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Genetic Sex Differences in Early Human Neuronal Development

An Investigation in Embryo Tissue and Embryonic Stem Cells

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Abstract
Pottmeier, P. 2023. Genetic Sex Differences in Early Human Neuronal Development. An Investigation in Embryo Tissue and Embryonic Stem Cells. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 2263. 84 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1795-3.

Sex differences in the human body affect many different organs and tissues, some of them have an effect on the human brain and its development. In the developing nervous system, sex differences can bias the number or functionality of neurons, glial cells or synapses. As a result, neural networks might develop with a sex-specific bias. A number of neurodevelopmental diseases, such as Tourette-Syndrome or Attention-Deficit/Hyperactivity Disorder, show sex differences in symptoms, onset and prevalence. It seems likely that sex differences in brain development contribute to differences in neurological disease susceptibility between males and females. In my work, I am investigating sex differences in gene expression during neuronal development in human embryo brain tissue, embryonic stem cells and neural stem cells. Of particular interest for sex differences are the genes of the sex chromosomes, since a large number of X-linked genes and even some Y-linked genes are implicated in neurodevelopment.

In our **first study**, we found that Y chromosome genes are highly expressed in fetal brain tissues and 5 X/Y homologous genes have an increased gene dosage in male samples. We suggest 6 novel long non-coding RNAs that were expressed in previously unannotated regions of the Y chromosome in male fetal brain tissue. In our **second study**, we identified an increased rate of proliferation in male neural stem cells but similar neuronal differentiation trajectories in cells of both sexes. An increased expression of DCX and DLG4 suggests a faster differentiation of male neural stem cells, but sex differences disappeared after 14 days. Male cells overexpressed MASH1 and RELN, markers for Cajal-Retzius neurons, and the two demethylases KDM5D and UTY. Female cells overexpressed RMST a long non-coding RNA critical for neurogenesis. In the **third study**, sex-biased gene expression was investigated in human embryonic stem cells during 37 days of neuronal differentiation. Male and female cell lines showed sex-biased expression of genes involved in neurodevelopment, suggesting a sex difference in differentiation trajectory. We propose 13 sex-biased candidate genes that could strongly affect neuronal development. In addition, we confirmed the gene dosage compensation of X/Y homologs escaping XCI through the Y-homolog and identified a significant expression of the Y-homologs TXLNGY and UTY after 37 days of neuronal differentiation. We have also measured a significant increase of the Y-linked genes PCDH11Y, UTY and USP9Y during differentiation. The **fourth study** was an investigation of sex differences in H3 methylation and acetylation marks in embryonic stem cells. We found that H3K4me3, a transcription activation mark, was enriched at promotor sites of major pluripotency genes and related pathways, in female cell lines.

In conclusion, we confirm the importance of Y chromosome genes for neuronal development and show that sex differences in gene expression exist during neuronal differentiation.

Keywords: sex chromosome, gametolog, x/y homolog, male, female, sex differences, sex bias, embryonic stem cells, neurodevelopment, neuronal differentiation, neural stem cells, genetics, neurodevelopmental disorders

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To my family and all my nearest and dearest,

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I.   Johansson MM, **Pottmeier P**, Suciu P, Ahmad T, Zaghlool A, Halvardson J, Darj E, Feuk L, Peuckert C and Jazin E (2019). Novel Y-Chromosome Long Non-Coding RNAs Expressed in Human Male CNS During Early Development. *Frontiers in Genetics* 10, 891.

II.   **Pottmeier P**, Doszyn O, Peuckert C and Jazin E (2020). Increased Expression of Y-Encoded Demethylases During Differentiation of Human Male Neural Stem Cells. *Stem Cells and Development* 29, 1497-1509.

III.   **Pottmeier P,** Nikolantonaki D, Lanner F, Peuckert C and Jazin E (2023). Sex-biased gene expression during neural differentiation of human embryonic stem cells. M*anuscript*.

IV.   **Pottmeier P**, Hetey S, Yuk Wong Yung P, Jazin E and Elsässer S (2023). Increased H3K4me3 at promoter sites of pluripotency genes in female human embryonic stem cells. *Manuscript*.

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Content

Abbreviations

mbp	megabase pair
CNS	Central nervous system
NRY	Non-recombining region of Y
SRY	Sex determining region of Y
XIST	X inactivation specific transcript
XCI	X chromosome inactivation
GSEA	Gene set enrichment analysis
DEG	Differentially expressed genes
lncRNA	long non-coding RNAs
Y-lncs	Y chromosome long non-coding RNAs
Gametologs	X/Y homologs
R / arg	Arginine
K / lys	Lysine
H3	Histone 3
me3	Tri-methylation
ac	Acetylation
H3K4me3	Histone 3 lysine 4 tri-methylation
H3K27me3	Histone 3 lysine 27 tri-methylation
H3K27ac	Histone 3 lysine 27 acetylation
hESC	Human embryonal stem cells
mESC	Mouse embryonal stem cells
iPSC	Induced pluripotent stem cells
pcw	Post conception week
$A\beta$	Amyloid-beta
FCG	Four core genotype mouse model

Introduction

The prevalence, age of onset, and clinical symptoms of many neurological disorders differ between males and females. Disorders such as Parkinson's disease and autism spectrum disorder are for example more common in males, while depression and anxiety spectrum disorder are more frequent in females (more examples and references in **Table 1**). Many sex-biased neurological disorders are believed to be of neurodevelopmental origin. Neurodevelopmental disorders arise from deviations in the normal developmental trajectory of the brain, which can result in an abnormal neuronal architecture or function. The development of the human brain starts already soon after conception and continues into early adulthood. During this time, any difference between the male and female physiology could contribute to the acquisition of sex differences in the developing brain.

One of the most investigated and best documented factors leading to sex differences are sex hormones. Sex-specific hormones are released after the differentiation of the gonads and act on hormone receptors, which in turn regulate a wide range of trophic effects in cells of the developing embryo, including cells of the developing brain. But sex hormones are not the only factors that have an effect on the developing brain. Also, genetic components, such as genes of the sex chromosomes or the autosomes, can be expressed in a sex-specific manner and thus contribute to a sex-biased development of the brain. The effect of sex-biased gene expression is most notable in the early developmental period of sex determination, before sex hormones are synthesized and released. After this point, sex hormones and sex-biased gene expression work in combination to contribute to sex differences.

Relatively little is known about sexual differential gene expression in early brain and neuron development. In my work I am investigating sex-biased gene expression in early embryo tissue and in human embryonic, as well as neuronal stem cells that differentiate into neurons. With this, I am contributing to the understanding of the magnitude and potential effects of sex-biased gene expression during early brain and neuron development. Ultimately, I hope that my results also add to the identification of sex differences and equalities in neuron development, as well as to the identification of factors leading to the sex-biased susceptibility of neurological disorders.

Sex differences and sexual dimorphisms

The terms sex difference and sexual dimorphism both describe differences between females and males of the same species. Although, the term sexual dimorphism is often used to describe morphological differences, it can also be used to describe any other biological process that differs between the sexes. In my work, I will use these terms interchangeably.

In mammals, sexual dimorphisms can manifest in many ways, such as in differences in size, weight and color or the development of different secondary sexual characteristics. Apart from the previously mentioned anatomical and physiological differences, sex differences can also lead to differences in behavior, such as courtship or mating. In fact, the best documented sexual dimorphisms are often related to reproduction and lead to e.g. difference in reproductive organs and reproductive behaviors.

In humans, the most obvious sex differences are also related to reproduction, such as the difference in sex organs and secondary sexual characteristics. Contemporary research however, shows that sex differences in humans go far beyond those of reproduction. Sex differences between females and males are reported in many disciplines of life-science, such as immunology, genetics and epigenetics, cancer research, behavioral research and neuroscience [1–5].

Especially in the field of developmental biology and neuroscience, researchers have been working extensively to understand sex differences and their origin. The importance of understanding sex differences in these fields is highlighted by many neurological and neuropsychiatric diseases that show sex biases in disease prevalence, symptoms and age of onset [6].

The origins of sex differences are not easily deciphered. Scientists have discovered a multitude of factors that each contribute to the development of sexual dimorphisms. They can roughly be categorized into genetic, genomic, epigenetic, hormonal and environmental factors. The contribution of each of these factors will be discussed in later chapters of this work 'Sources for sexual differential gene expression'. However, in principle any of these factors can contribute to the sexual dimorphic differentiation of tissues during human development.

Sex differences in neurological disorders

A surprisingly large number of neurological disorders show differences in prevalence, age of onset or symptoms between males and females (**Table 1**). Neurological disorders arise from deviations in neuroanatomy and neural circuitry, often as a result from disruptions in normal neurodevelopmental trajectories, and lead to a cascade of downstream effects across physiology, cognition, behavior, and affect. Based on the manifestation of the symptoms, their effect and their physiological cause, disruptions in the nervous system are then classified into different neurological disorders. Some of the most predominant neurological disorders that display sex differences are listed in the table below.

Table 1: Neurological disorders with sex biases in prevalence, age of onset or symptoms

Disorder	Prevalence ratio M:F	Sex Bias
Alzheimer's Disease (AD)	1:2	Almost twofold higher prevalence in women [7–11]. Faster cognitive decline in women [12,13].
Depression	1:2	Twofold higher prevalence in women [14,15]. Depression and anxiety are often present together [16].
Anxiety spectrum disorders	1:2	Prevalence for anxiety spectrum disorders twice as high in women [17–20].
Eating disorders	1:3-10	Higher prevalence for eating disorders in females [21–25].
Parkinson's Disease (PD)	2:1	Prevalence and age of onset higher in males [26–29]. Faster cognitive decline in males [30]. Symptoms differ among male and females [31–33].
Autisms spectrum disorders (ASD)	4:1	Prevalence higher in males [34–37].
Schizophrenia (SCZ)	1.4:1	Higher prevalence in males [38,39]. Symptoms differ between males and females [40,41]. Age of onset earlier in men, lower chance of full recovery, and poorer prognosis in men, anatomical brain differences between male and female patients [42,43].
Attention-deficit hyperactivity disorder (ADHD)	2:1 - 10:1	Prevalence higher in males [44–47]. High comorbidity with ASD [48].
Tourette's syndrome	3-4:1	Prevalence is higher in males [49].

Interestingly, many male-biased neurological conditions are early-onset neurodevelopmental disorders, such as autism, attention-deficit hyperactivity disorder (ADHD), conduct disorder, specific language impairment, Tourette syndrome, dyslexia, schizophrenia or cerebral palsy. Symptoms of some early onset neurodevelopmental disorders can already be seen in infants and latest in toddlers. Female-biased neurological disorder on the other hand, are often so called emotional disorders such as depression, anxiety disorder, and eating disorders, which usually start during puberty or later in life [6,50–52]. The term emotional disorder describes a set of chronic and often recurring psychiatric disorders that come along with significant impairments in quality of life, productivity, and interpersonal functioning.

The presence of so many sex differences in neurological disorders and especially the neurodevelopmental component in male-biased early onset disorders, indicates that the developing nervous system includes components that differ between male and females. These differences are not insignificant, as they seem to provide females with a protective advantage against neurodevelopmental disorders, underlining the importance of sex differences in neuroscience and developmental biology.

Sexual differentiation in humans

To understand how and why sex differences arise in the human body, it is essential to understand the concept of sexual differentiation. Sexual differentiation is the process in which cells and tissues develop differently between the sexes. For a long time genetical sex determination and the subsequent release of sex hormones was thought to be the main driver of sexual differentiation. This theory is referred to as the classical theory. Today however we know that more factors including gene expression from sex chromosomes and autosomes a well as epigenetic and environmental factors, are involved in sexual differentiation.

The classical theory of sexual differentiation

In the classical theory of sexual differentiation, genetical sex determination sets the foundation for all other sexual differentiations. In humans, the sex chromosomes (X and Y) are responsible for genetic sex determination. The presence of a Y chromosome in the genome of the zygote leads to the differentiation of the primordial gonads into testes later in development. The absence of a Y chromosome, on the other hand, leads to the development of ovaries. Our current understanding of these processes is still limited but in principle it is the influence of the gene SRY, which is present on the Y chromosome, that leads to the development of testes [53–55], while the factors that are expressed when a Y chromosome is absent, such as FOXL2, lead to the development of ovaries [56–58]. Researchers have found that the expression of SRY, and thus also the beginning of sexual differentiation, starts as early as week 6 in the human embryo development [59].

According to the classical theory of sexual differentiation, sex differences in humans arise as a result of different concentrations in circulating steroid hormones (sex hormones). The male sex hormones (androgens) are first secreted in the beginning of week 12 of development, after the primordial gonads differentiated into testes [60]. This leads to high levels of androgens (testosterone, androstenedione) in the male embryo while the fetal ovaries of female embryos promote an elevation of estrogen concentrations. Sex hormones bind to androgen or estrogen receptors in the developing tissues and can lead to permanent organizational effects (irreversible) or temporary activational

effects (reversible), which in turn lead to the sexual differentiation of tissues or whole regions [61]. It is believed, that sex hormones convey their major effects in specific hormone sensitive periods [62]. The first of such periods, is for example, the previously mentioned period in which sex hormones are first secreted and cause the differentiation of the primordial gonads (week 12 to 14) in embryo development. Another such period is the so called 'mini-puberty', which stretches across the first 3-6 month after birth and that is characterized by a testosterone surge in males, as well as increasing estradiol levels in females [63,64]. The last well described period of increased tissue hormone sensitivity is puberty, at the age of 10-16 years [65–67].

This knowledge about sex hormones and sex chromosomes has led to the classical two-stage concept of sexual differentiation. In a first stage, sex chromosomes initiate the genetic sex determination. Once the male and female gonads have formed, stage two begins and is characterized by the sexual differentiation of tissues through the effect of differences in sex hormone concentrations [68].

A change in sexual differentiation theories

Today, the classical two-stage hormone-centered theory is considered as incomplete. Researchers have found undisputable evidence that demonstrate the presence of sex differences before the production of sex hormones. First evidence came from developmental studies in rats. It was found, that male rat embryos weighed more than female rat embryos before sexual differentiation had occurred [69]. Another study in rats displayed a higher number of tyrosine-hydroxylase-immunoreactive dopaminergic neurons in female rat embryos before hormone release [70]. At a similar time, a number of experiments in mice have demonstrated that different numbers of Y chromosomes in the same mouse strain lead to different aggressive behaviors [71,72]. The study of a rare bilateral gynandromorphic zebra finch has led to some remarkable implications regarding the role of genes in sex differences. This bird was showing female-typical phenotypes in the left side of its body and male-typical phenotypes in the right side. It was shown that one half is entirely genetically female (ZW) and the other entirely genetically male (ZZ) [73]. Since the different sex-typical phenotypes, including brain differences, were expressed in the same body, they were exposed to the same sex hormones. This speaks for a major involvement of genetics in the development of sexual differentiation. Similar work on zebra finches [74], gynandromorphic chickens [75] as well as tammar wallabies [76] strengthened this implication. An outstanding approach of demonstrating the involvement of sex chromosomes on sexual differentiation was done by the creation of the so called 'Four Core Genotype' (FCG) mouse model. The main principle in this approach was the

removal of the gene *Sry* from the Y chromosome (Y^{-Sry}) and the translocation onto an autosomal chromosome (+*Sry*). This allowed the generation and comparison of the following four distinct genotypes: 1) 'XY^{-Sry} +*Sry*' *and* 2) 'XX +*Sry*' which are both gonadal male, as well as 3) 'XY^{-Sry}' and 'XX' which are both gonadal female [77]. By comparing the phenotypes of these mice, effects of hormones and sex chromosomes can be dissected. This has led to new insights into XX and XY difference of behavior, gene expression, and disease susceptibility, that are not mediated by gonadal hormones [78]. Apart from the FCG mouse model, there is the XY* mouse model, which is also frequently used to decipher the effect of sex chromosome genes [79].

