. There are four fundamental processes for sex variations in gene expression during brain development that are independent of the gonads.

The first mechanism is due to SRY (Sex-determining region of the Y), a single gene on the Y chromosome that codes for the testis-determining factor protein (TDF) and causes the bipotential gonadal anlage to develop into a testis [41]. The gonadal precursor will develop into an ovary If the SRY gene is absent or mutated. Both testicular and ovarian development are influenced by genetic regulation [42,43] (**Figure 3**).

The second mechanism is the X gene dosage in which random inactivation of one of the X chromosomes in female cells occurs [44–46] to prevent sex differences in X chromosome gene dosage, but at the end causes another degree of sex difference in gene expression. Some genes escape X inactivation (termed as X escapees), and therefore are expressed more in XX than XY cells, which could lead an increase in the expression of certain X chromosome-linked genes in the female cells [47–49] (**Figure 3**).

The third mechanism is the XX mosaicism due to parental imprint. In males, the X chromosome carries only maternal imprints — i.e., epigenetic modifications made by the parent in generating the sex cells—which alter the expression of genes in the offspring.

Because females inherit both parents' X chromosomes, they have maternal and paternal imprints, which target a different set of genes [44] (**Figure 3**).

Lastly, the fourth mechanism refers to sex differences in gene expression due to genomic imprinting that are extended to the autosomes [50]. These imprinted genes exhibit sex-specific and tissue-specific expression and in turn directly influence the neural development of sex differences and the risk of neurodevelopmental disorders [51].

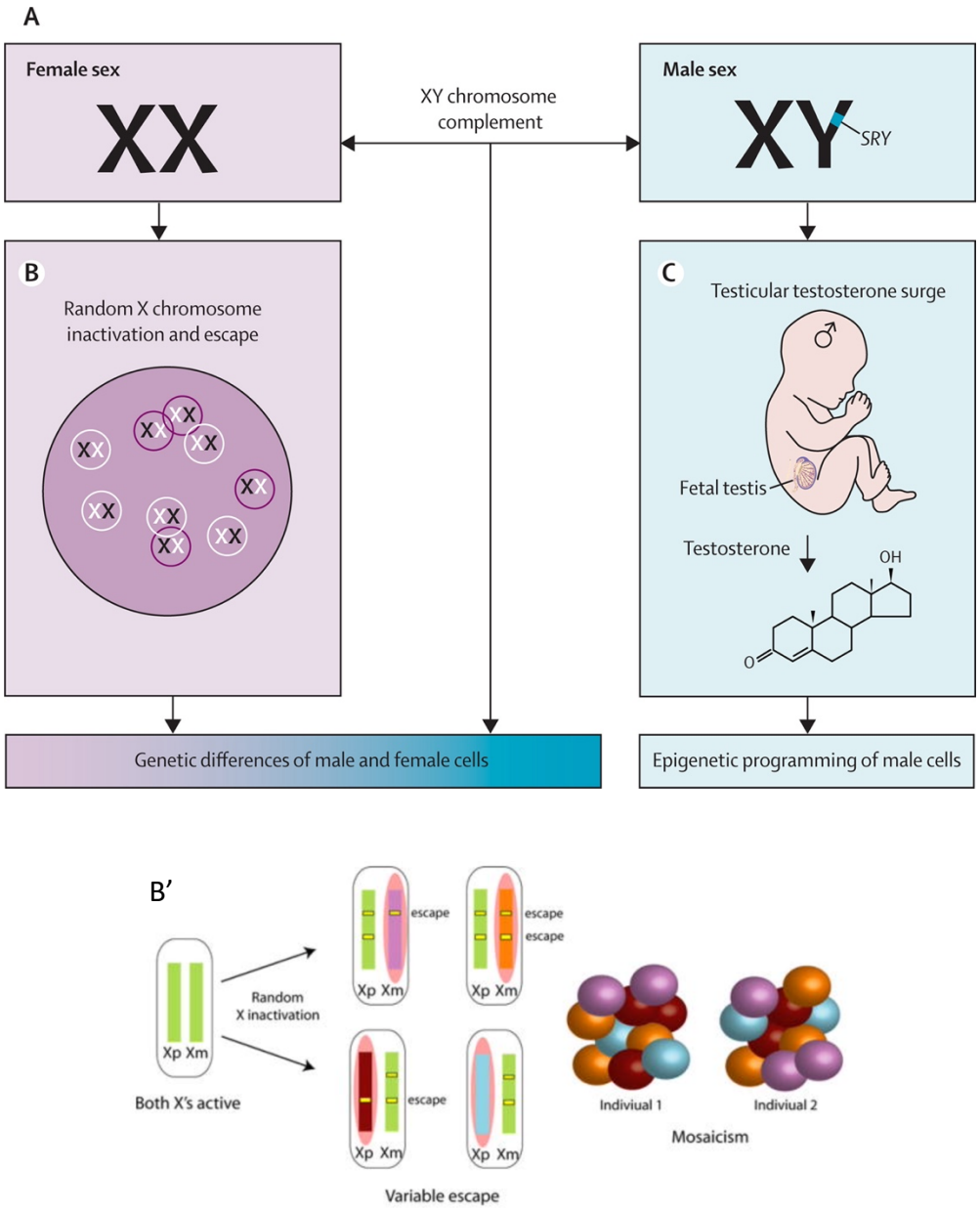

Figure 3. Sex-specific mechanisms in gene expression during early brain development. A. Cells carrying either the XX or XY chromosomes result in genetic sex

differences. **B.** In female cells, random inactivation of one X chromosome creates a new level of sex differences in gene expression. **B'.** The paternal X (Xp) and maternal X (Xm) chromosomes are active before differentiation. Random X inactivation is due to the covering of one X chromosome with Xist RNA (pink cloud). This will become the inactive X, while the other X will continue to function normally (green chromosome). Other genes are able to evade X inactivation in a subset of cells within a tissue, resulting in mosaicism in escape patterns. **C.** In males, the SRY gene on the Y chromosome drives the formation of a testis, which produces a peak of testosterone during the perinatal period. Through epigenetic remodelling, the testosterone surge programmes cellular gene expression and tissue structure in various organs of males. Illustration adapted from Mauvais-Jarvis et al., 2020 [52] and Berletch et al., 2011 [47].

1.3.2. Contribution of sex hormones to sex differences in the brain

Sex hormones are another important component that causes sex differences due to complex actions on all levels of the brain. There is evidence for gonadal hormone modulation of neurogenesis [53,54], apoptosis [55], synaptogenesis [56–58], synaptic pruning [59], neurotransmitter systems [60], myelination [61], microglia and astrocytes [62,63] and other important mechanisms of neural plasticity [64].

Sex hormones are steroid compounds that are primarily generated from cholesterol in the testes, ovaries, and adrenal cortex. Therefore, the biosynthetic pathway for male sex hormones (androgens) and female sex hormones (estrogen and progesterone) is shared (**Figure 4**) [64]. All steroids start off as cholesterol that undergoes a series sequence of enzymatic processes to produce progestins, glucocorticoids, mineralocorticoids, androgens, or estrogens. In particular, testosterone is a precursor to estradiol, which must be aromatized by the enzyme aromatase, also known as Cyp19a [65]. The aromatase enzyme is expressed by neurons, which allows them to locally synthesize estradiol from androgen precursors in circulation secreted by the embryonic testis. Aromatase expression is not distributed randomly, but is concentrated in certain nuclei and regions, with levels differing across brain areas and sexes [66]. Estradiol initiates a cascade of events that defines the hallmarks of a male brain [67].

As mentioned, sex hormones can also be further metabolized to different neurosteroids or be synthesized *de novo* within neurons and glial cells [68]. Given the nature

of this project, only gonadal sex hormones were considered in the experimental design of this project.

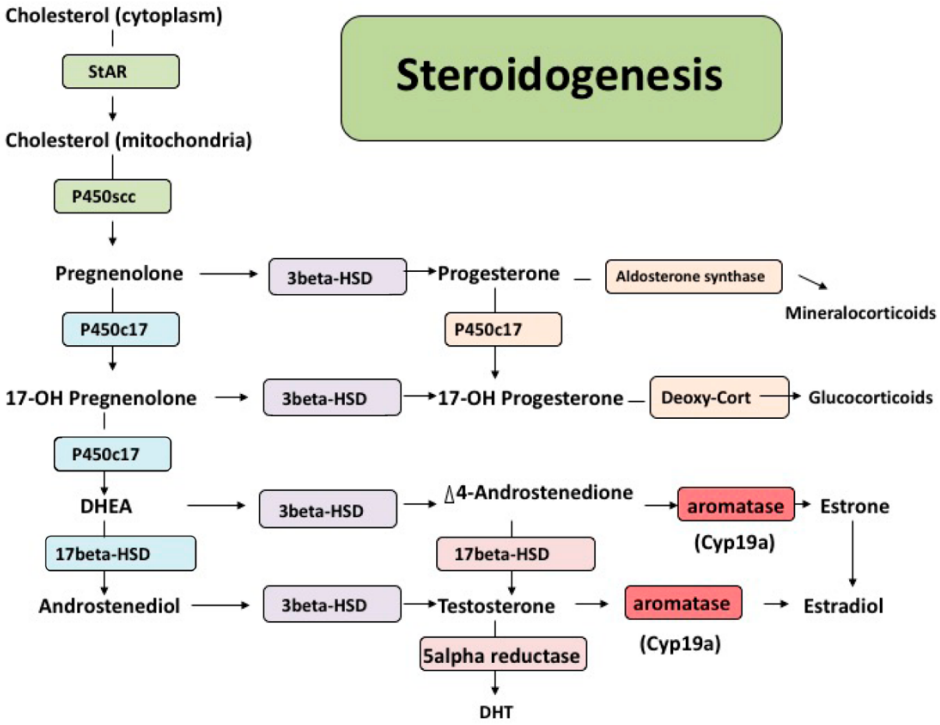

Figure 4. Steroidogenesis. All steroids start off as cholesterol and then are transformed into progestins, glucocorticoids, mineralocorticoids, androgens, or estrogens by a sequence of enzyme processes that usually remove hydroxyl groups and carbons. The following enzymes are involved in the production of these steroids: P450 side-chain cleavage enzyme, 3β-hydroxysteroid dehydrogenase, 17-hydroxysteroid dehydrogenase, 5α-reductase, aromatase, aldosterone synthase. Not all intermediate steroids, pathways, and enzymes are included. Diagram adapted from McCarthy, 2018 [66].

1.3.3. Organizational and Activational effects of gonadal hormones

The brain, like the gonads, is originally a bipotential organ, with the ability to be either masculinized or feminized. The sex differences in the brain are organized during the critical period in early brain development and then activated by circulating hormones

after puberty, characterizing the organizational-activational hypothesis of the brain and behavior [69–74].

The critical period for masculinization begins with the production of androgens late in gestation in rodents (third week of pregnancy) and about mid-gestation in humans and ends within the first few days of postnatal life or prior to birth, respectively [75]. The masculinization process is triggered by sex hormones exposure, in which testis of male embryo synthesizes androgens at levels near to adult levels [66]. Furthermore, proper development of the male rodent brain also requires completion of another process known as defeminization. Defeminization refers to the loss of the ability to respond to the activational effects of estradiol and progesterone in inducing feminine sex behavior. Both processes oppose the process of feminization that leads to adult female-typical behavior [76,77] (**Figure 5**). On that note, despite feminization occurring with no significant levels of sex hormones as a neonate, it is a very much active process in which brain feminization is maintained by the active suppression of masculinization via DNA methylation [78].

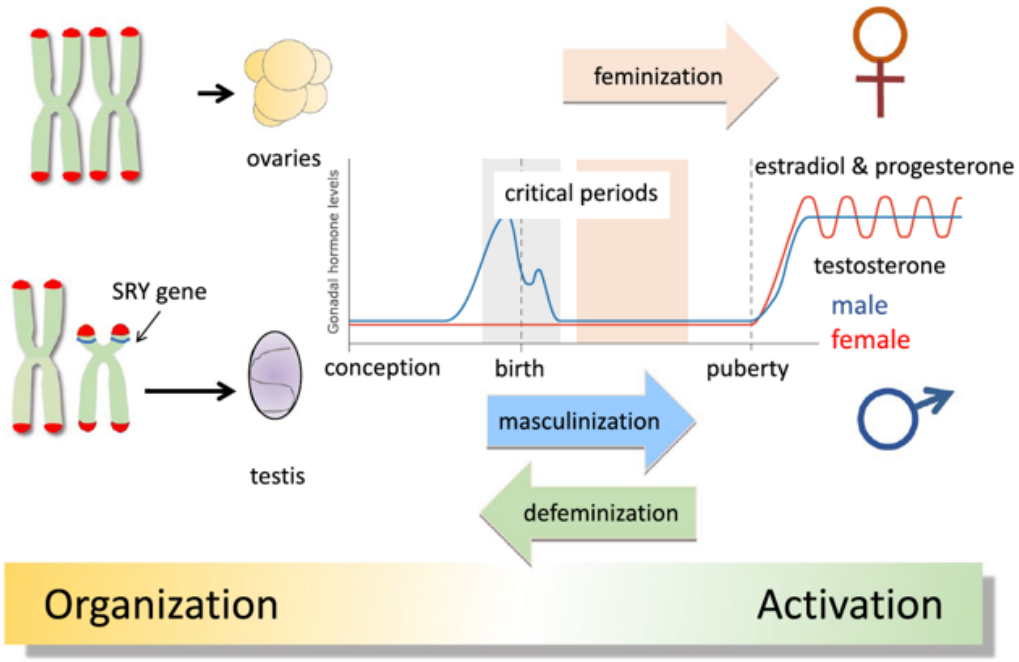

Figure 5. Classic view of sexual differentiation of the brain in rodents. In males, the SRY gene in the Y chromosome directs the differentiation of a testis from the bipotential gonad. The generated testis will begin producing testosterone in late gestation and early postnatal phase of the rodent and masculinize select male brain areas during a so-called critical period (grey area). A separate sex defeminization process reduces males' aptitude

for female-like receptive behaviour. In females, the ovary develops without SRY-induced gene expression cascade signals and remains dormant until adolescence, when cyclical estradiol and progesterone production begins. There is evidence that feminization has a sensitive period that occurs slightly later in development (orange area). The masculinizing effects of testosterone and its aromatized by product, estradiol, during development are considered organizational and are also required for hormone activation on many reproductive parameters during puberty. Illustration adapted from McCarthy, 2020 [79].

In females, there is a sensitive period that differs from a critical period in that it involves exogenous steroid sensitivity. Exogenous testosterone or its aromatized product, estradiol, from embryonic day 18 until about postnatal day 10 in rodents, can override female development and generate a phenotypic male brain in females. After postnatal day 10, the critical period is over and testosterone treatment in females has no effect on the organization of the brain [58,79] (**Figure 6).**

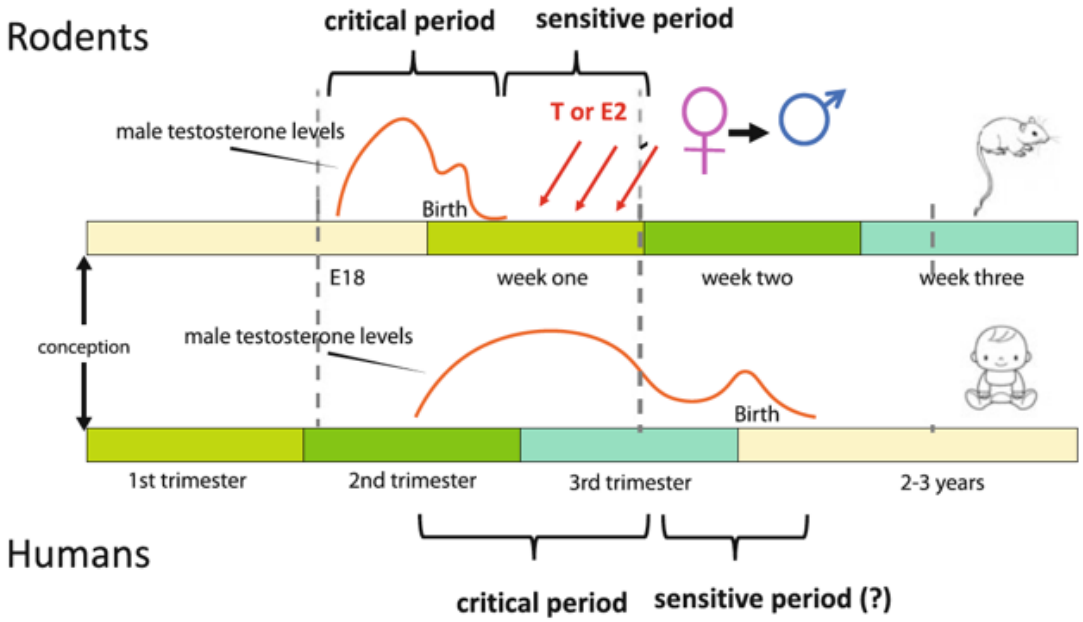

Figure 6. Critical and sensitive periods for sexual differentiation in rodents and humans. The masculinization of the brain begins at embryonic day 18 when endogenous testosterone production from the embryonic testis begins in male rats. The critical time ends shortly after birth when the process of masculinization is irreversible. Female rats also remain sensitive to exogenous testosterone treatment for up to 1 week after birth.

Around 7–10 days, the process of feminization is irreversible. Because of the unique synthesis of testosterone in males but the shared sensitivity of both sexes to this steroid hormone, males have a short critical period whereas females have a longer sensitive period. This developmental profile of the rat is shifted from humans in that a newborn pup is roughly equivalent to a mid- to late-gestation human fetus. In humans, the critical period begins during the 2nd trimester with fetal androgen production and probably ends prior to birth although this conclusion is constrained by a lack of experimental data. Also, it is not known when or if there is an analogous sensitive period. Illustration adapted from McCarthy and Wright, 2017 [80] .

1.3.4. Cellular and molecular mechanisms of sex hormones in the brain

The actions of gonadal hormones on the cells of the nervous system are mediated by two distinct mechanisms: genomic (intracellular steroid receptors) and non-genomic (ion channels and membrane receptors) [64]. The first mechanism is via binding of the sex steroid molecules to associated receptors: estrogen receptor (ER) α/β, androgen receptor (AR), progesterone receptor (PR), which then bind to discrete response elements on DNA and mediate transcription or repression of target genes [81]. These genomic effects require co-regulators for specific binding of the ligand-bound hormone receptor to hormone receptor response elements on genes [82]. These effects become apparent over several hours or days and can be observed in expression of proteins, alterations in receptors and cell structural elements [82,83] (**Figure 7**).

The second mechanism of action of gonadal hormones is via binding of the steroid molecules to membrane-associated receptors which signal through a variety of intracellular signaling cascades [82,84] (**Figure 7**). This mechanism of action results in rapid, short-term adaptation of cellular processes to the cellular milieu and the duration of the effect will depend on the kinetics of the ligand-receptor binding and the specific pathways that are activated or inhibited. Examples are related to the modulation of the γ-aminobutyric acid (GABA) type A receptor [56], rapid changes in neuronal firing [85], and estrogen-mediated enhancement of long-term potentiation in the hippocampus [86]. The non-genomic effects of sex steroids on intracellular signaling cascades might affect the

regulation of genes that do not contain steroid response elements, thus representing an indirect mechanism by which steroid hormones might exert a genomic effect [87,88].

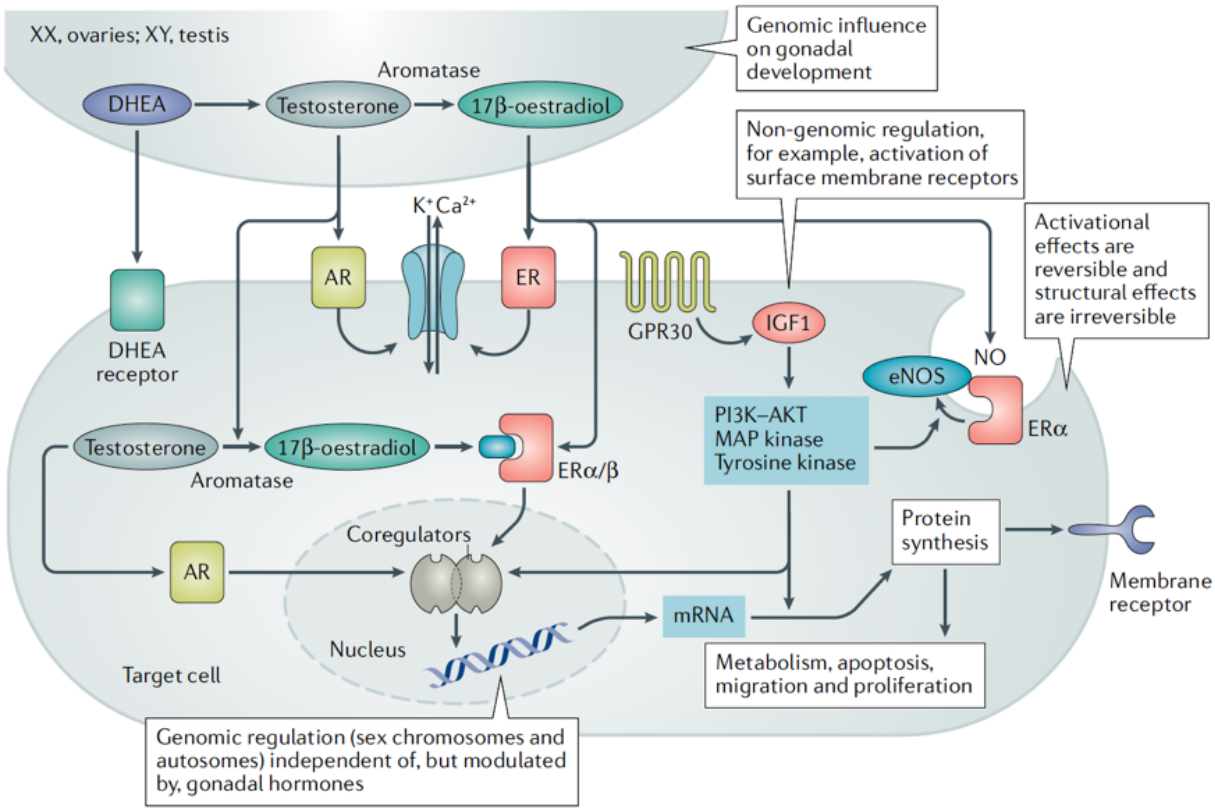

Figure 7. Sex steroids mechanisms through both genomic and non-genomic regulation. Sex steroids may alter membrane receptor, enzyme, and structural protein synthesis by binding to hormone response regions on specific genes, i.e., genomic regulation. Another sex mechanism may directly affect ion channel or enzyme activity, i.e. non-genomic regulation. Interestingly, changes in intracellular enzyme activity may affect genes that do not contain hormone response elements, characterizing an indirect influence of sex hormones on gene regulation. AR: androgen receptor; ER: estrogen receptor; AKT, RACα serine/threonine-protein kinase; GPR30, G protein-coupled estrogen receptor; IGF1 insulin-like growth factor 1; PI3K, phosphoinositide 3-kinase. Not all intermediate steroids, pathways and enzymes are included. Illustration adapted from Mielke and Miller, 2021 [82].

1.4. Sex differences in the GABA circuit

1.4.1. GABA signaling components

Gamma-aminobutyric acid (GABA), the principal inhibitory neurotransmitter, plays an important role in maintaining the inhibitory tone that counterbalances neuronal excitation. The GABA signaling system in the brain is comprised of GABA synthesizing enzymes, transporters, GABAA and GABAB receptors ($GABA_AR$ and $GABA_BR$) and chloride co-transporters.

In the brain, GABA is synthesized from glutamate by the glutamic acid decarboxylase enzyme (GAD), and it is packed into synaptic vesicles by the vesicular GABA transporter (VGAT) [89,90]. Once the membrane is depolarized, GABA is released from the vesicles and into the synaptic cleft, where it binds to either the ionotropic GABAA receptors (GABAARs) or the metabotropic GABAB receptors (GABAB receptors) (GABABRs) [91,92]. Membrane GABA transporters (GATs) are responsible for clearing excess GABA from the synaptic cleft [93]. The strength and polarity of $GABA_A$ receptor-mediated neuronal inhibition is determined by intracellular chloride concentrations, which are mainly controlled by two cation–chloride cotransporters: K–Cl cotransporter (KCC2) and Na–K–2Cl cotransporter (NKCC1) [94–96] (**Figure 8**).

The inhibitory action of GABA is important to maintain the excitatory-inhibitory balance within the brain, which is vital to normal brain function and the expression and function of the GABAergic signaling components are key to optimal GABAergic inhibition in the brain. Existing evidence suggests that sex plays a role in altered neurotransmission and GABAergic signaling components [97–99], which might contribute to sex-specific vulnerability to different neurological disorders [100].

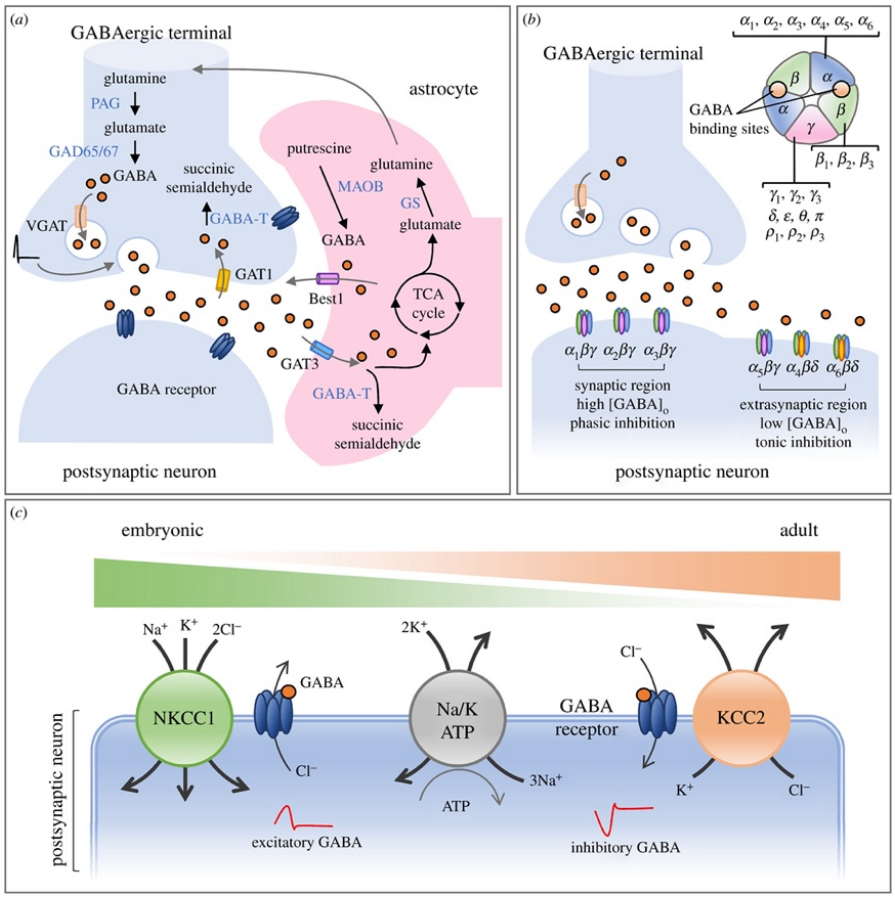

Figure 8. GABA signalling components. A. GABA homeostasis is regulated by neurons and astrocytes. GABA is synthesized by GAD65/67 from glutamate in neurons, while astrocytic GABA is synthesized through MAOB. The reuptake of GABA is mediated through GAT1 in neurons and GAT3 in astrocytes. The metabolism of GABA is mediated by GABA-T in neurons and astrocytes. **B.** GABAA receptors are heteropentameric complexes assembled from 19 different subunits. The compositions of different subunits determine the subcellular distributions and functional properties of the receptors. **C.** The excitatory or inhibitory response of GABA is driven by the chloride gradient across cell membranes, which can be determined via two cation–chloride cotransporters (NKCC1 and KCC2). Not all pathways, components and enzymes are included. Illustration adapted from Hsu, Chang and Chern, 2018 [97–99].

1.4.2. Maturation of the GABA network

Aside from being critical for the sexual differentiation of the brain, the perinatal period is also critical for the fate determination and development of inhibitory network activity as the GABAergic switch from depolarizing to hyperpolarizing, i.e. excitatory to

inhibitory [101–106]. GABA maintains this dual identity due to the transmembrane Cl− gradient. The GABAA receptor allows Cl− flux in either direction as a function of the Cl− concentration ([Cl−]) gradient. During development, increased intracellular [Cl−] results in a GABAA reversal potential that is positive relative to the resting membrane potential, while as development progresses, intracellular [Cl–] is lower that the reversal potential for GABAA is maintained just negative to the resting membrane potential [107]. Consequently, opening of the receptor results in hyperpolarization of the neuronal membrane [108–110] (**Figure 9**).

The developmental switch from depolarizing to hyperpolarizing GABA activity is dictated by the relative levels of the chloride co-transporters, NKCC1 and KCC2 [111]. The developmental excitatory action of GABA appears to be universal, having been documented in cortex [112], hippocampus [113], hypothalamus [114], cerebellum [115] and spinal cord [116]. Also, these characteristics allow GABA to serve as a trophic factor, and to tightly regulate neurogenesis, neuronal migration, neurite extension, synapse formation and apoptosis [117–120].

