

Sex steroids and social behavior: From mouse to human

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“I don't want to believe. I want to know”

Carl Sagan

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ABSTRACT

Social behavior is a classification of various behaviors such as sexual behavior, aggressive behavior, social memory and sociability. These behaviors are under the regulation of the brain, and the sex steroids influence important brain regions that control these behaviors in both humans and other animals. To behave and respond adequately in social situations is crucial for interacting with others in an appropriate way. Several disorders, e.g. autism spectrum disorders (ASD), display deficits in social abilities and, interestingly, show differences between females and males in prevalence and symptoms. Moreover, testosterone is hypothesized to be one of the causative factors behind the etiology of ASD. Both the androgen and estrogen receptors are of known importance for sexually dimorphic social behaviors such as sexual and aggressive behaviors. Furthermore, estrogen receptors are essential for social memory in rodents, and human studies show a female bias towards better social memory. Less is known about the role of androgen receptors.

The aims of this thesis were to elucidate if sex, sex steroids and the androgen receptor are involved in social memory and sociability in mice, if genes and proteins in brain regions regulated by sex steroids, with known importance for social behavior, show sexual dimorphisms or are regulated by androgen receptors, and if genetic variations in sex steroid-related genes are involved in social memory and/or autistic-like traits in human.

The results show that a) testosterone is not crucial for social memory or sociability but for the persistence of social investigation, b) estrogen is important for social memory but not for sociability, c) only when the conspecifics were male did the androgen receptor appear to be involved in social memory, d) a small number of genes involved in sex steroid synthesis, were

regulated by the androgen receptor but e.g. the oxytocin receptor show androgen receptor dependant sexual dimorphic expression, e) proteins expressed in postnatal day 8 old mice did not show great differences between the sexes, or between males with or without the androgen receptor, f) genetic variations in the estrogen receptors were associated with better social memory in women, and g) genetic variations in the transport protein of sex steroids were associated with language disabilities, specifically in boys.

The main findings presented in this thesis are thus that sex steroids are implicated in different manners in sociability and social memory in mice, and that genetic variation in sex steroid-related genes are associated with social abilities in humans.

Keywords: Sex steroids, sex steroid receptors, social memory, sociability, face recognition, genetic variations

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LIST OF PAPERS

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- I. **Sara Karlsson**, Kaltrina Haziri, Evelyn Hansson, Petronella Kattunen, Lars Westberg. Effects of sex and gonadectomy on social investigation and social recognition in mice. *BMC Neuroscience* 16(1):83 (2015).
- II. **Sara Karlsson**, Erik Studer, Petronella Kettunen, Lars Westberg. Neural androgen receptors modulate gene expression and social recognition but not social investigation. *Frontiers Behavioral Neuroscience* 10:41 (2016).
- III. Anna Zettergren, **Sara Karlsson**, Erik Studer, Anna Sarvimäki, Petronella Kettunen, Annika Torsell, Catarina Sihlbom, Lars Westberg. Proteomic analyses of limbic regions in neonatal male, female and androgen receptor knockout mice. *Submitted*.
- IV. **Sara Karlsson**, Susanne Henningson, Daniel Hovey, Anna Zettergren, Lina Jonsson, Diana Sanchez Cortes, Jonas Melke, Petri Laukka, Håkan Fischer, Lars Westberg. Social memory associated with estrogen receptor polymorphisms in women. *Social Cognitive Affective Neuroscience* (2016).
- V. Anna Zettergren, **Sara Karlsson**, Daniel Hovey, Lina Jonsson, Jonas Melke, Henrik Anckarsäter, Paul Lichtenstein, Sebastian Lundström, Lars Westberg. Further investigations of the relation between polymorphisms in the sex steroid related genes and autistic-like traits. *Psychoneuroendocrinology* 68:1-5 (2016).

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Abbreviations

ALTs	Autistic like traits
AOB	Accessory olfactory bulb
AR	Androgen receptor
ASDs	Autism spectrum disorders
A-TAC	Autism, Tics, AD/HD and other Comorbidities inventory
AVP	Vasopressin
AVPV	Anteroventral periventricular nucleus
BNST	Bed nucleus of the stria terminalis
ER α	Estrogen receptor alpha
ER β	Estrogen receptor beta
FFA	Fusiform face area
FSH	Follicle-stimulating hormone
GABA	Gamma-aminobutyric acid
GnRH	Gonadotrophin releasing hormone
GPGR	G protein-coupled estrogen receptor
LH	Luteinizing hormone
MeA	Medial amygdala
MeA	Medial amygdala
MOE	Main olfactory bulb
NOR	Novel object recognition
OB	Olfactory bulb
OT	Oxytocin
PCR	Polymerase chain reaction
PGR	Progesterone receptor
SCN	Suprachiasmatic nucleus
SND-POA	Sexual dimorphic nucleus – preoptic area
SNP	Single nucleotide polymorphism
SR	Social recognition
StAR	Steroidogenic acute regulatory protein
VMH	Ventrolateral hypothalamus
VMH	Ventromedial hypothalamus
VNO	Vomer nasal organ

PREFACE

Through evolution, humans have developed excellent abilities in encoding and interpreting social information, such as identity, sex, age and emotional state of other persons. There are several lines of evidence showing that these abilities are generally more developed in girls and women as compared to boys and men. Most children attend to faces and establish eye contact already at the day of birth. Very early in infancy girls attend more to faces and look more into the eyes of others compared to boys. Girls are also superior to boys with regard to face recognition memory, at least for recognition of female faces. These sex differences extend throughout life.