A modern framework for sexual differentiation

The classic theory of sexual differentiation is relatively simple. It is based on the effects of sex chromosomes that affect gonadal differentiation and subsequently the sex hormone concentrations in males and females. A modern theory of sexual differentiation however, must incorporate a multitude of factors, such as gene expressions from sex chromosomes as well as autosomes; epigenetic effects of imprinting, DNA methylation, histone modification and X-inactivation; hormonal influences, as well as environmental influences including sociocultural factors. While the classical theory is a relatively linear model, a new model should resemble more of a branched network with back-and-forward interactions that altogether shape the sexual differences in human tissues and organs. The following figure (**Figure 1**) summarizes the different factors that should be considered in a modern theory of sexual differentiation.

While previously, it was believed that the point of gonadal differentiation was also the key point for sexual differentiation, contemporary research has come to a point at which sex-biased differential gene expression has already been identified in the germ line [80,81] and as early as in the 2-8 cell stage of embryonal development [82–88]. This is long before the differentiation of the gonads and before the influence of fetal sex hormones. This vast evidence proves that sexual differential gene expression occurs basically as soon as the transcriptional machinery is active.
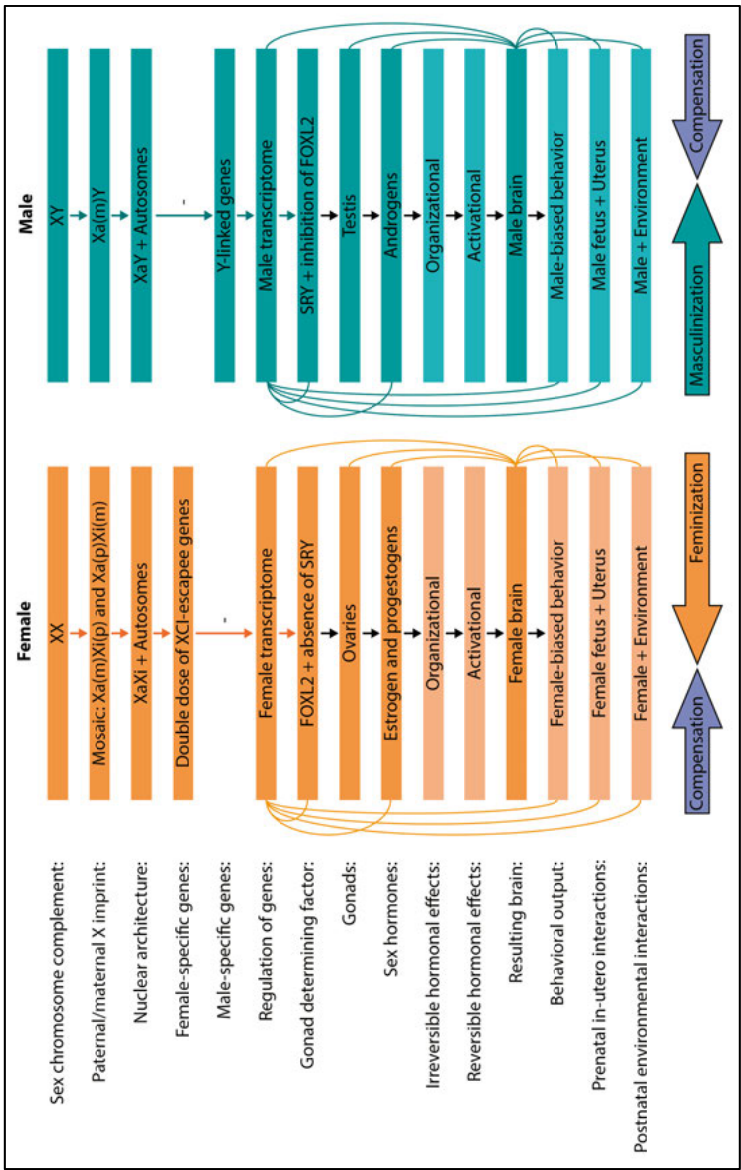
Figure 1: Schematic structure of sexual differentiation of the mammalian brain. Based on a similar figure created by Björn Reinius and published in his PhD thesis [89]. The figure displays genetic, hormonal, parental and environmental influences on the sexual differentiation of the brain. The vertical arrows represent processes that lead to sexual differentiation of the brain. Black arrows illustrate processes of the classical model and bows represent the forward and backward interactions beginning from the transcriptome and the brain. Horizontal arrows depict the possibility of sex differences as a form of compensational force to prevent even stronger feminization or masculinization.

Sources for sexual differential gene expression

There is a consensus among neuroscientists that the development of the human brain continues into peoples 20's. During most of the time sex hormones as well as genetic factors simultaneously and in combination lead to the sexual differentiation of the brain. However, there is a particular interesting period in the early point of human embryo development, which stretches from the point of fertilization until week 12 of development. In this period sex hormones are not released yet [60,90]. This means that any sex differences at this point are a result of genetic factors, since embryonic gene expression is already activated hours after fertilization, in a process called maternal to zygotic transition [91,92]. This early period in human embryo development is particularly interesting from a genetic point of view, as a number of critical events that initiate the development of the early nervous system, such as neural plate formation, neural tube closure, neural crest differentiation and the beginning of neurogenesis, are taking place (**Figure 2**). Sexual differential gene expression in this period of development can therefore be decisive for the development of sex differences in the brain. In fact, the presence of sex-biased gene expression this early has already been documented [85,87,93–95]. The most obvious genetic factors, are the sex chromosomes (XX or XY). However, even genes on the autosomes display sexual differentially expression [96–98]. The presence of differential gene expression suggests, that there are also sex differences in the mechanisms that regulate gene expression. However, these mechanisms are numerous and complex and thus not easy to decipher. Epigenetic and genetic features, such as non-coding RNAs, genomic imprinting, X-inactivation, DNA methylation and histone modifications are all able to modify gene expression, alone or in combination, in a sex-specific manner.

In the following section, I am listing the currently known sources for sexual differential gene expression.
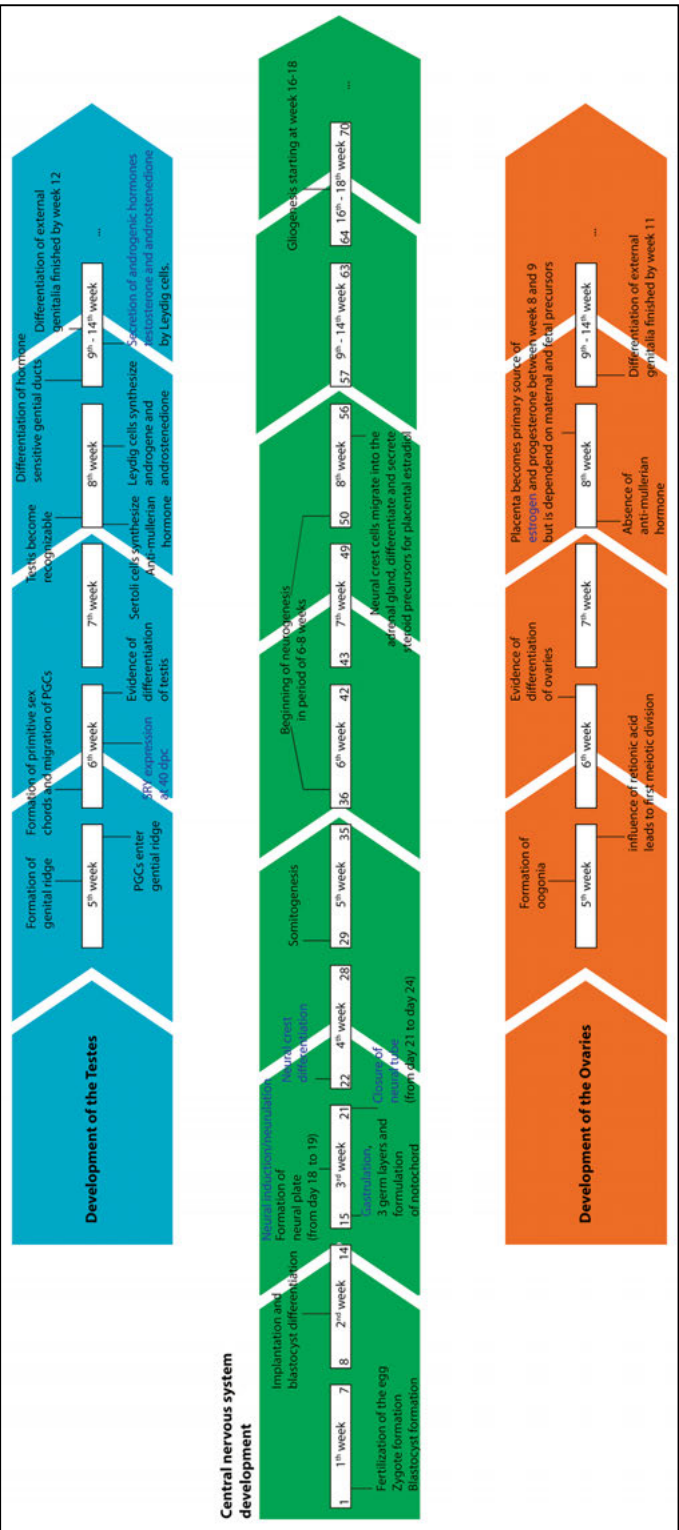
Figure 2: Timeline highlighting critical events of central nervous system development in human embryos and visualizing the first timepoints of sex hormone secretions in male and female embryos.

Sex chromosomes

The human genome is not exactly the same between males and females. The largest difference, and therefore theoretically the largest contributor to sex differences, is presented by the so-called sex chromosome complementation, the presence of a Y chromosome or an additional X chromosome in the genome of an individual. However, since the X and Y chromosomes have originated from a common ancestor autosomal pair, they also share a number of genes, the so-called homologous genes of X and Y aka. gametologous genes, which are discussed in a later section 'Gametologous genes, gene dosage and X-inactivation'. Over the course of evolution, the Y chromosome has lost a lot of its genetic content. Only about 3 % of the original ancestral genes survived [99,100] compared to 98% of ancestral genes on the X chromosome [101]. It seems like the Y chromosome has mainly preserved genes that were beneficial for males and harmful (or had no effect) for females. Today, the Y chromosome consists of approximately 60 mbp (accounts to ca. 2 % of the genome) from which almost half is constitutive heterochromatin which is considered inactive and without genes. Nevertheless, the Y chromosome encodes approximately 48 protein-coding genes, from which 28 are unique to the male genome. In large, these unique genes are coding for proteins and factors involved in reproduction such as testes development and sperm production. Apart from the protein coding genes, the Y chromosome also encodes 122 long-noncoding RNAs (lncRNAs) and approximately 379 pseudogenes (**Table 2**).

The X chromosome is comprised of ca. 155 mbp (accounts to ca. 5 % of the genome) and harbors approximately 859 protein-coding genes, 715 lncRNAs and 873 pseudogenes (**Table 2**). Unlike the Y chromosome, the genes on the X chromosome are involved in many different biological processes. The large number of pseudogenes on the X and Y chromosome might be surprising at first, for a long time pseudogenes have been considered as 'junk' DNA, however recent studies challenge this narrow perception and highlight the possibilities of pseudogenes to contain elements that allow the regulation of protein coding genes [102,103].

Table 2: Number of genes and non-coding elements of the X and Y chromosome and across the average autosome

	X-chromosome	Y-chromosome	Gametologous	Average autosome*
Protein-coding	859	48	21	886
Pseudogenes	873	379	13	606
lncRNAs	715	122	?	96
Total	2447	549	34+?	1588

* *calculated as average number across all autosomes*

Recombination events (crossing-overs) on the sex chromosomes are restricted to two narrow regions called pseudoautosomal regions 1 and 2 (PAR), **Figure 3**. These regions are located at each end of the chromosomes and are 2.6 mbp and 0.32 mbp in size. Within these regions genetic material is exchanged between the X and the Y chromosome, the rest of the chromosomes are so called non-combining regions. The non-combining region of the Y chromosome (NRY) is of special interest since theoretically, any gene or non-coding RNA expressed from this region is only present in males and should therefore directly contribute to sex differences.

It has been shown, that genes on the sex chromosomes contribute to sex differences in behavior (addiction, pain, learning, feeding, parental, sleep, social), in brain phenotypes and diseases, and in mouse models of various diseases including autoimmune, aging, neural tube closure defects, cardiovascular diseases (hypertension, cardiac ischemia/reperfusion injury, stroke, hypertension, atherosclerosis, and abdominal aortic aneurysms), immunity, metabolic disease and many more [79].
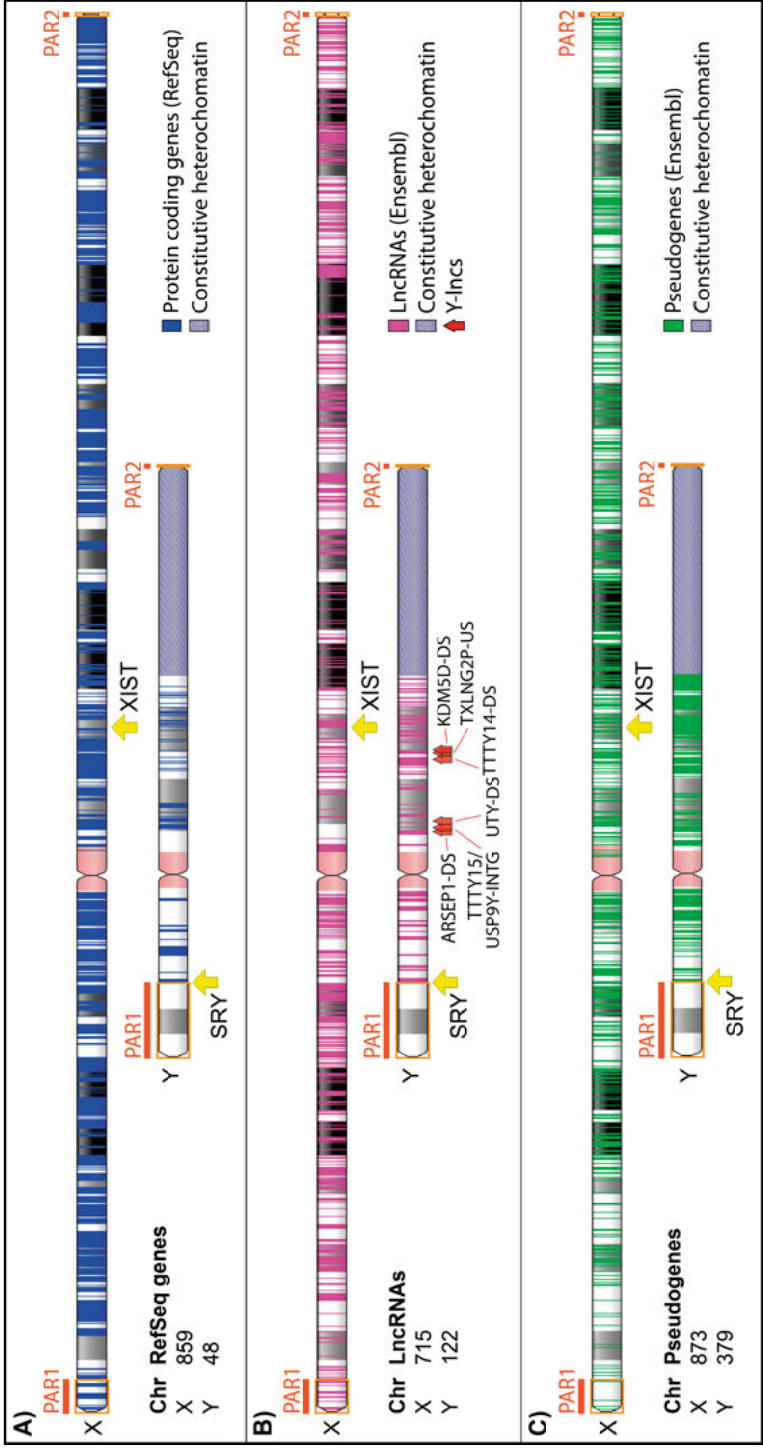
Figure 3: X and Y chromosome ideogram with G-banding at a resolution of 850 and PAR regions marked in orange. Y chr. long non-coding RNAs from Paper I are marked in red and the regions for SRY and XIST are marked with yellow arrows. **Subfigure A)** displays the location of all protein-coding genes described in the *RefSeq* assembly, **B)** shows all long non-coding RNAs and **C)** displays all pseudogenes on the X and Y chromosome. All data is extracted from the BioMart dataset: Ensembl Genes 109, GRCh38.p13, accessed March 2023.