The development and maintenance of the neuronal chloride gradient is vital for the efficacy of GABAA-mediated inhibition [121]. The maintenance of low intracellular chloride levels is critical for GABAergic inhibition and prevents hyperexcitability, such as seizures [122]. Disruption of the established balance between KCC2 and NKCC1 expression likely underlies the sexually dimorphic seizure susceptibilities observed in a diverse group of neurological disorders [123].

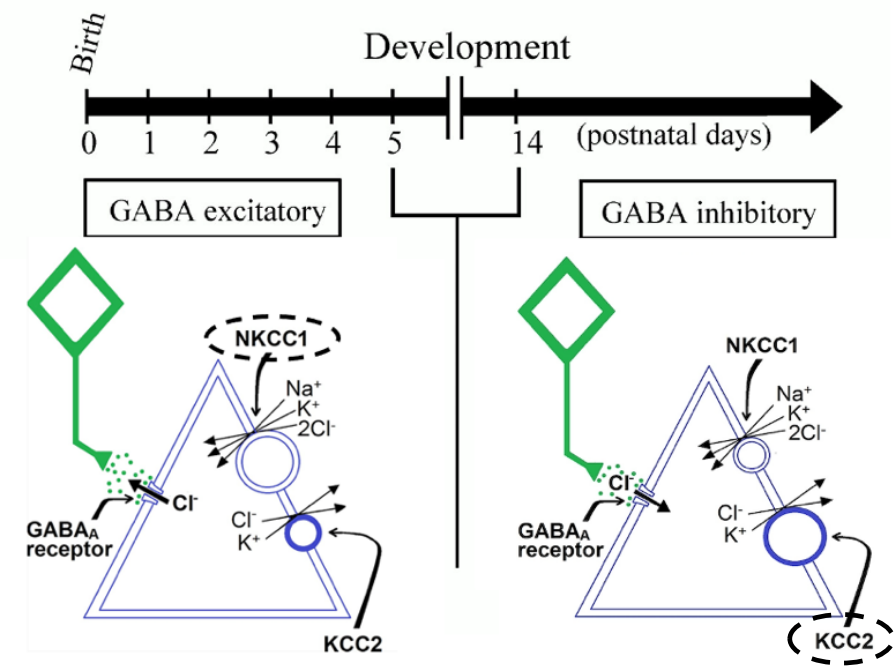

Figure 9. Developmental GABA switch. The direction of the flow of Cl^- ions through $GABA_A$ receptors depends on the electrochemical Cl^- gradient. *Left:* In the immature brain, the intracellular Cl^- concentration is relatively high, as Cl^- transport over the membrane is dominated by NKCC1. Activation of $GABA_A$ receptors results in an outflow of Cl^- resulting in membrane depolarization. *Right:* During development, intracellular Cl^- levels decrease, due to increased expression and activity of KCC2. As a result, activation of $GABA_A$ receptors leads to an entry of Cl^- and GABAergic signaling results in hyperpolarization of mature neurons. Illustration adapted from DiCristo, 2018 [124].

1.4.3. Sex differences in KCC2 expression

Excitation is a hallmark of male brain development, including the depolarizing actions of GABA, which are stronger and endure longer in developing males than females [125–127]. A striking sex difference found in the neonatal hippocampus of rats is the response to the neurotransmitter GABA, which is largely depolarizing at birth and gradually shifts to a hyperpolarizing response over the first two weeks of life in both males and females [128–132]. More specifically, at P1, KCC2 protein levels are significantly higher in females than in males in the rat hippocampus [133]. Consistently, CA1 pyramidal neurons show more hyperpolarized GABAergic postsynaptic currents in females from P4 to P14 [132].

Dependent or independently of its cotransporter activity, KCC2 is implicated in several neuronal processes such as mature inhibitory GABAergic responses, neuron migration, dendritic outgrowth, synapse formation and maturation [134,135]. Interestingly, seizure susceptibility is strongly influenced by sexual hormonal difference between sexes in the developing brain, which can regulate the excitation threshold and thus regulate seizure generalization [130,136–138] (**Figure 10**).

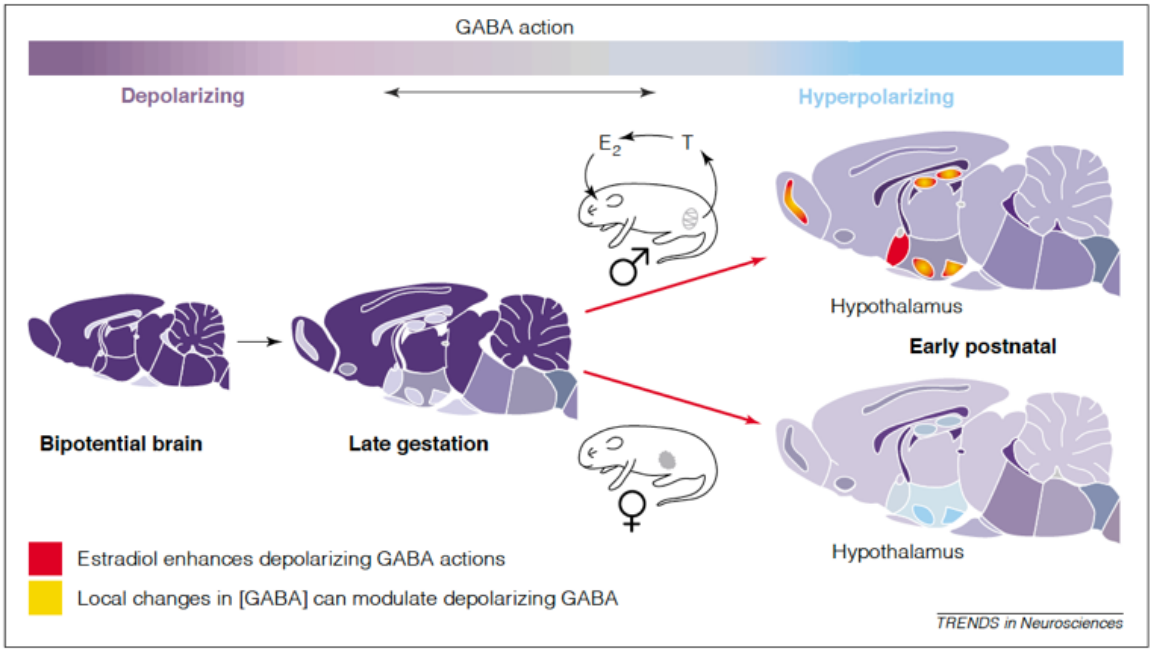

Figure 10. Sex hormones modulate GABA switch in developing neurons. The depolarizing to hyperpolarizing change in GABA activity s regions specific, and appears to occur particularly early in the hypothalamus, a region characterized by a high degree of sexual dimorphism. The rodent brain has the ability to develop into a male or female phenotype very early in the development. The onset of testicular activity in the male just before birth results in high levels of circulating testosterone (T), which is aromatized to estradiol (E2) within neurons, responsible for the process of the brain masculinization. Transiently high levels of E2 coincide with intrinsic shifts in GABA activity from depolarizing to hyperpolarizing in important brain areas like the hypothalamus, along with changes in GABA concentration ([GABA]). These factors may induce an excitatory GABA action foci during male brain development by modifying neurite extension, synaptogenesis, and cell death patterns in a sex-specific manner. Illustration adapted from McCarthy, 2002 [124].

1.4.4. Sex hormones, BDNF and GABAergic circuit

Brain-derived neurotrophic factor (BDNF) plays an important role in the survival, growth and differentiation of neurons during development [139–142], mediates the activity dependent modifications in synaptic strength [143,144], regulates dendritic and axonal growth [145,146] and the efficacy of synaptic transmission at excitatory and inhibitory synapses on hippocampal neurons [147,148], providing evidence that BDNF is essential during development.

ER have been shown to co-localize with neurons that express BDNF which suggests a relationship between sex hormones and the neurotrophic factor [149,150]. BDNF and estradiol may interact via several mechanisms. The BDNF gene contains a sequence with close homology to the estrogen response element [151]. In addition, estradiol and BDNF may stimulate similar downstream pathways, e.g., MAPK [152]. Estradiol may also regulate BDNF expression via ER-independent mechanisms which involve disinhibition of GABAergic neurons [153]. Lastly, given that both steroid hormones and BDNF regulate and modulate KCC2 expression [134] (**Figure 11**), these possible interactions have important implications in the susceptibility to early-life seizures [132,154] and will be explored in the following chapters.

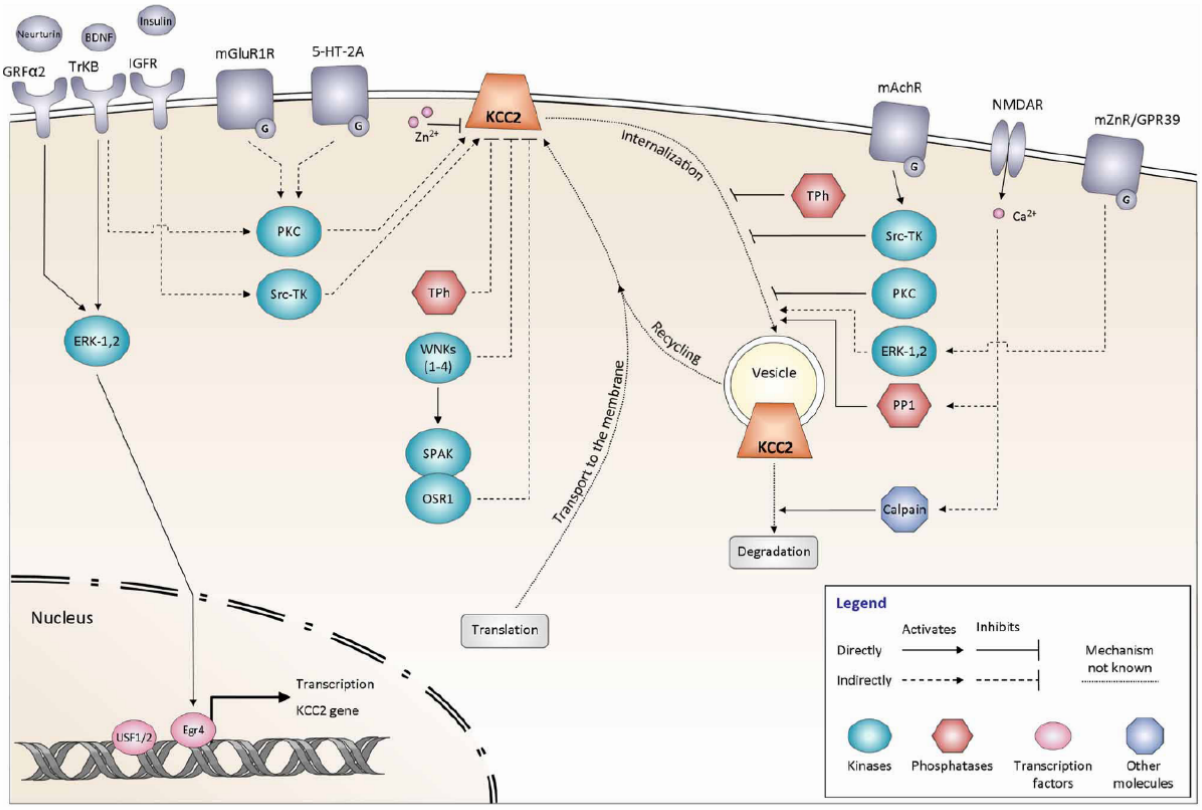

Figure 11. Signaling pathways involved in KCC2 function. KCC2 activity is regulated by a variety of proteins, including kinases and phosphatases. It influences either the steady-state protein expression at the plasma membrane or the recycling of the KCC2 protein. All different pathways are explained and discussed in detail on [XXX]. Illustration adapted from Medina et al., 2014 [133].

1.5. Sex differences in the neurobiology of stress

1.5.1. Homeostasis, Stress and Resilience

In physiology, resilience allows a host to remain healthy despite a stress or to recover faster from a stress by maintaining normal function (i.e., homeostasis). In more detail, homeostasis is a self-regulating process by which biological systems maintain constant internal conditions, while adjusting to changing external conditions. Homeostasis allows for adaptation and therefore survival in varying environments. It is also re-established by physiological and behavioral adaptive responses [155,156].

On the other hand, Hans Selye [157,158] the founder of modern stress research, introduced stress as the 'non-specific response of the body to any homeostatic demand'. Nowadays, McEwen [159] presented stress as a 'real or perceived threat to an individual's physiological or psychological integrity that results in physiological and/or behavioural responses'. A physiological or psychological stressor activates neuronal and endocrine systems, allowing for rapid adaptation and restoration to homeostasis [160].

Resilience is the ability to achieve a positive outcome in the face of adversity [161,162]. Resilience is defined as the amount of disturbance that a system can absorb before changing or losing its normal function, or the time it takes to return to homeostasis [163]. Although resilience may appear to be a return to normal behaviour after the disruption of homeostatic mechanisms by a stressor, it is actually a proactive process that involves utilising the host's adaptive potential to produce a positive outcome [164].

1.5.2. The hypothalamic-pituitary-adrenal (HPA) axis

The stress response is mediated by the autonomic nervous system (ANS) and the hypothalamus – pituitary – adrenal (HPA) -axis [165,166]. In particular, the brain serves as the primary organ for both the recognition of and the response to stressors and the central response to stress is a highly integrated process in which diverse neuronal systems are involved [167,168]. Here we focus on the HPA-axis and its glucocorticoid end products (i.e., cortisol in humans and corticosterone in rodents)

The HPA axis controls a sequential release of hormones from the brain and periphery [169] (**Figure 12**). The activation of the neurons in the parvocellular region of the paraventricular nucleus of the hypothalamus (PVN) by a stressor result in the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into portal blood system at the median eminence of the hypothalamus [170,171]. Both CRH and AVP are carried to the anterior lobe of the pituitary gland by the hypophyseal portal system, where they stimulate the release of adrenocorticotropic hormone (ACTH) into the general circulation. Although CRH is considered to be the main activator of ACTH, its effects are

amplified by AVP [172,173]. In the adrenal gland, ACTH stimulates the synthesis and secretion of glucocorticoids in the general circulation [174].

In order for glucocorticoids to exert their effect, they bind to mineralocorticoid and glucocorticoid receptors (MR and GR, respectively) [175], both of which are expressed throughout the body and the brain but differ in their distribution and affinities for glucocorticoids [176,177]. Although MR is found throughout the body [178], it is mostly seen in limbic regions like the hippocampus [179]. Glucocorticoids, on the other hand, bind the GR in the body and the brain [180]. Central GR is more extensive in the brain than MR and are more expressed in the hippocampus, PVN, hypothalamic nuclei, cortex, amygdala, and brainstem, which are regions involved in the regulation of the behavioral and endocrine stress response [179].

In regard to glucocorticoids affinity [181,182], MR and GR present different pharmacological properties with MR having a 10-fold higher affinity for corticosterone than GR [177]. Consequently, MR are already occupied at basal circulating corticosterone levels. MR regulate basal circadian and ultradian rhythms and are important in dictating HPA axis activity with respect to time of day. On the other hand, additional GR occupation at the circadian peak of after a stress response is essential for the HPA axis to return to homeostasis [180,183]. Lower-affinity GR stimulate gene expression, and it is speculated to mediate glucocorticoid effects on mobilisation of energy stores (liver, fat, and muscle), inflammation, and brain function (among others), along with norepinephrine and epinephrine [169,180]. The balance of actions mediated by these two receptors is vital for neuronal excitability, stress response, and behavioural adaptability. This imbalance increases the risk of neurological diseases [176,184,185].

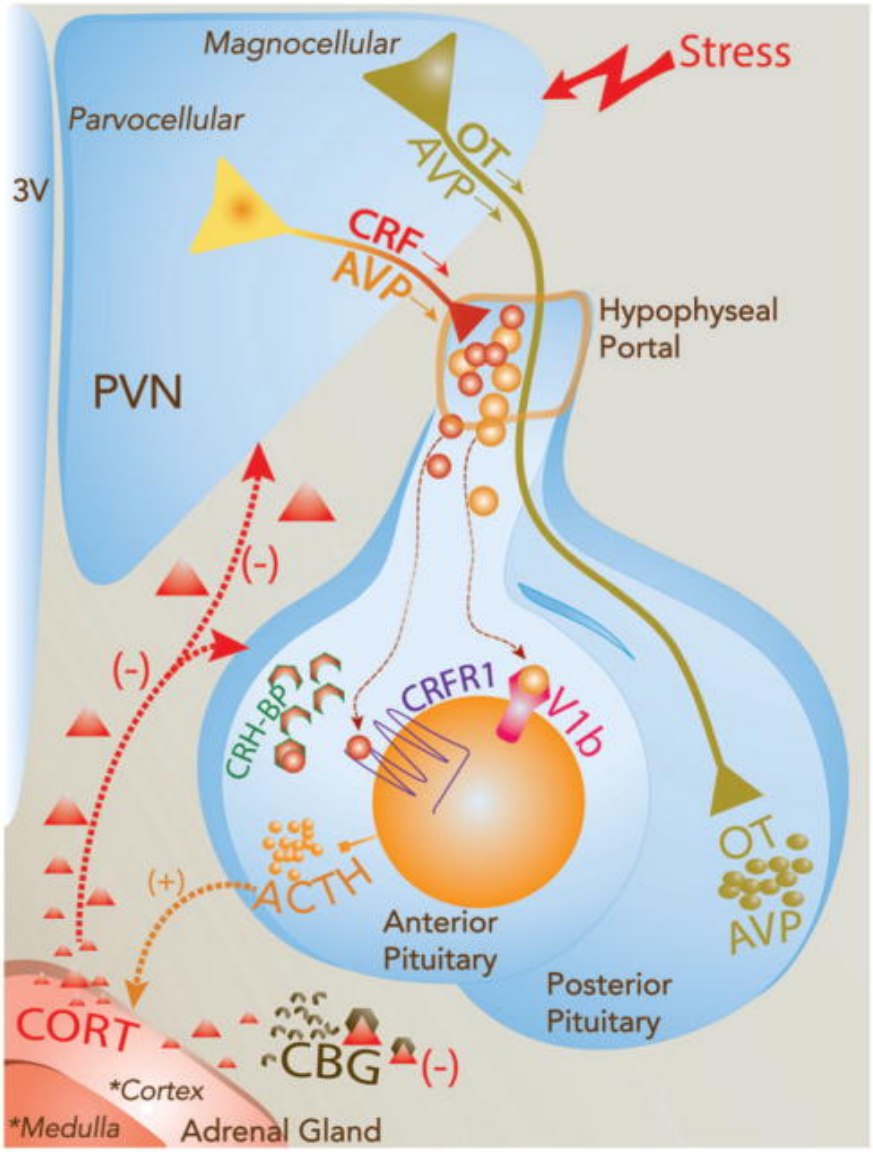

Figure 12. HPA axis and factors controlling the neuro-endocrine response to stress. When neurons in the parvocellular area of the PVN are activated by a stressor, CRH. and AVP are released into the portal blood stream at the hypothalamus's median eminence. The hypophyseal portal system transports both CRH and AVP to the anterior lobe of the pituitary gland, where they induce the release of ACTH into the general circulation. ACTH then increases the production and release of glucocorticoids in the systemic circulation in the adrenal gland. The activity of circulating CORT is in part regulated by CBG. PVN: paraventricular nucleus of the hypothalamus; CRH: corticotropin-releasing hormone; AVP: arginine vasopressin; ACTH: adrenocorticotropic hormone; CGB: CORT binding globulin. Not all intermediate and different pathways are included. Illustration adapted from Oyola and Handa, 2017 [217].

Glucocorticoids act on GRs at various levels of the HPA axis and upstream corticolimbic brain areas such as the hippocampus and medial prefrontal cortex (mPFC) to terminate the HPA axis stress response [186–188]. This glucocorticoid-mediated stress response termination is critical because it prevents extended exposure to high levels of glucocorticoids, which can have a deleterious effect in different brain regions and in the body. To prevent the production of excessive amounts of corticosterone by the HPA axis, glucocorticoids produce a negative feedback control on various levels in the cascade of a stress response, thereby reducing their own secretion [189,190] (**Figure 12**). This is due to the normal functioning of this system being necessary for survival, and therefore, tight regulation of the initiation and termination of glucocorticoid release is a high priority for all organisms.

1.5.3. Sex differences in response to stress

Responses to a particular stress can vary greatly between individuals or even from one time to another within the same individual. In terms of behavior, the survival mechanism of 'fight or flight' is well known [191], but it has typically been applied to both males and females as a universal truth [156,192]. Taylor *et al.* [192] suggested that male and female survival strategies, and hence stress coping, are fundamentally different, with males more prone to "fight or flight" response and females more prone to 'tend and befriend' response. The latter relates to nurturing behavior aimed at protecting oneself and offspring, as well as improving safety and minimising distress, and maintaining social networks [192]. Considering evolution, the 'fight or flight' response would not have been adaptive for females who were pregnant, lactating, or with offspring, as well as those who lacked the physical strength to survive. Studies in animal and humans show that females are more likely than males to associate with each other in stressful situations. Males, on the other hand, prefer a more autonomous and individualistic approach [192–194].

In terms of physiology, sex differences occur in the rodent's HPA axis response to stress [195–197]. Several studies have been focusing on sex differences on the physiological

HPA axis response of a healthy adults to a single, acute stressor, finding that females had a greater baseline level of CORT and a more robust CORT and ACTH response. Female rats also showed a longer time to return to baseline levels of ACTH and CORT, implying sex differences in the HPA axis' negative feedback regulation [198–201]. Following acute constraint, females had lower neuronal activity in limbic areas known to engage inhibitory inputs to the HPA axis, such as the frontal cortex and hippocampus, compared to males [202]. Furthermore, most corticosteroids are bound by corticosteroid-binding globulin (CBG), a liver glycoprotein, while in circulation and it is responsible for realising corticosteroids when it reaches its target [176]. Interestingly, sex differences in basal CBG levels are found and they are nearly double in females compared to males [203]. As a result, higher CBG levels in females may reduce CORT's availability for negative feedback, contributing to the increased HPA axis response to stress seen in female rodents.

On the other hand, sex differences in the activity and regulation of the rodent HPA axis can depend on severity, frequency, duration of the stressor, characterizing chronic stress, as well as the stage of life during which the stressor occurs. However, sex differences in response to chronic stress have not been thoroughly investigated, and only a few studies have found that females showed more pronounced HPA axis dysregulation after chronic stress, with higher corticosterone release [204,205]. Furthermore, there appear to be sex-specific differences in the appraisal/perception of how stressful a given experience is perceived, therefore sex differences are likely to rely on the type of the stressor. Overcrowding, for example, has been demonstrated to cause more stress in males, but social isolation causes more stress in females, which corroborates with ‘’flight-or-fight’ and ‘tend-and-befriend’ survival mechanisms [192,206–208].

1.5.4. Stress hypo-responsive period (SHRP)

During early development not every component is fully developed yet, which can influence the effects of glucocorticoids. The stress hypo-responsive period (SHRP) refers to the first two weeks of a rodent’s life, during which the HPA-axis activity is quickly

relapsed [209,210]. In rodents, it lasts from postnatal day (P) 4 to P14 in rats [210–212] and from P1 to P12 in mice [213] and rodent pups maintain very low circulation levels of glucocorticoids under basal conditions, and stress elicits little to no increase in corticosterone levels [214,215]. This is due to hypo-responsiveness of adrenal gland output rather than hypothalamus or pituitary function during this phase. It is important to highlight that control of glucocorticoids dynamics could be a potential neuroprotective mechanism against stress-induced excessive activation by GRs, given that greater levels of CORT are detrimental to central nervous system development [216,217].

Sex differences in the HPA axis have been documented as early as the neonatal period, at all components of the HPA axis, suggesting that gonadal hormones modulate development of HPA axis stress responsivity during early life development [218]. Sex differences in HPA axis functionality during SHRP have been observed in only a few preclinical studies and almost no clinical studies. Immune challenge at P3 increased HPA axis activity in intact female rats significantly more than in male rats. But when gonadectomy was performed at birth, the sex difference was reversed [219]. Female rats at P8 have been shown to have a higher ACTH responsivity to ether inhalation than male rats, but when given a testosterone injection at birth, which characterizes the sensitive period during development, they had a similar ACTH response to male rats [220]. Perinatal cortical abnormalities in males, such as a freezing lesion (focal microgyric lesion) at P1 combined with hyperthermic-seizures (seizures induced by increase of body temperature) at P10, resulted in a surge in plasma CORT levels in male pups and testosterone-treated females, as well as the development of mTLE. Untreated females were unaffected despite being subjected to identical brain injuries [221]. Furthermore, male neonates had a larger cortisol response than female neonates in a clinical investigation looking at stress reactivity in healthy term neonates, implying neonatal sex differences in physiological stress reactivity prior to the period of socialization [222]. As a result, the sex effect varies depending on the type of stressor used, and further research is needed to understand not only possible sex differences, but also how disruptions of normal HPA axis development during the SHRP are likely to cause long-term changes in neuroendocrine functioning and behavior.

1.5.5. Interactions between the HPA and hypothalamic-pituitary-gonadal (HPG) axes

Sex differences in neuroendocrine, i.e., HPA axis responses to stress arise as a result of the organizational and activational actions of gonadal hormones, implying a reciprocal link between the HPA and the hypothalamic-pituitary-gonadal (HPG) axes [196,218]. The HPG axis drives the germ cell development and maturation, as well as the synthesis and regulation of gonadal hormones in both, male and female gonads [218,223]. Gonadotropin-releasing hormone (GnRH) is secreted by GnRH hypophysiotropic neurons in the hypothalamus into the hypothalamo-hypophyseal portal vasculature at the median eminence where it is transported to the anterior pituitary. GnRH stimulates the anterior pituitary hormones production (i.e., luteinizing hormone (LH) and follicle stimulating hormone (FSH)). LH and FSH control steroidogenesis and gametogenesis in the ovary and testis [223]. Once produced, steroid hormones via feedback loops to the HPG axis and other brain regions via the anterior pituitary and steroid-sensitive hypothalamus neurons, regulating its activity and modulating reproductive behaviours and functions, including HPA axis activity [218] (**Figure 13**).

As previously mentioned, there are significant sex differences in the HPA axis, and bidirectional interactions between the HPA and HPG axes in both sexes [196,218]. Increased HPA axis activity, for example, can have an inhibitory impact on all levels of the female HPG axis [224], indicating that it is an evolutionary strategy used to restrict reproductive function during times of stress. Conversely, the female HPG axis influences HPA function on several levels in which adult females have higher basal corticosterone concentrations throughout the circadian cycle and show a greater ACTH and corticosterone response to acute stressors when compared to adult males, as previously described (Section 1.5.3). [198,225–228]. In general, the literature shows that androgens inhibit the HPA axis in males whereas estrogens stimulate the HPA axis in females [196]. On the other hand, progesterone studies suggest an inhibitory effect on HPA axis reactivity and may counteract some of estrogen stimulatory effects on the HPA axis [226]. Much research has been conducted at baseline or in reaction to acute stressors, rather than within animal models of chronic stress and epilepsy. To that aim, it remains to explore further the sex differences in the activity of the hypothalamic–pituitary–adrenal (HPA) axis in response to chronic stress

during, as well as the underlying contributions of gonadal hormones to epilepsy during development.