Interestingly, a core symptom of autism spectrum disorders (ASD) - several-fold more common in boys than in girls - is a deficiency in social communication skills, including an inability to attend to and interpret social signals from other individuals, which often extends to problems with identity recognition. Moreover, infants later diagnosed with ASD were recently seen to exhibit a decline in eye fixation from 2 to 6 months of age, a pattern not observed in infants who do not develop ASD.

Emerging evidence suggests that prenatal testosterone released in males may increase ASD risk. This does not seem farfetched since the sex hormone testosterone is crucial for the sexual differentiation of the brain, and hence responsible for many sexual dimorphisms seen in brain function and behaviors.

The main questions this thesis tries to resolve are the following:

- Are sex steroids involved in the sex differences seen in social attention, social recognition and the risk of ASD?
- Which receptors, downstream targets and neural mechanisms are involved in the effects of sex steroids on social attention, social recognition and the risk of ASD?

To answer these questions we have a) established behavioral mouse models for sociability/social attention and social recognition, b) generated conditional knockout mice lacking androgen receptors specifically in the neural system, c) employed quantitative gene expression and proteomics methodology to understand molecular mechanisms, and d) genotyped large samples of individuals assessed for social recognition and ASD risk.

INTRODUCTION

SOCIAL BEHAVIOR

Social behaviors are behaviors oriented towards other individuals, and comprise e.g. sexual behavior, aggression, parental care, and pair-bonding behaviors as well as sociability and social memory. Individuals living in social groups are dependent on the ability to recognize, encode and recall social and socioemotional information from conspecifics in order to behave in a suitable way. Neuronal networks in the brain that connects perception to emotions, and subsequently to behavioral reactions are required for social behaviors to be adaptive.

As most complex traits, social behaviors vary between individuals and are shaped by both genetic and environmental factors [1]. The contribution of genes to phenotypical variation arises from mutations in the DNA molecule, which comprises the genes that encode the proteins. There are several types of mutations, including single nucleotide variants, repeats, copy number variations, i.e., chromosomal deletions or insertions. Mutations present in more than 1% of the population are referred to as polymorphisms, and these common genetic variations are considered as the main genetic contributors to individual differences in human complex traits. In recent years a number of polymorphisms, and rare mutations, has been proposed to contribute to social behaviors [2-4] and to the risk of autism spectrum disorders [5].

Social memory and sociability

The phenotypes of interest in this thesis are *sociability* and *social memory*. *Sociability* is defined as showing an interest towards a conspecific individual, which is often measured as the preference for spending time with an individual compared to an object. *Social memory* is defined as the ability to remember an already encountered conspecific, which is often measured as the preference for spending time with a novel individual compared to a familiar one. These two important behavioral phenotypes are central for survival in social structures.

Social memory and sociability in rodents

Rodents generally live in social groups and, just as humans, they need to communicate and recognize each other in order to create and maintain social stability. In their natural habitat, mice typically live in social groups with a single dominant male that has polygamous relations with several females. Thus, mice prefer to spend time with other conspecifics rather than being alone – i.e. they express sociability. Moreover, they depend on the ability to differentiate between encountered and novel conspecifics, hence they exhibit social memory [6]. Rodents exhibit both short-term and long-term social memory, partly regulated by different neural mechanisms. Surprisingly, mice remember conspecifics for at least 24 hours, whereas the social memory of rats, according to most, but not all [7], studies, only lasts a couple of hours [6].

There are several paradigms developed for testing social memory and sociability in rodents, all exploiting the spontaneous investigatory behavior that animals show towards conspecifics, as well as the innate drive to prefer exploration of novel over familiar items. The first social recognition paradigm was developed by Holloway and Thor in 1982 [8]. They showed that when an unfamiliar conspecific is introduced into the home cage of an adult male rat, the resident persistently investigates the novel individual. If the animal is removed and then reintroduced to the same resident male a short time later, it will receive far less investigation during the second meeting. This paradigm was further refined to include the discrimination between a familiar stimulus and a novel stimulus at the same time, and was called the social discrimination test [9]. This paradigm is most appropriate for measuring long-term social memory. A third paradigm is the habituation-dishabituation paradigm. In this setup the focal animal is repeatedly presented to an unfamiliar stimulus conspecific, usually in four separate trials during which familiarity is detected by reduced investigation on each trial. In the fifth trial a novel stimulus animal is introduced to rule out the possibility that the test animal's reduced investigation is due to fatigue. Overall, this paradigm needs shorter test times than the others but it may be less sensitive since the focal mouse can be sensitized to the test procedure. Nevertheless, all these three paradigms are widely used.