X-linked genes

The study of gene expressed on the X chromosome (X-linked genes) has attracted a lot of attention in recent years since X chromosome genes have been extensively linked to functions and development of the nervous system [104,105]. On the X chromosome, the number of genes involved in neurodevelopmental and neurophysiological processes is predicted to be six-fold higher than on autosomes [106–108]. In addition, studies in human and mice have revealed, that X-linked genes are expressed at higher levels in the nervous system than in other tissue [108–110]. Mutations in X-linked genes are also often associated with disorders characterized by cognitive impairment such as Rett, Fragile X and Börjeson-Forssmann-Lehman syndrome [111–113]. Some of the X-linked genes that are of special importance for neurodevelopment and neurophysiology are KDM6A and KDM5C, MECP2, HDAC8, MSL3, MORF4L2, as they encode genes that function as epigenetic regulators of transcription. Others encode transcription factors such as PHF6 and others are proteins involved in translation and mRNA metabolism such as DDX3X, EIF2S3X and FMR1. There are modulators of protein activity through post-translational modifications such us deubiquitinase USP9X, ubiquitin ligase MID1 and the glycosyltransferase OGT. And at least there are also integral membrane proteins such us TMEM47/BCMP1 and the synaptic vesicle-protein synaptophysin (SYP).

Y-linked genes

In contrary to studies of X-linked genes, studies of the Y-linked genes in the sexual differentiation of the brain are not as popular. This is most likely due to the fact that the Y chromosome is believed to encode just a handful of protein-coding genes from which the most are associated with male reproduction. Some studies have, however, demonstrated that genes such as Uty, Ddx3y and Nlgn4y are critical for the development of the brain in males [114–118]. In addition, the potential of the non-coding regions of the Y as well as the X chromosome should not be underestimated. As mentioned previously, non-coding RNAs and pseudogenes display huge gene regulatory potential [119].

Gametologous genes, gene dosage and X-inactivation

Not all genes present on the Y chromosome are unique to males. In fact, a large part of the protein-coding genes 20 of 48 are also present on the X chromosome. The shared genes are referred to as homologous genes of the sex chromosomes or gametologous genes, **Figure 4 A**. These genes exist because the X and Y chromosome evolved from a common ancestor autosomal pair. The shared genes are highly conserved among mammals [120], but a number of gametologous genes on the Y chromosome have accumulated mutations

that have rendered their gene products unfunctional or have changed their function to cater to male specific biological processes. Gametologous genes have been shown to be associated to a large number of essential biological processes including cell signaling, expression of structure proteins, regulation of gene expression and histone modification (**Table 3**).

One approach to analyze sexual differential gene expression, is to compare the gene dosage between males and females. It is generally believed that there should be a balanced gene dosage between the two sexes [121–123]. Since females have two copies of the X chromosomes, they theoretically express twice the number of X chromosome genes. However, there is an epigenetic mechanism that prevents an increased X-linked gene dosage, as this kind of aneuploidies would be lethal to a human embryo. The mechanism is called X chromosome inactivation (XCI) and is mediated by the long non-coding RNA *Xist* which coats one X chromosome and recruits protein complexes to facilitate gene repression [124]. XCI leads to the packaging of the DNA into heterochromatin and therefore disables most parts of the second X chromosome. In this way, a balanced gene dosage of approximately one X is maintained. However, there are several mechanisms that can have an effect on this X-inactivation mediated gene dosage balance. One of them is the escape of X-inactivation which is controlled through an epigenetic mechanism of chromatin modification. Through this mechanism, some genes of the X chromosome are escaping the X-inactivation and are continuously able to be expressed from both X chromosomes. These genes are referred to as XCI-escapees. About 15 % of the genes on the human X chromosome escape X-inactivation (ca. 127 genes) and most of them are located in the PAR regions [125], **Figure 4 B**. In this context, gametologous genes play a special role. Since males possess an X and a Y chromosome they are subjected to gametologous gene expression from each of these two chromosomes. Females on the other hand, have two X chromosomes of which one undergoes XCI. By escaping XCI, some of the genes have demonstrated to compensate for the increased gametologous gene expression in males [110,126]. In general, most genes of the X/Y homologs in the PAR region are XCI-escapees. Genes that are located outside of the PAR and escape XCI are important candidates in eliciting sex differences as they are higher expressed in females [127], **Figure 4 B**. It has been shown that XCI differs among tissues and among individuals, which adds another level of complexity [128–130]. The escapees state can be switched on and off in certain genes, making them so called variable escapees [131,132]. In summary, X-inactivation and escapee mechanisms are complex and important regulators of X chromosome gene expression and dosage compensation.
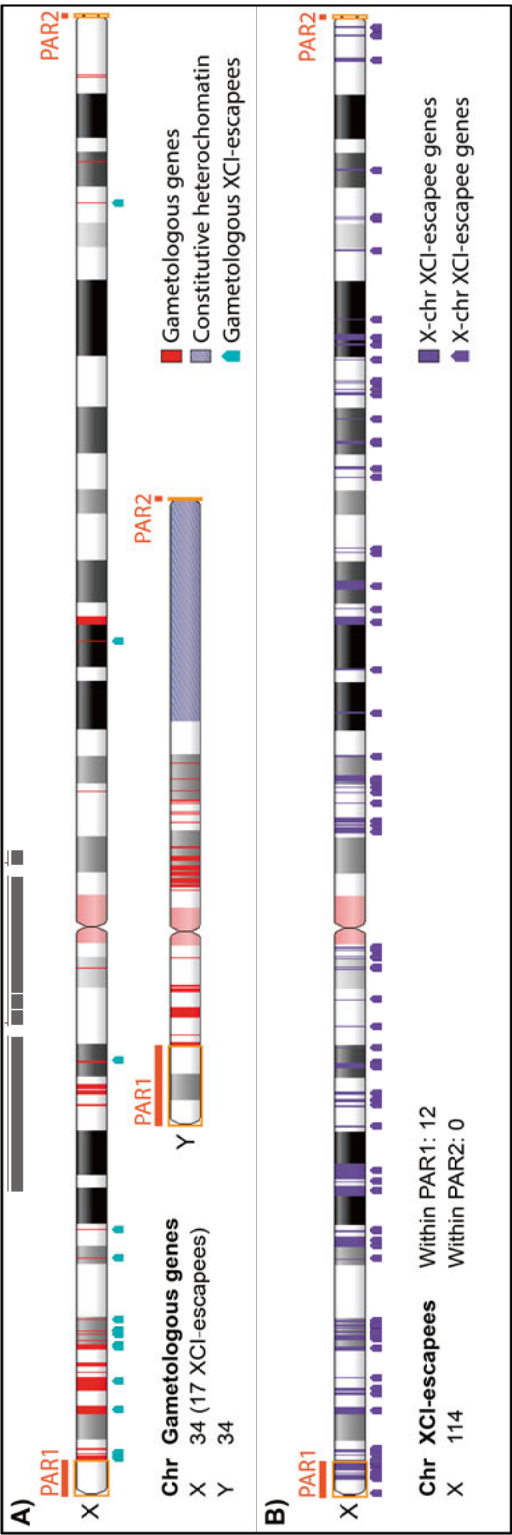
Figure 4: X and Y chromosome ideogram highlighting gametologous and XCI-escapee genes. Subfigure **A)** displays the homologous genes on chromosome X and Y. From the 34 gametologous genes, 17 are XCI-escapees, labelled with a teal mark below the chromosome (TBL1X, ZFX, ARSD, PRKX, NLGN4X, STS, TXLNG, EIF1AX, USP9X, DDX3X, KDM5C, RPS4X, PCDH11X, GYG2, TMSB4X, OFD1). Figure **B)** shows all X-linked XCI-escapee genes on the X chromosome, 12 of them are located within the PAR1 region (PLCXD1, CSF2RA, ASMTL, IL3RA, SLC25A6, P2RY8, AKAP17A, TCONS_00017125, DHRSX, TCONS_00017281, CD99, XG).

Table 3: Gametologous genes, their function and expression level in hESC

Gene expression in hESC was measured as an average of DEseq2 normalized counts in undifferentiated cells and in cells differentiating to neurons. Gene expression with counts <50 was classified as 'none', >50-200 as 'low', >200-2000 as 'medium' and >2000 as 'high'.

Y-gene	Gene/protein information	X-gene	Gene/protein information
GYG2P1 [Pseudo]	Protein is 3' truncated compared to its X chromosome paralog, leading to a non-functional protein. Expression in hESC: none	GYG2 [Coding]	Encodes a self-glycosylating protein involved in the initiation reaction of glycogen biosynthesis in the liver. Protein is involved in blood glucose levels. Expression in hESC: medium
ARSDP1 [Pseudo]	No information regarding gene function available. Expression in hESC: none	ARSD [Coding]	Encodes a member of the sulfatase family, which is essential for the composition of bone and cartilage matrix. Expression in hESC: low
ARSLP1 [Pseudo]	No information regarding gene function available. Expression in hESC: none	ARSL [Coding]	Encodes a member of the sulfatase family, which is essential for the composition of bone and cartilage matrix. Expression in hESC: low
ARSFP1 [Pseudo]	No information regarding gene function available. Expression in hESC: none	ARSF [Coding]	Encodes a member of the sulfatase family, which is essential for the composition of bone and cartilage matrix. Expression in hESC: none
MXRA5Y [Pseudo]	No information regarding gene function available. Expression in hESC: none	MXRA5 [Coding]	Encodes a matrix-remodeling associated protein. MXRA5 has recently been found to be involved in the MAPK pathways which plays a key role in the regulation of cell proliferation, survival, differentiation and apoptosis and is involved in signal transduction from the cell membrane to nucleus in response to a wide range of stimuli. Expression in hESC: none
PRKY [Pseudo]	Lost a coding exon resulting in nonsense-mediated decay of all transcripts. Abnormal recombination of Y and X gametologs is common cause for sex reversal disorder (XX males and XY females). Expression in hESC: low	PRKX [Coding]	Codes for a serine threonine protein kinase, which are regulated by cAMP signaling in cells. Implicated in several developmental processes, epithelial and endothelial cell differentiation, migration and vasculogenesis. Expression in hESC: medium

Gene	Description	Gene	Description
NLGN4X [Coding]	Encodes a type-B carboxylesterase/lipase protein, a neuronal cell surface protein that act as splice site-specific ligands for beta-neurexins. They may be involved in the formation and remodeling of CNS synapses and are thus involved in cell-cell interactions. Protein interacts with DLG4. Expression in hESC: high	NLGN4Y [Coding]	Encodes a type I membrane protein that belongs to the neuroligins. It is a neuron specific cell adhesion protein that is present at the postsynaptic side and is essential for cell-cell interaction. Expression in hESC: medium
STS [Coding]	Encodes a multi-pass membrane protein. Belongs to sulfatase family and hydrolyzes 3-beta-hydroxysteroid sulfates that serve as precursors for estrogens, androgens and cholesterol. Expression in hESC: low	STSP1 [Pseudo]	No information regarding gene function available. Expression in hESC: none
VCX [Coding]	Is a member of the VCX/Y gene family. Protein has unknown function but is exclusively expressed in male germ cells. Implicated in spermatogenesis and sex ration distortion. Different individuals have different numbers of VCX genes. Expression in hESC: none	VCY (BPY1) [Coding]	Is a member of the VCX/Y gene family. Protein has unknown function but is exclusively expressed in male germ cells. Implicated in spermatogenesis and sex ration distortion. Expression in hESC: none
VCX2 [Coding]	Is a member of the VCX/Y gene family. Protein has unknown function but is exclusively expressed in male germ cells. Implicated in spermatogenesis and sex ration distortion. Different individuals have different numbers of VCX genes. Expression in hESC: none	BPY2 (VCY2A) [Coding]	Is a member of the VCX/Y gene family. Protein is located in NRY and is expressed specifically in the testis. It interacts with protein ligase E3A and may be involved in male germ cell development. Expression in hESC: none
ANOS1 (KAL1) [Coding]	Encodes a glycoprotein of the extracellular matrix. Implicated in cell-adhesion, axonogenesis and neuron migration. Mutations in the gene are responsible for Kallmann syndrome. Expression in hESC: high	ANOS2P (KALP) [Pseudo]	Inactive homolog of ANOS1 due to frameshift resulting in premature stop codons. Expression in hESC: medium
TBL1X [Coding]	The encoded protein has sequence similarity with WD40 repeat-containing proteins. This protein family mediates protein-protein interaction and are involved in signal transduction, RNA processing, gene regulation, vesicular trafficking, cytoskeletal assembly and differentiation. The protein has been found to be a subunit in the corepressor SMRT complex together with histone deacetylase 3. Expression in hESC: low	TBL1Y [Coding]	The gene is believed to have a similar function due to high sequence similarity. However, studies suggest differences in function between the Y paralog and other members of the TBL1 gene family. Expression in hESC: low

X-linked gene	Description	Y-linked gene	Description
GPR143 (OA1) [Coding]	Encodes a protein that binds to heterotrimeric G proteins. It is thought to be involved in intracellular signal transduction. Until recent, the protein was thought to be only targeted to melanosomes of pigment cells, but it has now been found as a receptor for L-DOPA. The gene is widely expressed in the central and peripheral nervous system and is also located in Lewy-bodies. Expression in hESC: low	GPR143P (OA1P) [Pseudo]	No information regarding gene function available. Expression in hESC: none
SHROOM2 (APXL) [Coding]	The gene is expressed within endothelium and is implicated in amiloride-sensitive sodium channel activity. May be involved in endothelial cell morphology and regulate the biogenesis of melanosomes in retinal pigment epithelium. Expression in hESC: low	SHROOM2P1 (APXLP) [Pseudo]	No information regarding gene function available. Expression in hESC: none
AMELX [Coding]	Encodes a member of the amelogenin family which is involved in biomineralization during tooth enamel development. Expression in hESC: none	AMELY [Coding]	The gene is believed to have a similar function as its X chromosome paralog. Expression in hESC: none
TMSB4X [Coding]	Encodes an actin requesting protein which plays a role in the regulation of actin polymerization. Involved in the organization of the cytoskeleton, cell proliferation, migration and differentiation. Expression in hESC: high	TMSB4Y [Coding]	The gene is believed to have a similar function as its X chromosome paralog. Expression in hESC: low
OFD1 [Coding]	Encodes a centrosomal protein, which is involved in mother daughter cell centrioles length and in biogenesis of the cilium. Has a pseudogene on chromosome 5 and 15 on chromosome Y. Expression in hESC: medium	OFD1P1Y [Pseudo-gene]	No information regarding gene function available. Expression in hESC: none
TXLNG (CXorf15) [Coding]	Encodes a protein of the taxilin family, which binds to C-terminal coiled-coil region of syntaxin family members. May play a role in intracellular vesicle trafficking, cell cycle progression and bone mass density through an ATF4-dependent pathway. Expression in hESC: medium	TXLNGY (TXLNG2P) [Pseudo]	No information regarding gene function available. Expression in hESC: medium
EIF1AX [Coding]	Encodes an essential eukaryotic translation initiation factor require for protein biosynthesis at ribosomes. The	EIF1AY [Coding]	The gene is located in the NRY. It exhibits a similar function as the X chromosome paralog.