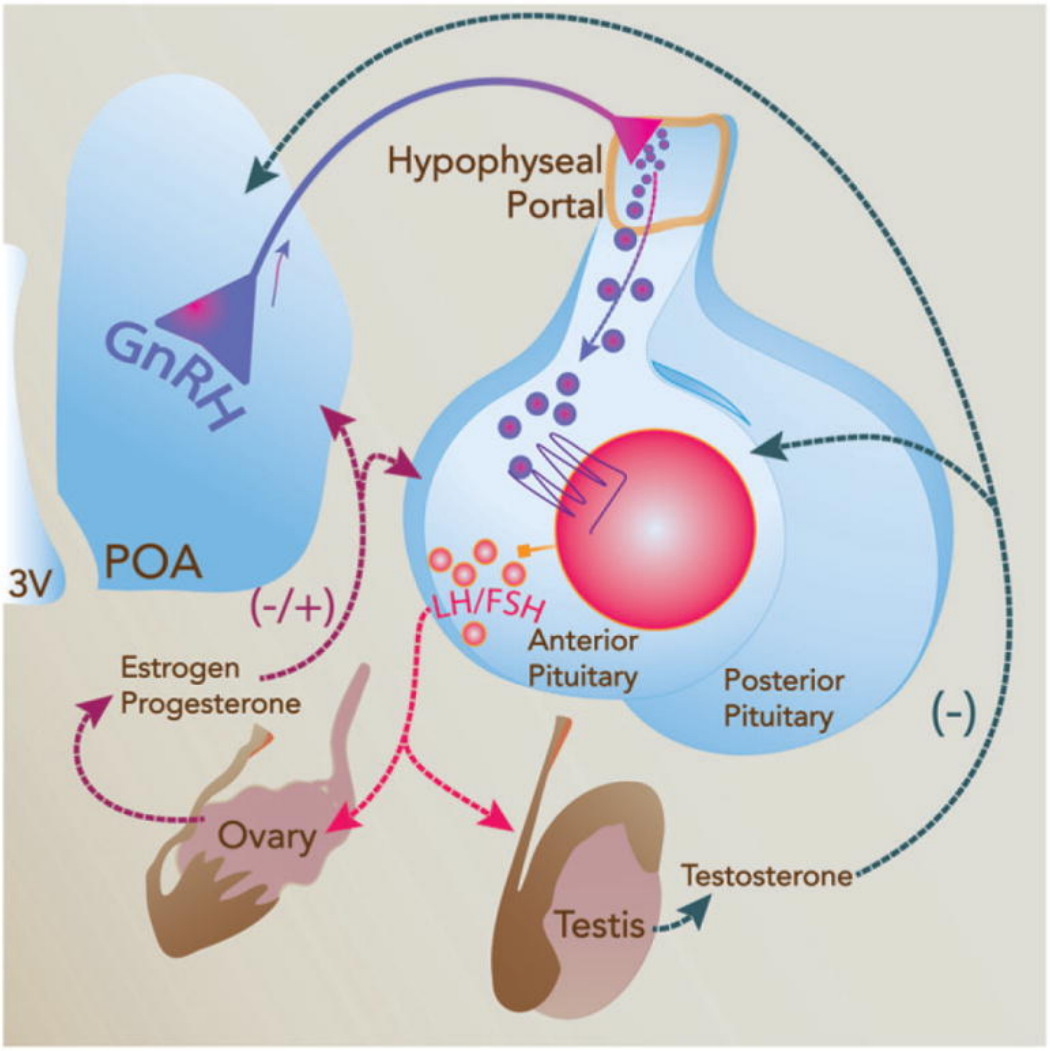

Figure 13. HPG axis and factors controlling its regulation. GnRH is released by GnRH hypophysiotropic neurons in the hypothalamus and delivered to the anterior pituitary via the hypothalamus-hypophyseal portal vasculature at the median eminence. The anterior pituitary hormones, LH and and FSH) are stimulated by GnRH. Once released, steroid hormones regulate the activity of the HPG axis and other brain areas via feedback loops that involves the anterior pituitary and steroid-sensitive hypothalamic neurons. GnRH: Gonadotropin-releasing hormone; LH: luteinizing hormone; FSH: follicle stimulating hormone; POA: preoptic area. Illustration adapted from Oyola and Handa, 2017 [217].

1.6. Sex, Stress and Epilepsy

1.6.1. Epilepsy and Mesial Temporal Lobe Epilepsy

Epilepsy is one of the common chronic neurological disorders with a prevalence of about 1% of disease-affected population worldwide [229]. Epilepsy is characterized by unprovoked recurrent seizures. Seizures are most associated with certain regions of the brain and may remain limited to these regions or may engage the cerebral hemispheres. Epilepsy is thought to be most prevalent in the hippocampus formation and cerebral cortex, which are two of the brain's most epileptogenic areas [230,231]. Epilepsy is a broad term that refers to a big set of disorders that have a variety of causes, with each of them involving network ensemble hyperexcitability and synchrony as a result of dysfunctional neuronal mechanisms [232].

Recent research has shown that epileptogenesis is caused by developmental factors such as congenital brain abnormalities, altered neuronal signaling, early life stress, and impairments in postnatal maturation of neuronal networks, resulting in the concept of epilepsy as a neurodevelopmental disorder [232]. In fact, the risk for having seizures during postnatal development is higher than that at other times in life [123], with an incidence of 1.5–3.5/1000 in term babies and as high as 130/1000 in preterm newborns, therefore neonatal seizures are the most common clinical symptom of central nervous system dysfunction in the newborn [233–235]. This vulnerability is speculated given that the developing immature brain has different physiological characteristics, such as longer-lasting and less selective neuronal ionic currents. This increases the likelihood of immature neurons spiking activity and allows them to connect and fire at the same moment, i.e., synchronise, thus making the developing brain more prone to seizures [236,237].

Epidemiological studies suggest that prevalence and incidence of seizures and epilepsy is slightly higher in males than in females [38,238,239]. During childhood, seizures and epilepsy syndromes more likely affect boys than girls, although some epilepsies are significantly more common or exclusive in girls than in boys (**Table I**). Sex-related differences have been described also in patients with temporal lobe epilepsy (TLE), with

respect to distinct regional distribution of brain dysfunction during interictal periods, seizure generalization, lateralization, as well as the extent of neuronal damage [240,241].

Table I. Sex differences in epilepsy syndromes during childhood. Table adapted from Velíšková and DeSantis, 2012, [237].

Some epilepsy/seizure syndromes more common in females	Some epilepsy/seizure syndromes more common in males
Idiopatic generalized epilepsy (Christensen et al., 2005) Aicardi syndrome (Ryan et al., 1997) Rett syndrome (Ryan et al., 1997)	Ohtahara syndrome (Clarke et al., 1987) Infantile spasms (Luthvigsson et al., 1994) Lennox-Gastaut syndrome (Trevathan et al., 1997) generalized myoclonic epilepsies (Nordli, 2005) Landau-Kleffner syndrome (Mouridsen, 1995) Febrile seizures (Forsgren et al., 1990; Tsuboi, 1984)

Mesial temporal lobe epilepsy (mTLE) is the most common type of epilepsy, accounting for one-third of all cases and typically refractory to treatment [242]. The mesial limbic regions of the temporal lobe, such as the hippocampus, the entorhinal cortex, and the amygdala, are the most epileptogenic areas in the brain. With regards to prevalence, mTLE accounts for 40% of all epilepsy disorders [242]; up to 40% of mTLE patients are refractory to medical treatment [243], 30-40% continue to have seizures following ressection of the epileptogenic zone, even after undergoing surgery [243,244]. mTLE often manifests itself in teenagers and even adults after a latent period; however, it is believed that an initial brain insult, such as febrile seizures (FS) [245], occurs during the early stages of development of the nervous system and have a role in the mTLE.

1.6.2. Febrile seizures in early postnatal life

Febrile seizures (FS) are a neurological abnormality characterized by neuronal hyper-excitability that occurs in infancy or childhood associated with fever of 38.0 ℃ or

higher, and presents without evidence of any definite causative diseases, such as central nervous system (CNS) infection or metabolic abnormality [246,247]. FSs are the most common convulsive events in children aged 3 months to 6 years [248,249] with a peak incidence between 12 and 18 months [250,251], and a prevalence of 2–14% depending on the population studied [252]. There are two types of FS: simple and complex FS. Simple FS are brief isolated seizures, generalized at onset, with rapid recovery; further, children with simple FS typically do not have additional neurologic abnormalities beyond the initial seizure and have normal brain development [249,253,254]. On the other hand, complex FS are prolonged (>15 min), can have multiple episodes within 24 h, are lateralized and are associated with post-ictal neurological deficits. In fact, it is now known that 30 to 50% of patients with mTLE during adulthood have history of complex FS occurring early in childhood [255] and display hippocampal injury visible on MRI [256–259]. In regard to sex differences, males have consistently emerged as having a higher frequency of FS (male to female ratio of 1.6 to 1) [260,261]. However, longitudinal studies have shown no sex-based differences [254].

Despite clinical data suggesting that complex FSs are the most prevalent pathology linked with mTLE [262–264], not all patients develop mTLE, indicating that additional factors have a role in epileptogenesis and mTLE (**Figure 14**). Studies show that complex FS can arise as a result of an anatomical [265,266] and/or genetic insult [267], as well as environmental stressors, such as early life stress [268], which can cause long-term alterations in the limbic system, including an increased susceptibility to epileptogenesis/mTLE [269]. Therefore, in order to investigate how these different insults affect FS and possibly mTLE, we rely heavily on experimental rodent models to gain a better understanding of the pathophysiology of FS and its possible consequences in the brain [269,270].

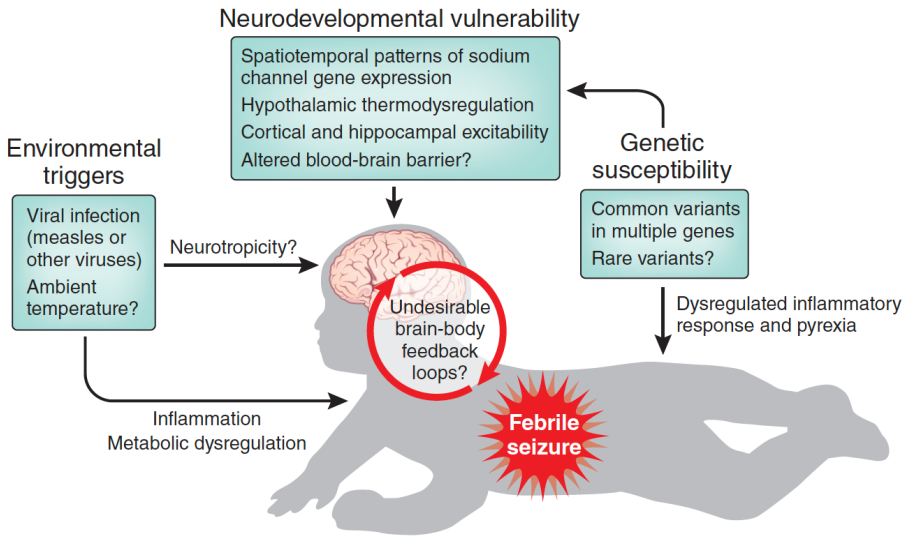

Figure 14. Environmental, genetic and developmental factors to febrile seizures. Under certain circumstances, febrile seizures can recur or initiate latent epileptogenesis. Illustration adapted from Sisodiya, 2014 [271].

1.6.3. Animal models of febrile seizures

The main challenges of conducting studies on the pathophysiology of FS are difficulty in recruiting, individual variability, and compliance, among other things, as well as ethical considerations that may occur with human trials [272]. As a result, several animal models have been developed to mimic FS. An approach that has improved our understanding of the pathophysiology of FS has been to study the effect of hyperthermia-induced seizures (HS), as a model of FS [273–276]. Similar to FS in humans, HS show age-dependent susceptibility peaking at P10-11 in rats (or P14-15 in mice), when hippocampal development is equivalent to the first year of human life. Furthermore, EEG recordings support a limbic origin of HS [273,275–277] (**Figure 15**).

In regard to experimental paradigms, different heating sources can artificially evoke hyperthermia-induced seizures (HS) to determine how FS are generated [278]. The most reliable and well-known model is hyperthermia produced by hot dry air [273,275,276]. With limited or no mortality, this paradigm produces highly stereotyped generalised seizures that are repeatable and simple to define [279–281]. Like in humans, this paradigm results in

the production of spontaneous seizures later in adulthood, particularly in extended HS models. Alternative paradigms for inducing HSs in rats include exposure to infrared light [282], infrared rays [283], microwaves [284], a heated pad [285], or warm water [286]. However, owing to high morbidity, mortality, and clinical heterogeneity, the utilisation of these experimental paradigms is restricted. Overall, these studies have shown that the HS paradigm is clinically relevant to FS.

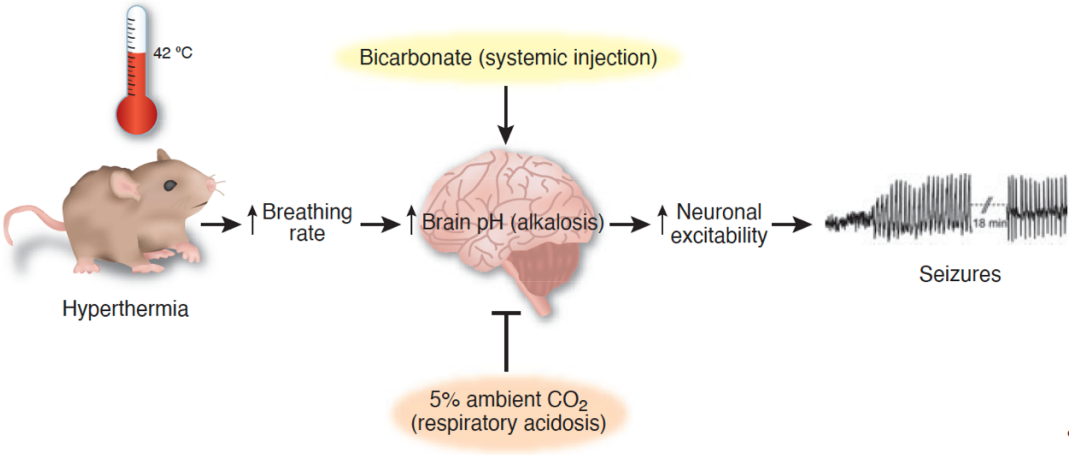

Figure 15. Events leading to seizures after hyperthermic-induced seizures in rat pups. Hyperthermia causes an increase in respiratory rate which enhances elimination of CO_2, resulting in brain alkalosis. Alkalosis causes hyperexcitation of neurons in the cortex and hippocampus, resulting in convulsions and epileptiform electrographic discharges recorded in the brain. The inspiration of ambient CO_2, which generates acidosis, prevents brain from alkalosis and thus from seizing. Illustration adapted from Tapia, 2006 [287].

1.6.4. Role of stress in mTLE

mTLE has been seen as a multistage process wherein early life brain insults cause a cascade of neurobiological events that eventually culminate in an epileptic state characterized by spontaneous recurring limbic seizures [288], which is common referred as a 'two-hit' model (**Figure 16**). In a retrospective study from Dr. Carmant's group, it was revealed that 66% of mTLE patients had a history of febrile seizures associated with focal cortical dysplasia (FCD), a cortical malformation [265]. Based on these observations, the

Carmant laboratory developed a two-hit rodent model in which a cortical malformation at P1 was induced and lead to enhanced susceptibility to the HS at P10 [273,289]. Results showed that rats that had undergone both insults developed focal limbic SRS ~P90 at a rate of 83-100% per litter, whereas littermates subjected to either one or another insult alone failed to exhibit SRS [274]. The results have clinical significance because we have shown that FSs in our lesioned model, occurs only in a vulnerable hippocampus [269,290] and suggests that FS represent the second insult leading to mTLE [221,274,291].

However, over the years, it became evident that a structural lesion was not mandatory to predispose the hippocampus to a second-hit and that early-life stress could be a significant first-hit in our model, which could also explain why not all children who develop MTLE have an associated cortical malformation. To study the physiology and biological substrates of stress as a predisposing factor in MTLE, we have modified, the two-hit rat model of mTLE (see Chapter III) (Figure X) [292]. Previous studies show that unlike naïve controls who never develop epilepsy, 66% of sham-operated male pups could develop epilepsy following HS. Furthermore, it was observed that female pups exposed to the brain lesion at P1 and HS at P10 never developed spontaneous seizures as adults, despite suffering prolonged HS, while combining both injuries in males lead to epilepsy in 100% of cases [221]. To investigate the role of stress in our two-hit model, CORT levels were measured at P1 and P10 before and after each insult. The results demonstrated that lesioned and a sham males had a significant rise of CORT levels at P1 and were the only ones to develop epilepsy; while females had higher baseline CORT levels but neither displayed a rise in these levels after the lesion nor developed epilepsy. Interestingly, this sex difference could be reversed by androgenizing females with testosterone injections during perinatal period [221].

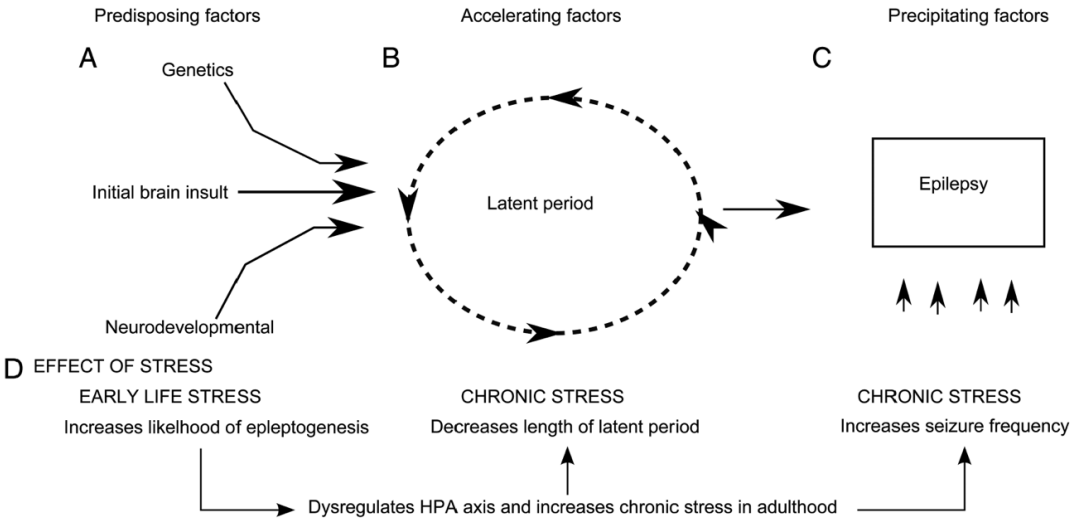

Figure 16. Factors involved in epileptogenesis. Epileptogenesis is the process through which a previously normal brain becomes functionally changed and biased towards the development of aberrant electrical activity, which is required for persistent seizures. Seizures may accompany the first underlying brain injury (e.g., genetic, neurodevelopmental, trauma, infection, etc.). The brain then undergoes a latent phase during which increasing cellular and network alterations modify the brain, resulting in an epileptic brain. The latent phase might span days, weeks, or even years, and or in some instances be indistinguishable from the insult. Each of these phases can be affected by stress. Diagram adapted from Galtrey, Mula and Cock, 2016 [293].

Furthermore, there are studies that indicate that early changes of function and structure in the brain following stress contribute to the susceptibility to FS and to epilepsy [294,295]. Moreover, FS and mTLE generally arise from the hippocampus, a structure known to be affected by stress biology. Thus, among the early-life insults that could modulate the outcome of FS, stress may be an important contributor. In epileptic patients, emotional stress is commonly reported to provoke seizures or increase seizure frequency. Animal studies have similarly shown that there exists strong evidence that chronically elevated glucocorticoid levels promote hyperexcitability, cause structural hippocampal changes and decrease seizure threshold. For instance, administering exogenous corticosterone accelerates kindling [296–298], which can be reversed by using GR and MR antagonists [297]. Kindling can also be slowed in adrenalectomized or hypophysectomized rats [299–301]. However, a definite causal relationship between early-life stress and epileptogenesis has not been demonstrated yet. Both the glucocorticoid stress hormones corticosterone

(cortisol in humans) and corticotropin releasing factor (CRF) appear to exert potent proconvulsive effects on limbic structures in the developing brain [302–304]. Although there is no clear evidence that early-life stressors induce epileptogenesis, the anatomical and physiological changes produced by stress hormones could clearly predispose the developing hippocampus to a second-hit, as we have demonstrated by creating an extra-hippocampal lesion [221,274,291].

In regard to sex differences, pre- or postnatally stress experimental paradigms has been shown to have sexually dimorphic effects on seizure susceptibility in rodents and humans. Pre-natal stress on gestational day 18 leads offspring to be more susceptible to kainic acid induced seizures, with males showing a greater susceptibility than females [305]. Clinical studies have shown that infants who experienced prenatal stress presented exacerbated FS duration and intensity [306], with males having a 1.75 times greater risk of experiencing a complex seizure. Furthermore, postnatal stress increased the rate and duration of tonic-clonic pilocarpine-induced seizures and showed more elevated CORT levels in P18–19 in males compared to female rats [307,308]. Similarly, prenatal chronic postnatal cross-fostering stress during a similar period (*i.e.*, P1-P23), in which rats raised either by their own mother, a foster mother from the same strain, or a foster mother from the opposing strain, enhanced kindling rates in adult male rats [309].

Together, results from clinical and animal studies strongly encourage continued studies on sex-specific biological effects on the development of epilepsy, especially as they pertain to the developing brain. Whether and how sex hormones regulate GABAergic circuits, stress responses and circuit hyper-excitability in the developing brain was yet to be established and it was the focus of my graduate work.

1.7. Research Objectives

1.7.1. Rationale

Sex-dependent signaling during normal development is critical and it forms the foundation for understanding sex differences in disease predisposition, manifestation, and response to treatment. Sex is associated with molecular, cellular, and structural changes in the brain leading to functional changes, cognitive differences, and increased vulnerability to neurological disorders.

Several animal studies have demonstrated molecular and cellular effects of developmental factors on the hippocampus that appear to be affected by sex and persist into adulthood. The abundance of data relating the hippocampus to sex-specific brain function encoded in childhood suggests that the time of brain sexual differentiation, which is driven by testosterone and its metabolites as one of the pathways, has significant impacts on the hippocampus. However, the cellular mechanisms by which steroid hormones enduringly modify hippocampal formation and function are poorly understood.

Furthermore, although increased glucocorticoid levels in response to stress are beneficial to an organism, chronic exposure to high levels of glucocorticoids can also have harmful effects. An increasing body of evidence suggests that early-life stress responses may also influence the developing brain's predisposition to seizures and epilepsy, with possible sex-based discrepancies in adulthood. Of interest is that neurodevelopmental disorders disproportionately afflict males, and the possibility that sex differences in the early hormone environment contribute to this disparity in relative risk cannot be ignored.

Therefore, based on the important role of sex hormones in normal regulation and function of the hippocampus, the research objectives of my thesis are to explore 1) sex differences in hippocampal inhibitory network development and, 2) sex differences in stress response, which could be implicated in seizure susceptibility. To this end, we systematically examined the effects of sex hormones manipulation on hippocampal GABAergic system components (Chapter II), as well as sex differences in a stress-based model of epilepsy (Chapter III). Below are the hypotheses of the experiments described in Chapters 2 and 3:

1.7.2. Hypotheses

CHAPTER II:

In order to provide a compelling picture of early postnatal sex differences in neuronal excitability, we analyzed GABAergic circuit functional markers in male, female, testosterone-treated female, and testosterone-insensitive male rats after the first postnatal week and in young adults. Our hypothesis is as follows: **Neonatal testosterone signaling regulates sex differences in the development of GABAergic system.** The present study demonstrates the importance of neonatal testosterone during CNS early development.

CHAPTER III:

Adverse early life events can program the susceptibility of the stress system for the development of epilepsy/mTLE later in life. Whether and how sex differences promote seizures or protect the brain from seizures in a stress experimental model is the focus of the work described on Chapter 3. We used a previously modified “double-hit” model, which consists of inducing injections of corticosterone from P1-P9 to recapitulate stress (first hit), followed by triggering experimental FS, i.e., hyperthermic-induced seizures (second hit) at P10 in rats. Our hypothesis is the following: **sex hormones early in life affect the stress responses, which then affect vulnerability to develop epilepsy.** The present study shows that early-life stress leads to hippocampal hyperexcitability rendering pups vulnerable to a second insult in a sex-specific manner.

– CHAPTER II –

FIRST PAPER

Sex-specific differences in KCC2 localisation and inhibitory synaptic transmission in the rat hippocampus

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Author's contributions: DCW and NTS designed the study. DCW, TS, ARE, ASFN performed experiments. DCW, JSC and AOSC analysed data. DCW, GDC, AGW wrote the manuscript. All authors reviewed the manuscript.

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2.1. ABSTRACT

Sexual differentiation of the brain is influenced by testosterone and its metabolites during the perinatal period, when many aspects of brain development, including the maturation of GABAergic transmission, occur. Whether and how testosterone signaling during the perinatal period affects GABAergic transmission is unclear. Here, we analyzed GABAergic circuit functional markers in male, female, testosterone-treated female, and testosterone-insensitive male rats after the first postnatal week and in young adults. In the hippocampus, mRNA levels of proteins associated with GABA signaling were not significantly affected at postnatal day (P) 7 or P40. Conversely, membrane protein levels of KCC2, which are critical for determining inhibition strength, were significantly higher in females compared to males and testosterone-treated females at P7. Further, female and testosterone-insensitive male rats at P7 showed higher levels of the neurotrophin BDNF, which is a powerful regulator of neuronal function, including GABAergic transmission. Finally, spontaneous GABAergic currents in hippocampal CA1 pyramidal cells were more frequent in females and testosterone-insensitive males at P40. Overall, these results show that perinatal testosterone levels modulate GABAergic circuit function, suggesting a critical role of perinatal sex hormones in regulating network excitability in the adult hippocampus.

KEYWORDS: Sex differences, Testosterone, Estradiol, GABAergic circuits, Hippocampus, KCC2, BDNF

2.2. INTRODUCTION

Female animal models have been consistently excluded from most basic neuroscience studies for decades [1–4] despite the well documented sex bias observed in many neurological and neuropsychiatric conditions, such as autism spectrum disorders (ASD), epilepsy, mood disorders, multiple sclerosis, Alzheimer's and Parkinson's disease [5–9]. Therefore, considering sex as a biological variable (SABV) in the design and analysis of basic and clinical research is extremely important when making treatment decisions for both men and women [3,10,11].

Hormonally influenced sex differences are caused by either activational or organizational effects of hormones [12–16]. Briefly, circulating gonadal hormones can have acute and transitory effects throughout life (activational effects), whereas exposure to gonadal hormones during developmental phases might result in persistent sex differences (organizational effects) [17]. Many molecular and cellular processes are influenced by gonadal hormones in the developing brain, including gene expression, cell birth and death, neurite outgrowth, synaptogenesis, and synaptic activity [18]. Extensive clinical observations suggest that males have a two-to-four times higher risk of being affected by neurodevelopmental disorders than females [19]. It has been suggested that gonadal hormones lead to functional difference in neuronal circuit development, thus contributing to sex bias in neurodevelopmental disorders [20,21].

The balance between excitatory and inhibitory circuits is fundamental for all aspects of brain function [22]. Gamma-Aminobutyric acid (GABA), the principal inhibitory neurotransmitter, plays an important role in maintaining the inhibitory tone that counterbalances neuronal excitation. The inhibitory action of GABA relies on the inflow of chloride ions (Cl^-), which hyperpolarises neurons in the brain. However, in early development, GABA signaling induces outward Cl- currents, resulting in membrane depolarization. The postnatal shift in GABA function relies on the developmentally regulated expression of the Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1) and the K^+-Cl^- cotransporter 2 (KCC2). Immature neurons accumulate Cl^- due to a high level of

NKCC1, a Cl^- importer, resulting in Cl^- efflux and membrane depolarization upon $GABA_AR$s opening. Conversely, mature neurons express higher levels of the Cl^- exporter, KCC2, which results in lower intracellular Cl^- concentration which drives Cl^- influx through $GABA_AR$s and leads to hyperpolarization [23,24]. Interestingly, recent studies showed that the timing of KCC2 and NKCC1 expression, and consequently the onset of mature inhibitory GABAergic neurotransmission during brain development, is sex dependent and occurs earlier in females compared to males [25–28]. However, it is unknown whether the observed sex difference in chloride transporter expression is dependent on perinatal hormones.

In this study, we explored the role of perinatal testosterone on GABAergic inhibition, by analyzing the expression of different molecular determinants of GABAergic neurotransmission and recording spontaneous and miniature GABAergic currents in hippocampal CA1 pyramidal neurons in males, females, masculinized females, and testosterone-insensitive males. Exogenous testosterone administration during sensitive periods of brain development induces adult male sexual behavior and brain anatomical patterns in female rodents [29,30]. On the other hand, the testicular feminization mutation (Tfm) is a naturally occurring point mutation of the gene encoding the androgen receptor (AR) that renders the Tfm male rat insensitive to physiological levels of androgens [31]. This latter approach allowed us to explore the organizational sex differences under baseline conditions, which are often undetected.

2.3. RESULTS

2.3.1. Profile of neonatal testosterone and estradiol at birth

In rats, testicular testosterone production peaks in the late gestational period (E18) and is high throughout the first few postnatal days [18]. Therefore, to characterize the levels of plasma testosterone and estradiol in male, female, testosterone-treated female, and testosterone-insensitive male rats, samples were taken from spontaneously delivered

pups within 24 hours after birth, i.e., P0. We found that *plasma testosterone levels were elevated in male,* testosterone-treated female, *as well as in testosterone-insensitive male rat pups when compared to female rat pups at birth (Figure 1A;* One-way ANOVA with Tukey's post hoc analysis, F x M: $p=0.028$; F x A: $p<0.001$; F x TFM: $p<0.001$). Conversely, there were no differences in plasma estradiol levels across the groups (Figure 1B; One-way ANOVA with Tukey's post hoc analysis, F x M: $p=0.995$; F x A: $p=0.080$; F x TFM: $p=0.140$).

2.3.2. Perinatal testosterone determined sexual developmental markers

Sexual developmental marker analysis revealed that testosterone affected the anogenital distance (AGD) length and secondary sexual characteristics from birth until puberty (Figure 2). At P1, we found smaller AGDs in females and testosterone-insensitive males, and larger AGDs in males and testosterone-treated females (Figure 2A, D; One-way ANOVA with Tukey's post hoc analysis, F x M, F x A TFM x M, TFM x A: $p<0.0001$). At P15, we found that areolas were either absent or normal (prominent). All females and testosterone-insensitive males displayed normal areolas. Conversely, areolas were not detected in any male or testosterone-treated female offspring (Figure 2A). At P35, sexual maturity was assessed in female and testosterone-treated female rats by inspecting the vaginal opening (VO). Testosterone treatment during the perinatal period affected the VO. Dissection of testosterone-insensitive male offspring at P40 revealed testes, confirming their male genotype (Figure 2B, C). Overall, these results showed that the presence of androgens at the time of birth, like it occurs in normal males or in females treated perinatally with exogenous testosterone, resulted in the development of male-like phenotypes, which include development of secondary sexual characteristics. These results are in accordance with previous data showing that, while the Tfm mutation does not render AR completely non-functional, the decrease in function by 85–90% [31] is enough to generate an entirely feminine external phenotype [32]. Notably, gonadal steroid

production is intact in the Tfm male, resulting in testosterone levels that are rather high yet within the normal range [33] as show in Figure 1 A.