The three chamber paradigm developed by Crawley *et al.* [10] measures sociability in mice. The test arena consists of three chambers, one with an empty corral, one empty mid-chamber and one with a corral containing a stimulus animal. The focal mouse has the free choice to spend time in any of the

three compartments and to more closely investigate the corrals with or without an unfamiliar conspecific. Consequently, the main measures are the time spent in the social versus the non-social chamber as well as the time spent sniffing a novel mouse or an object – the empty corral. This way it is possible to observe if the focal animal has a social preference but also to gauge the persistence of investigating. In many studies the sociability session is followed by a test of preference for social novelty where a novel mouse is introduced into the empty corral. The three chamber paradigm was mainly developed as a model of the social deficits seen in patients with, for example, ASD [11]. Since then it has been widely used in order to study the neurobiology underlying genetic contribution to ASD [12].

Rodents receive social information from others via volatile and non-volatile (urine, skin and reproductive secretions) cues, and it is believed that each individual has their own special scent, a so called “olfactory signature” [6], i.e. a combination of volatile and non-volatile molecules [13, 14]. The social cues reach the nasal cavity which contains chemosensory neurons located in the vomeronasal organ (VNO), predominantly sensitive to nonvolatile molecules, and in the main olfactory epithelium (MOE), mainly sensitive to volatile molecules but also to some volatile peptide chemosignals [15]. The chemo signals are further processed either via the VNO to the accessory olfactory bulb (AOB), and further to specific nuclei of the amygdala and hypothalamus, or via the MOE to the main olfactory bulb regulating regions of the main olfactory pathway [16] that further transmit the social information to brain areas involved in social behaviors, including the hypothalamus [6, 17]. Although the hippocampus is crucial for general memory function its importance for social recognition is not fully understood. Some studies showed no involvement of the hippocampus in social memory on mice (no active *c-fos* cells) [18-20] whereas other studies indicate that the hippocampus [21, 22], and more recently the hippocampal area CA2, is crucial for long-term social memory [23].

Social recognition and sociability in humans

The abilities to retain and interpret information from social cues such as facial expression, vocal tone, body posture and gestures are extremely well developed in humans. Even newborn children make eye contact and imitate facial gestures indicating that these are, to a large extent, innate abilities [24]. In contrast to rodents humans receive social information mainly from visual and auditory cues. The role of pheromones – chemosignaling – in human social communication is

debated. The human VNO degenerates before birth [25] and no AOB structures are found in humans [26]. Consequently, if pheromonal cues influence human behavior, current evidence suggests that chemical signaling is mediated by the main olfactory pathway [27].

Face recognition is a substantially heritable trait in humans, and interestingly, most of the genetic influence is unique and not shared with other cognitive abilities [28]. There are individuals with excellent skills in face recognition [29], and there are individuals with face recognition disabilities, so called prosopagnosia, displaying face blindness [30]. Remarkably, persons with prosopagnosia have intact memory for objects [31, 32] and show normal intellectual abilities. This disorder also has a genetic component, and, at least in some cases, is inherited as an autosomal dominant disorder [33]. Interestingly, there is a large amount of evidence showing that patients with autism often suffer from deficits in face recognition [34].

Studies using neuroimaging show that the occipitotemporal regions, including the occipital face area and fusiform face area (FFA) as well as the anterior temporal regions show stronger activity for face than objects [24, 35]. Moreover, humans show robust skills for familiar face recognition, and increased familiarity increases identification accuracy. Familiarity is also related, not only to features of a face, but also to representations of the person's mental state, personality, and the emotions an observer experience on seeing the face [36]. The face recognition ability involves the occipital gyrus and temporal areas including the fusiform gyrus and superior temporal sulcus reviewed in [37]. Furthermore, the FFA influences the amygdala response to emotional faces [38].

AUTISM SPECTRUM DISORDERS

Characteristics of autism spectrum disorders

Autism spectrum disorders (ASD) are neurodevelopmental disorders characterized by deficits in social communication, language impairments as well as restricted and repetitive behaviors. ASD affects approximately 1% of the general population [39], and is 2-4 times more prevalent in boys than in girls [40]. There is currently no available treatment for the core symptoms of ASD - only pharmacological treatment of comorbid symptoms such as aggression and stereotypic behaviour [41, 42]. ASD represents the upper extreme of autistic-like traits (ALTs) that are continuously distributed in the general population

[43]. ALTs can be assessed with self-assessment questionnaires such as the Autism spectrum quotient (AQ) that measures the continuum from ASD to normality [44], or with parental interviews, including the Autism Tics, AD/HD, and other Comorbidities inventory (A-TAC) [45] based on the DSM-IV (released 1994) criteria.

In 2002 Baron-Cohen proposed the Empathizing-Systematizing (E-S) theory to explain the sex differences in cognition. In the general population males have a stronger drive to systemize while females tend to have stronger drive to empathize [46]. A further development of the E-S theory is the extreme male brain (EBM) theory of autism, suggesting that individuals with autism display an extreme profile in empathizing-systematizing where females score higher on the empathy quotient (EQ) [47] and males score higher on the systemizing quotient (SQ)[48, 49].