X-linked gene	Description	Y-linked gene	Description
	... protein is required for the binding of the 43S complex to the 5' end of capped RNA. Expression in hESC: high		Expression in hESC: medium
ZFX [Coding]	Encodes a member of the krueppel C2H2-type zinc-finger protein family. Involved in the regulation of self-renewal and differentiation of stem cells. Expression in hESC: medium	ZFY [Coding]	Might act as a transcription factor by binding to the consensus sequence 5'-AGGCCY-3'. Expression in hESC: low
BCOR [Coding]	Encodes a protein that interacts as a corepressor of BCL6 which ihibits apoptosis. Class I and II histone deacetylases have been shown to interact with this protein. Expression in hESC: medium	BCORP1 [Pseudo]	No information regarding gene function available. Expression in hESC: none
USP9X [Coding]	Encodes a member of the peptidase C19 family with deubiquitinase activity, processing ubiquitin precursors and ubiquitinated proteins. May play a role in regulating protein degradation and is an essential component of TGF-beta/BMP signaling cascade. Deubiquitinates SMAD4 and the mTORC2 complex component RICTOR. Involved in axonal growth and neuronal cell migration. Expression in hESC: high	USP9Y [Coding]	Is thought to exhibit a similar function as its X chromosome paralog. Expression in hESC: high
DDX3X [Coding]	Encodes a protein that is member of the large DEAD-box protein family and has ATP-dependent RNA helicase activity. Nuclear roles include transcriptional regulation, mRNP assembly, pre-mRNA splicing, and mRNA export. In the cytoplasm, this protein is thought to be involved in translation, cellular signaling, and viral replication. Facilitates HNF4A acetylation. Expression in hESC: high	DDX3Y [Coding]	This protein shares high similarity to DDX3X, on the X chromosome, but a deletion of this gene is not complemented by DDX3X. The protein is believed to have a similar function as its X chromosome version of this gene is expressed only in male germ cells and is implicated in sperm cell differentiation and proliferation. Expression in hESC: high
CASK [Coding]	Encodes a calcium/calmodulin-dependent serine protein kinase. The protein is a scaffold protein and is located at synapses in the brain. Catalyzes the phosphotransferase from ATP to proteins such as NRXN1 and plays a role in synaptic transmembrane protein anchoring and ion channel trafficking. Contributes to neural development	CASKP1 [Pseudo]	No information regarding gene function available. Expression in hESC: none

Y Gene	Y Description	X Gene	X Description
			and regulation of gene expression via interaction with the transcription factor TBR1. Binds to cell-surface proteins, including amyloid precursor protein, neurexins and syndecans. Expression in hESC: medium
UTY (KDM6C) [Coding]	The protein is believed to have a similar function as its X chromosome paralog. Expression in hESC: medium	KDM6A (UTX) [Coding]	Encodes a histone demethylase which contains a JmjC-domain and catalyzes the demethylation of 'Lys-27' of histone H3. In addition, it methylates 'Lys-4' of histone H3, and regulates the recruitment of the PRC1 complex and monoubiquitinating of histone H2A. Plays a central role in regulation of posterior development by regulating HOX gene expression. Expression in hESC: high
TSPY1 [Coding]	Encodes a gene that is specific to testis and may be involved in sperm differentiation and proliferation. Many functional paralogs and pseudogenes of this gene are found in human, but only a single, nonfunctional orthologous gene is found in mouse. Expression in hESC: none	TSPYL2 (TSPX) [Coding]	Encodes a protein that is localized to the nucleolus where it is functioning in chromatin remodeling and as an inhibitor of cell-cycle progression. The protein is part of the CASK/TBR1/TSPYL2 transcriptional complex which modulates gene expression in response to neuronal synaptic activity. May inhibit cell proliferation by inducing p53-dependent CDKN1A expression. Expression in hESC: low
KDM5D (SMCY) [Coding]	Is functionally similar to its X chromosome paralog. A short peptide derived from this protein is a minor histocompatibility antigen which can lead to graft rejection of male donor cells in a female recipient. Expression in hESC: medium	KDM5C (SMCX) [Coding]	The gene is member of the SMCY homolog family and encodes a protein with one ARID, JmjC and JmjN domain and two PHD-type zinc fingers. Encodes a histone demethylase that targets 'Lys-4' of histone H3. Contributes in transcriptional repression of neuronal genes by recruiting histone deacetylase and REST at neuron-restrictive silencer elements. Expression in hESC: high
RPS4Y1 [Coding]	Functional equivalent to its X chromosome paralog but not identical in sequence identity. Disease association includes Turner Syndrome. Expression in hESC: high	RPS4X [Coding]	Encodes the ribosomal protein S4, a component of the 40S subunit. Disease association includes Turner Syndrome and spermatogenic failure. The ribosomal control seems to be important in stem cell differentiation. Expression in hESC: high

Gene	Description
TGIF2LX [Coding]	Encodes a TALE/TGIF homeobox of transcription factors. The gene is expressed predominately in testis and may play a role in spermatogenesis. Expression in hESC: none
PCDH11X [Coding]	Encodes a protocadherin, a protein responsible for cell-cell adhesion. Protocadherins form adherens junctions between cells and regulate cytoskeletal complexes. They are predominantly expressed in the developing nervous system where they control axon guidance and dendrite arborization. PCDH11X is implicated in the regulation of differentiation and proliferation of neural stem cells. Expression in hESC: low
RBMX [Coding]	Encodes an RNA binding motif protein. The protein plays a role in pre- and post-transcriptional processes. The X chromosome gene variant is widely expressed in different tissues. It is implicated in tissue specific regulation of gene transcription and alternative splicing of pre-mRNAs. Expression in hESC: high
SOX3 [Coding]	Encodes a member of the SOX family of transcription factors. The protein is involved in the regulation of embryonic development (sex determination and formation of hypothalamus-pituitary axis) and in determination of cell fate. May function as a switch in neuronal development, and keeps neural cells undifferentiated by counteracting activity of proneural proteins and suppresses neuronal differentiation. Expression in hESC: low
HSFX1 and HSFX2 [Coding]	Encodes a heat shock transcription factor protein located in the nucleus. Is predicted to enable DNA-binding transcription factor activity involved in the regulation of transcription by RNA polymerase II. Expression in hESC: none
TGIF2LY [Coding]	Functionally similar to its X chromosome paralog. However, the C-terminus is divergent, suggesting the protein to act as a regulator of its X chromosome paralog. Expression in hESC: none
PCDH11Y [Coding]	The encoded protein is believed to be functionally similar to its X chromosome paralog. Expression in hESC: low
RBMY1A1 [Coding]	Encodes an RNA binding motif protein. The Y chromosome gene variant is expressed specifically in testis and is implicated in splicing regulation during spermatogenesis. Expression in hESC: none
SRY [Coding]	Encodes a protein that acts as a transcription factor and is the testis-determining factor, which initiates male sex determination. Is involved in different aspects of gene regulation including promoter activation or repression. In male adult brain it is involved in the maintenance of motor functions of dopaminergic neurons. Expression in hESC: none
HSFY1 and HSFY2 [Coding]	Functionally similar to its X chromosome paralog, but the Y chromosome variant is exclusively expressed in the testis and diseases associations include spermatogenic failure and azoospermia. Expression in hESC: none

Genomic imprinting

Genomic imprinting is an epigenetic mechanism that leads to the silencing of one of the two alleles of a gene. It is facilitated through DNA methylation and histone modifications [133]. Imprinting is a complex mechanism that has not been fully understood yet. Imprinting marks are accumulated throughout the lifetime of an organism and can be passed down from parents to their children. When we speak of an imprinted gene, one allele of the gene is silenced. Which allele that is, depends if it is maternally or paternally imprinted. If a gene is maternally imprinted, the copy of the imprinted gene from the mother is silenced. The opposite is true for paternally imprinted genes. About 100 autosomal genes are known to be imprinted and many of these imprinted genes are expressed in early-stage embryos, placenta and brain [134]. Aberrant imprinting can lead to disorders such as the behavioral and neurodevelopmental disorders Prader-Willi and Angelman syndromes [135].

Imprinting plays a special role in sex chromosomes. In females paternally derived X chromosomes are preferentially X-inactivated, while maternal X chromosomes are imprinted to not undergo X-inactivation [136]. Genomic imprinting is believed to have evolved to control the dosage of a subset of genes that play critical roles in the reproduction-related physiology and behavior [137]. Genes that are parentally imprinted, i.e. expressed unequally from the maternal and paternal allele, are thus possible sources of sex differences [138].

Non-coding RNAs

Non-coding RNAs are known to play an important role in gene regulation by interacting with e.g. translation, splicing or DNA replication [139,140]. In this way they contribute significantly to the way genes are expressed. Especially long non-coding RNAs have been implicated in reproduction, development and cell differentiation [141–143]. The average autosome has approximately 96 non-coding RNAs and 606 pseudogenes. While chromosome X encodes 715 non-coding RNAs and 873 pseudogenes, chromosome Y encodes for 122 and 379 subsequently. Compared to the number of coding sequences on the Y chromosome (28 unique genes), the number of non-coding elements is relatively high. Even though, the Y chromosome is represented to large part by non-coding regions, we still know very little about them and about the non-coding RNAs originating from it.

DNA methylation and histone modifications

DNA methylation and histone modification are two major epigenetic mechanism that can affect gene expression. In the following, I will explain the relevance of these two mechanisms for the regulation of sex-biased gene expression.

DNA methylations

DNA methylation is a biological process in which methyl groups from *S*-adenosyl-l-methionine (SAM) are added to a cytosine of a CpG island immediately following DNA replication. The methylation can change the activity of a DNA segment. Methylation in the DNA of a gene promotor or transcription factor, will for example repress gene transcription. DNA methylation is associated with a number of key processes such as genomic imprinting, X chromosome inactivation, repression of transposable elements and carcinogens. The addition and subtraction of methyl groups is tightly regulated by antagonizing enzymes, such as the DNA methyl transferases (DNMT) which add methyl groups. DNMT1 is expressed in at high levels in all tissue and plays a role in the maintenance of cytosine methylation following progression through the cell cycle, DNMT3a and 3b are involved in the de novo initiation of methylation patterns. The ten-eleven translocation (TET) family of proteins is a methylcytosine dioxygenases and can reverse the methylation actions of DNMTs by oxidizing 5mC. Methyl-CpG-binding domain proteins (MBDs) bind to methylated DNA and mediate the effects of DNA methylation on gene transcription and other processes.

Dynamic changes in DNA methylation are involved in modulating cell-, tissue-, and developmental stage-specific gene expression. Different DNA methylation profiles are also linked to a broad spectrum of processes in neurodevelopment, such as synaptic functioning, homeostasis, and plasticity [144–146], as well as in the sex-biased risk for psychiatric disorders [147].

Histone modifications

The nucleosome, the basic packaging unit of DNA, consists of an octamer of core histones that contains two of each histone H2A, H2B, H3, and H4. These histone proteins largely control chromatin architecture, nucleosomal positioning, and ultimately access to DNA for gene transcription. Modifications to the histone proteins can affect the condensation of the chromatin into transcriptional active euchromatin or inactive heterochromatin. At least nine different types of histone modifications have been discovered, from which methylations, acetylations, phosphorylation and ubiquitination are the most common and best understood.

Histone methylation describes the process in which a methyl group is attached to a nitrogen atom in amino acid side chains and/or at the amino termini. Methylation occurs at various sites of histone proteins but primarily on lysine (Lys or K) and arginine (Arg or R) residues. Based on the affected residue and the genomic context of the methylation, it can have an activational or repressive effect on gene expression. The primary site of histone methylation is H3, although other core histones also show methylations. Histone methylations can exist in different states, such as mono- di- or tri-methylations. Di- and tri-methylations at H3K4 are typically gene-activating with tri-methylations (H3K4me3) targeting promoters [148]. Mono-methylation of H3K4 is an activating mark unique to enhancers [148]. H3K9 and H3K27 methylations are generally gene-repressive [148] but both serve a unique function. H3K27me3 marks dynamically regulated genes and is highly reversible [149]. It is therefore, especially important in development where genes are switched 'on' and 'off' based on periods of growth or differentiation. H3K9me3 is a characteristic of heterochromatin and leads to silencing of gene expression and is used to prevent chromosomal instability, whereas H3K9me2 is found more commonly at silent or lowly expressed genes in euchromatin [150]. Often, H3K27me3 and H3K4me3 are present together near gene promoters, creating a 'bivalent state' where genes are 'poised' for activation by the removal of H3K27me3 or repression by the removal of H3K4me3 during development [151] ref). Methylation-based histone modifications are regulated by different enzymes: 'writers' methyltransferases (KMT) which add modifications and 'erasers' demethylases (KDM) which remove modifications, as well as 'readers' chromodomain and bromodomains which recognize modifications and influence gene expression [152,153]. A number of KMTs and KDMs are encoded on the sex chromosomes (**Table 4**).

Next to histone methylations, histone acetylations are also implicated in gene transcription. Histone acetylation is the process by which the lysine residue at the N-terminal tail that protrudes from the histone core is acetylated. Acetylations are usually associated with transcriptional activation. Important histone acetylation marks are for example, H3K27ac a mark that is associated with the upregulation of genes as it is deployed at poised and active gene enhancers often in combination with H3K4me1 [154]. H3K27ac and H3K27me3 modifications are sharing the same location on the histone and therefore antagonize each other [155]. H3K9ac and H3K14ac have been shown to be part of the active promoter state. They are also present over bivalent promoters and active enhancers [156]. Active H3K4me3 and H3K9ac are deposited in the promoters of genes in neurons related to neuronal functions in two modes: *de novo* establishment or increase from existing levels in NSCs. In addition, changes of H3K27ac and H3K9ac in promoters and enhancers synergistically upregulate genes with functional enrichment for neuron differentiation and

downregulate genes with functional enrichment for neural progenitor cell-related pluripotency [157].

Similar to histone methylations, acetylations are modulated by two opposing groups of enzymes: histone acetyl transferases (HAT), which are responsible for adding acetyl groups and histone deacetylases (HDAC), which in turn remove acetyl groups. Like KMTs and KDMs, HATs and HDACs are also expressed from the human sex chromosomes (**Table 4**).

Table 4: Gene name and chromosome of gene products with histone modification activity (methylation and acetylation)

Chr.	Modifiers of methylation		Modifiers of acetylation	
	KMT	**KDM**	**HAT**	**HDAC**
X	*SUV39H1*	*KDM5C (esc)*	*MSL3 (esc)*	*HDAC6*
X	-	*KDM6A (esc)*	*TAF1*	*HDAC8*
X	-	*PHF8*	-	*MORF4L2*
X	-	-	-	*RBBP7(esc)*
X	-	**-**	**-**	*TBL1X (esc)*
Y	-	*KDM5D*	*CDY2A*	*TBL1Y*
Y	-	*UTY (KDM6C)*	*CDY1*	-
1-22	67	26	40	33

*esc marks XCI-escapees

Since the histone modifiers can have such a wide-ranging effect on basically all DNA-dependent processes, the possibility of a sex-biased expression from the X and Y chromosomes makes them highly valuable for the investigation of sex differences. In the nervous system, Kdm6a is involved in the determination of neural stem cells and their subsequent differentiation into glial cells and neurons through removal of H3K27me3 at promoter regions [158,159]. KDM5C is involved in sexual differentiation of the brain by actively contributing to a repression of gene expression through H3K4 demethylation, further it is involved in the control of the transcriptional programs within neurons to impact their differentiation, neurite growth and synaptic activity [160–166]. The Y chromosome homolog to UTX, the histone demethylase UTY, has been implicated in brain development of males [114,115]. *Uty* appears to be functionally similar to *Utx* [167] but their expression levels and patterns differ in female and male brains, suggesting that they may mark genes in a sex-specific manner [168].

Research models for the identification of genetic sex differences

For centuries scientists have tried to understand how disorders affect the brain and what causes them. The result is a sheer endless list of biological processes that are involved in the development and maintenance of the nervous system. Some of the processes are for example, neurogenesis, gliogenesis, cell migration, differentiation of neurotransmitter specific cells, synaptic functioning, morphological differences, neuronal projections, neurotransmitter generation, release and uptake through receptors and last but not least cell death. It is possible that these processes are also affected by sex differences in one or the other way. Unfortunately, the study of sex differences is a relatively modern field of science and thus a lot of work remains to be done. The interplay between genetic, hormonal and environmental factors makes it hard to identify the origin of sex differences. However, a number of model system have been developed with the goal to distinguish genetic from hormonal sex differences.