2.3.3. Perinatal testosterone signaling did not affect mRNA levels of major molecular determinants of GABAergic neurotransmission

Previous studies have shown fluctuations in expression levels of GABA signaling components between sexes [34,35]. Therefore, to characterize the GABA signaling components in our four experimental groups, we quantified by RT-qPCR the mRNA expression levels of GABA-A receptor subunits (α1, α5, β2, γ2), GABA transporter (GAT-1), GABA synthesis (GAD65 and GAD67) and Chloride co-transporters (NKCC1 and KCC2) in hippocampal samples collected from the four groups at P7, when GABAergic circuits are still developing, and P40, when GABAergic transmission is considered mature. We found no significant difference in mRNAs expression levels for any GABA signaling components in all sex groups at both ages (Figure 3 A, B; One-way ANOVA with Tukey's post hoc analysis, $p>0.05$, fold change >2 or <0.5 for all comparisons). Therefore, testosterone signaling at birth seemed to not have any effects on the transcription of major molecular determinants of GABAergic signaling.

2.3.4. Testosterone signaling delayed membrane localisation of KCC2 during the first postnatal week

The increase of KCC2 expression during the first two postnatal weeks underlies the onset of powerful inhibitory neurotransmission in the hippocampus [22–24]. To evaluate the impact of testosterone on KCC2 expression, we quantified the monomeric (140KDa) form of KCC2 at two developmental ages, P7 and P40, by western blot. We did not observe any significant differences in KCC2 expression levels in whole cell lysates between any of the groups for KCC2 monomer band (Figure 4A, S1, B, C; One-way ANOVA with Tukey's post hoc analysis, $p=0.926$ at P7; One-way ANOVA with Tukey's

post hoc analysis, p=0.355, at P40) in contrast to what previously reported [25,26]. While KCC2 protein can be found both in cytoplasmic and plasmalemmal compartments, KCC2-dependent Cl- extrusion in neurons is mostly reliant on KCC2 localization at the membrane. Therefore, to investigate whether KCC2 localization at the membrane was sex dependent, we quantified KCC2 monomer levels in the membrane fractions from hippocampi of P7 and P40 rats (Figure 4D, S1). At P7, we found that KCC2 monomer levels in the membrane fractions were significantly higher in female when compared to males and testosterone-treated female rats (Figure 4E; One-way ANOVA with Tukey's post hoc analysis, F x M: $p<0.001$; F x A: $p=0.001$). Furthermore, testosterone-insensitive male rats showed higher KCC2 monomer levels in the membrane fractions when compared to male and testosterone-treated female rats at the same age (Figure 4E; One-way ANOVA with Tukey's post hoc analysis, TFM x M: $p<0.001$; TFM x A: $p=0.001$). Conversely, at P40 we observed no significant difference in KCC2 monomer levels in the membrane fractions between groups (Figure 4F; One-way ANOVA with Tukey's post hoc analysis, p=0.121). Overall, our results showed that, during the first postnatal week, higher testosterone signaling was associated with lower KCC2 membrane localization.

2.3.5. BDNF expression was higher in females and testosterone-insensitive males during the first postnatal week

Several studies have suggested that BDNF may play a role in the developmental increase of KCC2 expression [36–38]. Sex steroid hormones may induce BDNF transcription, enhance CREB activity and modulate it epigenetically [39–41], which may account for the functional discrepancies in BDNF between different sexes. Since we found that KCC2 localization in the membrane of hippocampal neurons was hormone dependent, we asked whether BDNF expression levels in the hippocampus at P7 were hormone dependent as well. We found higher levels of BDNF in the hippocampus of females and testosterone-insensitive males compared to male rat pups (Figure 5 A, S2, B; One-way ANOVA with Tukey's post hoc analysis, F x M: p=0.009; TFM x M: p=0.007). Thus, these data show

that during the first postnatal week higher testosterone signaling was associated with lower BDNF expression levels.

2.3.6. Perinatal testosterone affects spontaneous GABAergic neurotransmission in the adult hippocampus

To determine whether perinatal testosterone had long term consequences on GABAergic neurotransmission, we recorded miniature and spontaneous inhibitory postsynaptic currents (IPSCs) from pyramidal cells in the CA1 region of the dorsal hippocampus in P40 rats. We did not find any significant difference in mIPSC frequency (Figure 6A, B; One-way ANOVA with Tukey's post hoc analysis, $p=0.895$) or amplitude (Figure 6A, C; One-way ANOVA with Tukey's post hoc analysis, $p=0.849$) in the four experimental groups, suggesting that GABAergic synapse numbers or strength were not significantly affected by testosterone signaling during the perinatal period. Conversely, at the network level, we observed that the mean frequency of sIPSCs was significantly smaller in pyramidal neurons from males compared to pyramidal neurons from females and testosterone-insensitive males (Figure 6D, E; One-way ANOVA with Tukey's post hoc analysis, F x M: $p=0.002$; TFM x M: $p=0.021$). We further observed a decreased frequency of sIPSC in testosterone-treated females compared to females and testosterone-insensitive males (Figure 6D, E; One-way ANOVA with Tukey's post hoc analysis, F x A: $p=0.004$; TFM x A: $p=0.030$). sIPSC amplitude was not significantly different between the four experimental groups (Figure 6D, F; One-way ANOVA with Tukey's post hoc analysis, $p=0.067$). Overall, these results suggest that perinatal testosterone signaling lead to overall reduced spontaneous inhibitory transmission in young adult rodents.

2.4. DISCUSSION

In the present report, we provide evidence that membrane KCC2 localisation is negatively regulated by perinatal testosterone signaling in the hippocampus during the first postnatal week. We also demonstrated that perinatal testosterone signaling leads to decreased spontaneous inhibitory transmission in adult hippocampus. These data urge caution in generalizing findings regarding the cellular and molecular mechanisms underlying GABAergic circuit development and function in the different sexes.

During early postnatal development, a shift from $GABA_A$-mediated excitation to inhibition occurs in a wide array of brain structures, including the hippocampus [42,43]. The developmental GABA switch in the hippocampus has traditionally been studied only in male rats. In recent years, research using female rats has revealed precocious $GABA_A$ transmission compared to male rats. In female rats, the GABA switch in CA1 and CA3 pyramidal neurons occurs between P4 and P7. In male rats, the transition occurs between P7 and P14 [23,26,44–50], suggesting a longer window of $GABA_A$-mediated excitation in males compared to females. The mechanisms underlying these sex differences are poorly understood. It has been suggested that the testosterone surge that occurs perinatally in males [51] promotes the expression and the activity of NKCC1, while decreasing the synthesis and activity of KCC2 [52], which would result in males experiencing the GABA shift later than females. This effect has been shown not only in hippocampus, but also in substantia nigra reticulata neurons in acute slices [53], and embryonic hypothalamic neurons in culture [54]. Our data show that testosterone signaling does not seem to alter KCC2 mRNA or protein levels, whereas it significantly limits KCC2 localisation at the membrane in males and masculinized females. Membrane KCC2 levels correlate with the development of inhibitory neurotransmission [55]; on the other hand, cytoplasmic KCC2 does not seem to affect the reversal potential, E_{GABA} [56]. Therefore, our results suggest that KCC2 transporter activity may play a larger role in influencing E_{GABA} in the female than in the male hippocampus during the first postnatal week. Functional evaluation of E_{GABA} in the different experimental groups would further support this hypothesis. Altogether, the changes we observed in KCC2 localisation show an organizational effect

of gonadal steroids. These effects might be brain region specific, since for example the GABA shift in Purkinje cells in the cerebellum is delayed in females compared to males [57]

One of the most powerful modulators of KCC2 activity is BDNF [38,58,59]. Here, we report that overall BDNF expression levels are significantly higher in females compared to males during the first postnatal week. Consistent with our data, quantification of BDNF concentrations showed higher levels of BDNF in the hippocampus, ventromedial hypothalamus, and cortex in female compared to male rats [60–63]. Lower BDNF levels may play a role in reduced KCC2 plasmalemmal localization in males because BDNF has been found to enhance KCC2 membrane confinement [36,59,64]. Further experiments are needed to determine whether different BDNF levels underlie the effects of perinatal testosterone signaling on KCC2 membrane localisation. Nevertheless, these data indicate that when investigating the effects of BDNF *in vivo*, the sex of the animal models employed is a crucial variable to consider.

Gonadal hormones have been shown to influence synaptic transmission via genetic processes as well as fast changes in cell-to-cell communication [65–69]. In gonadotropin-releasing hormone neurons, estrogens control GABA release and cause bursts in $GABA_AR$-dependent inhibitory postsynaptic currents [70]. Through its impact on $GABA_AR$, another ovarian hormone, progesterone, and its metabolite allopregnanolone, also regulate inhibitory neurotransmission [71,72]. Here, we report that CA1 pyramidal neurons from females and testosterone-insensitive males show higher sIPSC frequency at the end of adolescence, which indicates an increase of spontaneous basal inhibition. This could be due to increased GABAergic circuit excitability or/and increased drive of GABAergic circuits synapsing onto CA1 pyramidal cells. All together, these data suggest that organizational perinatal testosterone leads to long lasting effects on spontaneous GABAergic activity in post-adolescent brain. Higher sIPSC frequency may lead to an overall decrease, or enhanced stability, of neuronal excitability, which could in turn contribute to a more resilient state of female compared to male brains against pathological states, such as epilepsy [73,74]. Further studies will be critical to understand how gonadal hormones impact different aspects of neuronal development, thus contributing to sex bias in specific developmental and pathological conditions.

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2.5. METHODS

2.5.1. Experimental models and prenatal procedures

Sprague-Dawley wild-type (wt) rats and Sprague-Dawley rats carrying the *Tfm* allele were bred with commercially purchased Sprague-Dawley (Charles River, St. Constant, QC, Canada) and were group housed in our colony at Sainte-Justine Hospital Research Centre within a 12-hour light/dark cycle with free ad libitum access to food and water. Animal care, use and procedures were conducted in accordance with the Canadian Council on Animal Care regulations and conformed to the guidelines of protocols #617 and #696, which were approved by Comité Institutionnel de Bonnes Pratiques Animales en Recherche (CIBPAR) at Sainte-Justine Hospital Research Centre and Université de Montréal (Montreal, Quebec, Canada). This study also complies with the ARRIVE guidelines.

The testicular feminization mutation (Tfm) carriers were provided by Dr. Cindy Jordan (Michigan State University, MI, USA). Tfm is a naturally occurring point mutation of the gene that codes for the androgen receptor (AR), rendering the Tfm male rat insensitive to physiological levels of androgens [75]. Genotype was determined by extracting

DNA from ear punches followed by PCR to detect Tfm versus wild type (WT) alleles for androgen receptor (AR), and the presence or absence of the Sry gene, which is located on the Y chromosome [76] (Figure 2B). For this study, we used WT males, WT females that were not carrying the Tfm allele, and Tfm males. The primer sequences for genotyping are listed in Table 1.

Pregnant dam body weight was measured from gestational day (GD) 1 (day of plug) to 14 to explore the rate of daily body weight gain during pregnancy. At GD14, dams were randomly assigned to one of the following treatments:

- Vehicle: dams were injected s.c. with vehicle solution (sesame oil: 0.1 ml/rat) from GD14 to 19.
- Testosterone: dams were injected s.c. with a 1mg/0.1ml of testosterone propionate solution (Testosterone propionate, MP Biomedicals, cat: 218655, lot: 40272, dissolved in Sesame oil) from GD14 to 19. Testosterone regimen was based on Wolf et al. [32]. All injections were performed at 1 PM during the light phase of the light/dark cycle. On the day of parturition, anogenital distance (AGD) was recorded. Rats were weaned at P21. Male and female offspring were housed in separate cages, with no more than five pups per cage, with standard rat chow and water ad libitum.

2.5.2. Sexual developmental markers

Sexual developmental markers were measured as described in Pallarés et al. [77]. AGD was measured using a vernier-caliper at P1. On P15, pups were re-examined for sexual phenotype, their sex confirmed or reassigned if necessary, and males and females were checked for areolas in a blind fashion. Areolas were deemed as either faint or normal, i.e. prominent and easily identified. On P35, female offspring were checked for vaginal opening (VO) and male offspring were monitored for testicular descent as indicators of puberty. Tfm males were dissected at P40 to confirm the presence of testis internally.

2.5.3. Hormone assays

Trunk blood was collected at birth, centrifuged to separate the plasma, and analyzed for testosterone and estradiol levels. Testosterone and estradiol concentrations were measured using AlphaLISA kit (Perkin Elmer, cat: AL324, lot: 2477098) and ELISA kit (Abcam, cat: ab108667, lot: GR3214106-3), respectively, following the company's protocol.

2.5.4. RT-qPCR

Total RNA was extracted using the "Aurum Total RNA fatty and fibrous tissue" kit and following the steps outlined in "Section 8: Spin protocol" of the instruction manual (732-6830, Bio-rad). The quality of extracted total RNA was assessed using a Bioanalyzer (CHUSJ, Montreal, QC, Canada) and conformed with high purity and integrity standards to perform RT-qPCR. The reverse transcription of RNA into cDNA and the following qPCR were performed in collaboration with IRIC (Institute for Research in Immunology and Cancer, Montreal, QC, Canada). Three endogenous controls (ACTB, HPRT, GAPDH) were tested and GAPDH being the more stably expressed across samples was chosen for the final analysis as the reference gene. Details of RT-qPCR primer sequences are listed in Table 1.

2.5.5. Western blot

Whole lysate proteins and membrane protein fractions were extracted from hippocampal tissue at P7 and P40 following previously described protocols [59,78]. In particular, to obtain membrane protein fractions, samples were homogenized in 5 vol of HB (300 mM sucrose/10 mM Tris–HCl, pH 7.5/1 mM EDTA/protease inhibitor mixture) and centrifuged at 1000 × g for 10 min at 4 °C. The pellet was washed in 0.5 vol HB and used as the nuclear fraction. Supernatants were centrifuged at 17 000 × g for 15 min at 4 °C, yielding the mitochondria fraction. The supernatant was further separated by ultracentrifugation at 100 000 × g for 1 h. The pellet and supernatant were used as the membrane and cytosol fractions, respectively. Membranes were probed with the following

primary antibodies: anti-KCC2 1:1000 (rabbit polyclonal IgG; Cat. no. 07-432, Millipore), 1:200 anti-BDNF (BDNF, mouse monoclonal IgG, Cat. no. 327100, Icosagen), anti-glyceraldehyde-3-phosphate dehydrogenase 1:4000 (GAPDH, mouse monoclonal IgG; Cat. no. AM4300; Applied Biosystems) and anti-β dystroglycan 1:3000 (rabbit polyclonal IgG, Cat no. ab43125, Abcam). All samples were run simultaneously. Bands were quantified using ImageJ v.1.52 software (National Institutes of Health, USA, http://imagej.nih.gov/ij). The intensity of KCC2 and BDNF bands were normalized over the intensity of the GAPDH band for whole lysates or of the β dystroglycan band for membrane fractions, in the same lane (internal loading control). We did not quantify KCC2 dimer band because it was not detectable in all samples. Full gels of western blot with molecular weight markers are shown in Figure S1 and Figure S2. During all experimental steps, the experimenter was blinded to the treatment groups.

2.5.6. Hippocampal slice preparation and *in vitro* electrophysiology

Brain slices were prepared as previously described by Sanon et al. [79]. Briefly, WT rats aged 38-42 days old were anaesthetized with isoflurane (Baxter corp, Mississauga, ON, Canada) and rapidly decapitated. The brain was extracted and immersed in cold (4°C) and oxygenated (95% O_2, 5% CO_2) high sucrose cutting artificial cerebro-spinal fluid (ACSF) containing (in mM): 250 sucrose, 2 KCl, 0.5 $CaCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 7 $MgSO_4 \cdot 7H_2O$, 10 D-glucose. Transverse cortical slices (300µm) were cut using a vibratome (VT1000S, Leica Microsystems Inc., Buffalo, NY, USA) and placed in oxygenated ACSF containing (in mM): 126 NaCl, 3 KCl, 2 $CaCl_2$, 25 $NaHCO_3$, 1 NaH_2PO_4, 2 $MgSO_4 \cdot 7H_2O$, 10 D-glucose (pH 7.3-7.4; 300-310mOsm) at room temperature. Slices with coordinates -3.08 to -4.36mm posterior to bregma from both hemispheres were allowed to recover a minimum of one hour before individual slices were transferred in a submerged recording chamber with ACSF flowing at a rate of 2ml/min.

Whole-cell recordings were made using 1mm (outer diameter) borosilicate patch pipettes (A-M Systems, Carlsborg, WA, USA) yielding a series resistance of 4-7MΩ when filled with an intracellular solution containing (in mM) 140 CsCl, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.5 EGTA, 10 phosphocreatine, 2 ATP-tris, 0.4 GTP-Li (pH 7.2-7.3 adjusted with

CsOH, 290mOsm). Signals were acquired using an Axopatch 200B amplifier (low-pass filtering at 1KHz). Data acquisition, at a sampling rate of 5KHz was performed using a Digidata 1440A analog-digital converter (Molecular Devices, Sunnyvale, CA, USA) and pClamp10 software (Molecular Devices, Sunnyvale, CA, USA). We recorded from visually identified CA1 pyramidal cells of the hippocampus using an upright microscope (BX50WI, Olympus Canada, Markham, ON, Canada) equipped with differential interference contrast and infrared (DIC-IR) CCD video camera (KP M1U, Hitachi Denshi Ltd, Japan). To investigate the inhibitory activity onto hippocampal pyramidal cells, we recorded spontaneous inhibitory postsynaptic currents (sIPSC) in voltage-clamp mode at a membrane potential of -60mV and in presence of 6,7-dinitroquinoxaline-2,3-dione (DNQX, 40μM), 2-amino-5-phosphonopentanoic acid (D-AP5, 50μM), and routinely blocked these currents with bicuculline (BIC, 2μM). Miniature inhibitory post-synaptic currents (mIPSCs) were measured in the presence of 0.5 μM TTX. Intrinsic properties, such as resting membrane potential, input resistance and capacitance were also analyzed. During all electrophysiological recordings, the experimenter was blinded to the treatment groups

2.5.7. Statistical analysis

Statistical analysis and generation of figures was performed with RStudio (RStudio Inc., version 1.2.1335). Data was log-transformed to fit a normal distribution and assessed via one-way ANOVA for multiple comparisons between groups. Post-hoc Tukey's test was performed to identify statistically significant differences when significant main effects were detected. The accepted threshold of significance for all statistical tests was set at a two-tailed p-value <0.05. In particular, significance was considered as p-value <0.05 and fold change >2 or <0.5 for all comparisons of qPCR data. All data are presented as mean ± standard error of the mean (SEM).

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2.7. Figures and Legends

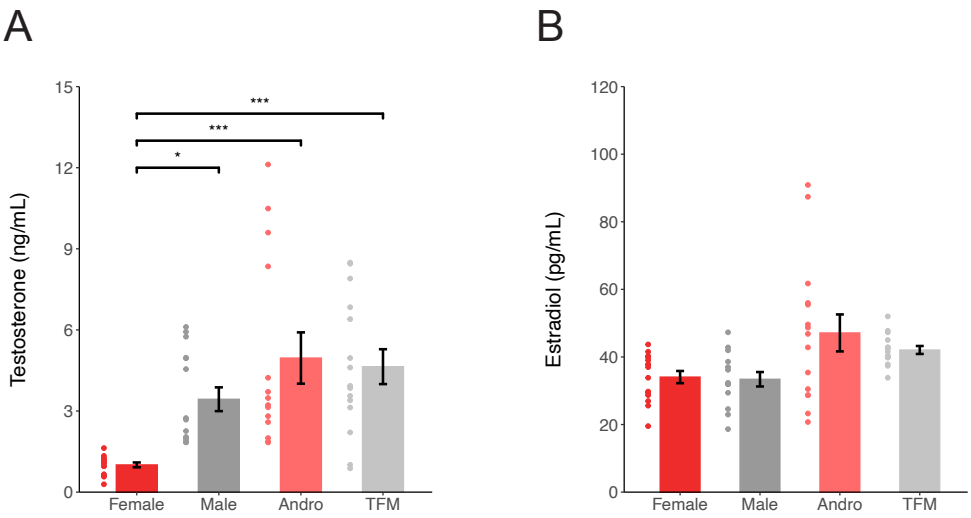


Figure 1. Testosterone is significantly lower in females whereas estradiol levels are similar between sex groups. A. Bar graph shows higher testosterone levels in all sexes, except for female rat pups (F: 1.01 ± 0.09 ng/ml, n = 15 rats from 3 litters; M: 3.44 ± 0.44 ng/ml, n = 15 rats from 3 litters; A: 4.96 ± 0.95 ng/ml, n = 14 rats from 4 litters; TFM: 4.64 ± 0.65 ng/ml, n = 15 rats from 5 litters; One-way ANOVA with Tukey's post hoc analysis, F x M: p=0.028; F x A: p<0.001; F x TFM: p<0.001). **B.** Bar graph shows no significant differences in levels of estradiol between all groups (F: 34.0 ± 1.81 pg/ml, n = 15 rats from 3 litters; M: 33.4 ± 2.14 pg/ml, n = 15 rats from 3 litters; A: 47.1 ± 5.47 pg/ml, n = 14 rats from 4 litters; TFM: 42.1 ± 1.18 pg/ml, n = 15 rats from 5 litters; One-way ANOVA with Tukey's post hoc analysis, F x M: p=0.995; F x A: p=0.080; F x TFM: p=0.140). Dots represent individual data points. M: males; F: females; A: andro/testosterone-treated females; TFM: testosterone-insensitive males. Graphs represent mean ± SEM.

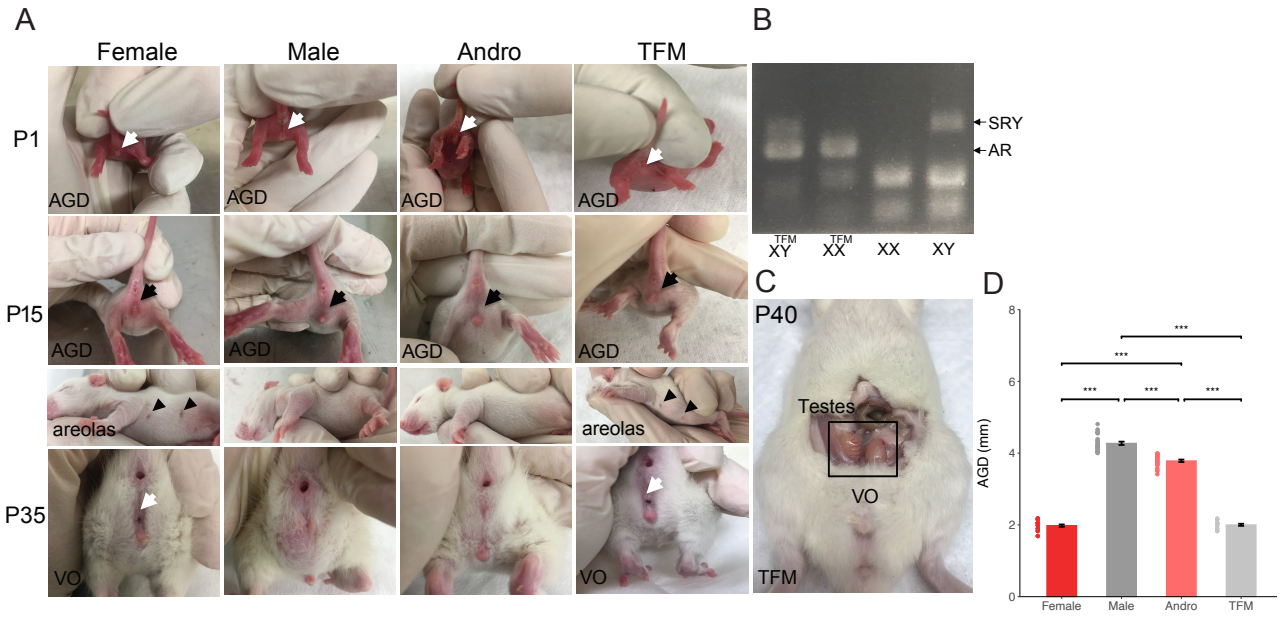


Figure 2. Testosterone levels affect sexual developmental markers. A. Photographs show representative anogenital distances AGD (white arrows) observed in male, female, testosterone-treated females, and testosterone-insensitive male rats at P1, 15 and 35. Presence or absence of areolas are represented by black arrowheads. Vaginal opening (VO) is observed (white arrows) at P35. **B**. PCR confirming the genotype of each animal (Tfm vs. wt alleles for androgen receptor (AR). **C**. Presence of testes in testosterone-insensitive males despite the feminine external phenotype. **D.** Bar graph shows significant differences in AGD length between sex groups (F: 1.98 ± 0.03 mm, n = 24 rats from 5 litters; M: 4.27 ± 0.05 mm, n = 25 rats from 5 litters; A: 3.79 ± 0.04 mm, n = 23 rats from 7 litters; TFM: 2.00 ± 0.03 mm, n = 17 rats from 6 litters; One-way ANOVA with Tukey's post hoc analysis, F x M, F x A TFM x M, TFM x A: $p<0.0001$). Dots represent individual data points. F: females; M: males; A: andro/testosterone-treated females; TFM: testosterone-insensitive males. Graphs represent mean ± SEM.

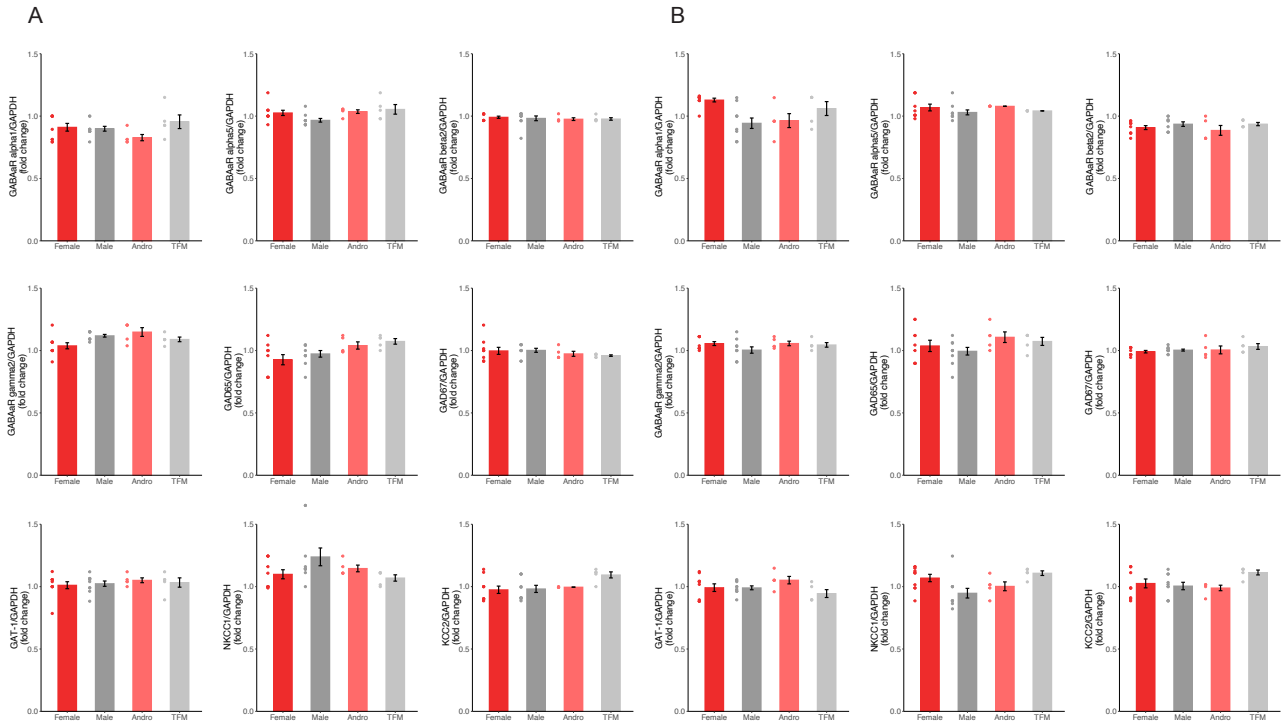


Figure 3. Testosterone does not affect mRNA levels of major GABAergic neurotransmission determinants. **A, B**. Fold-change values in mRNA expression of GABA signaling components extracted from rat hippocampus at P7 (A) and P40 (B) do not show significant differences between groups (F: n = 10 rats from 3 litters; M: n = 10 rats from 3 litters; A: n = 5 rats from 3 litters; TFM: n = 5 rats from 3 litters; One-way ANOVA with Tukey's post hoc analysis, $p>0.05$, fold change >2 or <0.5 for all comparisons at P7; One-way ANOVA with Tukey's post hoc analysis, $p>0.05$, fold change >2 or <0.5 for all comparisons at P40). Dots represent individual data points. F: females; M: males; A: andro/testosterone-treated females; TFM: testosterone-insensitive males. Graphs represent mean ± SEM.