ASD is very heterogeneous and patients may vary substantially with respect to their symptom profiles. Both ASD and ALTs are also highly heritable [50] and it was recently shown that ALTs below the threshold for a diagnosis and ASD share common genetic influences [51]. Hundreds of genes appear to be involved in ASD etiology [52]. Rare mutations in specific genes have recently been estimated to cause the disorder in approximately 20-25% of ASD patients, including already known genetic syndromes such as Fragile X, Rett syndrome and tuberous sclerosis. A large number of genes have already been shown to comprise mutations causing ASD symptoms, but none of the individual genes seem to explain the disorder in >1% of the patients. Hence, common genetic variants are crucial also for the pathophysiology of ASD [53]. Furthermore, the underlying mechanism for the male bias is not fully understood, but a female protective effect has been supported by several lines of evidence [54], including that girls with ASD showed higher mutational burden than boys [55].

SEX STEROIDS

Synthesis

Ovaries and testicles are developed during early fetal development and females (XX) are said to be the default developmental pathway while the development of the male phenotype requires the activation of the *SRY* gene located on the Y-chromosome. If no *SRY* gene is present, the gonadal ridge (precursor to gonads) develops to ovaries.

This process occurs between gestational weeks 6 and 12 [56]. Thus, the activation of the *SRY* gene initiates the development of testes which secrete testosterone into the blood stream where it acts on sex steroid receptors.

The cholesterol molecule is the precursor of all steroid hormones, including the sex steroids. Through enzymatic reactions, called the steroidogenesis, cholesterol is converted to the sex steroids testosterone, estrogen and progesterone (Figure 1) that are secreted from the gonads. Specific sex steroids are also synthesized in other tissues, including the adrenal glands, adipose tissue and the brain. The gonadal secretion of sex steroids is regulated by gonadotropin-releasing hormone (GnRH) which after release from the hypothalamus stimulates secretion of the gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland into the blood stream. LH stimulates the expression of steroidogenic acute regulatory protein (StAR) in the Leydig cells of the testes and in the theca cells of the ovaries.

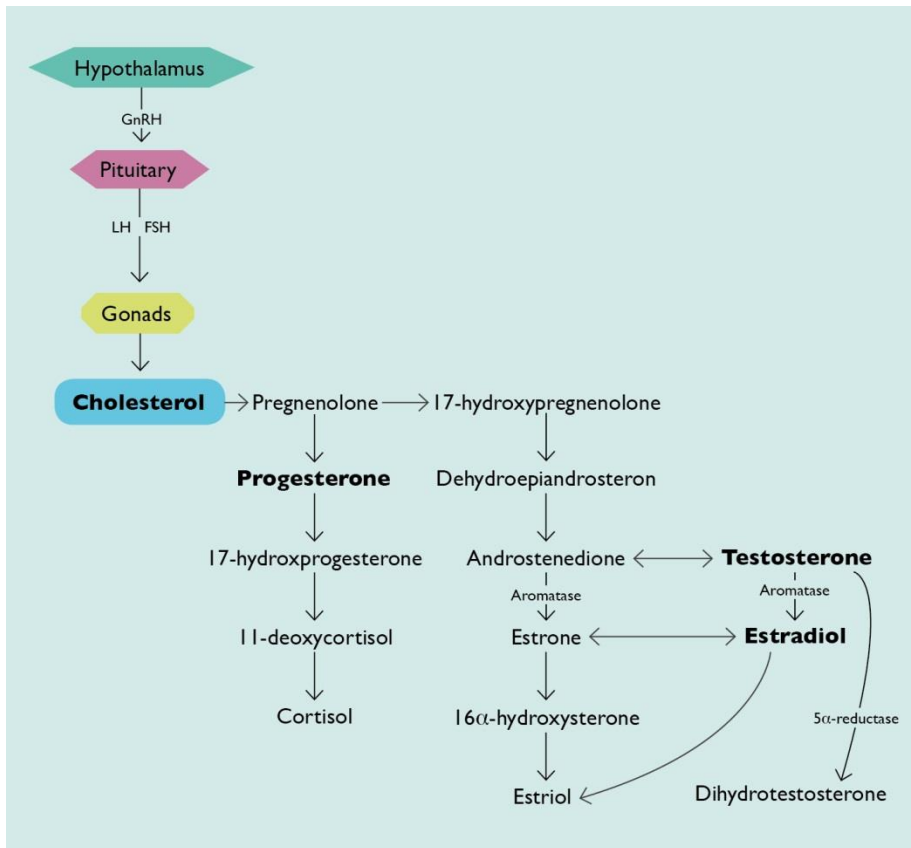


Figure 1, The regulation and synthesis of sex steroids.

The StAR protein, which is the rate-limiting step in the synthesis of sex steroids, stimulates the transfer of cholesterol into the inner membrane of the mitochondria to initiate the steroidogenesis. In the mitochondria the cholesterol molecule is converted to pregnenolone which in turn is converted to androgens, estrogens and progesterone. The synthesis of sex steroids is controlled by a feedback system where sex steroid receptors in the hypothalamus and pituitary have the ability to inhibit the secretion of GnRH, LH and FSH and in thus decrease the gonadal synthesis of sex steroids.