Cell culture systems

With the recent breakthroughs in the generation of induced pluripotent stem cells (iPSC) from something as simple as a skin cell, stem cells became widely accessible to the research community after 2007. This breakthrough was pioneered by the work of John B. Gurdon and Shinya Yamanaka [169,170], as well as many other scientists that also dedicated their research to the reprogramming of iPSCs. Since then, human or mouse stem cells are frequently used for the identification of gene expression differences in a multitude of tissues. Following one of the many protocols available, stem cells can be differentiated into the desired tissue and a subsequent RNA sequencing analysis can give insights into the transcriptome of the cells. Using this approach, we can also identify sex-specific difference. The crux however, is to distinguish between interindividual differences of cell lines from different donors and 'real' sex differences. One way to ensure that 'real' sex differences are identified, is to use an adequate number of cell lines from each sex. Fortunately, the popularity of stem cells as a research model has led to the rise of human stem cell banks in many countries and European wide initiatives like the ECACC and EBiSC, in which stem cells usually are readily accessible. A

factor that can contribute to interindividual difference of stem cell lines and can affect their comparability, is the way the lines are generated and maintained. Difference in reprogramming protocols can lead to different genetic and epigenetic baselines, so that certain stem cell lines differentiate more or less readily into certain cell types. Similarly, the composition of culture medium and treatment of cells during cultures can lead to differences between cell lines. However, these issue is are currently tackled with the introduction of globally acknowledged guidelines for characterization, reprogramming, handling and culture conditions of human pluripotent stem cells through ISSCR Standards Initiative for Pluripotent Stem Cell Research [171], expected to be released in the beginning of 2023.

The possibility to reprogram iPSCs has also made it possible to generate induced pluripotent stem cell from individuals with sex chromosome aneuploidies, such as Klinefelter or Turner syndrome (**Table 5**). These cell models are useful to investigate the effect of X and Y chromosome dosage abnormalities [172–174].

Table 5: Sex chromosome aneuploidies

Genotype	Genetic sex	Gonadal sex	Name
45, X0	Female Decreased X dosage	Female	Turner syndrome
46, XX	Female SRY present	Male	Testicular difference of sex development
47, XXX	Female Increased X dosage	Female	Trisomy X
47, XXY	Male Increased X dosage	Male	Klinefelter syndrome
48, XXYY	Male Increased X and Y dosage	Male	XXYY syndrome
48, XXXY	Male Increased X dosage	Male	XXXY syndrome
49, XXXXY	Male Increased X dosage	Male	XXXXY syndrome

The downside with any cell model is, that they are considered as quite 'artificial'. The cells are extracted from the body and are thus not subjected to the complex interactions within an organism. They therefore do not behave the same way they would do in the body. At the same time a certain simplicity is desired in a model system to be able to study biological processes at a basic level. Researchers have started to add complexity to the traditional 2D culture models by culturing cells floating or in scaffolds so that a self-organizing 3D growth is achieved. Such 3D stem-cell derived systems are typically referred to as organoids. The combination of multiple organoids from different cell types or primary tissue are called assembloids. In addition, a combination of cell culture and microfluidic systems called organ-on-a-chip has been

developed to increase the interaction between multiple tissue types. The system is used to co-culture different tissue types in defined compartments on a microfluidic chip. The compartments can be connected to each other so that it becomes an interactive system.

Scientists from Israel have taken a particularly interessting approach in minimizing interindividual differences of cell lines and the possibility to investigate genetic sex differences. Using somatic cells from a Klinefelter syndrome patient, they have generated an iPSCs line with different sex chromosome complements: 47,XXY/46,XX/46,XY/45X0. These nearly isogenic lines are ideal to identify genetic sex differences [174].

The four core genotype mouse model

One remarkable way how scientists have been identifying genetic sex differences, is the so called four core genotype (FCG) mouse model. In the model, the *Sry* gene can either be removed from the Y chromosome of a mouse, or inserted in an autosome. The absence of the Sry gene will lead to the development of ovaries. While a presence of the Sry gene will lead to the development of testes. Through mating it is then possible to generate XX as well as XY offspring with ovaries or testes (**Figure 5**). In this way, effects of sex chromosome complement can be distinguished from effects of gonadal hormones.
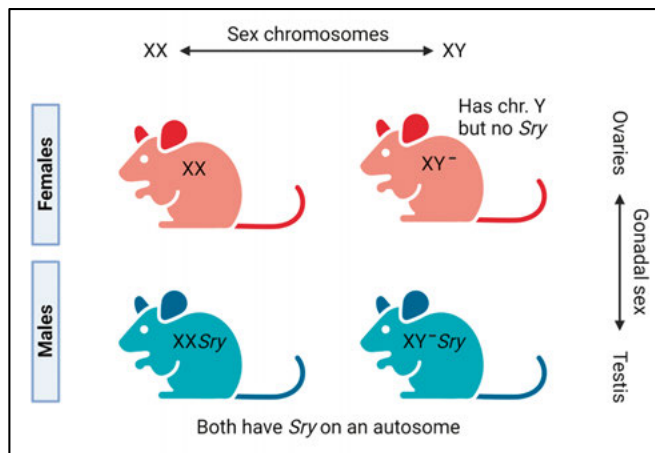

Figure 5: Schematic representation of the genotype options in the FCG mouse model. The options are: 1) normal XX genotype with ovaries; 2) XY genotype without *Sry* on the Y chromosome which will develop ovaries; 3) XX genotype with *Sry* on autosome which will develop testes; 4) XY genotype with *Sry* on autosome which will develop testes.

Relevant sex difference for neurodevelopment

Every cell of our body has a sex, it is either male or female, it has either an XY or XX sex chromosome set. Regardless of their sex, most of them can perform the same functions such as proliferation, differentiation or apoptosis. Liver cells perform glycolysis and kidney cells work together to retain water and regulate blood pressure. However, sometimes the sex of the cells makes a difference. It is clear that for reproductive tissue the sex chromosome complement has a huge impact. The presence of a Y chromosome commits cells to a testicular fate while the absence leads to an ovarian fate. However, in the following I would like to demonstrate how the difference in sex chromosomes can affect tissues and functions apart from reproduction, such as in the cells of the nervous system.

- Midbrain dopaminergic neurons from XY embryos show more expression of tyrosine hydroxylase, the rate-limiting enzyme of dopamine production, regardless of the gonad type [175].

- Aromatase expression in developing mouse brain embryos seems to be under control of sex chromosomes. Aromatase converts testosterone to estradiol. Studies found increased aromatase expression in the stria terminalis and anterior amygdaloid area of XY mouse embryos, as well as increased expression of estrogen receptor beta. Estradiol or dihydrotestosterone treatments increased aromatase expression in cell culture of amygdala neurons derived from XX mouse embryos. In both cases gonadal sex was irrelevant [176,177].

- Investigations of gene expression in the basolateral amygdala of mice has shown the following. A decreased expression of mood-related genes in XY mice weanlings. Adult XY mice under stress showed lower expression of the GABA related gene somatostatin (Sst). At the same time higher levels of Sst correlated with lower anxiety-like behavior [178]. Two other studies that investigated genes relevant to mood, found an increased expression of mood-related genes due to stress in the prefrontal cortex of XX mice [179,180].

- Somatotropin is an essential hormone in human embryo development, including the development of the brain. A study in adult mice has identified that in XX mice sex chromosomes but also estradiol led to

an increase of growth hormone (somatotropin) in the arcuate part of the hypothalamus [181]. Its release has been shown to be sex-biased in many mammalian species, including humans [182]. Another study that investigated neurons of the hypothalamus, showed that neurons of male mice showed lower expression of neurogenin 3 and decreased neurogenesis. The effect could be recovered through the effects of estradiol [183].

- Hypothalamic neuronal cultures from wild-type and transgenic FCG mice showed higher expression of X-linked genes *Kdm6a*, *Eif2s3x* and *Ddx3x* in XX neurons, regardless of gonadal sex. *Kdm6a* down-regulation using siRNA reduced axonal length and *Ngn3* expression only in female neurons. The results suggest *Kdm6a* as a key mediator of the higher axogenesis and *Ngn3* expression observed in XX neurons before the critical period of brain masculinization [184].

- Male mice showed higher density of vasopressin-immunoreactive fibers in the lateral septum and habenular nucleus [77]. Vasopressin acts as a neurohormone and is involved in social and parental behavior and stress response. Anomalies in vasopressin signaling have been observed in neuropsychiatric disorders.

- Dendritic spines are tiny protrusions from dendrites that form functional contacts with neighboring axons. Female rats in proestrus have a greater density of dendritic spines in CA1 of the hippocampus than males. Under stress the spine density is affected in opposite direction, increased in male and decreased in female hippocampus [185].

- Rat male and female oligodendrocyte precursor cells (OPCs) display sexual dimorphic properties *in vitro*. Female derived OPCs show higher cell proliferation and migratory properties, as well as a higher resistance to oxygen-glucose deprivation. Male OPCs in contrast, show a significant faster differentiation capacity and a higher rate of myelination [186].

- The previously mentioned group that created a 46,XX and a 46,XY isogenic iPSC cell line from a Klinefelter syndrome patient, have used their cell lines to identify sex-biased gene expression during neuronal differentiation. They found that, during neural differentiation, most of the differentially expressed genes are upregulated in the male cells. Among them *GRIN3A* and *SF3B*, two genes associated with schizophrenia and cerebral palsy. Additional upregulated genes in males were associated with amyotrophic lateral sclerosis, autism spectrum disorder, and Rett syndrome gene sets. All of the disorders are known for their sex-biased prevalence and phenotypes [174].

- Rats were infused with BrdU, a DNA synthesis marker that labels dividing neurons in the dentate gyrus (DG). Male rats showed a greater density of neural stem cells in the dorsal but not the ventral DG and had a higher level of cell proliferation than females. However, males showed a significant reduction in neurogenesis between one and two weeks after mitosis while neurogenesis in females was unchanged throughout the measured period of three weeks. In line with this, male adult-borne neurons also showed a faster rate of maturation after two weeks displayed by expression of NeuN. The results suggest a sex difference in the potential for neurogenesis in rats [187].

- An investigation of sex chromosome effects on the vulnerability of an Alzheimer disease mouse model (XY/XX-hAPP) showed that the presence of a Y chromosome results in worse mortality and deficits in the AD mice, while a second X chromosome, even in male mice, was beneficial. The resilience effect of a second X chromosome is potentially conferred through Kdm6a, among other genes. In cell culture experiments, they showed that XY neurons display greater cell death than XX neurons when exposed to the neurotoxin Aβ. However, Kdm6a overexpression slightly reduced, while a Kdm6a knockdown increased neurotoxicity in XX neurons. Similarly, mice engineered to express mutated forms of the human amyloid precursor protein (hAPP mice), individuals carrying a single X showed reduced longevity and worse spatial learning and memory performance compared to XX mice. When Kdm6a was overexpressed using lentivirus in the hippocampus of XY-hAPP mice, a significant improvement in learning and memory performance was observed in these animals compared to control XY-hAPP mice. Thus, the presence of a single X/Kdm6a copy consistently worsened hAPP/Aβ-related mortality, cognitive impairment and cellular viability compared to two X/Kdm6a copies. KDM6A expression in the human brain was higher in women than in men and in Alzheimer's disease patients compared to controls. Considering all these observations in the human brain and in mouse models of the disease, authors suggested that having two copies of Kdm6a compared to just one copy of the gene confers stronger resilience to the disease, and speculated that increased KDM6A in brains of people with Alzheimer's disease might be a neuroprotective, compensatory response [188].

- An investigation of sex-biased expression in primary neurons of mice showed a sexual dimorphic expression in microglia (264 genes), neurons (69 genes) and astrocytes (30 genes). The Y chromosome genes Ddx3y, Eif2s3y, Kdm5d, and Uty were highly expressed in the neurons and an overexpression of Eif2s3y led to increased synaptic

transmission specifically in male neurons and caused autism-like behaviours specifically in male mice [189].

- A study analyzing 120 human brains from fetus with an average age of 14 weeks post conception (pcw), identified the overexpression of 43 Y-linked, 48 X-linked genes, as well as 1377 autosomal genes in male samples. In contrast, in female samples they identified an overexpression of 107 X-linked genes and 1181 autosomal genes. They also found an enrichment in genes associated with neurodevelopmental disorders in male samples (13 genes overexpressed in male fetus tissue (ADNP , MED13L, TCF4 , EP300 , FOXP1, CDK13 , TBL1XR1 , KAT6B, CHD2, POGZ, EHMT1, CTCF, AUTS2) and 3 in female samples (SCN2A, COL4A3BP , DNM1) [95]. An investigation of the same data set from another group, identified a consistent sex bias in biological processes such as cell cycle, cell differentiation, energy metabolism and extracellular matrix organization [190].

- In a study investigating the gene expression profile of human brains at major developmental stages (prenatal: 8-24 pcw, early child: 4 mos - 4 yrs, puberty: 8 yrs - 19 yrs, adult: 21 yrs - 40 yrs) it was found, that in prenatal most brain regions show more male-biased genes, except for four brain regions (AMY, MD, STR and V1C). This confirms that the brain transcriptome bias between males and females is already present at the prenatal stage and that it is mainly driven by male-biased genes. Y chromosome gene expression of KDM5D, DDX3Y, ZFY, PCDH11Y, USP9Y, RPS4Y1, CYorf15B, TMSB4Y, NLGN4Y, UTY, EIF1AY and GYG2P was detected at all developmental stages, while TBL1Y and SRY were overexpressed prenatally. In addition, they observed a sharp contrast between male and female biased genes in relation to brain disorders. Male biased genes during prenatal time, are significantly enriched for diseases including OCD (obsessive compulsive disorder), schizophrenia, microcephaly, epilepsy, bipolar disorder, autism and Alzheimer's disease [191].

An ethical side note: Why neuroscience of sex differences is indispensable and how to prevent neurosexism

Sex differences is a topic that fascinates and inflames people but it is also easily misinterpreted and exaggerated by the laymen. Particularly sex differences in the brain seem to attract much controversy [192,193]. The confusion about sex differences in neuroscience has come so far, that some claim these studies defend essentialists or sexist beliefs or challenge the idea of real equality [194–196]. Even among specialists in the field, there is a debate about the way that research of sex differences is performed and interpreted [197–203], although much of the debate aims to improve the neuroscience of sex difference so it does not create neurosexism [204–208].

In this section I will elucidate why studying sex differences in neuroscience is an ethical imperative and how findings should be presented so they cannot be misinterpreted, extrapolated or misused.

A number of neurological disorders show sex differences in their prevalence, age of onset and symptoms. Examples for such disorders are Alzheimer's disease, Parkinson's disease, autism spectrum disorder and depression (Table 1). These neurological disorders are very complex and their origin and cause are hard to identify. Unfortunately, the complexity of the disorders and the limited regenerative capacity of the brain make a treatment very challenging. However, the fact that the disorders display sex differences, highlights the existence of differences in the male and female physiology that promote or impede the development of these neurological disorders. Some of the general factors that can contribute to the susceptibility for such disorders are our genes, our hormones, the environment we are exposed to and the life-style we are living. By dissecting and investigating each of the factors in the context of sex differences, researchers are able to identify what contributes to the susceptibility to neurological disorders of one sex and to the resistance of the other. In this way, studying sex differences contributes to the understanding of disorders and identified sex differences can serve as a starting point for additional research or the development of a treatment or therapy. In addition, the study of sex differences approaches an issue that did not receive enough attention in

research for a long time, the problem of sex-specific adverse effects of treatments [209]. This problem persists because too little research includes female animal models or cells in basic, as well as clinical research [210,211].

I therefore believe, that it is an ethical imperative to study sex differences in neuroscience. It leads to advances in the understanding of the human physiology in health and disease and thus contributes to a better public health.

In 2015, the American National Institute of Health (NIH) expanded its guidelines with a policy to include sex as a biological variable. This was done in an effort to counter the overrepresentation of male animals and cells in basic and preclinical biomedical research, as well as to address the problem of unknown sex-specific responses to medical treatments. It is now expected from every applicant for NIH funding to consider sex as a biological variable and adjust research designs, analyses, and reporting in vertebrate animal and human studies accordingly, or provide strong justification to study only one sex [212]. This policy has led to a dramatic increase in the use of female animal models and has also led to a rise in research that focuses on sex biases, including sex differences in neuroscience.