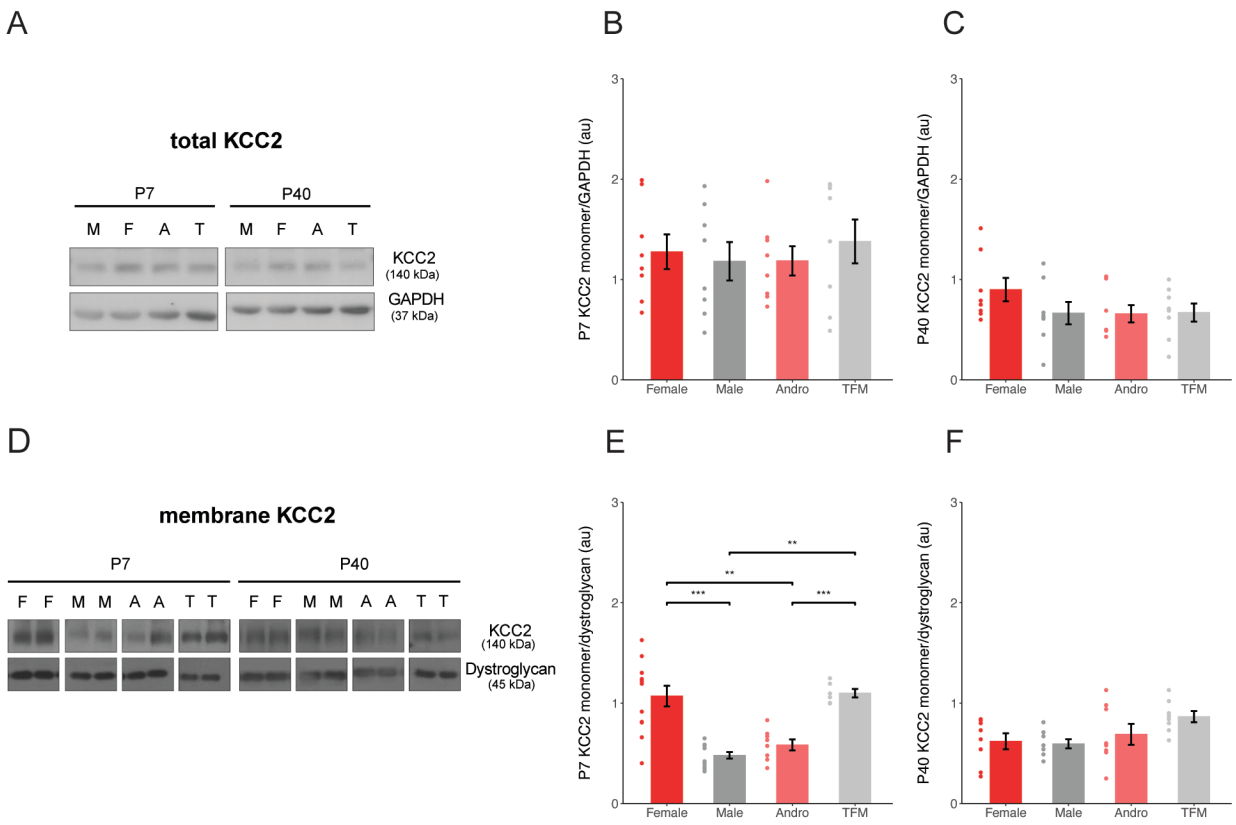


Figure 4. Perinatal testosterone limits KCC2 localisation at the membrane. **A.** Western blot membrane of total KCC2 (images were cropped from the same gel). **B, C.** Quantification of total KCC2 expression levels (monomer band at 140 kDa) in the hippocampus of different experimental groups do not show significant differences at P7 (B) (F: 1.28 ± 0.17 au, n = 8 rats from 3 litters; M: 1.18 ± 0.19 au, n = 8 rats from 3 litters; A: 1.19 ± 0.15 au, n = 8 rats from 3 litters; TFM: 1.38 ± 0.22 au, n = 8 rats from 3 litters; One-way ANOVA with Tukey's post hoc analysis, p=0.926) or at P40 (C) (F: 0.90 ± 0.12 au, n = 8 rats from 3 litters; M: 0.67 ± 0.11 au, n = 8 rats from 3 litters; A: 0.66 ± 0.09 au, n = 8 rats from 3 litters; TFM: 0.67 ± 0.09 au, n = 8 rats from 3 litters; One-way ANOVA with Tukey's post hoc analysis, , p=0.355). **D.** Western blot membrane of KCC2 in membrane fractions (images were cropped from different gels). **E, F.** Quantification of KCC2 monomer expression levels in the membrane fractions show sex differences in the hippocampus at P7 (E) (F: 1.07 ± 0.10 au, n = 12 rats from 4 litters; M: 0.48 ± 0.03 au, n = 12 rats from 5 litters; A: 0.58 ± 0.05 au, n = 8 rats from 3 litters; TFM: 1.10 ± 0.04 au, n

= 5 rats from 3 litters; one-way ANOVA with Tukey's post hoc analysis, F x M: $p<0.001$; F x A: $p=0.001$, TFM x M: $p<0.001$; TFM x A: $p=0.001$). Conversely, no sex differences were found at P40 (F) (F: 0.62 ± 0.08 au, n = 8 rats from 3 litters; M: 0.60 ± 0.04 au, n = 8 rats from 4 litters, A: 0.69 ± 0.11 au, n = 8 rats from 3 litters; TFM: 0.87 ± 0.06 au, n = 8 rats from 3 litters; One-way ANOVA with Tukey's post hoc analysis, p=0.121). Each lane represents a different animal. Blot shows representative samples of different sex groups. Dots represent individual data points. F: females; M: males; A: andro/testosterone-treated females; T: TFM/testosterone-insensitive males. Graphs represent mean ± SEM.

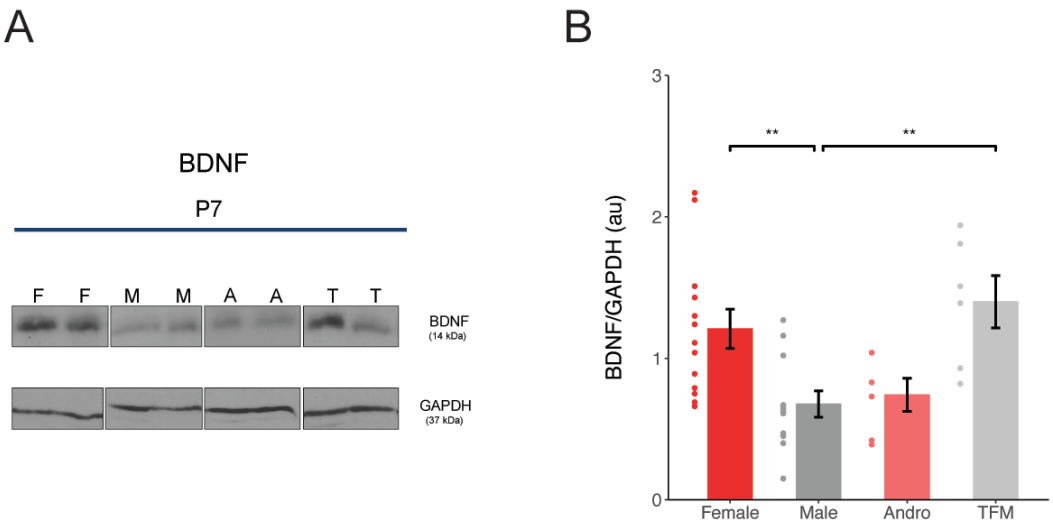


Figure 5. Perinatal testosterone negatively correlates with BDNF expression levels during the first postnatal week. A. Western blot analysis of mature BDNF (14 kDa) expression levels in the hippocampus of different experimental groups at P7. Each lane represents a different animal. Blot shows representative samples of different sex groups (images were cropped from different gels). **B.** Quantification revealed that the expression of mature BDNF is significantly lower in male when compared to female and testosterone-insensitive male rat pups (F: 1.21 ± 0.14 au, n = 13 rats from 4 litters; M: 0.68 ± 0.09 au, n = 10 rats from 4 litters; A: 0.74 ± 0.12 au, n = 6 rats from 3 litters; TFM: 1.4 ± 0.19 au, n = 6 rats from 3 litters; One-way ANOVA with Tukey's post hoc analysis, F x M: p=0.009; TFM x M: p=0.007). Dots represent individual data points. F: females; M: males; A: andro/testosterone-treated females; T: TFM/testosterone-insensitive males. Graphs represent mean ± SEM.

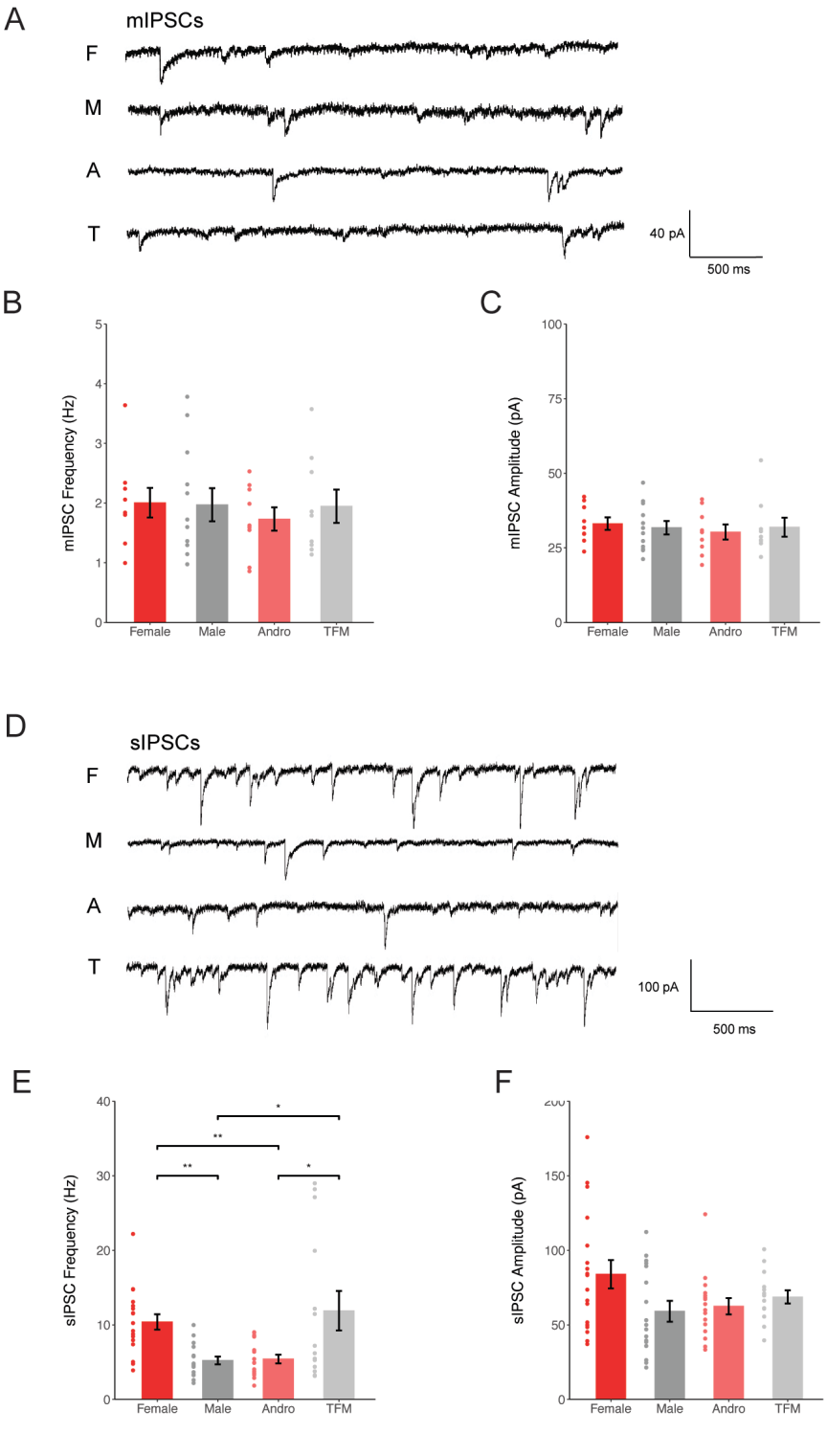
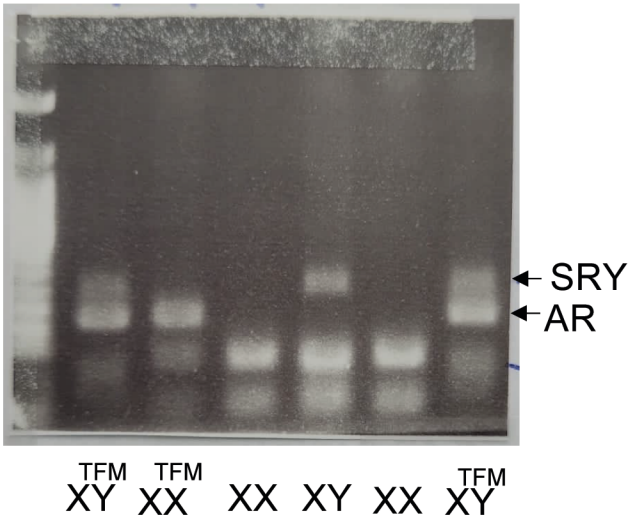

A
mIPSCs
F
M
A
T
40 pA
500 ms
B
mIPSC Frequency (Hz)
0
1
2
3
4
5
Female
Male
Andro
TFM
C
mIPSC Amplitude (pA)
0
25
50
75
100
Female
Male
Andro
TFM
D
sIPSCs
F
M
A
T
100 pA
500 ms
E
sIPSC Frequency (Hz)
0
10
20
30
40
*
**
**
*
Female
Male
Andro
TFM
F
sIPSC Amplitude (pA)
0
50
100
150
200
Female
Male
Andro
TFM

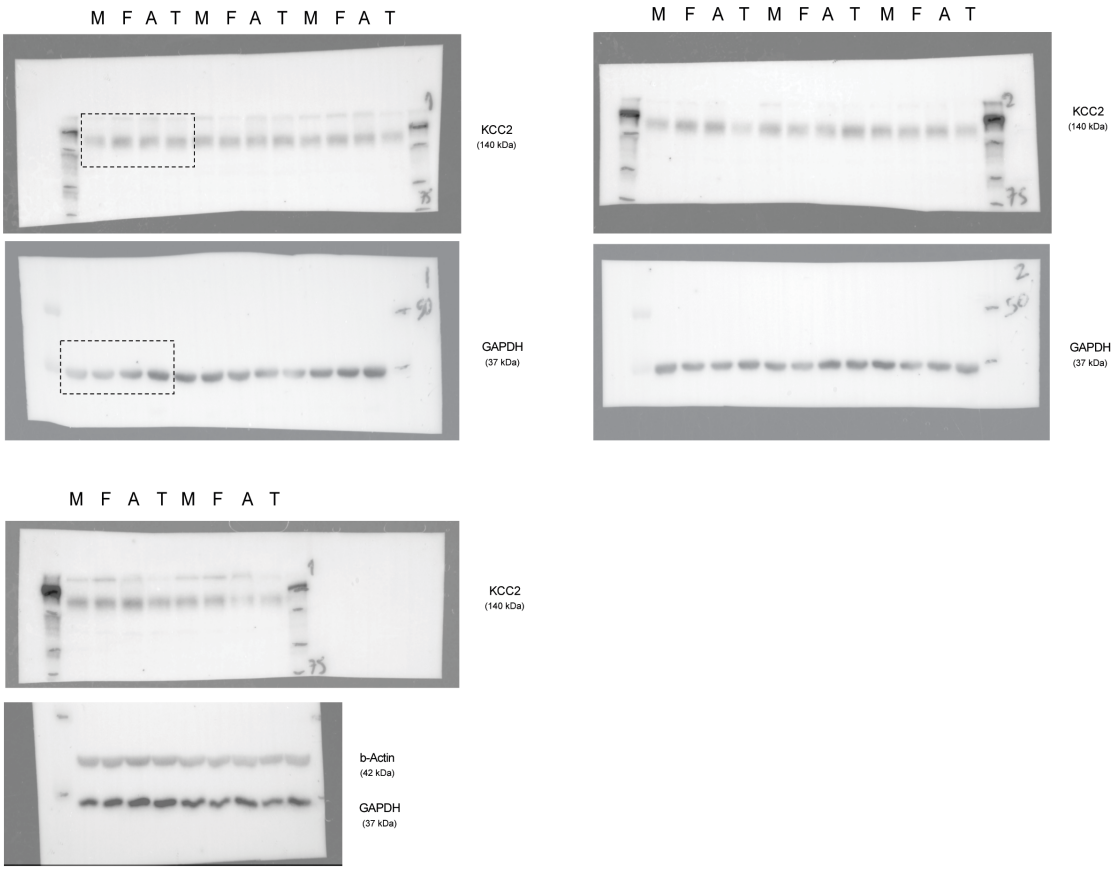
Figure 6. Testosterone signaling is associated with lower sIPSC frequency in young adult CA1 pyramidal neurons. A. Representative traces of mIPSCs from CA1 pyramidal neurons. **B.** mIPSC frequency (F: 2.01 ± 0.25 Hz, n = 9 cells, 4 rats from 3 litters; M: 1.97 ± 0.28 Hz, n = 12 cells, 5 rats from 3 litters; A: 1.73 ± 0.20 Hz, n = 9 cells, 4 rats from 3 litters; TFM: 1.95 ± 0.28 Hz, n = 9 cells , 4 rats from 3 litters; One-way ANOVA with Tukey's post hoc analysis, p=0.985) and **C.** amplitude (F: 33.10 ± 2.10 pA, n = 9 cells, 4 rats from 3 litters; M: 31.70 ± 2.27 pA, n = 12 cells, 5 rats from 3 litters; A: 30.28 ± 2.52 pA, n = 9 cells, 4 rats from 3 litters; TFM: 31.92 ± 3.20 pA, n = 9 cells,4 rats from 3 litters; One-way ANOVA with Tukey's post hoc analysis, p=0.849) did not differ in hippocampal CA1 pyramidal neurons between the sex groups. **D.** Representative traces of sIPSCs from CA1 pyramidal neurons. **E**. Sex differences were found in the frequency of sIPSC (F: 10.40 ± 1.03 Hz, n = 18 cells, 7 rats from 4 litters; M: 5.23 ± 0.52 Hz, n = 17 cells, 6 rats from 3 litters; A: 5.42 ± 0.58 Hz, n = 16 cells, 6 rats from 3 litters; TFM: 11.90 ± 2.64 Hz, n = 14 cells, 5 rats from 3 litters; One-way ANOVA with Tukey's post hoc analysis, F x M: p=0.002; TFM x M: p=0.021; F x A: p=0.004; TFM x A: p=0.030). **F.** Conversely, no sex differences were found in the amplitude of sIPSC (F: 83.98 ± 9.48 pA, n = 18 cells, 7 rats from 4 litters; M: 59.13 ± 7.00 pA, n = 17 cells, 6 rats from 3 litters; A: 62.50 ± 5.45 pA, n = 16 cells, 6 rats from 3 litters; TFM: 68.74 ± 4.43, n = 14 cells, 5 rats from 3 litters; One-way ANOVA with Tukey's post hoc analysis, p=0.067) in the four experimental groups. Dots represent individual data points. F: females; M: males; A: andro/testosterone-treated females; TFM: testosterone-insensitive males. Graphs represent mean ± SEM.

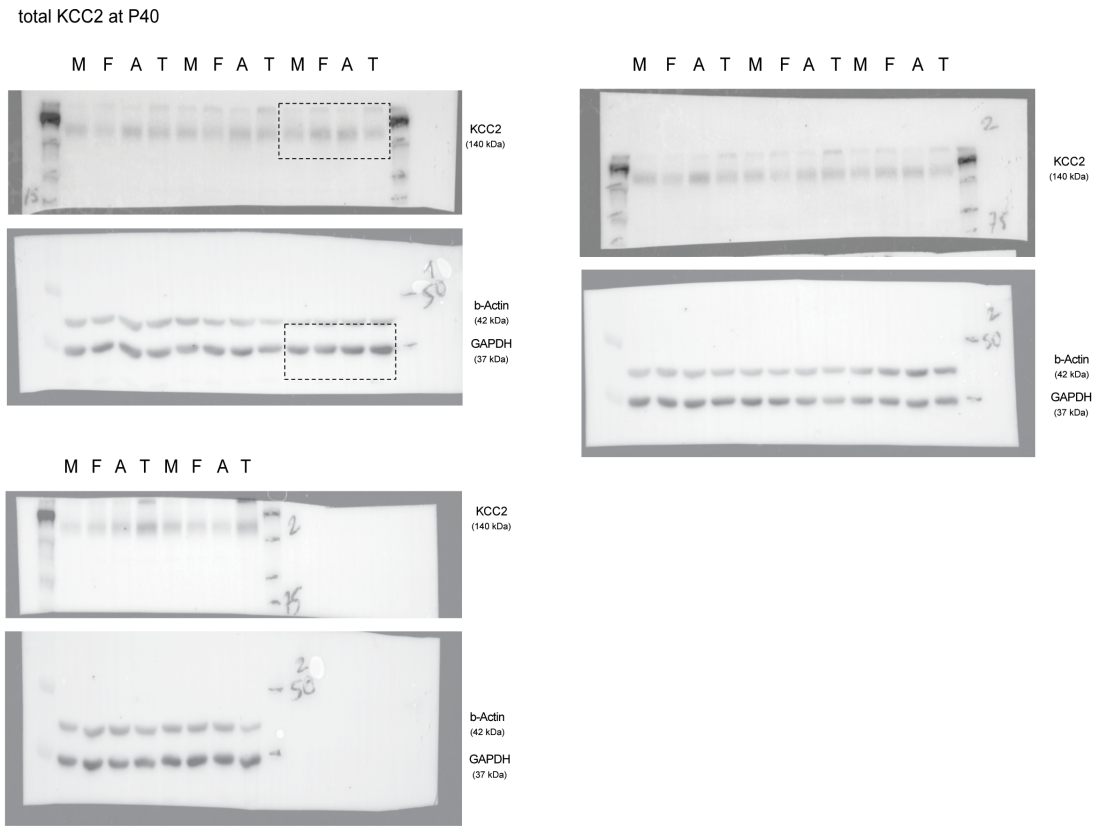
2.8. SUPPLEMENTARY FIGURES



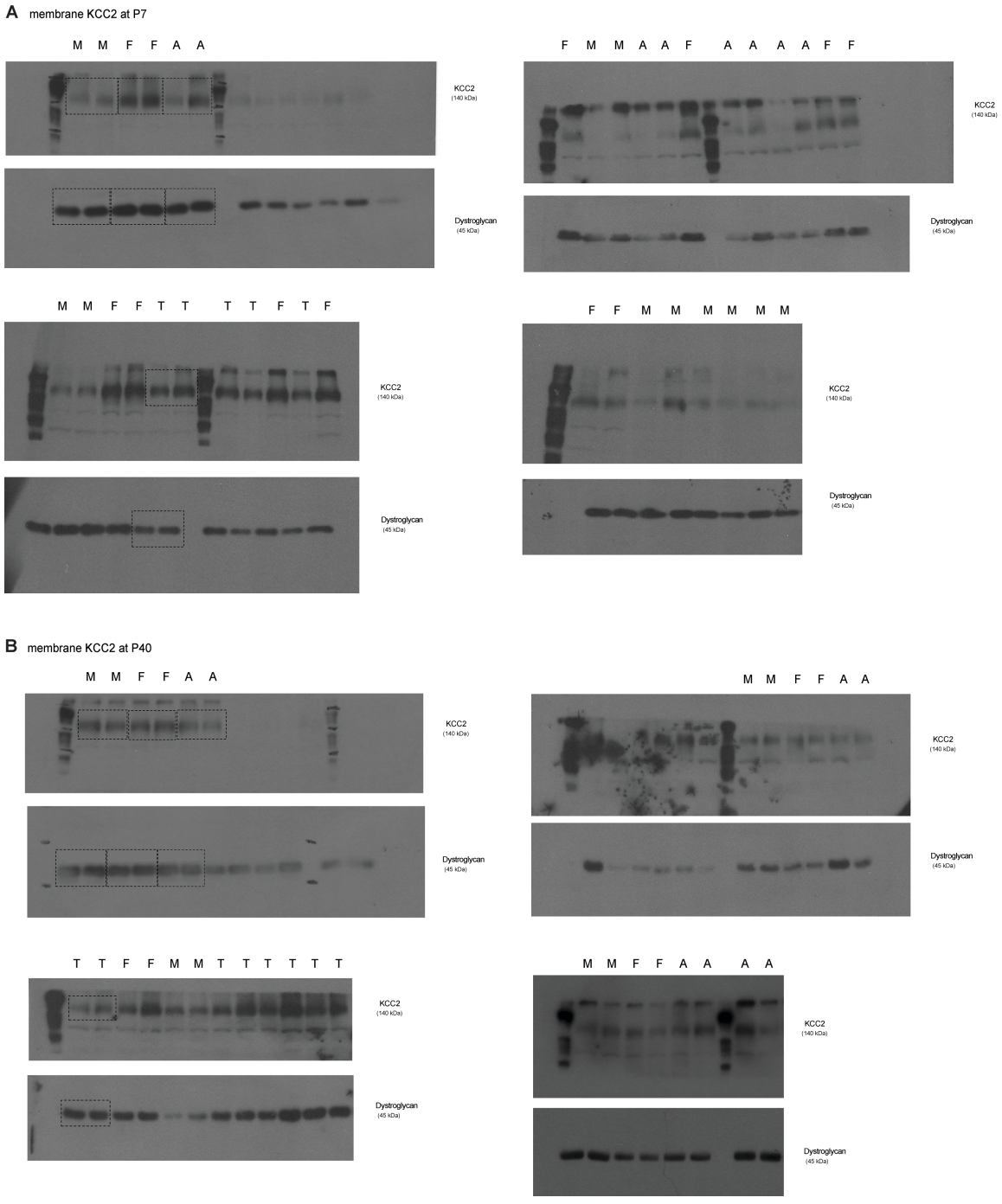
Supplementary Figure 1. Original full-length image of polyacrylamide gel with the amplification and digestions results of the AR and SRY genes in WT females, WT males, Tfm males and Tfm female carriers showed in Figure 2B of the manuscript.

A total KCC2 at P7



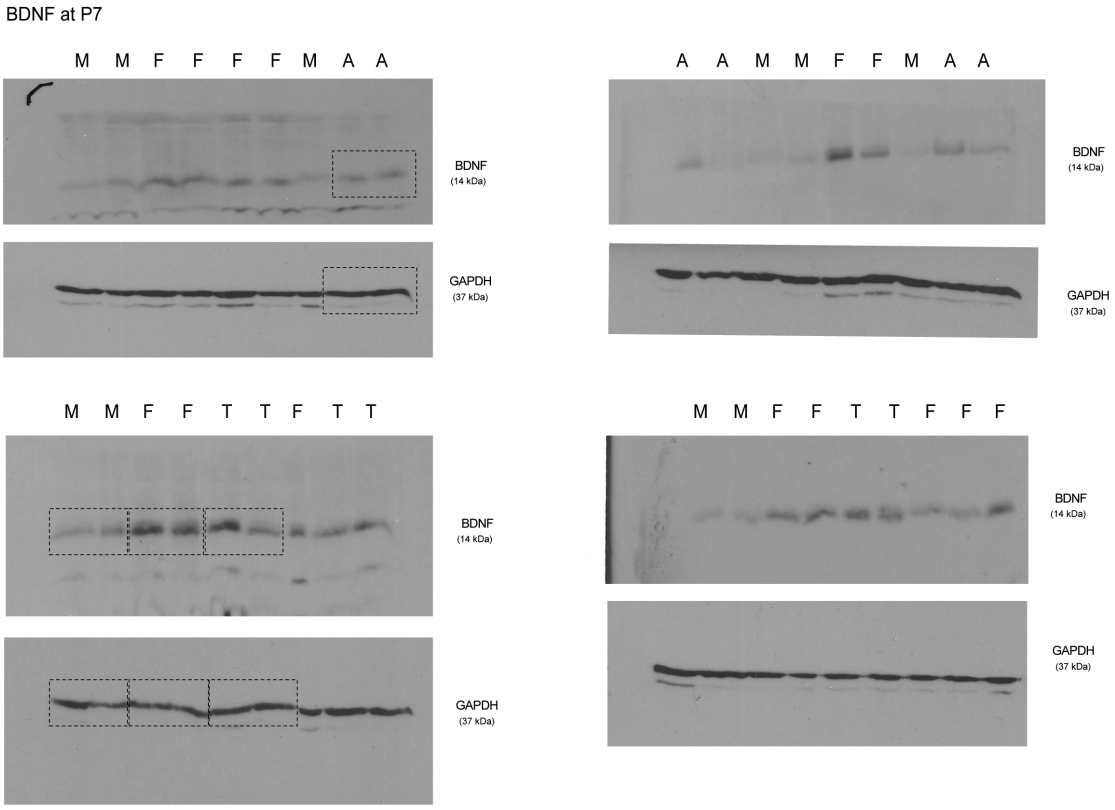


Supplementary Figure 2. A, B. Uncropped full-length pictures of western blot membranes used for the quantification of total KCC2 expression at P7 (A) and P40 (B). Dotted areas indicate lanes shown in Figure 4A. Blots were developed by chemiluminescence. Membranes were cut to enable blotting for different antibodies. Note that we did not quantify KCC2 dimer band because it was not reliably detectable in all samples. F: females; M: males; A: andro/testosterone-treated females; T: TFM/testosterone-insensitive males.