Two important enzymes in the sex steroid synthesis are 5α -reductase and CYP19/aromatase (Figure 1). A fraction of the testosterone is transformed by 5α -reductase to the more potent androgen, dihydrotestosterone. Testosterone can also be transformed into 17β -estradiol in tissues expressing aromatase, for example in the brain, gonads, bone and adipose tissue.

In females, 17 β -estradiol is the most prevalent and potent of the estrogens. 17 β -estradiol can be converted to estrone (predominant serum estrogen after menopause) or estriol (predominant serum estrogen during pregnancy).

Receptors

Most of the sex steroid actions are mediated by the nuclear sex steroid receptors, widely expressed in both the brain and peripheral organs. So far, one AR, two ERs, ER α and ER β , and one PGR have been identified. These nuclear sex steroid receptors are located inside the cell and are ligand-activated transcription factors; hence after ligand-binding they enter the cell nucleus, bind to specific sites of the DNA molecule and regulate transcription of genes. A G-protein coupled estrogen receptor (GPER), expressed in the endoplasmic reticulum, and a number of cell membrane bound progesterone receptors, mediate many of the non-genomic effects of estrogen and progesterone, respectively [57, 58].

Actions/Physiology

The sex steroids are mainly secreted from the gonads, and once secreted they are transported in the blood stream bound to transport proteins, i.e. the sex steroid binding globulin (SHBG) and albumin. Only a fraction, 1-2%, of the sex steroids is free in the blood stream [59].

In males the level of plasma testosterone peaks during three different time points; in utero, shortly after birth and during puberty, and the effects of testosterone are mediated by androgen receptors, and after aromatization to 17- β -estradiol, by the estrogen receptors (ER- α or ER- β). Testosterone effects can be classified as androgenic or virilizing, and anabolic. The testosterone peak during the second trimester is crucial for e.g. the development and formation of the male sex organs and the prostate. The function of the second testosterone peak during early infancy is the least understood. In humans the levels rise during the first weeks of life, remain in a pubertal range for a few months, and then fall to barely detectable levels until puberty. During puberty, testosterone, among other things, continues the virilization of the male gonads, induces a deepening of the voice and growth of the beard and axillary hair. It also exerts androgenic effects such as stimulation of increased muscle mass and bone density. In male rodents testosterone peaks just before birth (E18)[60], again on the day of birth, and then during puberty starting at about one month of age.

In females 17- β -estradiol and progesterone are the main sex steroids during the fertile period. They are cyclically released during the menstrual or estrus cycle, and are essential for e.g. reproductive tissues and fertility, development of breast as well as bone density. At between 45 to 55 years of age females enter menopause when estrogen levels decline, the menstrual period stops and as a result of the decline in estrogen most women experience menopausal symptoms and decreased bone mineral density. Males go through a similar process – the andropause – where the production of testosterone declines which results in muscle atrophy, hypogonadism and osteoporosis.

SEX STEROIDS, BRAIN AND BEHAVIOR

Sex steroids and behavior

As early as in the 1850's Arnold Adolph Berthold initiated studies in the field of endocrinology and specifically the influence of the gonads on secondary sexual characteristics. When observing chickens he discovered that male chickens, roosters, were aggressive, had a large comb and more developed muscles compared to female chickens. To explore if the gonads influenced these traits he subsequently removed the testicles from the chickens and discovered that the aggressive phenotype as well as comb and muscle size decreased. When he re-implanted or transplanted testes from other chickens into the abdominal cavity the testes established a connection with the blood and the behavioral and peripheral phenotypes were re-instated.

Later on the effects of gonadal hormones on mating and aggressive behaviors in roosters were proven to be due to actions of androgens and estrogens via the AR and ERs, respectively, and this turned out to be true also for many other species. The AR and ERs are expressed in many brain regions, including those known to regulate social behaviors, such as the hypothalamus and amygdala [61, 62]. A substantial amount of studies have also described effects of sex steroids on the expression of specific genes in the central nervous system [61, 63].

In more recent years the role of the specific steroid receptors for specific social behaviors has been further elucidated. Studies using ERKO α female mice show that these animals have a higher aggressive behavior and less pronounced sexual behavior [64]. ERKO α males showed less aggressive behavior which was not restored when treated with testosterone. A recent study in mice show that ER β is not important for male sexual behavior [65], but it is important in aggression

with ERKO β mice displaying higher aggressive behavior which could be further induced by estrogen treatment [66]. Furthermore, mice lacking ARs specifically in the brain display decreased mating behavior and aggression [67-71].