But sex differences in neurosciences has a problem. Research is often misinterpreted and extrapolated by the public. Misconceptions are fueled by statements in books such as *Men Are from Mars, Women Are from Venus (1992), Why Gender Matters (2005), The Female Brain (2006), Leadership and the Sexes (2008), A Gendered Choice (2010)*. Books like these are exploiting the fascination for sex differences, give seemingly easy explanations to in reality very complex subjects and engrave perpetuated sex-stereotypes into the minds of the reader. There is a lot of confusion among the public about sex differences and how results from publications should be interpreted. Therefore, every researcher that publishes sex differences should carefully consider how they present their findings. We have the responsibility to perform our experiments and phrase our conclusions in a way that they cannot be misinterpreted or misused for sexist or other discriminative purposes.

There are a number of perils and pitfalls that can be avoided before publishing. Some of the following advice might sound like simple *good research practice* but they are still often not being followed carefully enough:

1. **Translating results to other species or from simple/isolated to complex system**

   Even though some animal models are used in experiments to resemble certain features of the human organism as close as possible, results from experiments can still not be translated directly to humans. To draw conclusions between a human and a model organism, without informing about the limitations, is risky and can easily be misunderstood. The same goes for simplified or isolated systems such as cell models and their applications. Cell models, even if they are human cells, do not resemble the complexity of the human body e.g., the interaction between organs and lack systems such as the hormonal, immune, vascular and lymphatic system. Special care should also be taken when abnormal cell lines such as immortalized or cancer cell lines are used.

2. **Inferring a sex difference in behavior without enough evidence**

   Often, we infer an evolutionary or behavioral function of a sex difference in the brain without sufficient evidence to supports this. A common approach to link sex differences to human behavior includes three aspects, (I) the sex difference between men and women (in e.g., a brain structure), (II) the relation of the sex difference (brain structure) to a behavior or ability (multitasking) and (III) the presence of an alleged sex difference in the behavior (men and women differ in their ability to multitask). To deduct that there is a sex difference in the ability to multitask (III) because of the sex difference in the brain structure (I) is invalid (*false-cause fallacy: a real or perceived relationship between two things means that one is the cause for the other*). Only through the link of (II) the claim is valid (**Figure 6**). If we are to imply a sex difference in behavior from a sex difference in brain structure, like in the example above, enough evidence for all three aspects needs to be available.
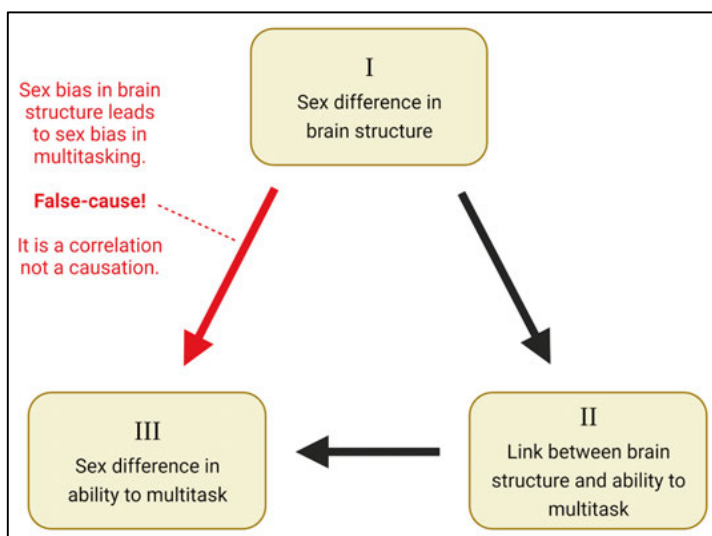
Figure 6: False-cause fallacy example. Sex difference in a behavior such as the ability to multitask cannot be deduced from a sex difference in brain structure without evidence that links the brain structure to the ability to multitask.

Evidence that a structure in the brain plays a defined role in a certain behavior is often rare [213]. Likewise, well supported evidence for a sex difference in a behavior are often scarce. The temptation to fall back to male and female stereotypes like, the ability to multitask, language skills, empathy, spatial orientation, mathematics skills or map reading, is large. Without sufficient evidence, inferring from a sex difference in neuroscience to a difference in behavior should be made cautiously.

3. Presenting sex differences as hardwired and rigid and ignoring the socio-cultural cause of sex differences

When sex differences in the brain are identified and linked to behaviors, the plasticity of the brain should be taken into account. Today, we know the neural circuits of the brain are not fixed at birth. In fact, the brain is very plastic and can be shaped and reshaped by many factors over the course of a person's life. This plasticity is essentially the ability of the nervous system to modify itself in structure and functionality as a response to injury or experience. This means, that sex differences are not necessarily fixed or hardwired. In the absence of proof for genetic or hormonal influence, any sex difference in the brain could be shaped through experience, social-, physical- and sensory stimuli. Implying that sex differences in the brain are innate is often used to argue that gender stereotypes are rooted in biology. This makes gender-stereotypical behavior appear predetermined and inevitable and can lead people to fall for the logical fallacy

of the appeal to nature: something is good, ideal, justified, valid, or inevitable because it is 'natural'. As a consequence, people may feel powerless to change their own trajectory. However, sex stereotypes are largely formed through experience and culture, and are therefore not innate [214]. Sex-linked genes, sex hormones and neuroanatomy are all interlinked with sex-specific experiences and all of these factors need to be taken into consideration if sex differences are linked to behaviors.

The three points mentioned above describe only some of the many perils and pitfalls that an investigator can fall for. I have selected them because they were the three most important ones for me and my work and which I would like to share with my fellow researchers. More advice can be found in a lot of excellent literature that provide guidelines to help with the correct choice of experimental design, statistical tests and interpretational approach, to appropriately analyze sex differences [5,201,215–218]. However, during my literature research, I have come across a message that all of the scholarly work has in common, the call for a cautious and considerate execution and interpretation of experiments that identifies sex differences. This is, to avoid a consolidation of gender stereotypes and the misuse of sex difference research for sexist and discriminative agendas. Studying biological sex should contribute to our health and foster the understanding of diseases etiology, manifestation, progression and its treatment.

Summary of the papers and manuscripts

The overall aim of my work was to investigate the presence of genetic sex differences and the contribution of X and Y chromosome gametologs, as well as Y chromosome genes, in early human neuronal development.

Paper I

Aims

The identification of Y chromosome gene expression and sexual differential gene expression of X and Y chromosome gametologs in early human embryonal CNS development, before maturation of gonads and production of sex hormones.

Methods

To detect and quantify expressed transcripts at a stage of early CNS development in humans, brain tissue samples were obtained from 2 male and 2 female embryos in week 10-11 of development. Total RNA and poly(A) RNA from the midbrain and medulla were analyzed and RNA-seq tracks were searched for regions of high expression in previously non-annotated regions to detect novel genes or non-coding RNAs. qPCR experiments were used to confirm lncRNA expression on the Y chromosome in human and chimpanzee samples.

Results

<u>Many Y chromosome genes are differentially expressed in human early embryo development (Table 2)</u>

The RNA sequencing resulted in dgene expression of 36 genes detected in embryo samples of 11-12 pcw. Of these genes 15 were Y-linked (KDM5D, DDX3Y, EIF1AY, PRKY, TXLNG2P, TTTY15, NLGN4Y, RPS4Y1, TMSB4Y, ZFY, NCRNA00185, USP9Y, TTTY14, UTY, TBL1Y), one was X-linked (XIST), and 22 were autosomal genes. Among the autosomal genes, 9 are classified as estrogen and/or androgen responsive, and another 6 code for proteins that play a role in developmental processes. A second analysis of the data with stricter read mapping, revealed an additional 8 differentially expressed X-linked genes (PAGE4, MAGEC3, CAPN6, APLN, ZFX, VGLL1,

GYG2, and XIST), of which 5 are known XCI-escapees (MAGEC3, XIST, ZFX, VGLL1, GYG2).

Y chromosome genes contribute to gene dosage differences and balances in early embryo development (Figure 2)

Of the 15 Y-linked genes, 13 have X chromosome homologs. We have used the combined gene expression of X and Y homologs in males to calculate the gene dosage and compared it to the gene dosage in the females. In 9 cases, the X-linked gene expression was similar in females and males. In 5 of those, the Y-linked gene expression resulted in a significant higher overall gene dosage in males (EIF1A, RPS4, USP9, KDM6A/UTY, TXLNG). In three cases (NLGN4, KDM5C, ZFX/Y), the gene expression was balanced by the Y gametolog expression. In one case (TMSB4), the overall gene expression was higher in males.

RNAseq reads indicate the expression of conserved long non-coding RNAs on the Y chromosome (Figure 3 and 4)

While inspecting the Y chromosome sequencing reads, we have noticed six regions with high expression in non-annotated areas. The regions are located downstream of KDM5D, downstream of TTTY14, downstream of UTY, in-between TTTY15 and USP9, downstream of ARSEP1, and upstream of TXLNG2P. The presence of poly(A) positive reads in some of the regions (Figure 3 A/C/E) indicate the presence of functional non-coding RNAs. With the help of the RNA sequencing results of one male and one female chimpanzee brain sample (Figure 4), we have confirmed the conservation of these Y-lncRNAs in 4 of the 6 cases (KDM5D-DS, TTTY14-DS, UTY-DS, TTTY15/USP9-intg). To look for possible annotations in the Ylnc regions, we have consulted the database NONCODE, which lists currently described non-coding RNAs. We did not find annotations that exactly matched our sequences regarding length, genomic position, and tissue expression. In conclusion, comparisons with currently available databases indicated that we have found six possible Y-lncs with relative high expression in the CNS.

Conclusion

The study demonstrated that a number of Y chromosome genes and X/Y gametologs are expressed in the CNS of human embryos during week 10-11. Publicly, available expression databases [219] and RNA-seq results [220] confirm the expression of these genes in early embryo samples. Indicating their importance in this stage of the male embryo development.

In addition, we found that the gene dosage of 9 of 13 gametologs are not balanced at this stage of embryo development. The Y chromosome gametolog thus contributed to a higher overall gene dosage. Recent microarray studies

on human brain during development [221] and adulthood [97] also describe a lack of compensation. This implicates that Y chromosome genes could be involved in more than merely reproduction physiology and behavior. In addition, it fosters the importance of gametologous genes in gene dosage regulation.

We have demonstrated the presence of conserved regions of Y-encoded long non-coding RNAs via RNAseq and qPCR on human and chimpanzee samples. Several other studies have also located long-non coding RNAs close to the regions we have described here [222,223]. Long-non coding RNAs have been implicated in regulatory processes for reproduction, development and cell differentiation [141–143]. Thus, it is not unlikely that these Y-lncs could serve an important role in early embryonal CNS development.

Paper II

Aims

The exploration of gene expression differences relevant for neurodevelopment, in neural stem cells of male and female origin (hNSC-H14 and H9 respectively). A special focus was put on gene expression of X/Y chromosome homologs.

Methods

We have studied proliferation and morphology of the neural stem cell lines H14 (XY) and H9 (XX) in monolayer cultures with defined seeding densities on geltrex substrate. Growth curves were created by counting the cell number every 24 hours. To analyze the differentiation capacities of the NSCs, the cells were allowed to differentiate for a total of 14 days by removing FGF2 in the culture medium. Total RNA samples were obtained on day 0, 4 and 14 of differentiation. The experiment was repeated three times to account for variability. Gene expression was measured using qPCR. Immunohistochemistry with anti TUJ1, DCX and NEUN antibodies was used to confirm similar neural differentiation of the cell lines.

Results

Growth curves indicates faster growth in hNSC-H14 and morphology suggests similar differentiation trajectories in both cell lines

Figure 1: The average duplication time across different seeding densities revealed a significant faster growth of hNSC-H14 cells. HNSC-H9 cells took on average 8.7 days to reach confluency, while hNSC-H14 cells needed 5.3 days. **Figure 2**: In an undifferentiated state the cell lines were morphologically undistinguishable. During differentiation the two lines developed similarly and

developed bipolar, pyramidal-shaped and multipolar cells with relatively long and branched neurites within 7 days. A general increase in neurite formation, branching and fasciculation together with ongoing cell migration and clustering was observed until day 14 of differentiation.

qPCR results display differences in marker gene expression during neural differentiation of H9 and H14 cells

Figure 3: A housekeeping gene analysis revealed HPRT1 and PPIA as optimal calibrator for the qPCR gene expression analysis. **Figure 4:** The subsequent qPCR analysis detected gene expression differences in H14 and H9 cells during a 14 day-long neural differentiation. Neural marker genes, such as the microtubule-associated protein 2 (MAP2), post synaptic density protein (DLG4 or PSD95), synaptophysin (SYP), doublecortin (DCX) and neuron-specific class III tubulin (TUJ1) displayed similar neural differentiation in both NSC lines after 14 days. However, differences in gene expression between the cell lines were found during early differentiation. At day 4, the genes DCX, DLG4 and TUJ1 were significantly higher expressed in H14 cells. For DCX and DLG4, this difference disappeared at 14 days of differentiation, however the difference remained for TUJ1. H9 cells on the other hand presented a 32-fold increase in RMST, a long noncoding RNA, which is indispensable during neurogenesis. This difference was already present before differentiation, and also at D4, but disappeared at D14. The most striking expression differences between H9 and H14 cells after 14 days of differentiation were found for RELN with almost 100-fold higher levels in male cells and MASH1 (also called ASCL1), with more than 1000-fold higher expression in male cells at D14.

qPCR analysis reveals increased gene expression of two X and Y encoded demethylases in H14 cells (Figure 6)

To identify whether X- or Y- encoded genes could have an effect on the differentiation of neural stem cells, we studied the expression of X/Y homologous genes. One of the most noticeable sex differences in expression was observed for the demethylase pair UTX/UTY (aka. KDM6A/KDM6C). The expression in male cells (H14) for UTX/UTY was significantly larger than the expression of UTX in female cells (H9), with an 18-fold difference between the sexes at 4 days and 24-fold difference at 14 days of differentiation. This difference was due to increased expression of UTY at day 4 and 14 compared to the NSC stage. The expression of UTX did not increase significantly over these time points. Similarly, another pair of demethylases, KDM5C/KDM5D, was significantly increased in male cells during differentiation, due to larger expression of the Y-encoded gametolog in male hNSC-H14. In this case the difference was already significant at day zero and remained over the rest of the differentiation period (day 4 and day 14).

<u>Two long non-coding RNAs (Y-lncs) show increased expression during neural differentiation (Figure 7)</u>

As mentioned in paper I, we have previously found six novel non-annotated long non-coding RNAs on the Y chromosome (Y-lncs) with expression in early human embryos. To evaluate whether any of the six Y-lncs are also expressed in neural stem cells before or during differentiation, we measured their expression in hNSC-H14 cells. In addition to the six Y-lncs, we have investigated an additional long non-coding RNA, located on the gene anosmin 2 (ANOS2P also known as KALP). This Y-encoded gene is gametologous to the gene for Kallman Syndrome (KAL) located on the X chromosome, and it was first described as a non-processed pseudogene [224]. Only two of the novel Y-lncs described in paper 1, the one located downstream of UTY and the one located downstream of KDM5D, displayed modified expression during differentiation, with significantly higher values already after four days of differentiation. Thus, the Y-lncs may be involved in the upregulation of the expression of UTY and KDM5D, which are also increased during the differentiation of male cells.

Conclusion

We have used a male and a female NSC line to analyze gene expression during neural differentiation. The cell lines showed differences in growth rate (faster growth in male line) and gene expression, but same differentiation tendencies. Male cells showed a faster differentiation indicated by the mature neuronal marker DCX and DLG4 but the differences resolved after 14 days of differentiation. Most prominent was the female-biased expression of RMST, a long non-coding RNA promoting neurogenesis, after 4 days of differentiation, and the male-biased expression of MASH1 and RELN which are both relevant for the differentiation of Cajal-Retzius neurons. In general, we believe that the gene expression differences point towards a tendency of the cells to differentiate in a different way in this undirected neural differentiation. This should be considered when cells are used for research or medical purposes. Unfortunately, with our experimental setup we are not able to distinguish sex differences from individual differences of the cell lines. Thus, all claims for sex difference should be done carefully.