Supplementary Figure 3. A, B. Original full-length pictures of western blot membranes used for the quantification of membrane KCC2 expression at P7 (A) and P40 (B). Dotted areas indicate lanes shown in Figure 4D. Blots were developed using X-rays films.

Membranes were cut to enable blotting for different antibodies. Note that we did not quantify KCC2 dimer band because it was not reliably detectable in all samples. F: females; M: males; A: andro/testosterone-treated females; T: TFM/testosterone-insensitive males.



Supplementary Figure 4. Original full-length pictures of western blot membranes of BDNF expression at P7. Dotted areas indicate lanes shown in Figure 5A. Blots were developed by X-rays films. Membranes were cut to enable blotting for different antibodies. F: females; M: males; A: andro/testosterone-treated females; T: TFM/testosterone-insensitive males.

2.9. TABLE

Table 1. Sequence of primers used in the RT-qPCR and genotyping.

Target Genes: RT-qPCR	Primers
α1	**F:**5'-CGATCCTCTCTCCCACACTT-3' **R:** 5'-TCTTCATCACGGGCTTGTC-3'
α5	**F:**5'-CACCCAACAAGCTGCTGA-3' **R:** 5'-GACACTCAGCAGAGATCGTCA-3'
β2	**F:**5'-CTGGGTCTCCTTTTGGATCA-3' **R:** 5'-TCATCGTCAGGACAGTTGTAATTC-3'
γ2	**F:**5'-TGCTCACTGGATCCGACTC-3' **R:** 5'-GTAATTGCAACTGGCACTCG-3'
GAD65	**F:**5'-AGGCTCTGGCGATGGAAT-3' **R:** 5'-TTATAGCGGGCAATGAGCA-3'
GAD67	**F:**5'-GCGAGAGATCATTGGATGGT-3' **R:** 5'-GCCATGATGCTGTACATGTTG-3'
GAT-1	**F:**5'-GATTGGCCTCTCTAACATCACC-3' **R:** 5'-CAAAGAAAACCAGGAACAGCA-3'
KCC2	**F:**5'-GGAGTCCTTCTGCATGGTCT-3' **R:** 5'-ATGGAAATGGCTGTGAGCAT-3'
NKCC1	**F:**5'-CAGCAATAGCTACCAATGGATTC-3' **R:** 5'-TGGCCCTAGACTTCTAGAGATTAAA-3'
Reference genes	**Primers**
Actb	**F:**5'-CCCGCGAGTACAACCTTCT-3' **R:** 5'-CGTCATCCATGGCGAACT-3'
Gapdh	**F:**5'-CCCTCAAGATTGTCAGCAATG-3' **R:** 5'-AGTTGTCATGGATGACCTTGG-3'
Hprt	**F:**5'-GACCGGTTCTGTCATGTCG-3' **R:** 5'-ACCTGGTTCATCATCACTAATCAC-3'
Target Genes: Genotype	**Primers**
AR	**F:**5'-GGCTGTGTGAGGGCCAAATT-3' **R:** 5'-GGACCAAAGGCTGATCACAAG-3'
SRY	**F:**5'-GGGAGGAGGGATGAATAT-3' **R:** 5'-CATTGCAGCAGGTTGTACAGT-3'

– CHAPTER III –

SECOND PAPER

Sex differences in the developing brain impact stress-induced epileptogenicity following hyperthermia-induced seizures

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Author's contributions: DCW, SD, NTS, designed the study and wrote the manuscript. DCW, SD, NTS, CMB, PA, AS, SD, MTS, MS, GAB performed experiments. DCW, SD, JSC analysed data. DCW, JSC, LME, ECL, AGW reviewed manuscript.

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3.1. ABSTRACT

Febrile seizures (FS) are common, affecting 2-5% of children between the ages of 3 months and 6 years. Complex FS occur in 10% of patients with FS and are strongly associated with mesial temporal lobe epilepsy. Current research suggests that predisposing factors, such as genetic and anatomic abnormalities, may be necessary for complex FS to translate to mesial temporal lobe epilepsy. Sex hormones are known to influence seizure susceptibility and epileptogenesis, but whether sex-specific effects of early life stress play a role in epileptogenesis is unclear. Here, we investigate sex differences in the activity of the hypothalamic–pituitary–adrenal (HPA) axis following chronic stress and the underlying contributions of gonadal hormones to the susceptibility of hyperthermia-induced seizures (HS) in rat pups. Chronic stress consisted of daily injections of 40mg/kg of corticosterone (CORT) subcutaneously from postnatal day (P) 1 to P9 in male and female rat pups followed by HS at P10. Body mass, plasma CORT levels, temperature threshold to HS, seizure characteristics, and electroencephalographic *in vivo* recordings were compared between CORT- and vehicle (VEH)-injected littermates during and after HS at P10. In juvenile rats (P18-P20), *in vitro* CA1 pyramidal cell recordings were recorded in males to investigate excitatory and inhibitory neuronal circuits. Results show that daily CORT injections increased basal plasma CORT levels before HS and significantly reduced weight gain and body temperature threshold of HS in both males and females. CORT also significantly lowered the generalized convulsions (GC) latency while increasing recovery time and the number of electrographic seizures (>10sec), which had longer duration. Furthermore, sex-specific differences were found in response to chronic CORT injections. Compared to females, male pups had increased basal plasma CORT levels after HS, longer recovery time and a higher number of electrographic seizures (>10sec), which also had longer duration. Sex-specific differences were also found at baseline conditions with lower latency to generalized convulsions and longer duration of electrographic seizures in males but not in females. In juvenile male rats, the amplitude of evoked excitatory postsynaptic potentials, as well as the amplitude of inhibitory postsynaptic currents, were significantly greater in CORT rats when

compared to VEH littermates. These findings not only validate CORT injections as a stress model, but also show a sex difference in baseline conditions as well as a response to chronic CORT and an impact on seizure susceptibility, supporting a potential link between sustained early-life stress and complex FS. Overall, these effects also indicate a putatively less severe phenotype in female than male pups. Ultimately, studies investigating the biological underpinnings of sex differences as a determining factor in mental and neurologic problems are necessary to develop better diagnostic, preventative, and therapeutic approaches for all patients regardless of their sex.

Keywords: sex differences, corticosterone, chronic stress, febrile seizures, SHRP, HPA

3.2. INTRODUCTION

Epilepsy has a prevalence of 1% and is the most common neurological disorder worldwide (Asadi-Pooya et al., 2017). Mesial temporal lobe epilepsy (mTLE), the most widespread form of drug-resistant epilepsy, results in neuro-cognitive impairment and reduced quality of life (Berg, 2008; Engel J., 2001). While mTLE is a heterogeneous disorder that often becomes symptomatic in the second decade of life or later, a unified theory of its pathophysiology resides on limbic epileptogenesis occurring through a multi-stage process that begins early in life from an external insult combined with intrinsic susceptibility (Scharfman, 2007; Spagnoli et al., 2015). While multiple external insults in early life have been identified, such as perinatal anoxia, birth trauma, and infection, by far the most prevalent is the occurrence of FS during childhood (Sanon et al., 2012; Scharfman, 2007). FS are induced by fever and are the most common neurological disorder observed during childhood ("Guidelines for Epidemiologic Studies on Epilepsy: Commission on Epidemiology and Prognosis, International League Against Epilepsy," 1993). The overall estimated prevalence of FS is between 2 to 5% in North America, occurring primarily in children aged 3 months to 6 years (Berg and Shinnar, 1996; Hauser, 1994). FS are divided into simple or complex FS. Simple FS are brief isolated seizures, generalized at onset, with rapid recovery; further, children affected by simple FS usually do not suffer further neurologic issues beyond the initial seizure, and typically show normal brain development (Berg and Shinnar, 1996). Comparatively, complex FS are prolonged (>15 min), can have multiple episodes within 24h, are lateralized and are associated with post-ictal neurological deficits. Complex FS are a major risk factor for the subsequent development of mTLE, which develops in 30-50% of patients with a history of FS (Hamati-Haddad and Abou-Khalil, 1998; Nelson and Ellenberg, 1976). However, not all patients with complex FS develop mTLE suggesting that other variables likely play a role in leading to epileptogenesis and mTLE.

Environmental stressors, such as early life stress, are known to harbor persistent changes to the limbic system, including increased vulnerability to epileptogenesis (Huang et al., 2002; Jones et al., 2009; Kumar et al., 2011; Lai et al., 2006; Salzberg et al., 2007). Several animal models have shown that postnatal stress, through maternal separation or

exogenous corticosterone administration, promote limbic epileptogenesis, possibly through HPA axis hyper-reactivity in adulthood (Huang et al., 2002; Koe et al., 2014). The HPA axis is activated in response to real or perceived stressors and culminates in the production and secretion of glucocorticoids, such as CORT, by the adrenal glands (Munck et al., 1984). Stress can alter hippocampal function and structure, which may render an individual susceptible to FS, and possibly mTLE (Wood et al., 2004). Prenatal stress and exposure to FS studies exhibited a decrease in hippocampal mass, increase in cell death, as well as advanced stages of seizure resulting in a heightened seizure response (Qulu et al., 2015, 2012). Furthermore, postnatal stress studies have shown that chronically elevated CORT levels promote hippocampal hyperexcitability and structural changes, decrease seizure threshold, and increase interictal epileptiform activity in adult animals (Castro et al., 2012; Kumar et al., 2007).

While previous experimental studies have demonstrated that patient-related factors, such as cortical malformations, may partially explain certain individuals' predisposition to both complex FS and mTLE (Bocti et al., 2003), which is similarly observed in animal models (Scantlebury et al., 2005, 2004), sex hormones may also play a role (Awad et al., 2016; Desgent et al., 2012). Current studies indicate that sex differences in the developing brain are likely caused by changes in steroid exposure, and that these differences account for changes in neuronal excitability during early development (Kight and McCarthy, 2014). These variations include (among others) the timing of the shift from depolarizing to hyperpolarizing GABA (Galanopoulou and Moshé, 2003; Wolf et al., 2019), differences in neuroimmune activation (Schwarz et al., 2012; Wynne et al., 2011), higher susceptibility to ischemic neuronal death in males (Hill and Fitch, 2012) and differences in epigenetics (Kight and McCarthy, 2014). Sex differences are apparent in patients with FS, which have a male-to-female ratio of approximately 1.6 to 1 (Millar, 2006; Leung and Robson, 2007). Sex hormones have been shown as a predisposing factor to epileptogenesis in a two-hit mTLE model following HS; the experiment used a freeze lesion to mimic focal cortical dysplasia on the first day of birth. This lesion, combined with HS at P10, led to the development of mTLE in male pups and testosterone-treated females, but not in untreated females (Desgent et al., 2012; Seale et

al., 2005). Overall, these findings further support that these differences in sex hormones may influence seizure severity and outcome in patients with a history of complex FS.

An increasing body of evidence suggests that early-life stress responses may also influence the developing brain's predisposition to seizures and epilepsy, with possible sex-based discrepancies in adulthood (Brummelte and Galea, 2010; Desgent et al., 2012; Gallagher et al., 1984; Kumar et al., 2011; Salzberg et al., 2007). While it is well established that early life stress has long-term consequences, these studies focus mainly on sex differences during adulthood. To date, a relationship between chronic stress and mTLE has not been clearly established in the developing brain; further, despite the HPA axis exhibiting sex-biased activity (Goel et al., 2014; Heck and Handa, 2019), few preclinical studies have reported sex differences in HPA axis functionality during the stress hyporesponsive period (SHRP) (Hary et al., 1986; Shanks et al., 1994). Therefore, the current study seeks to further define the sex-dependent activity of the HPA axis during SHRP following chronic stress and using a HS experimental model.

3.3. MATERIAL AND METHODS

3.3.1. Animal subjects

Time-pregnant (non-primapara) Sprague-Dawley female rats were obtained from Charles River laboratories (St. Constant, QC, Canada) at gestational day 10. The pregnant dams were left to be accustomed to the animal facility environment for thirteen days (i.e., until parturition), with 12-hour light/dark cycle and *ad libitum* food and water availability. All animal-related procedures were conducted in accordance with the Canadian Council on Animal Care regulations and conformed to the guidelines of protocol #617, which was approved by the Comité Institutionnel de Bonnes Pratiques Animales en Recherche (CIBPAR) at Sainte-Justine Hospital Research Centre affiliated to Université de Montréal (Montreal, Quebec, Canada). All efforts were made to minimize the number of animals used and their suffering. The final data are derived from 188 Sprague-Dawley rat pups, 94 females and 94 males sampled from 15 litters. The animals were used for the

different endpoints described on the experimental design (Figure 1). However, 45 females and 32 males were used in a different set of long-term experiments that are not included in the current study.

3.3.2. Daily postnatal CORT injections

At P1, the newborn male and female rat pups were identified by sex and randomly assigned to one of two treatment groups: 1. VEH: vehicle-injected; 2. CORT: corticosterone-injected. Using a 50:50 ratio approach, the rat pups from each litter were separated in two groups in which both sexes were equally represented, controlling for litter effects. From P1 to P9, each rat pup received daily subcutaneous CORT (40 mg/kg, Sigma-Aldrich Canada, Oakville, Ontario) injections (10µl/g of body) suspended in saline containing 0.1% dimethyl sulfoxide (DMSO) or VEH with 0.1% DMSO in saline. The CORT solution was sonicated 30 seconds before each use to ensure an even suspension of the hormone micelles throughout the solution. The body mass of all pups was measured daily, and the dose adjusted accordingly. The injections were administered at the same time of the day, during the light phase of the light/dark cycle.

Despite endogenous stress hormones being greatly reduced and minimally responsive to increases in stressful stimuli during the SHRP, which occurs between P4 and P14 in rats (Sapolsky and Meaney, 1986; Walker and Scribner, 1991), the decision of injection during this period was made due to study goal of investigating the effects of high CORT levels of a pharmacological range (Claflin et al., 2017) on the well-established HS experimental model at P10 (Dubé et al., 2009; Scantlebury et al., 2005). Consequently, a high CORT dose was chosen in the attempt to investigate any possible sublet sex differences.

3.3.3. Hyperthermia-induced seizures (HS)

Pups at P10 (i.e., 24 hours after the last CORT injection) were exposed to HS as previously described (Scantlebury et al., 2005). To minimize the effects of circadian variation, all experiments started at 1PM, the dam was withdrawn, and the offspring were weighed. Then, each pup was individually placed in a Plexiglass box equipped with heated airflow to increase body external temperature to 44-46ºC (i.e., corresponding to 40-42ºC internally) (Sanon et al., 2017). Each pup was monitored until the onset of a GC characterized by loss of posture, lying on the side and tonic body flexion lasting 10 consecutive seconds. Seizure induction was coupled with real-time vEEG recordings for each pup rat. At this point, they were immediately removed from the box for further behavioral observations and vEEG monitoring of the pursuing generalized seizure. Between experimental sessions the box was cleaned, dried, and cooled down to maintain a starting temperature at around 23ºC.

3.3.4. vEEG recordings of HS at P10

To determine the prevalence of HS, animals were implanted at P7 with a stainless-steel bipolar electrode of 125 µm in diameter (Plastics-1 Inc., Roanoke, VA, USA) which was positioned into the right dorsal hippocampus in *cornus ammonis* region one (CA1), at the following coordinates with reference to bregma: AP = -3.0, ML = -2,4, DV = -2.0, under constant general anesthesia with isoflurane. At P10, following implantation of hippocampal electrodes, animals were placed in individual Plexiglas cages surrounded by a Faraday cage to undergo vEEG recordings before, during and after HS, like what was previously described (Desgent et al., 2012). EEGs and behavior were recorded simultaneously with a Stellate Harmonie system linked to a 32-channels Lamont amplifying unit and an infrared analogical video camera positioned 1.5 meter in front of the cages (Stellate Systems v 6.2e, Victoria, Montreal, Qc, CAN). Using the Harmonie Software, with EEG data acquired at 200Hz, 5-minute windows containing electrographic activity tracings were collected

every 10 minutes for the first 30 minutes after GC occurred. These data point windows were imported to MATLAB (MathWorks, MATLAB 9.1) for further analysis.

Electrographic seizures were defined via MATLAB as the occurrence of discrete episodes of uninterrupted high voltage spike and/or poly-spike discharges lasting at least 10 seconds, with a mean frequency higher than 1Hz and amplitude higher than a mean voltage threshold that was calculated from a 15 second segment of baseline activity for each case. The categories were: overt electrographic seizures (>10 seconds), poly-spike bursts (>3 and <10 seconds), and interictal spikes (<3 seconds). The severity of the HS was assessed by measuring the number and total duration of ictal events before return to baseline. Furthermore, to confirm seizure activity, epileptic behaviours corresponding to score levels of 3 to 5 on the modified Racine scale (Racine, 1972) had to be present simultaneous to seizure activity on the EEG. EEG monitoring was pursued until the recovery of baseline activity and exploring behaviour. Recovery from seizures was defined as disappearance of both clinical GC and epileptic activity, given that spikes persist after the GC and recovery correlates with the disappearance of the epileptic activity. Animals that did not recover within the first 120 minutes post GC were attributed this maximal time value as recovery latency. Three separate observers blinded to the treatment groups, reviewed the vEEG recordings to detect epileptiform events and clinically associated behaviours.

3.3.5. Thermographic measurements during HS

To gather body temperature data non-invasively during HS, we used a ThermoVision A40M (FLIR systems, Inc.) thermographic camera and computer vision Matlab tools, in a subgroup of animals. The camera allowed us to obtain every $1/30^{th}$ of a second, temperature measurements as 320 x 240 pixels greyscale images, where each pixel corresponded to a temperature measurement in the camera field of view. The thermographic camera lens was placed through a hole pierced in the box cover, giving an overhead view of the pup. The camera was set with a linear measurement range between

20 and 53°C. Temperature data was analyzed by taking measurements every 7 seconds (Bilodeau et al., 2015; Sanon et al., 2017).

3.3.6. Plasma CORT level measurements

Plasma CORT levels were sampled in P10 rats for each treatment and sex groups. Baseline values were obtained 30 minutes before HS via a blood sample (between 20-30µL) collected through rapid saphenous vein puncture with a capillary. Two hours after the first blood sample and the HS, the pups were quickly beheaded, and a second sample was taken. Whole blood samples were collected into commercially available anticoagulant-treated tubes (EDTA-treated) and centrifuged immediately (10 minutes at 1,000–2,000 x g using a refrigerated centrifuge). Plasma was collected and kept in an Eppendorf tube at -80°C until further processing. CORT levels were measured directly in plasma with a commercially available radioimmunoassay kit (Corticosterone ^{125}I RIA Kit, Medicorp inc., Montreal, Canada). This assay enabled us to reliably measure CORT levels superior to 0.022 nmol/ml.

3.3.7. Electrophysiological recordings

Patch-clamp recordings has been described previously (Sanon et al., 2010). Briefly, hippocampal slices were prepared from P18-P22 rats anesthetized with isoflurane. Brain tissue was quickly removed and placed in cold artificial cerebrospinal fluid (ACSF) containing in mM: 126 NaCl, 3 KCl, 2 $MgSO_4$-H_2O, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 10 D-Glucose, bubbled with 95% O_2/5% CO_2, with pH and osmolarity adjusted to 7.3-7.4 and 300-310 mOsm respectively. The 300µm thick hippocampal slices were cut using a vibratome (Leica Microsystems VT1000S, Concord, On, Canada) and transferred to a room temperature chamber with oxygenated ACSF. After one-hour incubation period, individual slices were placed into a recording chamber and continuously superfused with oxygenated and heated ACSF at ~32°C using a

temperature controller (Warner CL-100 Bipolar Temperature Controller, Hamden, CT, USA).

Hippocampal CA1 pyramidal cells were visualized using an upright microscope (Olympus BX50WI, Richmond Hill, ON, Canada.) fitted with differential interference contrast (DIC) optics and infrared (IR) video camera (Hitachi Kokusai Electric Canada, St-Laurent, QC, Canada). Recording patch pipettes were pulled from borosilicate glass tubing with filament (World Precision Instruments, Sarasota, FL, USA), with resistance ranging from 5 to 7 MΩ when filled with intracellular solutions containing in mM: 140 K-gluconate or CsCl, 5 NaCl, 2 $MgCl_2$, 10 Hepes buffer, 0.5 EGTA, 10 phosphocreatine, 2 ATP-Tris, 0.4 GTP-Li. CsCl based solution also included the Na^+ channel blocker N-(2,6-dimethyl-phenylcarbamoylmethyl) triethylammonium bromide (QX314, 2mM) to internally block Na^+ channels (Sigma, St-Louis, MO, U.S.A.). The pH and osmolarity were adjusted to 7.2–7.3 with KOH or CsOH, and 280-290mOsm respectively. After tight seals (> 1 GΩ) on pyramidal cell bodies and whole-cell configuration was attained, recorded signals were amplified using an Axopatch 200B, low-pass filtered at 1 kHz, digitized with a Digidata 1440A A/D converter and acquired at a sampling rate of 10kHz using the pClamp software (versions 8 and 10; Molecular Devices, Sunnyvale, CA, USA).

Evoked excitatory postsynaptic potentials (eEPSP) were elicited at -60mV by electrical stimulation (50% of the maximal stimulation intensity 0.1-0.5mA; 0.2 ms), using a bipolar electrode placed in the *stratum radiatum* layer, in current-clamp mode in ACSF. Spontaneous excitatory and inhibitory currents (sEPSC and sIPSC) were recorded using a CsCl based intracellular solution in voltage-clamp mode in the presence of bicuculline methiodide, a $GABA_A$ receptor antagonist (1μM) for sEPSC, and with N-methyl-d-aspartate (NMDA) receptor antagonist d-(-)-2-amino-5-phosphonovaleric acid (D-AP5; 50mM) and the non-NMDA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10mM) for sIPSC. All drugs were purchased from Sigma-Aldrich (Oakville, ON, Canada).

3.3.8. Statistical analysis

Data was log-transformed to fit a normal distribution and subsequently analyzed using a two-way analysis of variance (ANOVA) with Tukey's post hoc test to assess how continuous outcomes varied across multiple experimental groups. An independent unpaired two-tailed Student's *t*-test was used to identify significant differences between two groups. Non-linear regression curves (Gaussian fit) were traced to establish differences between data sets. Finally, the Kolmogorov-Smirnov test was used to establish differences between cumulative probability curves. All statistical analyses were carried out with RStudio 1.2.1335 with a two-tailed p-value < 0.05 used as the threshold for concluding statistical significance. All data are presented as mean ± standard error of the mean (SEM) except when specified otherwise.

3.4. RESULTS

3.4.1. Validation of CORT-treatment as a chronic stress in male and female rat pups

The progression of weight gain in male and female rat pups was monitored during CORT injection experiments from P1 to P9. Both VEH and CORT-treated male and female pups gained weight over the course of the 9 injection days (Figure 2 A, B). No mortalities were observed. On P1, there were no significant differences in mean body weight for male pups (p=0.998). However, starting at P4, male CORT pups gained weight at a slower rate than their VEH littermates. (two-way ANOVA with Tukey's post hoc analysis, p=0.002), the differential weight gain being sustained until P9 (two-way ANOVA with Tukey's post hoc analysis, p<0.001) (Figure 2 A). Data suggest that at young ages, male pup weight gain is altered by chronic CORT treatment.

Female pup development followed a similar trajectory, with no significant differences between VEH and CORT-treated rats at P1 (p=0.999). Differences in weight

were again first noticed at P4 (two-way ANOVA with Tukey's post hoc analysis, $p = 0.002$) and sustained until P9 (two-way ANOVA with Tukey's post hoc analysis, $p<0.001$) (Figure 2B). On P9, there were no significant sex differences in body weight within the VEH ($p=0.973$) and the CORT (two-way ANOVA with Tukey's post hoc analysis, $p=0.999$) groups. Overall, data suggest that chronic CORT treatment significantly affects weight gain in both males and females, as seen by the significant main effect of CORT treatment in both male ($F[1,767]=262.8$, $p<0.001$) and female cohorts ($F[1,758]=259.53$, $p<0.001$).

At P10, 24h after the last injections of CORT and before the HS, basal plasma CORT levels in the CORT group were expectedly significantly greater than the VEH group in both male (two-way ANOVA with Tukey's post hoc analysis, $p=0.001$) and female (two-way ANOVA with Tukey's post hoc analysis, $p=0.019$) cohorts (Figure 2C). However, only CORT-injected males had significantly greater plasma CORT levels than their VEH counterparts after HS (two-way ANOVA with Tukey's post hoc analysis, $p=0.009$). Plasma CORT levels in CORT-injected females after HS did not significantly differ from VEH-injected females ($p=0.059$) (Figure 2C). These results indicate a sex-dependent impact of postnatal chronic stress treatment on plasma CORT levels between males and females.

During hyperthermia in the heating apparatus at P10, CORT-injected male pups reached the critical body surface temperature of 45^{o}C (corresponding to a core temperature of 40.2 - 41.2^{o}C) significantly faster than their VEH littermates (two-way ANOVA with Tukey's post hoc analysis, $p=0.008$). This time a discrepancy was also observed in female pups (two-way ANOVA with Tukey's post hoc analysis, $p=0.013$) (Figure 2D, E). Therefore, the chronic CORT treatment lowers the temperature threshold to generalized convulsions (GC) allowing these animals to reach the critical body temperature faster as compared to the VEH-treated counterparts during hyperthermia.

3.4.2. Sex-specific effects of CORT-treatment on clinical seizures

Regarding the clinical manifestation of GC during hyperthermia, representative EEG traces of VEH and in CORT-injected male and female rats show basal activity,

epileptiform events, and full-blown seizures after HS. The clinical manifestations in animals typically included freezing or head bobbing, myoclonic hind limb jerks and abrupt loss of posture (Figure 3A, B). The latency to GC was significantly shorter in CORT males relative to VEH-treated control males (two-way ANOVA with Tukey's post hoc analysis, $p<0.001$) (Figure 3C). This was also the case for the female population, with CORT female rats seizing faster than VEH female rats (two-way ANOVA with Tukey's post hoc analysis, $p<0.001$). VEH-treated male rats also seized more rapidly than their VEH-treated female littermates (two-way ANOVA with Tukey's post hoc analysis, $p=0.019$). However, latency to GC was not significantly different between CORT males and CORT females ($p=0.245$) (Figure 3C). Overall, chronic CORT treatment had a significant main effect on the mean latency for GC in both sexes ($F[1,119]=54.622$, $p<0.001$).

After GC, VEH-treated rats recovered significantly faster than their CORT treated counterparts in both male (two-way ANOVA with Tukey's post hoc analysis, $p=0.001$) and female (two-way ANOVA with Tukey's post hoc analysis, $p=0.005$) cohorts (Figure 3D). The VEH female group recovered particularly quickly, with some individuals taking less than 30 minutes. Recovery time did not significantly differ between sexes for the VEH cohort ($p=0.244$). However, CORT-injected males took significantly longer to recover than CORT-injected females (two-way ANOVA with Tukey's post hoc analysis, $p=0.029$) (Figure 3D).

To study whether postnatal CORT injections altered the pattern and occurrence of epileptiform events following hyperthermia-induced GC, three main types of activity based on duration were quantified: interictal spikes, polyspike bursts and electrographic seizures. CORT-injected male animals had significantly more frequent epileptic discharges than their VEH littermates (two-way ANOVA with Tukey's post hoc analysis, $p<0.001$) (Figure 3E). Similarly, CORT-injected female also had significantly more frequent epileptic discharges than their VEH counterparts (two-way ANOVA with Tukey's post hoc analysis, $p<0.001$) (Figure 3F). When comparing the frequency of electrographic seizures and polyspike bursts during the first 30 minutes following the HS between different sexes of the same treatment, males were significantly more affected than females. VEH males and CORT males both had a greater proportion of electrographic

seizures than VEH females (two-way ANOVA with Tukey's post hoc analysis, $p<0.001$) and CORT females (two-way ANOVA with Tukey's post hoc analysis, $p<0.001$), respectively. Overall, the CORT male group was the most affected.

Looking only at seizure frequency (>10 sec), seizures occurred more frequently in the CORT male group compared to the others. The CORT male rat group experienced significantly more seizures than VEH males (two-way ANOVA with Tukey's post hoc analysis, $p<0.001$) and CORT females (two-way ANOVA with Tukey's post hoc analysis, $p=0.014$). The CORT female group was also significantly different from VEH female group (two-way ANOVA with Tukey's post hoc analysis, $p=0.011$) (Figure 3G). Sexual dimorphism in seizure frequency was only detected between the CORT-treated groups, with no significant sex differences in baseline conditions ($p=0.691$). Furthermore, the CORT-treated male and female groups experienced significantly longer seizures than their VEH counterparts (two-way ANOVA with Tukey's post hoc analysis; male, $p=0.004$ and female, $p=0.003$), and a sex-dependent difference in seizure duration was present in both VEH-treated (two-way ANOVA with Tukey's post hoc analysis, $p=0.029$) and CORT-treated groups (two-way ANOVA with Tukey's post hoc analysis, $p=0.043$) (Figure 3H).