Sex steroids and sexual differentiation

A vast amount of evidence supports the notion that sex steroids are, to a great extent, responsible for sexual dimorphism in the anatomy and function of the brain. In 1959 Phoenix et al, proposed the organizational-activational hypothesis of hormone-driven sex differences in the brain and behavior. They established that sex steroids during critical periods i.e. in utero, after birth and to some extent during puberty [72] exert organizational and permanent effects on specific neural circuits. If testosterone is absent or the receptors are lacking, the brain is feminized. On the other hand, if testosterone is released during the critical periods it masculinizes behaviors, such as mounting behavior, and defeminizes behaviors typical for female rodents, such as lordosis. Moreover, the actual execution of most sex steroid-dependent behaviors, including mounting and lordosis, are dependent on the activational i.e. acute and reversible. effects of sex steroids in adulthood [61, 73]. Since testosterone's virilizing effects to a large extent is mediated by its metabolite estradiol, pregnant females have developed a way to bind high levels of estradiol during pregnancy - the α -fetoprotein. It binds the maternal estrogen, and hence protects the female fetus from masculinization [74]. However, in humans no α -fetoprotein is present but instead SHBG serves the same purpose [75].

Transcriptional effects during development are believed to be mainly responsible for sex differences with respect to neuronal numbers by differentially inducing apoptosis in the sexes [76-80] while other important sex differences regarding neuronal morphology, such as dendritic spines, dendrite length and the number of synapses are likely modulated both during development as well as later in life [81-86]. Moreover, both neuroimmunological [72] and epigenetic processes [81, 87, 88] are thought to contribute to sex differentiation of these neural circuits.

SEX STEROIDS, SOCIABILITY AND SOCIAL MEMORY

Rodents

Thor and co-workers reported that intact male rats more persistently investigated pups and pre-pubertal conspecifics in their home cage than intact females as well as castrated males and females did [89]. The differences disappeared when castrated males and females were treated with testosterone [8, 89]. Moreover, neonatal androgenisation of females further increased the sensitivity to exogenous testosterone of female rats as measured by social investigation in adulthood [90]. Later studies in mice and rats have also reported sex differences in the duration of social investigation [91-93]. In line with the rat studies, exogenous testosterone treatment increased social investigation in mice [94]. None of these studies used the three-chambered apparatus test measuring sociability and social investigation, which is relevant since this test is commonly used in mice models of ASD having an evident sex difference in prevalence.

It is not fully clarified to what extent social recognition is sexually dimorphic. Early studies reported that female rats displayed a better long-term but not short-term social recognition memory [95]. Although castration reduces the duration of olfactory investigation that a male rat display toward both novel and familiar conspecifics, it does not affect its capacity to recognize previously encountered individuals [96]. The fluctuating levels of ovarian hormones across the estrous cycle in female rats, are not crucial for social recognition [97, 98]. Ovariectomy does not diminish short-term social recognition performance in rats, but treatment with ovarian hormones seems to have a subtle facilitatory effect detected with the habituation–dishabituation procedure [96, 99, 100]. However, estrogen treatment seems crucial for the long-term social memory in ovariectomized female rats [101, 102]. In line with these results long-term memory in female mice was only seen when learning occurred during proestrus, (when estrogen levels are highest). A Study of male mice carrying a mutation in the gene coding for the aromatase showed decreased performance in social recognition. Furthermore, when they were given both dihydrotestosterone and estrogen the social memory abilities were restored [103], indicating an influence of both ARs and ERs.

Experiments conducted in knockout mice show that both ER α and ER β are important for social memory in mice, ER α playing an essential role [98, 104,

105], whereas ER β playing a lesser, modulatory role for the social recognition. In addition, the G-protein coupled estrogen receptor (GPER), mediates rapid enhancing effect of estrogen on social as well as object recognition in female mice [106]. The long-term, but not short-term social recognition was affected in male mice lacking ER α [98]. Interestingly, the progesterone receptor has been suggested to inhibit social recognition in male rat [107]. To, date it is however not known if ARs is involved in social preference or sociability.

Human

Already in early infancy girls attend more to faces than boys do [108], and this sex difference extends throughout childhood and adulthood [109]. Girls and women engage longer in eye-to-eye contact [110, 111], and are better at recognizing and interpreting facial expressions [112, 113] as well as emotions expressed via body language [114], in comparison to boys and men.

As expected from these results, girls and women perform better than boys and men do in tasks measuring face recognition memory [109]. More specifically, women recognize female but not male faces better than men do. Hence, women show an own-gender preference in face recognition paradigms [109, 115], which is not shown in men. Additionally, women have improved skills in processing information from neutral faces [116].

A number of studies using functional magnetic resonance imaging (fMRI) support the notion that females have a general advantage over men in the processing and recognition of faces. For example, girls and women display more pronounced face-specific activations in the fusiform gyri, including FFA compared to boys and men [117, 118] and more pronounced face-specific activations of this area has been linked to higher face recognition performance [119]. In relation to the own-gender preference in face recognition, higher activity in the left FFA was shown to be correlated with better face memory for the own gender [120].

Sex steroids seem to influence processing and memory of faces in humans. Men treated with estrogen receptor modulators performed better in a face memory task [121], and estrogen levels correlated with performance in a face recognition paradigm [122]. Most of the studies on face recognition in women have investigated the potential influence of the menstrual cycle. Although quite conflicting results have been reported, there is some evidence for improved face

recognition during the follicular phase, when progesterone levels are low compared to estrogen levels [123, 124]. Additionally, when given progesterone women perform worse in a face recognition paradigm [125]. Although, it is rather established that progesterone decrease face recognition memory, it does seem to influence cognitive function in visuospatial and verbal tasks [126]. Females given testosterone showed lower trust and empathy [127], and testosterone also influences response to face expressing emotions [128]. Studies on the role of testosterone for social recognition in humans is however sparse.