When looking at gametologous gene expression, we saw that most genes had a balanced gene dosage throughout the differentiation. However, there were large differences in two gametologs that are expressing demethylases. The male gene of the gametolog pairs KDM5C/KDM5D and UTX(KDM6A)/ UTY(KDM6C) were significantly higher expressed in all timepoints during differentiation in male NSCs. Demethylases play an important role in epigenetic mechanisms due to their effect on histones. Specifically the UTX gene has long been known to be involved in development, due to its effect on germ layer fate of mouse ESCs [225] as well as on the fidelity and lineage

specificity of neural progenitor cells [226]. It was long believed that the UTY gene has lost its demethylase activity but results in knockout mice have demonstrated that this is not the case [227]. Thus, we believe that the UTY and UTX gametolog pair can perform similar demethylase functions.

Similarly to UTX and UTY, the demethylase KDM5D of the gametolog pair KDM5C/KDM5D was upregulated in male NSCs. KDM5C has shown to be important during development, and mutations in this gene caused X-linked cognitive disability [162]. Genomic analyses and functional assays demonstrated that Kdm5c plays a critical role as a repressor responsible for the developmental silencing of germ line genes during cellular differentiation, and in fine-tuning activity-regulated enhancers during neuronal maturation [228]. In none of the two studies described above a possible effect of the intact Y gametologous KDM5D was taken into consideration nor discussed. Interestingly, KDM5D, can specifically demethylate Lys-4 of histone H3. Taken together, we believe that the genes UTY and KDM5D with its demethylase ability can serve an important role in neural differentiation or neural cell fate. Interestingly, not only UTY and KDM5D were increased during differentiation in this study, but also two Y-lncs located close to these genes were upregulated early during differentiation. These results suggest that these Y-lncs may be involved in the control of expression of Y-encoded demethylases, which should be investigated in the future.

Paper III (Manuscript)

Aims

The investigation of genetic sex differences during neural differentiation using multiple human embryonic stem cell lines of each sex in an *in vitro* model of neuronal differentiation.

Methods

A differentiation of human embryonic stem cell lines of both sexes (4 male and 4 female) was performed using dual SMAD inhibition and small molecules in 2D cultures. This was followed by bulk total RNA extraction and Illumina sequencing of multiple timepoints during differentiation (D0, D4, D9, D17, D27, D37). The subsequent differential gene expression analysis was based on RNAseq data using DEseq2 and the gene set enrichment analysis was performed with the GSEA/MSigDB software. Differential gene expression was confirmed by qPCR analysis for additional timepoints and all available cell lines. The differentiation experiments were repeated three times to be able to estimate the variability of the differentiation via qPCR.

Results

The differentiation protocol and differentiation capacities of the cell lines have proven to be robust, displayed by low gene expression variability in experiment replicates and equal neuronal marker gene expression in male and female cell lines. Differential gene expression analyses identified sex-biased gene expression already at an undifferentiated stage (D0). After stringent filtering of sex-biased differentially expressed genes (DEG) to exclude false-positives, we identified a large contribution of sex chromosome genes to the sex-biased expression at D0. With increasing neuronal differentiation, the number of differential expressed genes increased, and with it the contribution of autosomal genes increased, especially in male cell lines (**Table 2**).

At the end-point of neuronal differentiation (after 37 days), functional neurons were present, detected by marker gene expression. However, a substantial number of genes involved in neurodevelopment were found to be expressed in a sex-biased manner. Of 148 sex-biased genes (104 overexpressed in males and 44 in females), 32 were overexpressed in males and linked to neurodevelopment, and 7 were overexpressed in females that also showed an involvement in neurodevelopment. From these genes we selected 13 candidate genes that possess the ability to influence neurodevelopment. The selection is based on the genes' GO-term association in neurodevelopment, upregulation during 37 days of differentiation, appearance in clusters of neuronal processes in GSEA, expression level and appearance in literatures that attributes an involvement in neurodevelopment. The following genes have been proposed as candidates: NHLH2, EBF1, SLC17A6, RUNX1T1, KIF5A, AKAP12, MDGA1, ONECUT2, P2RX3, LMX1B, SYAP1, AMOT, PAK3.

Since, the gene dosage balance of X/Y homologs play a special role in sex differences, we have invested their gene expression. We noticed that a large number of X/Y homologs that escape XCI are balanced through Y-homolog gene expression. In addition, the Y-homolog TXLNGY and UTY are highly upregulated in male cells during differentiation.

Conclusion

Human embryonic stem cells are a robust research model for human neuronal differentiation. Male and female cell lines differentiate similarly without large difference, indicated by relatively low fold-change of differentially expressed genes. Nevertheless, the presence of sex-biases in expression in a large number of genes related to neurodevelopment indicates potential sex-biases in neural differentiation trajectories. The suggested 13 candidate genes have the potential to be involved in the development of sex-biases during human neuronal development. Most X/Y homologs displayed a balanced gene dosage during neuronal differentiation. In X/Y homologs that escape XCI, the Y-homolog robustly leads to balance in gene dosage.

Paper IV (Draft Manuscript)

Aims

Investigation of sex differences of common epigenetic modifications affecting gene expression in human embryonal stem cell lines.

Methods

Cultivation of 3 male and 4 female human embryonal stem cell lines in a pluripotent stage, subsequent RNA extraction and total RNA sequencing. Cell pellets of 2 mio cells per cell line were used for quantitative ChIP-seq (MINUTE-ChIP). H3K4me3 peaks were identified and differential expression analysis was performed with DEseq2 based on expression of gene promoters.

Results

We detected a hypermethylation (H3K4me3) of promoters of essential pluripotency genes (SOX2, OCT4, NANOG) and genes of corresponding signaling pathways such as, TGF-beta, MAPK/ERK, PI3K-Akt and Wnt, in female cell lines. Pluripotency pathways were identified by an overrepresentation assay (enrichR) using all significantly sex-biased H3K4me3 hypermethylated promoters. According to the RNA sequencing data, the increased H3K4me3 signal at promoter sites of pluripotency genes did not lead to an overexpression of the affected genes.

Conclusion

Female mammalian embryos develop later than their male counterparts. Related to this, is a shift towards naïve pluripotency that has been detected in female human stem cell lines. We hypothesize, that the increased H3K4me3 signal at promoters of pluripotency genes leads to an increased maintenance of cell identity. This can be achieved through an increased transcriptional response at pluripotency genes due to higher H3K4me3 signal at associated promoters. As a consequence, female cell lines resist differentiation cues longer than their male counterparts, until a threshold is reached.

Future perspective

During my investigation I have noticed that sex differences are still not routinely investigated even though samples and data from different sexes are available in a study. Often the studies in question are not interessted in sex difference but have other aims. As a result, data from both sexes are combined and analyzed as one. In this way, sex differences or similarities are disregarded and will never be identified. It is in everyone's interest that we reveal sex differences and similarities wherever possible. This is a plea to my fellow scientists, even though you might not specifically be looking for sex differences, it is still worth taking the time to do a comparison between your male and female data and to write a small abstract about your findings. If this can be introduced routinely into every scientific publication, we can make enormous advances in the identification of critical sex differences, from which we all will profit in the long run when it comes to the prediction, prevention and treatment of sex-biased disorders.

To investigate the effect of sex-biased gene expression during human early neurodevelopment is not trivial. The main reason for this is, that non-invasive sampling of human embryo brain tissue during development is not possible and even imaging techniques comes to their limitations. Luckily, we live in a time at which the field of stem cells is developing at lightspeed and new, more physiological differentiation techniques and models of embryo development are established by the minute, at least that is how it feels. To clearly identify sexual differential gene expression, a large number of male and female samples are needed in order to rule out inter-individual differences. The first large cell banks stocking human induced pluripotent stem cells and other stem cell types have been established in Europe and readily supply stem cells for medical research. With these vast improvements, I am positive that the presence and effect of sex-biased gene expression in neurodevelopment and its contribution to sex-biased neurodevelopmental disorders, will soon be unriddled.

In regards to the findings in my studies, most of the time, genes and their proteins have more than one function and I have the feeling that often only a fraction of the functions are known and listed in databases. This applies especially to the genes and proteins of the X and Y chromosome, but also to genes involved in neurodevelopment. During my studies I have regularly come across genes that are expressed in an intriguing pattern during

neurodevelopment in research models, but lack a function in current databases. This highlights how much there is still to discover. In all of my studies, and literature researches, I have noticed the large contribution of X chromosome genes to sex-biased expression in neurodevelopment. In fact, a large number of X-linked genes have already been implicated in neurodevelopment. Therefore, I believe, that X-linked genes are highly rewarding targets for future investigations of sex differences. Furthermore, is the X chromosome inactivation of utmost importance in stem cell models of the future. XCI is highly linked to gene dosage and also to pluripotency stages in female cell lines, both are important for differentiation capabilities of stem cells. It was only a few years ago that I asked a representative of a large US stem cell bank regarding their characterization for XCI status and state of pluripotency, and they have replied that its effect is not evident and therefore not regarded, while today, it is a hot topic in the generation of stem cells. The Y-linked genes have potential to a much smaller extend but should not be underestimated. As mentioned before, genes of the Y chromosome are often only annotated with a single function, an that is within reproductive processes, but in my opinion, this is due to the lack of functional investigations in fields other than reproduction. Especially from an epigentic point of view, the Y chromosome gene has a number of interessting chromatin modifiers that can have wide ranging effects even beyond the Y chromosome. Also, non-coding genes harness a great potential and these are highly represented on the Y-chr gene. The importance of X/Y homolog gene dosage is still elusive and needs more investigation. Contradictory reports about the effect of gene dosage balance of X/Y homologs question its relevance in disease or dysfunction. Enough data from previous studies of X/Y homologs is available that should attract more functional investigations, especially with the increasing availability of CRISPR screens.

In conclusion, more thorough investigations, with high number of samples, are necessary to detect sex-biased gene expression in neuronal development. X and Y chromosome genes, as well as non-coding RNAs are targets with high potential.

Popular science summary

The term 'sex differences' describes the differences between biological males and females. Sex differences, are present in many different forms and shapes in the human body. Some of them are well known, such as the difference between size or muscle capacity. Others are not so prominent such as sex differences in the brain. While the exact differences between the brain in males and females, and the molecular mechanisms that lead to them are not easily investigated, the result of sex differences can relatively easy be seen in a sex bias in susceptibility of males and females towards neurological disorders. Males are more susceptible to neurodevelopmental disorders, such as Autism Spectrum Disorders or Tourette's syndrome, while females are more prone to develop so called emotional disorders such as, Depression or Anxiety Spectrum Disorders. These differences in disease susceptibility suggest that there are some differences in the development of the nervous system that, for example, make females more resilient to neurodevelopmental disorders. To be able to identify, monitor and treat patients with neurological disorders adequately, it is of great importance to understand what can cause sex differences in the development of the brain.

The largest and well investigated factor that influences the sex-biased development of the human brain are the sex hormones (e.g. testosterone and estrogen). However, there are other factors that are not as well understood and studied. One of these factors is the effect of genes on the development of the brain. Genes are the blueprints from which the body can create proteins, which in turn are required for the structure, function, and regulation of the body's tissues and organs. The genes of the sex chromosomes (X and Y chr) play a special role in sex differences. In general, almost every cell in the body, whether male or female, has 44 chromosomes called autosomes that do not differ in content between males or females. But apart from that, female cells possess two X chr, while male cells have one X and one Y chr. This rather small difference can have a large impact, a single gene on the Y chr for example is the reason why males develop testes and not ovaries. Even though the Y chr is very small it still possesses some genes that seem to influence the development of neurons. At the same time a lot of genes on the X chr have also been shown to influence the development of the brain.

In my studies, I am investigating the presence and effects of sex chromosome genes in the development of the brain. In addition, I am studying if genes from the sex chromosome or even autosomes are expressed in a sex-biased manner (expressed = decoded and put into use) and if they then could affect neurodevelopment. To study this, I am analyzing gene expression in male and female samples such as human fetal brain tissue and human stem cells that develop into neurons.

In a first study, we confirmed that a lot of Y chr genes are expressed in the fetal brain as well as in human stem cells that develop into neurons. This confirms, together with studies from other scientists, that Y-linked genes are involved in neurodevelopment. Previously, Y chr genes have been associated mainly with function in tissues involved in reproduction. We have also identified that Y chr genes contribute to a balanced gene dosage of so-called X/Y homolog genes (aka. gametologs). These genes are special because they exist in one copy on the X chr and in one copy on the Y chr. Since the X chr is present twice in females, the X/Y homolog genes can have a higher gene dosage in females. An imbalance in gene dosage can lead to dysfunctions in the body. The Y chr homolog of these genes however, seems to successfully work towards a balanced gene dosage in fetal brain tissue as well as in stem cells developing to neurons.

In a study that investigates neural stem cells, cells that develop into neurons and that are essential for the development of the brain, we have found that male cells reproduce faster than female cells. We also found that male cells at day 4 in a neuron-development experiment have higher levels of genes involved in neuron and synapse development but after 14 days of differentiation the gene expression was at an equal level in male and female cells. These results suggest a sex difference in early neuron development. Further, we have noticed that the gene expression of two genes (UTY, KDM5D) involved in the unwrapping of the DNA is increasing sharply during neuron development in male cells. The unwrapping of DNA is essential for the decoding (transcription) of genes into functional units. These unwrapping and rewrapping mechanisms are regulated by functional units that leave marks on the DNA. These marks are then used to either unwrap the DNA and make it accessible for decoding, or to rewrap the DNA so it is tightly packed and does not use so much space. A whole field of science has developed around it, called Epigenetics.

Since we noticed a high activity of genes that express epigenetic factors in the male cells, we have conducted a study that looks for sex differences among these factors in male and female stem cells. The study is not finalized yet, but we have noticed a female sex bias in epigenetic marks that increase the activity of genes involved in pluripotency. Pluripotency is the ability of stem cells to develop into almost all other tissue types. An increase in marks at pluripotency

genes suggests that female stem cells stay longer in a pluripotency state than male cells. As a consequence, female stem cells eventually develop into neurons later or need a stronger signal to start developing.

In another study, we used male and female stem cells and provoked them to develop into neurons. We analyzed sex differences in gene expression and found that male and female cells show slight differences in a number of biological processes already before developing into neurons. One of such processes is an increase in ribosome activity in male cells, which could lead to an increased growth of male cells. This is in line with the results we described earlier in human neural stem cells. We also noticed that at 37 days of neuron development there is a large number of genes that contribute to neuron growths and function that are expressed in male cells but not in female cells. At the same time, there is a large number of X chr genes that are expressed in female cells but not in male cells. This suggests that male and female cells show a sex difference in gene expression during the development of neurons and that X chr genes contribute to this. We also identified 10 candidate genes that are likely to provoke a sex difference in neuron development in male cells and 3 candidate genes in female cells.

In summary, this thesis has contributed to the identification of genetic sex differences and investigated mechanisms that alleviate and contribute to sex differences in human neuron development.

Populärvetenskaplig sammanfattning

Begreppet "könsskillnader" beskriver skillnaderna mellan biologiska män och kvinnor. Könsskillnader förekommer i många olika former i människokroppen. Vissa av dem är välkända, som t.ex. skillnaden mellan storlek eller muskelkapacitet. Andra är inte så framträdande, t.ex. könsskillnader i hjärnan. De exakta skillnaderna mellan hjärnan hos män och kvinnor och de molekylära mekanismer som leder till dem är inte lätta att undersöka, men resultatet av könsskillnader kan relativt lätt ses i form av en könsbias när det gäller mäns och kvinnors känslighet för neurologiska sjukdomar. Män är mer mottagliga för neurologiska utvecklingsstörningar som autismspektrumstörningar eller Tourettes syndrom, medan kvinnor är mer benägna att utveckla så kallade känslomässiga störningar som depression eller ångest. Dessa skillnader i sjukdomskänslighet tyder på att det finns vissa skillnader i nervsystemets utveckling som t.ex. gör kvinnor mer motståndskraftiga mot neurologiska utvecklingsstörningar. För att kunna identifiera, övervaka och behandla patienter med neurologiska störningar på ett adekvat sätt är det av stor vikt att förstå vad som kan orsaka könsskillnader i hjärnans utveckling.