3.4.3. Neuronal circuit alterations in male juvenile rats following chronic CORT-treatment in an *in vivo* model of limbic epileptogenesis

After identifying stronger effects in males relative to females, we investigated the effects of CORT on hippocampal hyperexcitability specifically in the more susceptible male juvenile rats. Even though spontaneous recurrent seizures do not develop until the third postnatal month (Scantlebury et al., 2005), we have previously shown that neural circuit abnormalities are already evident at P20 (Ouardouz et al., 2010). At the cellular level, evoked excitatory postsynaptic potentials (eEPSPs) in CA1 pyramidal cells yielded significantly greater amplitude responses in CORT-treated rats relative to VEH (unpaired Student's *t*-test, $p=0.043$) (Figure 4A, B). Furthermore, these greater responses were obtained at most stimulation intensities (Non-linear regression, $p=0.0006$) (Figure 4C).

Despite an increase in the synaptic efficacy, intrinsic properties including input resistance (p=0.745) (Figure 4D), resting membrane potential (RMP) (p=0.573,) (Figure 4E), and action potential (AP) threshold (p=0.495) (Figure 4F) were not different in the CORT group relative to VEH animals.

Studies indicate that stress can lead to epilepsy which may converge to perturb the excitation/inhibition (E/I) balance, due to the dysfunction of excitatory and inhibitory circuits in different brain regions. Therefore, to examine the impact of CORT treatment, spontaneous excitatory/inhibitory postsynaptic currents (sEPSC/sIPSC) from CA1 pyramidal neurons were recorded. Representative traces of sEPSC (Figure 4G) showed that the mean amplitude (p=0.274) (Figure 4H) and frequency (p=0.997) (Figure 4I) were not significantly different between VEH and CORT groups. Cumulative amplitude (p=0.985) (Figure 4J) and inter-event interval (IEI) (p=0.876) (Figure 4K) distribution plots for sEPSCs recorded in CORT slices overlapped the distribution plots for events recorded in VEH slices, confirming there was no significant difference between CORT and VEH slices in that respect. However, looking at the inhibitory activity onto CA1 pyramidal cells, representative traces of sIPSC (Figure 4L) in pyramidal cells showed that the mean amplitude of sIPSCs was significantly greater in cells from CORT relative to VEH animals (unpaired Student's t-test, p=0.018) (Figure 4M), while the mean frequency of these currents was unchanged (p=0.785) (Figure 4N). Cumulative amplitude distribution plots for sIPSCs recorded in CORT slices exhibited a rightward shift relative to VEH (Kolmogorov-Smirnov test, p=0.005) (Figure 4O) indicating an increase in the proportion of large amplitude events. Cumulative IEI distribution plots for sIPSCs showed no significant difference between CORT and VEH slices (p=0.569) (Figure P). All events were blocked with $GABA_A$ antagonist bicuculline. In summary, our findings revealed an increase in evoked potential responses, as well as an increase in the inhibitory drive in the CA1 pyramidal cells.

3.5. DISCUSSION

In this series of experiments, we examined the effect of chronically elevated CORT levels on hyperthermia-induced seizure in male and female rat pups during development. The data demonstrated this chronic stress model affect weight gain, basal plasma CORT levels before HS, body temperature threshold of HS, GC latency, recovery time, number, and duration of electrographic seizures in both male and female rat pups. Sex-specific differences were found in basal plasma CORT levels after HS, recovery time, number, and duration of electrographic seizures in CORT animals. Interestingly, sex-differences were also observed in baseline conditions in GC latency and duration of electrographic seizures. Overall, while also affected by CORT, female rat pups were more resistant than males to this chronic stress model and appeared significantly less likely to develop FS that could possibly lead to mTLE. Consequently, effects of CORT were observed in evoked potential responses, as well as spontaneous inhibitory currents in the more susceptible male juvenile rats.

3.5.1. CORT administration as a chronic model of stress

Glucocorticoids (CORT in rodents and cortisol in humans) are the primary end product of the HPA axis, and chronic stress states are associated with a sustained elevation of this hormone into the bloodstream (Munck et al., 1984). In the present study, daily postnatal injections of CORT in rat pups mimic a chronically stressed state during development. The use of CORT administration was chosen because it reduces inter-subject variability as physical, environmental or emotional stress paradigms can all cause individual differences in the HPA axis regulation (Kott et al., 2016; van Campen et al., 2018), as well as mimic normal temporal variation in hormone levels (Claflin et al., 2017). Although the current approach is not a natural physiological stressor, it standardizes the effects of constant or elevated exposure to glucocorticoids *in vivo*. Moreover, the current injection regimen maintained a CORT increase pattern that was

comparable to those found following psychosocial or maternal deprivation stress; these models represent better models of early-life stress in rodents, where a hormone increase between two to four folds of basal physiological level values is expected (Brummelte and Galea, 2010; Desgent et al., 2012; Kumar et al., 2007; Morales-Medina et al., 2009).

3.5.2. Stress Hyporesponsive Period (SHRP)

The secretion of glucocorticoids in rats begins during the fetal period and before birth; the basal levels of CORT are similar to those found during adulthood (Condon et al., 1998). However, they markedly decrease after the first two days of life to remain at very low levels until the end of the second postnatal week, a phase called stress SHRP (Dent et al., 2000; Levine, 1994; Schoenfeld et al., 1980). This suggests that the HPA axis of the neonate is not only less responsive to the stimulatory effects of stressors, but also to the inhibitory mechanisms that regulate the neuroendocrine response to stress for their life span. It has been suggested that the SHRP constitutes a protective mechanism as it ensures low and stable levels of glucocorticoids during the early postnatal brain development (Sapolsky and Meaney, 1986; Walker and Scribner, 1991), given that different lines of research evidence demonstrate that exposure to high levels of CORT during the neonatal period leads to irreversible changes that persist in adulthood (Shors, 2006).

To date, very few preclinical and clinicals studies have reported sex differences in HPA axis functionality during the SHRP. Our results indicate that males have higher levels of CORT whereas females undergoing similar brain insults do not have such changes in plasma CORT. These results corroborate previous studies demonstrating that perinatal cortical malformations, such as a freeze lesion combined with HS at P10, led to a rise in their plasma CORT levels and to the development of mTLE in male pups and testosterone-treated females. Despite undergoing similar brain insults, untreated females were not affected (Desgent et al., 2012). Furthermore, in a clinical study looking at stress reactivity in healthy term neonates, higher cortisol response was found in male neonates compared to the female ones, suggesting neonatal sex differences in physiological stress reactivity prior to socialization (Davis and Emory, 1995). However, different types of

stressors can affect females more than males. Evidence comes from models of stress due to an immune challenge at P3 in which results showed that HPA axis activity was greater in intact females relative to male rats, whereas this sex difference was reversed with gonadectomy on the day of birth (Shanks et al., 1994). Similarly, female rat pups at P8 exhibited an enhanced adrenocorticotropic hormone (ACTH) response to inhalation of ether when compared to males and when given a testosterone injection at birth, yielding comparable ACTH responsivity to males (Hary et al., 1986). Therefore, it is inappropriate to assume that circuits and regulatory processes are common to males and females. Our data suggest that sex differences during SHRP clearly indicate biological differences between males and females during the organizational period possibly because of prenatal gonadal hormones (androgens or estrogens) that exert a permanent, organizing effect on brain tissue.

3.5.3. Sex differences in HS: males are more affected than females

Sex differences have been observed in several animal models when combining diverse stress paradigms with experimental induction of seizures. Despite this, most animal models used in epilepsy have used only males, do not specify sex, or pool sexes. However, there are new National Institutes of Health (NIH) and Canadian Institutes of Health Research (CIHR) regulations in place to change this for pre-clinical and clinical studies(Health Canada, 2009; National Institutes of Health, 2015).

Alterations in hippocampal structure and function of CORT might be one of the results underlying hyperthermia-induced seizures in stressed rats. There is increasing evidence showing that chronic stressors that increase glucocorticoid levels lower the threshold for seizure induction, accelerate ictogenesis and promote epileptiform discharges in several animal models of epilepsy (Joëls et al., 2007a, 2007b, Joëls, 2009). For example, CORT in the WAG/Rij rat model of childhood absence epilepsy was associated with rapid increases in spike-wave-discharges (Schridde and Van Luijtelaar, 2004). Similarly, in the Kainic Acid (KA) model of TLE, pre-exposure to CORT led to

increased seizure susceptibility and frequency in male mice and rat species (Roberts and Keith, 1994). Furthermore, in offspring of unspecified sex from pregnant Sprague Dawley dams, results showed prolonged or more severe seizure response in the lipopolysaccharide (LPS) + KA-induced rat model of epileptogenesis in P14 rats that were exposed to prenatal stress in comparison to controls (Qulu et al., 2012). The amplitude of population spikes in the CA1 area was also increased in hippocampal kindled male Wistar rats after exposure to high CORT levels (Karst et al., 1999). Further evidence supporting the role of chronic stress in epileptic activity has been shown in experiments using postnatal stressors comparable to those used in the current experiment. For example, neonatal isolation for one hour per day between P2 and P9 enhanced plasma CORT and exacerbated the neurological consequences of status epilepticus induced by lithium-pilocarpine at P10 (Lai et al., 2006). However, this was done in Sprague Dawley rats of unspecified sex.

Recently, using the amygdala kindled model, O'Brien and colleagues highlighted a potential influence of sex and stress hormones in seizure susceptibility, which has been reported in several prior studies as well (Jones et al., 2013; Jones and O'Brien, 2013). For example, a study using hippocampal kindling in P14 Wistar rats found that mid- or late-gestational stress via prenatal restraint of the mother increased the rate of kindled seizure development in rat pups and later adult males, but not in female littermates (Edwards et al., 2002b, 2002a). Stress also increased the rate and duration of tonic-clonic pilocarpine-induced seizures on P18-19 more severely in males, which showed more elevated CORT levels and epileptic behaviors than female rats (Ahmadzadeh et al., 2011; Sadaghiani and Saboory, 2010). Similarly, increased kindling rates were also observed in adult male rats pre-exposed to chronic postnatal cross-fostering stress during a similar period (i.e., P1-P23) (Gilby et al., 2009). Furthermore, in adult female rats, adrenalectomy compared to CORT replacement or sham-operated controls can delay the kindling process, while testosterone in males can enhance it (Edwards et al., 2001, 1999). However, it should also be noted that certain circumstances do lead to increased seizure susceptibility in females more than males. For example, daily maternal separation (chronic stress) compared to handling (acute stress), between P2-14, was shown to lower seizure threshold and increase amygdala kindling rates in adult Wistar rats of both sexes, but with

more potent effects seen in females (Jones et al., 2009; Kumar et al., 2011; Salzberg et al., 2007).

Hence, our model corroborates with previous studies in which a resistance to HS may be due to the animal body size. More specifically, Barrett et al., 2016 found a positively correlation between body weight and seizure latency in a heat-sensitive transient receptor potential vanilloid-1 (TRPV1) KO mice (Barrett et al., 2016). Accordingly, a slower weight gain in our model of CORT increased seizure susceptibility when compared to VEH-treated animals. Furthermore, our results define a sex-dependent activity of the HPA axis in the SHRP and the male susceptibility for seizures may be influenced by different factors. Our results show that CORT-treated animals have a decreased latency to reach seizure threshold although no sex differences were found between groups. This suggests that CORT affects temperature regulation. Recent literature on FS and breathing reposes to HS shows that vagal TRPV1-driven thermal hyperpnea, a breathing pattern during HS characterized by an increase in tidal volume and breathing frequency, possibly rises susceptibility to HS in pups (Barrett et al., 2018). Therefore, CORT may produce sex-specific effects on breathing responses to HS leading to males being more affected than females, which corroborates with sex-based differences in the consequences of neonatal stress on cardio-respiratory system (Baldy et al., 2018; Tenorio-Lopes & Kinkead, 2021).

3.5.4. Possible compensatory alteration in the CORT juvenile male rats

While CORT increased synaptic efficacy as measured by eEPSP, the CA1 pyramidal cells into the epileptic environment received augmented inhibitory input with an increase of the amplitude of sIPSCs. Changes in $GABA_A$ receptor subunit composition, as well as enhanced sensitivity or activation of postsynaptic $GABA_A$ receptors, might explain the elevated inhibitory drive of CORT juvenile male rats. This is supported by studies demonstrating that $GABA_A$ receptors exhibit enhanced efficacy after SE (Brooks-Kayal et al., 1998; Cohen et al., 2003; Gibbs et al., 1997). We speculate that changes in

the amplitude of inhibitory currents may maintain homeostatic balance in the face of changing E/I balance and characterize a compensatory alteration in the CORT treated animals.

Two main limitations are noted in this study. First, the electrophysiology experiments performed at P18-P22, included only males. Even if previous studies showed a preferential KCC2 upregulation in a model of freeze lesion combined with HS probably due to sustained neural activity, which in turn may increase the risk for mTLE in juvenile male rats (Awad et al., 2016; Fiumelli et al., 2005; Fiumelli and Woodin, 2007), future studies in females will be needed to determine the implications of sex-specific susceptibility to the emergence of mTLE following FS. Second, this series of experiments are exploratory and suggest that males are more affected in the short term but long-term video-EEG recordings are needed to confirm the sex differences in the two-hit model of mTLE, as described in previous studies (Desgent et al., 2012).

3.5.5. Crosstalk between HPA and hypothalamic-pituitary-gonadal (HPG)

There exists a reciprocal relationship between the HPA and the HPG axes wherein the activation of one affects the function of the other and vice versa (Oyola and Handa, 2017). By using CORT administration as a chronic model of stress, our results suggest that this resistance could be due to a lower susceptibility of the female HPA axis to excessive CORT exposure during this specific postnatal window, supporting our hypothesis that sex hormones influence the HPA activity during development and seizure severity and outcome. Androgens generally exert pro-convulsant effects that can underlie sex differences in the expression of seizures, and these are mediated in part by their actions on the hippocampus, where HS originate (Frye, 2008; Goel and Bale, 2009; Hamed, 2008). Thus, this study suggests that the surge of sexual hormones (testosterone and its estrogenic metabolites) during the end of the embryonic period and until birth in male Sprague Dawley rat pups is involved in their more severe phenotype during HS and

that this effect is potentiated by the presence of excessive plasma CORT levels in male rat pups.

3.6. CONCLUSION

These results suggest that repeated exposure to elevated CORT levels exacerbates HS and that males are more affected than females. This provides additional evidence that an exposure to early life stress, by increasing glucocorticoid levels early in life, may act as a first hit to predispose to complex febrile seizures and possibly ensuing epileptogenesis. The results of this experiment are consistent with the two-hit hypothesis of epileptogenesis and suggest early-life stress is a significant first hit due to an increased hippocampal vulnerability to febrile seizures. However, further work is required to investigate the long-term outcome (e.g., at P90) using the current model. Furthermore, the results encourage continued studies on sex-specific biological effects on the development of epilepsy in animal models, especially as they pertain to the developing brain. Converging data indicate that elevated expression of glucocorticoids constitutes an important mechanism, through HPA axis modifications, for generating developmentally regulated alterations that could increase excitability in the hippocampus that in turn would trigger and worsen seizure responses early in life. Our data adds additional insights to the important relationship between sex, early-life stress and epileptogenesis, and may lead to refinement of our clinical surveillance and therapeutic strategies in the neonatal period.

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3.8. Figures and Legends

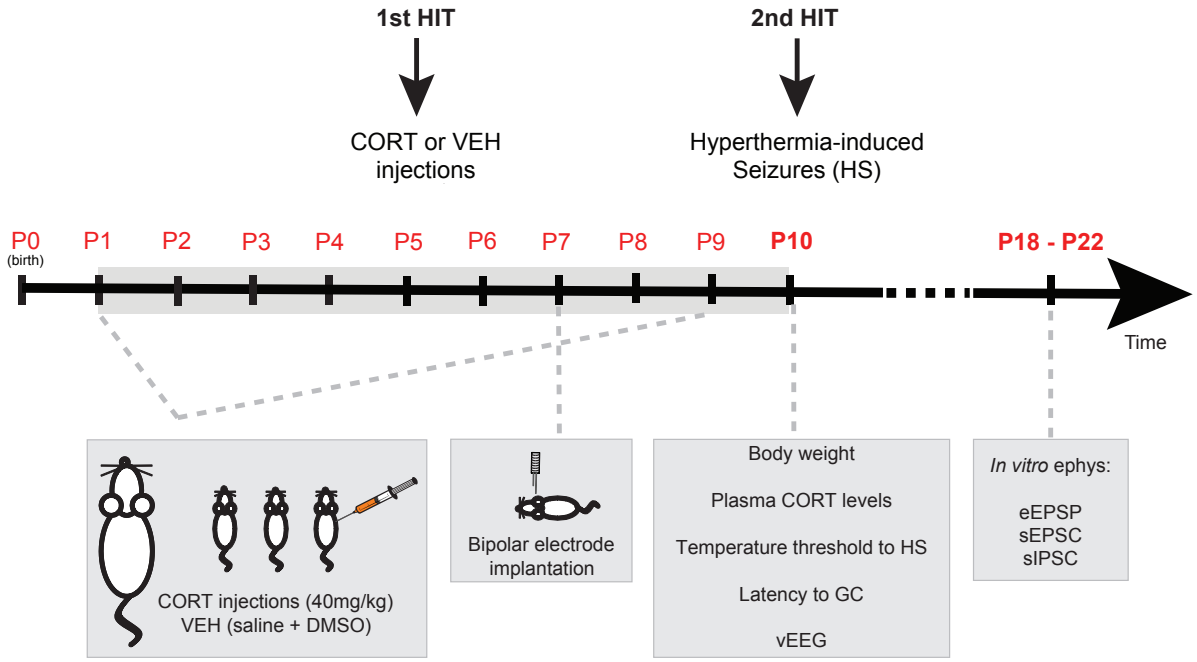


Figure 1. Experimental design. Starting at P1, subcutaneous CORT injections were given daily until P9. Weight gain was monitored everyday throughout the experiment. At P7, a subset of animals underwent a surgery to implant bipolar electrode used during vEEG recordings at P10. At P10, pups underwent HS. Plasma CORT levels, temperature threshold to HS and latency to GC were measured. *In vitro* recordings were performed around P18-P22. CORT = corticosterone, HS = hyperthermia-induced seizures, GC = generalized convulsions, vEEG = video and EEG recordings, eEPSP = evoked excitatory postsynaptic potentials, sEPSC = spontaneous excitatory postsynaptic currents, sIPSC = spontaneous inhibitory postsynaptic currents.

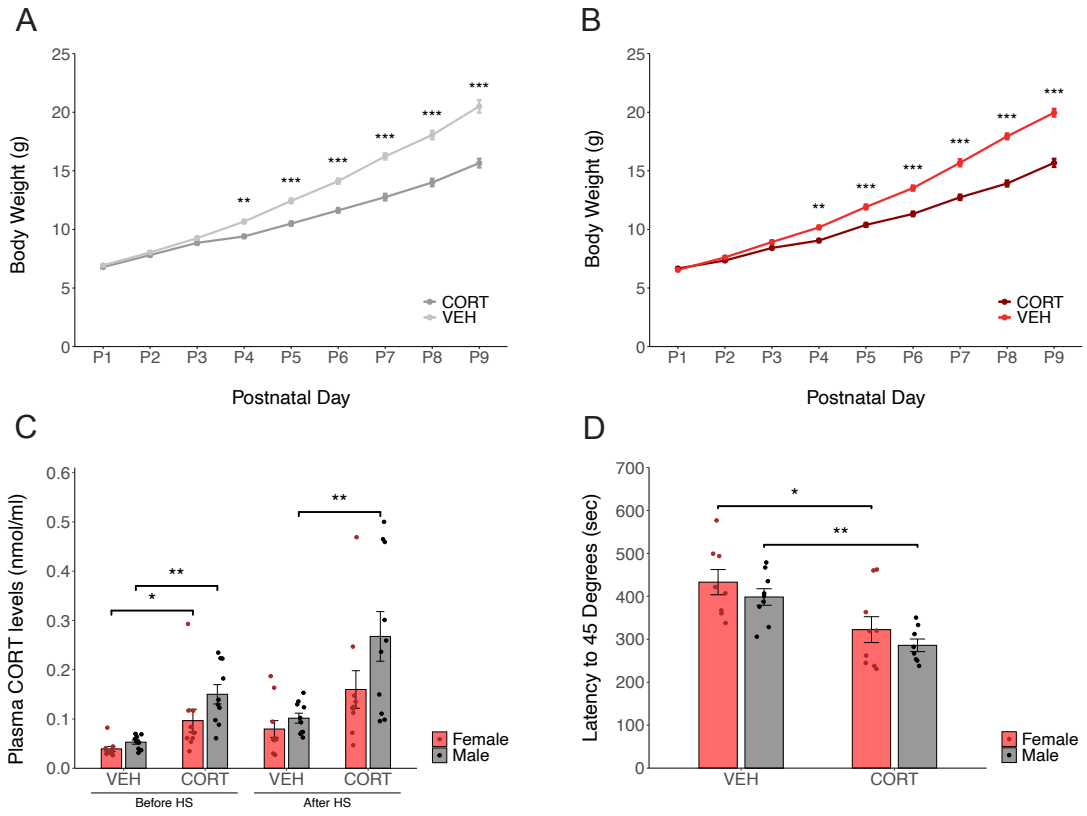
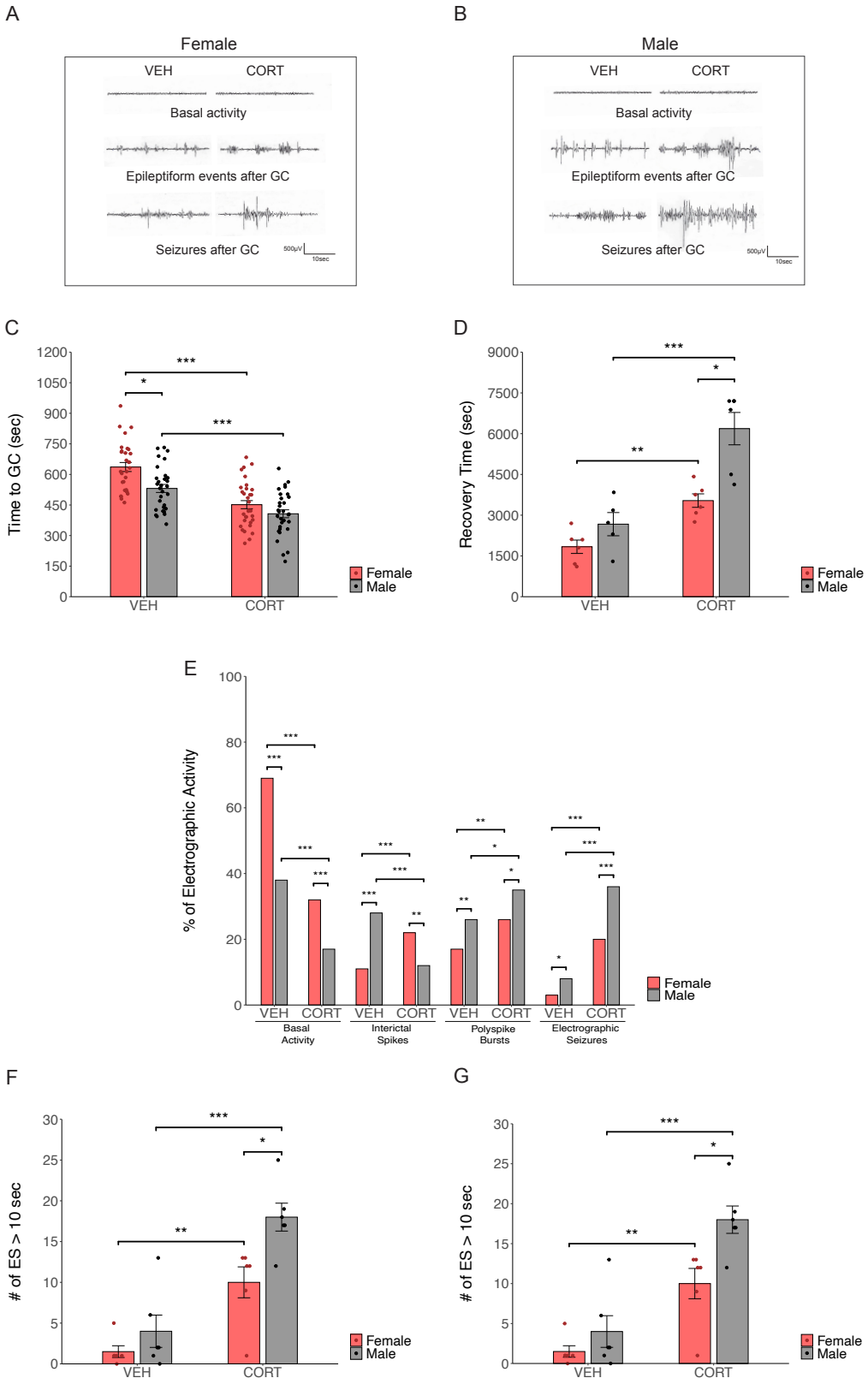


Figure 2. Temporal effects of postnatal CORT administration on weight gain, plasma CORT levels and temperature threshold of HS in male and female rat pups. **A.** Body weight curve from P1 to P9 during daily CORT injections shows a significant effect in male CORT-injected (n=48) relative to VEH-injected animals (n=46) (P1: VEH, 6.9 ± 0.1g vs. CORT, 6.8 ± 0.1g; P4: VEH, 10.7 ± 0.2g vs. CORT, 9.4 ± 0.1g; P9: VEH, 20.5 ± 0.5g vs. CORT, 15.7 ± 0.4g). **B.** Body weight curve of female pups (VEH n=51 and CORT n=43) during the daily CORT injections shows a significant decrease in weight like males (P1: VEH, 6.6 ± 0.1g vs. CORT, 6.7 ± 0.1g; P4: VEH, 10.2 ± 0.2g vs. CORT, 9.1 ± 0.1g; P9: VEH, 20.0 ± 0.3g vs. CORT, 15.7 ± 0.4g, $p<0.001$. On P9, there were no significant sex differences in body mass within the VEH (male, 20.5 ± 0.5g vs. female, 20.0 ± 0.3g) and the CORT (male, 15.7 ± 0.4g vs. female, 15.7 ± 0.4g) groups. **C.** Bar chart of basal plasma CORT levels in the CORT group (n=10) were significantly greater than the VEH group (n=10) in both male (VEH 0.05 ± 0.01nmol/ml vs. CORT, 0.15 ± 0.02nmol/ml) and female (VEH, 0.04 ± 0.02nmol/ml vs. CORT, 0.10 ± 0.02nmol/ml)

cohorts before HS. However, after HS, only CORT-injected males had significantly greater plasma CORT levels than their VEH counterparts (VEH, 0.10 ± 0.01nmol/ml vs. CORT, 0.27 ± 0.105nmol/ml). Plasma CORT levels in CORT-injected females after HS did not significantly differ from VEH-injected females (VEH, 0.08 ± 0.02nmol/ml vs. CORT, 0.16 ± 0.04nmol/ml). **D.** Bar chart of latency to reach the critical external temperature of 45^{o}C shows that CORT males (n=8) were significantly quicker to arrive at that point than VEH injected males (n=9) (VEH, 399 ± 19sec vs. CORT, 286 ± 15sec). This time discrepancy was also observed in female pups (VEH, 433 ± 29sec, n=8 vs. CORT, 322 ± 30sec, n=9). Dots represent individual data points. Graphs represent mean ± SEM. Significant differences are represented by * - $p < 0.05$; ** - $p < 0.01$, *** - $p < 0.001$.