SEX STEROIDS AND ASD

It has been purposed that high levels of testosterone during early fetal development may be a risk factor for ASD [129, 130]. As males are exposed to high testosterone levels during prenatal development this hypothesis may provide an explanation for the higher prevalence of ASD in boys compared to girls. In support of the hypothesis, several studies have showed correlations between fetal testosterone levels as well as indirect measures of prenatal androgenization, and autistic traits, duration of eye contact, vocabulary skills, restricted interests and empathy [131]. Furthermore, women with polycystic ovarian syndrome (PCOS), in which circulating testosterone levels are higher than normal, are more likely to have a child with autism than women without this condition [132]. Recently, Baron-Cohen and co-workers showed that individuals who later developed ASD had elevated levels of testosterone and other steroid hormones during fetal life [133]. Although the evidence is less convincing there are several studies indicating that elevated androgen levels in adulthood also influence autistic traits [134-136]. It may be speculated that adult testosterone may rather influence than cause autistic traits. Noteworthy in this context, some recent reports find associations between increased ASD risk and clinical conditions [137] as well as indirect measures related to low testosterone levels [136, 138]. Comparisons of brain size development between children with ASDs and typically developing children have lent further support to the hypothesis of an “extreme male brain” involved in the disorder. Infant males have, on average, larger brains than females and the brains of children with autism were reported to be even larger [139]. In addition, the amygdala in autistic children has been shown to be abnormally large.

This enlargement seems to persist through early childhood [140], during the period of sex-differential amygdala growth in typically developing boys.

Lombardo et al. [141] have shown that gray matter volume in brain areas of importance for autism, such as the right temporoparietal junction/posterior superior temporal sulcus (RTPJ/pSTS), and amygdala, was greater in males compared to females and influenced by fetal testosterone. An alternative approach in order to establish the relationship between endogenous sex steroids and ASD risk is to use human genetic studies. True associations between sex steroid-related genetic variants and ASD diagnosis or autistic traits would prove that endogenous sex steroids play a role in the pathophysiology of ASD. So far, some studies have indicated that genetic polymorphisms in the AR gene [142] as well as in other sex steroid-related genes [143, 144] may influence the risk to develop ASD or ALTs.

AIMS

Paper I:

The aim of this paper was to investigate the influence of sex and gonadal hormones on social preference and sociability in mice, and to investigate the importance of the hormonal status of the used stimulus animal.

Paper II:

The aim of this paper was to explore the influence of sex and the androgen receptor (AR) in the brain on social preference and sociability in mice, and to study if the sex of the stimulus animal influences the outcome in the social tests. Furthermore, the influence of AR on the expression levels of genes related to social behaviors was investigated.

Paper III:

The aim of this paper was to investigate if differences in protein levels in brain regions known to regulate social behavior are different between females, males and androgen receptor knockout mice during early development.

Paper IV:

The aim of this paper was to study if variants in sex steroid-related genes are associated with social recognition performance in humans.

Paper V:

The aim of this paper was to study if variants in sex steroid-related genes are associated with autistic like traits in a normal population.

MATERIAL AND METHODS

ANIMALS

Conditional knockout mice – AR^{NesDel} (paper II and III)

A knockout model can be used in order to study the effect of a specific gene in an organism. To study the effect of a gene in a specific tissue or cell-type instead of the whole organism, a conditional knockout mouse can be generated, using the Cre-LoxP system [145]. We used this method in order to generate mice lacking the androgen receptor in the central nervous system (AR^{NesDel}).

When generating a conditional knockout mouse line by the Cre-LoxP system a short genomic sequence (*loxP* sites) is introduced up- and downstream of an exon of interest by transgenic techniques in embryonic cells. These *loxP* sites are targets for a DNA recombinase (Cre) isolated from the bacteriophage P1. Cre recognizes the DNA sequence flanked by *loxP*-sites (floxed), and will cleave and remove the floxed sequence, resulting in a non-functional gene product. For tissue specificity, the expression of Cre can be driven by tissue-specific promoters in hybrid animals. Only cells where the promoter is actively expressed will produce the Cre protein. In our case, Cre was driven by the promoter for the *Nestin* gene, which is primarily expressed in the central nervous system during embryonic development [68, 146] and hence produce a knockout of the androgen receptor in the brain -AR^{NesDel} (Figure 2). The littermates generated in this breeding (wildtype ♂, AR^{flox/y} ♂, NesCre^{+/-} ♂ and also AR^{flox^{+/-}} ♀) were used as controls during the tests. The genotype and sex was confirmed using a polymerase chain reaction (PCR) with primers for *Sry* (a gene that is only present at the Y chromosome), *Nestin* and *floxP*. The AR gene is situated on the X chromosome; hence the males can be only hemizygotes.