Den största och mest väl-undersökta faktorn som påverkar den könsbundna utvecklingen av den mänskliga hjärnan är könshormonerna (t.ex. testosteron och östrogen). Det finns dock andra faktorer som inte är lika väl förstådda och studerade. En av dessa faktorer är genernas inverkan på hjärnans utveckling. Gener är de ritningar från vilka kroppen kan skapa proteiner, som i sin tur krävs för struktur, funktion och reglering av kroppens vävnader och organ. Generna i könskromosomerna (X och Y chr) spelar en särskild roll för könsskillnader. I allmänhet har nästan varje cell i kroppen, oavsett om den är manlig eller kvinnlig, 44 kromosomer som kallas autosomer och som inte skiljer sig i innehåll mellan män och kvinnor. Men bortsett från detta har kvinnliga celler två X chr, medan manliga celler har en X och en Y chr. Denna ganska lilla skillnad kan ha stor betydelse: en enda gen på Y chr är t.ex. orsaken till att hanar utvecklar testiklar och inte äggstockar. Även om Y chr är mycket liten har den ändå några gener som verkar påverka utvecklingen av nervceller. Samtidigt har många gener på X chr också visat sig påverka hjärnans utveckling.

I mina studier undersöker jag förekomsten och effekterna av könskromosomgener i hjärnans utveckling. Dessutom undersöker jag också om gener från könskromosomen eller till och med autosomerna uttrycks på ett könsbundet sätt (uttryckt = avkodad och tas i bruk) och om de då skulle kunna påverka neuroutvecklingen. För att studera detta analyserar jag genuttryck i manliga och kvinnliga prover, t.ex. mänsklig fosterhjärnvävnad och mänskliga stamceller som utvecklas till neuroner.

I våra studier bekräftade vi att många Y chr-gener uttrycks i fosterhjärnan och i mänskliga stamceller som utvecklas till neuroner. Detta bekräftar, tillsammans med studier från andra forskare, att Y-länkade gener är involverade i neuroutvecklingen. Tidigare har Y chr-gener främst förknippats med funktion i vävnader som är involverade i reproduktionen. Vi har också identifierat att Y chr-gener bidrar till en balanserad gendosering av så kallade X/Y homologgener (även kallade gametologer). Dessa gener är speciella eftersom de finns i en kopia på X chr och i en kopia på Y chr. Eftersom X chr finns två gånger hos kvinnor kan X/Y homologgener ha en högre gendosering hos kvinnor. En obalans i gendoseringen kan leda till dysfunktioner i kroppen. Y chr-homologen av dessa gener verkar dock framgångsrikt arbeta för en balanserad gendosering i hjärnvävnad hos foster och i stamceller som utvecklas till neuroner.

I en studie som undersöker neurala stamceller, celler som utvecklas till neuroner och som är viktiga för hjärnans utveckling, har vi funnit att manliga celler förökar sig snabbare än kvinnliga celler. Vi har också funnit att manliga celler vid dag 4 i ett experiment med neuronutveckling har högre nivåer av gener som är involverade i neuron- och synapsutveckling, men efter 14 dagars differentiering var genuttrycket på samma nivå i manliga och kvinnliga celler. Dessa resultat tyder på en könsskillnad i den tidiga neuronutvecklingen. Vidare har vi noterat att genuttrycket av två gener (UTY, KDM5D) som är involverade i utvikningen av DNA ökar kraftigt under neuronutvecklingen i manliga celler. Utvikningen av DNA är nödvändig för avkodning (transkription) av gener till funktionella enheter. Dessa mekanismer för att utvika och återvika generna regleras av funktionella enheter som lämnar märken på DNA. Dessa märken används sedan för att antingen packa upp DNA och göra det tillgängligt för avkodning, eller för att packa om DNA så att det blir tätt packat och inte tar så mycket utrymme i anspråk. Ett helt vetenskapsområde har utvecklats kring detta, kallat epigenetik.

Eftersom vi noterade en hög aktivitet av gener som uttrycker epigenetiska faktorer i de manliga cellerna har vi genomfört en studie som letar efter könsskillnader bland dessa faktorer i manliga och kvinnliga stamceller. Studien är inte färdig ännu, men vi har noterat en kvinnlig könsbias i epigenetiska markeringar som ökar aktiviteten hos gener som är involverade i pluripotens.

Pluripotens är stamcellernas förmåga att utvecklas till nästan alla andra vävnadstyper. En ökning av markeringar vid pluripotensgener tyder på att kvinnliga stamceller stannar längre i pluripotenstillstånd än manliga celler. Som en följd av detta utvecklas de kvinnliga stamceller så småningom senare till neuroner eller behöver en starkare signal för att börja utvecklas.

I en annan studie använde vi manliga och kvinnliga stamceller och provocerade dem att utvecklas till neuroner. Vi analyserade könsskillnader i genuttryck och fann att manliga och kvinnliga celler uppvisar små skillnader i ett antal biologiska processer redan innan de utvecklas till neuroner. En av dessa processer är en ökad ribosomaktivitet i manliga celler, vilket skulle kunna leda till en ökad tillväxt hos manliga celler. Detta stämmer överens med de resultat som vi tidigare beskrivit i mänskliga neurala stamceller. Vi noterade också att det vid 37 dagars neuronutveckling finns ett stort antal gener som bidrar till neuronernas tillväxt och funktion som uttrycks i manliga celler men inte i kvinnliga celler. Samtidigt finns det ett stort antal X chr-gener som uttrycks i kvinnliga celler men inte i manliga celler. Detta tyder på att manliga och kvinnliga celler uppvisar en könsskillnad i genuttryck under utvecklingen av neuroner och att X chr-gener bidrar till detta. Vi identifierade också 10 kandidatgener som sannolikt framkallar en könsskillnad i neuronutvecklingen i manliga celler och 3 kandidatgener i kvinnliga celler.

Sammanfattningsvis har denna avhandling bidragit till identifieringen av genetiska könsskillnader och undersökt mekanismer som lindrar och bidrar till könsskillnader i mänsklig neuronutveckling.

Populärwissenschaftliche Zusammenfassung

Der Begriff "Geschlechtsunterschiede" beschreibt die Unterschiede zwischen biologisch männlichen und weiblichen Personen. Geschlechtsunterschiede sind im menschlichen Körper in vielen verschiedenen Formen und Ausprägungen vorhanden. Einige von ihnen sind sehr einfach zu messen, wie der Unterschied in Größe oder Muskelkapazität. Andere sind weniger einfach zu erkennen, wie die Geschlechtsunterschiede im Gehirn. Während die genauen Unterschiede zwischen dem männlichen und dem weiblichen Gehirn, und die molekularen Mechanismen die zu diesen führen, nicht leicht zu erforschen sind, lässt sich das Ergebnis der Geschlechtsunterschiede relativ leicht in einer geschlechtsspezifischen Anfälligkeit von Männern und Frauen für neurologische Störungen erkennen. Männer sind anfälliger für neurologische Entwicklungsstörungen wie Autismus-Spektrum-Störungen oder das Tourette-Syndrom, während Frauen anfälliger für so genannte emotionale Störungen wie Depressionen oder Angststörungen sind. Diese Unterschiede in der Krankheitsanfälligkeit deuten darauf hin, dass es Unterschiede in der Entwicklung des Nervensystems gibt, die z. B. Frauen widerstandsfähiger gegenüber neurologischen Entwicklungsstörungen machen. Um Patienten mit neurologischen Störungen angemessen identifizieren, überwachen und behandeln zu können, ist es von großer Bedeutung zu verstehen, was diese Geschlechtsunterschiede in der Entwicklung des Gehirns verursacht.

Der meist- und am besten untersuchte Faktor, der die geschlechtsspezifische Entwicklung des menschlichen Gehirns beeinflusst, sind die Sexualhormone (z. B. Testosteron und Östrogen). Es gibt jedoch auch andere Faktoren, die weniger gut verstanden und untersucht sind. Einer dieser Faktoren ist die Wirkung der Gene auf die Entwicklung des Gehirns. Gene sind die Baupläne, aus denen der Körper Proteine herstellen kann, die wiederum für die Struktur, Funktion und Regulierung der Gewebe und Organe des Körpers erforderlich sind. Die Gene der Geschlechtschromosomen (X- und Y-Chromosomen) spielen bei den Geschlechtsunterschieden eine besondere Rolle. Im Allgemeinen hat fast jede Zelle im Körper, ob männlich oder weiblich, 44 Chromosomen, die so genannten Autosomen, die sich in ihrem Inhalt nicht zwischen Männern und Frauen unterscheiden. Abgesehen davon besitzen weibliche Zellen zwei X-Chromosomen, während männliche Zellen ein X- und ein Y-Chromosom besitzen. Dieser eher kleine Unterschied kann große Auswirkungen haben: Ein einziges Gen auf dem Y-Chr ist zum Beispiel der Grund dafür, dass

Männer Hoden und keine Eierstöcke entwickeln. Auch wenn das Y-Chr sehr klein ist, besitzt es doch einige Gene, die die Entwicklung von Neuronen zu beeinflussen scheinen. Gleichzeitig hat sich gezeigt, dass auch viele Gene auf dem X-chr die Entwicklung des Gehirns beeinflussen.

In meinen Studien untersuche ich das Vorhandensein und die Auswirkungen von Genen der Geschlechtschromosomen auf die Entwicklung des Gehirns. Außerdem untersuche ich, ob Gene der Geschlechtschromosomen oder sogar der Autosomen geschlechtsspezifisch exprimiert werden (exprimiert = dekodiert und genutzt) und ob sie die Neuroentwicklung beeinflussen könnten. Um dies zu untersuchen, analysiere ich die Genexpression in männlichen und weiblichen Proben, wie z. B. humanem fötalen Hirngewebe und humane Stammzellen, die sich zu Neuronen entwickeln.

In einer ersten Studie haben wir bestätigt, dass viele Y-chr-Gene sowohl im fötalen Gehirn als auch in humanen Stammzellen, die sich zu Neuronen entwickeln, exprimiert werden. Dies bestätigt zusammen mit Studien anderer Wissenschaftler, dass Y-chr-Gene an der Neuroentwicklung beteiligt sind. Bisher wurden Y-chr-Gene hauptsächlich mit Funktionen in Verbindung gebracht, die an der Fortpflanzung beteiligt sind. Wir haben auch festgestellt, dass Y-chr-Gene zu einer ausgewogenen Gendosierung von so genannten X/Y-homologen Genen (auch Gametologe genannt) beitragen. Diese Gene sind etwas Besonderes, weil sie in einer Kopie auf dem X-chr und in einer Kopie auf dem Y-chr vorhanden sind. Da das X-chr bei Frauen doppelt vorhanden ist, können die X/Y-homologen Gene bei Frauen eine höhere Gendosis aufweisen. Damit bei Männern eine gleichhohe Gendosis besteht, muss das Y-chr-homolog die Gendosis ausgleichen. Ein Ungleichgewicht in der Gendosierung kann zu Funktionsstörungen im Körper führen. Wir haben festgestellt, dass das Y-chr-Homolog dieser Gene jedoch erfolgreich eine ausgewogene Gendosierung in fötalem Hirngewebe sowie in Stammzellen, die sich zu Neuronen entwickeln, bewirkt.

In einer Studie über neuronale Stammzellen, Zellen, die sich zu Neuronen entwickeln und für die Entwicklung des Gehirns unerlässlich sind, haben wir festgestellt, dass sich männliche Zellen schneller vermehren als weibliche Zellen. Außerdem haben wir festgestellt, dass männliche Zellen, am 4. Tag eines Experiments zur Entwicklung von Neuronen, eine höhere Anzahl von Genen aufweisen die an der Entwicklung von Neuronen und Synapsen beteiligt sind, nach 14 Tagen der Differenzierung war die Genexpression jedoch bei männlichen und weiblichen Zellen gleich hoch. Diese Ergebnisse deuten auf einen Geschlechtsunterschied in der frühen Neuronenentwicklung hin. Außerdem haben wir festgestellt, dass die Genexpression von zwei Genen (UTY, KDM5D), die an der Entfaltung der DNA beteiligt sind, während der Neuronenentwicklung in männlichen Zellen stark zunimmt. Das Entpacken der DNA ist für die Dekodierung (Transkription) von Genen in funktionelle

Einheiten unerlässlich. Diese Entpackungs- und Verpackungsmechanismen werden durch funktionelle Einheiten reguliert, die Markierungen auf der DNA hinterlassen. Diese Markierungen werden dann verwendet, um entweder die DNA zu entfalten und sie für die Entschlüsselung zugänglich zu machen oder um die DNA neu zu falten, so dass sie dicht gepackt ist und nicht so viel Platz einnimmt. Ein ganzer Wissenschaftszweig hat sich zu diesem Thema entwickelt, die Epigenetik.

Da wir eine hohe Aktivität von Genen, die epigenetische Faktoren kodieren, in männlichen Zellen festgestellt haben, haben wir eine Studie durchgeführt, die nach Geschlechtsunterschieden bei diesen Faktoren in männlichen und weiblichen Stammzellen sucht. Diese Studie ist noch nicht abgeschlossen, aber wir haben einen Geschlechtsunterschied bei den epigenetischen Markierungen festgestellt, die die Aktivität von Genen erhöhen, die an der Pluripotenz beteiligt sind. Pluripotenz ist die Fähigkeit von Stammzellen, sich zu fast allen anderen Gewebetypen zu entwickeln. Eine Zunahme der Markierungen an Pluripotenzgenen deutet darauf hin, dass weibliche Stammzellen länger in einem Pluripotenzzustand bleiben als männliche Zellen. Dies hat zur Folge, dass sich weibliche Stammzellen später zu Neuronen entwickeln oder ein stärkeres Signal benötigen, um ihre Entwicklung zu beginnen.

In einer anderen Studie haben wir männliche und weibliche Stammzellen verwendet und sie stimuliert, sich zu Neuronen zu entwickeln. Wir haben die Geschlechtsunterschiede in der Genexpression analysiert und fanden heraus, dass männliche und weibliche Zellen bereits vor der Entwicklung zu Neuronen leichte Unterschiede in einer Reihe von biologischen Prozessen aufweisen. Einer dieser Prozesse ist eine erhöhte Ribosomenaktivität in männlichen Zellen, was zu einem verstärkten Wachstum der männlichen Zellen führen könnte. Dies steht im Einklang mit den Ergebnissen, die wir zuvor bei humanen neuronalen Stammzellen beschrieben haben. Wir haben auch festgestellt, dass am 37. Tag der Neuronenentwicklung eine große Anzahl von Genen, die zum Wachstum und zur Funktion von Neuronen beitragen, in männlichen Zellen, nicht aber in weiblichen Zellen exprimiert werden. Gleichzeitig gibt es eine große Anzahl von X-chr-Genen, die in weiblichen Zellen, nicht aber in männlichen Zellen exprimiert werden. Dies deutet darauf hin, dass männliche und weibliche Zellen während der Entwicklung von Neuronen einen Geschlechtsunterschied in der Genexpression aufweisen und dass X-chr-Gene dazu beitragen. Wir haben 13 Kandidatengene identifiziert, die wahrscheinlich einen Geschlechtsunterschied in der Neuronenentwicklung in männlichen und weiblichen Zellen hervorrufen.

Zusammenfassend lässt sich sagen, dass diese Arbeit dazu beigetragen hat, genetische Geschlechtsunterschiede zu identifizieren und Mechanismen zu untersuchen, die die Geschlechtsunterschiede in der menschlichen Neuronenentwicklung verringern aber auch zu ihnen beitragen können.

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