A
Female
VEH
CORT
Basal activity
Epileptiform events after GC
Seizures after GC
500μV
10sec
B
Male
VEH
CORT
Basal activity
Epileptiform events after GC
Seizures after GC
500μV
10sec
C
Time to GC (sec)
1200
1050
900
750
600
450
300
150
0
***
*
***
VEH
CORT
Female
Male
D
Recovery Time (sec)
9000
7500
6000
4500
3000
1500
0
***
*
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VEH
CORT
Female
Male
E
% of Electrographic Activity
100
80
60
40
20
0
VEH
CORT
Basal Activity
Interictal Spikes
Polyspike Bursts
Electrographic Seizures
Female
Male
F
# of ES > 10 sec
30
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Figure 3. Sex-dependent electrographic modifications of CORT-treatment during HS. A. Representative EEG traces in VEH and in CORT-injected female rats showing basal activity and the following epileptiform events: interictal spiking (top), mix of interictal epileptic spike and polyspikes (middle) and complex polyspike bursts (bottom). **B.** Representative EEG traces in VEH and in CORT-injected male rats Horizontal bar =10 seconds; vertical bar = 500 μV. **C.** Bar chart of latency to generalized convulsion (GC) during hyperthermia shows that CORT-treatment significantly shortens the latency to GC during hyperthermia in males (VEH, 531 ± 20sec, n=31 vs. CORT, 407 ± 19sec, n=31) and in female (VEH, 636 ± 22sec, n=30 vs. CORT, 452 ± 20sec n=32), but that the latency is already shorter in males than in females with VEH treatment (531 ± 20sec vs. 636 ± 22sec). **D.** Bar chart of recovery time that after GC, VEH-treated rats recovered significantly faster than their CORT treated counterparts in both male (VEH 2670 ± 427sec, n=5 vs. CORT, 6185 ± 595sec, n=6) and female (VEH, 1838 ± 246sec n=6 vs. CORT, 3537 ± 248sec, n=6) cohorts. However, CORT-injected males took significantly longer to recover than CORT-injected females (6185 ± 595sec vs. 3537 ± 248sec). **E.** Pie chart shows that CORT-injected male animals had significantly more frequent epileptic discharges than their VEH littermates. Electrographic seizures and complex polyspike bursts constituted 36% and 35% of the electrophysiological activity in CORT-injected males (n=6), relative to 8% and 26% of activity in VEH males (n=6). Similarly, pie chart shows that CORT-injected female also had significantly more frequent epileptic discharges than their VEH counterparts with electrographic seizures and polyspike bursts making up 20% and 26% of the electrophysiological activity in CORT-injected females (n=6) and 3% and 17% in VEH females (n=6), respectively. When comparing the frequency of electrographic seizures and polyspike bursts between different sexes of the same treatment, males were significantly more affected than females. **F.** Bar chart shows that the CORT male rat group experienced significantly more seizures than VEH males (VEH, 4 ± 2, n=6 vs. CORT, 18 ± 2, n=6). The CORT female group was also significantly different from VEH female group (VEH, 1 ± 1, n=6 vs. CORT 10 ± 3, n=6). Sex differences were found in between CORT groups (CORT male, 18 ± 2 vs. CORT female 10 ± 3). **G.** Bar charts shows that seizure duration of electrographic seizures was longer in CORT males compared to VEH males (VEH, 15 ± 1sec, n=6 vs. CORT, 25 ± 3sec, n=6). The

CORT female group was also significantly different from VEH female group (VEH, 10 ± 0.2sec, n=6 vs. CORT, 17 ± 2sec, n=6). Sex differences were found in between VEH (VEH male, 15 ± 1sec vs. VEH female 10 ± 0.2sec) and CORT groups (CORT male, 25 ± 3sec vs. CORT female 17 ± 2sec). Dots represent individual data points. Graphs represent mean ± SEM. Significant differences are represented by Significant differences are represented by * - $p < 0.05$; ** - $p < 0.01$, *** - $p < 0.001$.

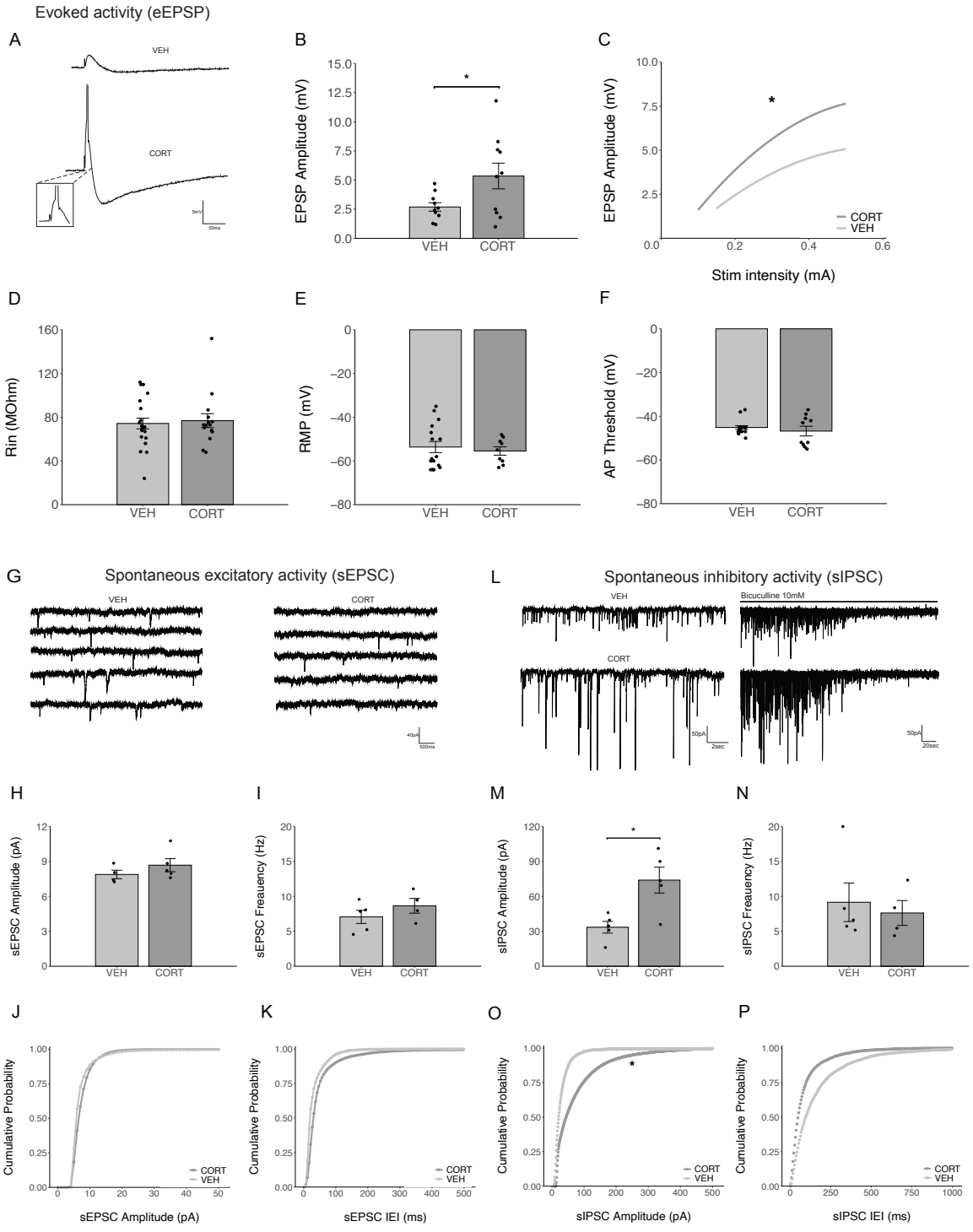

Figure 4. Intrinsic and synaptic properties of pyramidal cells in male rats submitted to CORT injections and HS. A. Representative traces of excitatory post-synaptic potentials (EPSPs) evoked on CA1 pyramidal cells upon Schaffer collateral stimulation in ACSF. Inset shows an action potential (AP) as present mostly in the CORT group. **B.** Bar

graph including all recorded cells from VEH and CORT showing a notable increase in EPSP amplitude in the CORT group relative to VEH (VEH 2.7 ± 1.1mV, n=10 vs. CORT, 5.4 ± 1.1mV, n=10). **C.** Gaussian fits of stimulus-response curves of EPSPs showing significantly greater amplitudes at all stimulation intensities in the CORT rats (n=10) relative to the VEH group (n=14). **D.** Bar graphs shows no differences between CORT and VEH groups in input resistance (VEH 74.26 ±, 22.42 MOhm, n= 21 vs. CORT, 76.89 ± 24.60 MOhm, n=15), **E.** resting membrane potential (RMP) (VEH, -53.63 ± -10.18mV, n=16 vs. CORT, -55.44 ± -5.73mV, n=9) and **F.** action potential (AP) threshold (VEH -45.14 ± -3.30 mV, n=16 vs. CORT, -46.80 ± -6.99mV, n=10). **G.** Representative traces of sEPSC in pyramidal cells. **H.** Bar graphs of mean amplitude (VEH 7.88 ± 0.73pA, n=4 vs. CORT, 8.67 ± 1.25pA, n=5) and **I.** mean frequency (VEH 8.65 ± 2.11Hz, n=4 vs. CORT, 7.06 ± 2.09Hz, n=5) show similar sEPSC in cells from VEH and in CORT groups. **J.** Cumulative probability distribution plots of sEPSC amplitude and **K.** IEI confirming previous findings. **L.** Representative traces of sIPSC in pyramidal cells. **M.** Bar graph of mean amplitude shows greater amplitudes in CORT group relative to VEH (VEH, 33.46 ± 11.22pA, n=5 vs. CORT, 73.92 ± 24.81pA, n=5). **N.** Bar graph shows no significant change in mean frequency (VEH, 7.62 ± 3.56Hz, n=4 vs. CORT, 9.15 ± 6.17Hz, n=5). All events were blocked with $GABA_A$ antagonist bicuculline. **O.** Cumulative probability distribution plots of sIPSC amplitude and **P.** IEI confirming previous findings. Dots represent individual data points. Graphs represent mean ± SEM. Significant differences are represented by Significant differences are represented by * - $p < 0.05$; ** - $p < 0.01$, *** - $p < 0.001$.

– CHAPTER IV –

GENERAL DISCUSSION

4.1. Overview

The overall objective of this thesis was to investigate the impact of sex hormones in the brain that may make it more prone to seizure development. In the first manuscript (Chapter II), I investigated how perinatal testosterone levels/signaling affect GABAergic circuit properties. In the second manuscript (Chapter III), I determined how sex-specific differences in the developing brain have an impact on stress-induced epileptogenicity after the induction of febrile seizures. Below, I will summarize our findings, describe study limitations, and outlines future perspectives of each study.

4.2. Perinatal testosterone levels/signaling affect GABAergic circuit function

The main finding of the first study is that females and testosterone-insensitive males show earlier KCC2 membrane localisation and higher BDNF levels during the first postnatal week, whilst enhanced spontaneous GABA synaptic transmission in adult hippocampus when compared to males and testosterone-exposed female rats.

The rat is considered a useful model to study sexual differentiation of the brain because manipulation of sex hormones during the organizational critical period can induce robust and reliable sex differences in the brain. In detail, during a so-called sensitive period, which is a parallel period in which a newborn female can be injected with testosterone, or its aromatized by product estradiol, for up to 6–10 days postnatally and still be masculinized [58,78]. Since it is relatively easy to manipulate the hormonal milieu and other physiological parameters in a newborn pup, this is proved to be a highly useful experimental tool for studying the process of masculinization using females because. On the other hand, this process begins in utero in males, and once it stars, it is very difficult to block or reverse it [66,75]. On the other hand, testicular feminization mutant (Tfm) male rats, in which TFM is a naturally occurring point mutation of the gene that codes for the androgen receptor (AR), rendering the Tfm male rat insensitive to physiological levels of androgens, may be used to investigate the involvement of the AR in various cellular responses, as well as to distinguish between the genomic and nongenomic effects of testosterone [310]

The development of the hippocampal formation occurs during the late gestational and early postnatal period concomitantly with the critical period of brain sexual differentiation, and thus the cytoarchitectural patterning of the hippocampus could be potentially influenced by gonadal steroid hormones. Although GABA is the primary inhibitory neurotransmitter in the adult CNS, it begins as an excitatory neurotransmitter in the early postnatal rodent brain [104,105]. The overall effect of $GABA_A$ receptor activation on cellular excitability is dependent on the chloride ion gradient, which is maintained by NKCC1 and KCC2 [107]. The developmental increase of KCC2 expression regulates inhibition [120] and accordingly, there is a negative shift in the reversal potential (E_{GABA}) for chloride ions with brain maturation [311,312]. In particular, previous studies have shown that are sex differences in regards to the developmental profile of KCC2 expression in the hippocampus of newborn rats [132,313]. Further, the GABA switch, characterized by the downregulation of NKCC1 and the upregulation of the KCC2 chloride transporter, occurs earlier in females and remains consistently higher in females during development compared to males [133]. My work showed that KCC2 membrane localisation, and thus likely function, is regulated by perinatal sex steroid hormone signaling [314,315]. KCC2 expression and function have been shown to regulate different aspects of brain development, including dendrite arborization [316], interneuron migration [317] and synapse formation [318–320]. Different temporal dynamic in the chloride gradient may thus contribute to differences in brain sexual differentiation. Further, my work demonstrated that perinatal hormones lead to differences in spontaneous inhibitory neurotransmission in the adult hippocampus. Differences in spontaneous GABAergic activity may in turn affect excitability by modulating the firing rate of the postsynaptic cells, i.e., modulating the efficacy of GABAergic inhibition [321]. The sex differences reported in this thesis have interesting implications given that alterations of GABAergic neuron function have a significant impact on the input–output functions of postsynaptic cells, with the potential to influence a broad variety of physiologically relevant behaviours such as network information processing features and seizure threshold changes [322,323]. It is possible that sex-based differences in hippocampal circuit excitability may play a role in the sex bias observed in several neurodevelopment disorders, such as, epilepsy, autism spectrum disorders (ASD), attention deficit/hyperactivity disorder (ADHD) and schizophrenia [324–326] (Section 1.2.4.).

One limitation of this study is that GABAergic circuit function was studied only in the adult brain. In order to study the maturation of inhibitory synaptic transmission, analysis of inhibitory transmission (sIPSC and mIPSC recorded from CA1 pyramidal neurons) should be performed at earlier stages of development as well. For instance, it could be studied at P7, the same stage used to assess KCC2 and BDNF expression, which coincides with the beginning of the GABAergic synapse formation [318]. Given that GABAergic synapse density increases during the first postnatal month [327], recordings at P14 and P28 would give a clearer picture of when spontaneous GABAergic activity starts to differ in males versus females. GABA-dependent signalling is considered to be one of the main driving forces responsible for the developmental GABA switch [126], thus the proposed experiments could shed light on the interaction between GABAergic transmission levels and proportion of KCC2 localised at the membrane, depending on perinatal sex hormones.

Previous studies indicate that increased BDNF expression may be responsible for the increased KCC2 localisation to the membrane of CA1 pyramidal neurons in the developing brain [318,328,329], which may translate into a greater role for the KCC2 transporter function during this developmental window. In order to prove a causal link between BDNF levels and membrane localisation of KCC2, we could investigate whether KCC2 expression and/or localisation are affected by BDNF treatment in males and testosterone-treated females, since our results show a lower level of BDNF compared to female or Tfm rats [330,331]. Accordingly, decreasing BDNF in females or Tfm males by AAV-mediated transfection of siRNA for BDNF [332,333] could indicate if the different BDNF levels between sexes promote the localisation in the plasmalemmal pool of already synthetized KCC2.

Another limitation of this study is that more experiments are needed to quantitively measure E_{GABA} or intracellular chloride levels in hippocampal pyramidal neurons to support the hypothesis that perinatal testosterone affects the developmental GABA switch. In particular, experiments measuring E_{GABA} by gramicidin-perforated patch of pyramidal CA1 neurons in P7 brain slices from the different sex groups included in this thesis would confirm when a hyperpolarizing shift of E_{GABA} occurs and the effect of the organizational sex hormones [134,311,312].

4.3. Perinatal sex hormones and GABAergic circuits: future perspectives

Our current knowledge of sexual differentiation is based on the organizational hypothesis, which states that the presence or absence of testosterone during early brain development in males and females, respectively, determines most of sex differences in the brain. However, this concept of sexual differentiation ignores any sex distinctions in the brain that are not a direct result of sex determination [334]. In fact, recent studies have identified genes encoded on the sex chromosomes that act directly on the brain to influence neural development and sex-specific behaviours [335]. Thus, sex differences are present at a molecular level and are due to the genetic variability between the X and Y chromosomes being present during the lifespan of an individual and being independent of sex hormones [78].

As a result, investigations on gonadal hormones as well as genetic sources of sex differences have recently been included to the field of sex differences. In order to separate gonadal sex from chromosomal sex, a model known as the 4-core genotype model [336,337], allows researchers to determine the relative contribution of sex chromosomes and hormones in sexual development. The model also permits investigating the interplay between the two, in order to differentiate gonadal sex from chromosomal sex. In this mouse model, the Sry gene is translocated to an autosome, where it controls normal testicular development outside of the Y chromosome. This allows for the creation of XX and XY animals that are either male or female depending on their gonadal status (genotypes: XX Sry–, XX Sry+, XY Sry+, XY Sry–) [336,337].

The 4-core genotype model is an interesting model because transcriptomics studies show unexpected differences in gene expression in specific regions of male and female brains that may be responsible for the the differences encountered between sexes. Those differences could also serve a compensatory role or provide latent sex differences that are only revealed in response to challenges. It would be interesting to explore the GABA circuit development in the 4-core genotype model and compare with the results of Chapter II to check to what extent sex chromosomes and sex hormones have an influence in the sexual differentiation of the brain and in the inhibitory network maturation. These

studies could provide a mechanistic insight into sexual dimorphism in neurodevelopmental disorders [338].

Two other interesting experimental models that could be used to study the role of sex hormones on GABAergic circuit function are: i) sex hormone blockers, in which androgen receptors (AR) and/or estrogen receptors (ER) antagonists are used to test whether the hippocampus is indeed a critical site of androgen and estrogen modulation through classical AR/ER [339,340]; and ii) gonadectomy, in which animals are bilaterally castrated to reduce circulating levels of gonadal hormones, with or without subsequent sex hormones treatment, during development [341] or in adulthood [342] (organizational vs. activational periods). These experimental paradigms would allow testing causality in animal models. Accordingly, differences in spontaneous GABAergic activity at P40 can be further investigate by exploring the effects of cyclical variation of sex hormones during estrous cycle, given that sex hormones have been suggested to contribute to fluctuation of brain GABA levels in healthy females [343].

4.4. Sex-specific differences affect stress-induced epileptogenicity after the induction of febrile seizures in neonatal rats

To determine the impact of stress on epileptogenesis, we used daily postnatal injections of CORT in rat pups to mimic a chronically stressed state during development, i.e., during the stress hyporesponsive period and induced hyperthermia febrile seizures. The results from our second article (Chapter III) showed that CORT injections increased basal plasma CORT levels before HS and reduced weight gain and body temperature threshold of hyperthermia-induced seizures in both male and female rat pups. After hyperthermia-induced seizures, CORT-injected males had greater plasma CORT levels than females. In addition, we found that repeated exposure to increased CORT levels exacerbates febrile seizures in rodents and male rats are more affected compared to the females. In particular, we found higher number and longer duration of seizures induced by hyperthermia in CORT-injected males compare to females. Sex-specific differences were also found at baseline conditions with lower latency to generalized convulsions and longer duration of electrographic seizures in males but not in females. Finally, we reported

greater amplitude of evoked excitatory postsynaptic potentials as well as spontaneous inhibitory postsynaptic currents in CA1 in CORT injected juvenile male rats compared to control males.

Males are more susceptible to seizures in the newborn stage than females, according to experimental and clinical findings [344–347]. Steroid hormones are produced and released by the ovary, testes, and adrenal glands, and they play an important role in the neuroendocrine regulation of neuronal excitability and seizure susceptibility [349]. As a result, it's possible that sex hormones alter newborn seizure susceptibility [137,138].

Complex febrile seizures are a major risk factor for the subsequent development of mTLE, which are characterized by spontaneous recurrent seizures (SRS), and which develops in 30-50% of patients with a history of febrile seizures [350,351]. Because the causality between hyperthermic seizures and SRS is not fully established, there are speculations that hyperthermic seizures (and perhaps febrile seizures) on their own may not be sufficient to predispose the hippocampus to epileptogenesis. In this context, certain clinical investigations suggest that a considerable percentage of mTLE patients with febrile seizures had neurologic or developmental brain problems prior to febrile seizures, including focal cortical dysplasia [266,351,352], which has been demonstrated by our lab [123,222,275], as well as early life stress, suggesting that when febrile seizures is preceded by a brain trauma, the likelihood of the development of mTLE is higher. Hence, animal models are important for investigating and testing this “two-hit” hypothesis.

Our two-hit model constitutes an important advancement to the field because there seems to be a response to chronic CORT administration and an impact on seizure susceptibility, proposing a connection between early-life stress and complex febrile seizures. Furthermore, the strength of this study resides in the inclusion of both sexes to study epileptogenesis. Overall, these findings further support that these differences in sex hormones may influence seizure severity and outcomes in patients with a history of complex febrile seizures. Interestingly, our current data demonstrate that sex differences are present at least 3 months before the development of SRS [275]. In conclusion, CORT alone renders the hippocampus vulnerable to a second insult leading to a reduction of the induction threshold of subsequent seizures together with eliciting synaptic function functional alterations. Based on these findings, our experimental two-hit models support

the hypothesis that the presence of early-life stress and febrile seizures may have additive (or maybe synergistic) epileptogenic effects on the brain, allowing or even forcing the brain to seize.

One limitation of this study is that, although sex differences were investigated in most of the experiments in this study, we did not record spontaneous inhibitory and excitatory post-synaptic currents (sIPSCs and sEPSCs) onto CA1 pyramidal cells collected from females at P18-P22, i.e., in a young adult age in which we have previously shown that neuronal circuit alterations are already present [291]. Possible sex differences in the experimental two-hit model can speculate about resilience and vulnerabilities to chronic stress and epileptogenesis between sexes [162].

Furthermore, total CORT levels are used to draw conclusions about animals' stress status and response. But following their release from the adrenal gland, most corticosteroids are bound by corticosteroid-binding globulin (CBG). Therefore, only free CORT leaves the circulation and has biological effects on CORT-sensitive tissues [176]. Our study measured total CORT levels, which is a shortcoming of our study, given that only free CORT levels can provide a more reliable information about an animal's stress response.

Lastly, because early life stress is known experimentally to have both short-term and long-term neurodevelopmental effects on limbic structures [222,353], another limitation of our experiments is related to the investigation of the SRS and consequently emergence of mTLE, which was not analyzed in the present study. However, it can be explored in freely-moving animals from different experimental groups after the implantation of bipolar electrodes at P90 and video-EEG recordings. Accordingly, our group has previously reported that freeze-lesion followed by prolonged febrile seizures at P10 resulted in sex-based differences in the long-term vulnerability to developing epilepsy, in which male rats developed mTLE from P90 onwards, whereas female rats did not [222]. Male rats were also found to have elevated levels of the stress hormone corticosterone after the P1 lesion compared to female rats. A similar result was observed in female rats treated with testosterone [222]. In addition, the latent phase between the two early-life insults and the development of epilepsy can be used to further investigate the occurrence of several

neuronal circuit alterations that occurs well before seizure onset, as demonstrated by the currently study.

4.5. Sex-specific differences of stress response: future perspectives

There appear to be sex-specific differences in the appraisal/perception of how stressful a given event is at the moment, therefore sex differences are likely to rely on the type of the stressor. Overcrowding, for example, has been demonstrated to cause more stress in males, but social isolation causes more stress in females [207–209]. As a result, sex-specific vulnerabilities to chronic stress are likely to come from the essentially distinct techniques utilized to cope with the stressor, rather than a lack of ability to cope with the stressor per se [354,355].

These observations validate the investigation of CORT injection effects in regulation of the stress response in rodents as good experimental model, however an interesting approach would be to use a more physiological/natural model of stress. For this purpose, in rodents the most frequently used paradigms are daily handling [356–358], repeated maternal separations for 3-6 hours [359–361] and a single 24-hour maternal deprivation [210,362,363]. These paradigms are applied during the stress hypo-responsive period or during the period from birth until weaning. The outcome of each of these treatments is different depending on age, sex and strain of the pup and also on the duration and frequency of the separation. In addition, pre-natal manipulations in pregnant rodents are also interesting and several paradigms can be used, such as crowding , noise, saline injections, or restraint stress [364–367]. An interesting model of prenatally stressed febrile seizure rat model, as shown by Qulu *et a.l*, was developed by subjecting pregnant rats to restraint stress (1 hour daily, from gestational day 14 to 20). Following the birth of the rat pups, febrile seizures were induced on postnatal day 14 (intraperitoneal injection of 200 µg/kg lipopolysaccharide (LPS), followed by an intraperitoneal injection of 1.75 mg/kg kainic acid 2.5 hours later) [368]. The development of this particular model provides a means to conduct further sex-specific investigations on prenatal stress and febrile seizures.

Lastly, it is well established that KCC2 activity is important to maintain a hyperpolarizing GABAergic neurotransmission. Low KCC2 activity, on the other hand, should result in depolarizing and, under some circumstances, excitatory GABAergic transmission [107,120]. Changes in KCC2 expression and activity have been associated to epilepsy, which is not surprising considering KCC2's crucial function in regulating the inhibitory drive [123,124]. In order to further explore the results described in Chapter II and combine with experiments done in Chapter III, it would be interesting to study expression levels and function of KCC2 in both male and female rats in the two-hit model consisting of early life stress followed by hyperthermia-induced seizures. Revealing the alterations of KCC2 expression and function may further our understanding of the subsequent circuit-based alterations, which are different between sexes (Chapter II).

4.6. Further considerations: sex and gender in health research

4.6.1. Disease Prevalence

When it comes to cardio-vascular disease, women have have a lower incidence of hypertension than age-matched males over the majority of their lives [369], but after menopause, hypertension in women equals or exceeds that in men [370]. Nonetheless, according to Anastos *et al.*, 65% of the studies addressed research on males, none on females (sex bias), 10% covered both sexes, and 25% failed to indicate sex (sex omission) [371]. In regard to neurological and neuropsychiatric disorders, despite the fact that women are 1.5 times more often than men to develop numerous clinical pain conditions [32], 79 % of the research exclusively looked at males accordingly to [372]. With regards to affective disorders, women are diagnosed with anxiety disorders 2.25 times more often than men [13], but the majority of animal studies on anxiety and anxiolytic drugs

focus on male rats [209], whilst about twice as many women as men experience depression [30], but still models of depression were developed using male rodents [373]. On the other hand, multiple sclerosis is approximately 3 times more prevalent in women than men [374] and accordingly to Voskuhl and Palaszynski, 85% of the studies addressed research on female mice or rats (sex bias), whereas none was done in males, and 15% left sex unspecified (sex omission) [375]. In this instance, when it comes to illness susceptibility, focusing on females makes sense when the objective is to unravel the factors that contribute to women's increased disease vulnerability.

4.6.2. Adverse Effects to Medications

With regards to epidemiological and clinical studies, men and women respond to various medications differently, as evidenced by sex differences in reaction to several drugs [376], in which adverse drug responses are 1.5–1.7 times more common in women than in males [377]. Unfortunately, information on sex differences in side effects is not included in product descriptions available to patients [378], despite well-established sex differences in pharmacokinetics and pharmacodynamics from medications. Therefore, to ensure the safety and efficacy of therapy, it is vital to understand the sex variations in medication reactions [376]. In response to that, 8 out of 10 prescription drugs were withdrawn in the United States in 2005 due to women-specific side effects and health issues [379].

4.6.3. Sex and gender in health research is a persisting data gap

Despite some recent effort and progress, sex and gender in health research is still a persisting data gap mainly because of interrelated challenges that remain with acknowledging and understanding the impact that these concepts can have on the evidence produced by health research [380]. The challenges are as follow:

1. The terms sex and gender are frequently misunderstood by the general public and in publications with conflicting language [1,2]. To determine if biological or social aspects are being discussed, clarity is essential. In certain cases, interactions between sex and gender are difficult to separate, but in general, it is important to rectify misused terminology.

2. Failure to recognize the impact of sex and gender on research design and outcomes, difficulties in applying the concepts and challenges with data collection and datasets, most of the times restrict the inclusion and analysis of both sexes in animal and human studies [381]. However, researchers should do their best to ensure that studies account for potential sex differences in order to move towards a more personalized approach in health research and make it a standard practice. Although, it is needed to be recognized that the fact of recruiting 50:50 human participants for a sex-skewed disease can be challenging and not always possible.

3. Impossibility of recruiting enough of the underrepresented sex or gender in order to draw robust conclusions [382,383]. The recommendation would be to oversample of the underrepresented sex so that the sample size will have enough power to draw statistically valid conclusions.

4. Lack of data disaggregation [384]. Raw data should be presenting by sex or gender, in order to help future meta-analyses.

5. Continue using the argument that females are more variable because of their estrous cycle. According to a recent meta-analysis of almost 10,000 published measurements of molecular and behavioural features in male and female mice found that there was no difference in variability across genders for any endpoint; in fact, for some measures, the variability was higher in males [385]. This finding has been reached by similar meta-analyses on other species as well [372,386,387].

4.6.4. Plan of action

Our collaborative efforts must be strengthened to promote the inclusion of sex and gender as normal practice in health research in order to enhance health. Innovative and relevant health research can only be achieved by studying the impact of sex and gender on health. While a number of resources have been developed towards this purpose (as mentioned in Section 1.2.2), researchers and reviewers need more comprehensive techniques and methods for integration and assessment if we are to improve the inclusion of sex and gender in health research to promote stronger evidence. This goal has a significant impact on how policies and planning decisions are made with respect to the implications that this might have on men and women.

Lastly, it is important to recognize that failure to take into account these concepts and analysis exposes one to considerable sources of error, putting patient safety at risk [379]. Sex and gender are also important considerations in the development of personalized medical therapy, which is a very important contribution in light of the increased emphasis on patient-centered care [388,389].

– CHAPTER V –

CONCLUSION

Based on the results outlined in this thesis, we showed the complex role of sex hormones during development and in young adult rats of both sexes. In fact, the inclusion of both sexes associated with manipulating sex hormones provided an opportunity to compare GABAergic circuit development and stress responses associated with hyperthermic-induced seizures. In Chapter II, our results highlighted sex-related GABAergic differences in the hippocampus during development and in young adults. In Chapter III, our data illustrated how sex hormones represent a substantial factor in seizure susceptibility, especially during specific developmental time-windows. In pursuit of a better understanding of the role of sex hormones in different neurological disorders models, it is imperative that future studies carefully consider both sexes in their studies, even when considering diseases that afflict men and women with equal prevalence, because there might be sex-related differences in the underlying mechanisms and/or risk factors. Further experiments can help expand the knowledge base of male and female biology and inform appropriate individualized care for women as well as men, improving the quality of life of the patients.

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