In paper III we examined 8 day old (P8) AR^{NesDel} ♂, AR^{flox/y} ♂, AR^{flox^{+/-}} ♀ and NesCre^{+/-}. At postnatal day 8 the mice pups were decapitated, the brains were removed, the hypothalamus together with the amygdala were subsequently collected. Protein was extracted from the two areas for further analysis.

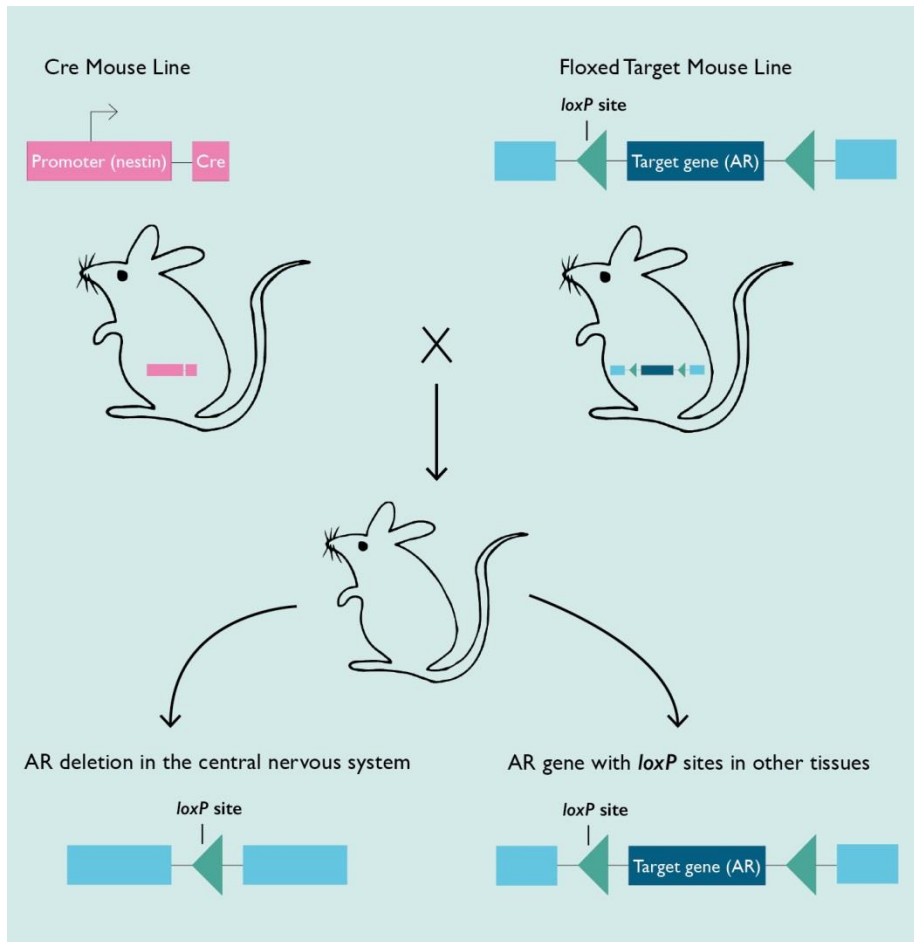


Figure 2, Mice with the Cre protein expressed in a specific cell type (central nervous system) are bred with mice that contain a target gene (androgen receptor gene) surrounded by *loxP* sites. When the mice are bred, cells carrying Cre will cause those cells to lose the target gene creating a knockout model specifically of the androgen receptors in the central nervous system, AR^{NesDel}.

Test/Focal mice (paper I)

The wild type female and male mice used in the behavioral tests in paper I were adult C57Bl/6N mice purchased from Charles River (Denmark). To study the differences between females and males, and the influence of gonadal hormones in the sexes, half of the male and female groups were sham-operated (minimizing any confounding influence of surgery on behavior) while the remaining animals were gonadectomised (GDX) three weeks prior to the study. Consequently four groups were generated: intact males, intact females, GDX males and GDX females.

Female estrus cycle

To determine the phase of the estrus cycle in the female control group in paper II, vaginal smears were taken directly after the social tests (both the three-chambered test and the social recognition test). Smears were obtained by gently flushing the vagina with PBS solution. In order to count and discriminate the cells, each smear was stained with tryptophan blue and further inspected with light microscopy. Determination of the estrus cycle stages (metestrus, estrus, diestrus and proestrus) was done according to Byers *et al.*, [147]. In proestrus mostly nucleated and some cornified epithelial cells are visible, and possibly a few leukocytes. In estrus mostly cornified epithelia is present. In metestrus cornified epithelia and polymorphonuclear leukocytes are observable, and in diestrus more polymorphonuclear leukocytes and a few epithelial cells. For the behavioral analyses the intact females were divided into two groups: “estrus” (proestrus and estrus) and “non-estrus” (metestrus and diestrus).

Stimulus mice (paper I and II)

The stimulus mice used as the social stimuli throughout the social tests in paper I and II were *C57Bl6/6N* mice ordered from Charles River (Denmark). In order to assess only the social interest of the focal mice, the stimulus females were gonadectomised and placed in corrals [148] throughout the tests to prevent the focal mice from interacting with the stimulus animals in other ways than sniffing (i.e. sexual or aggressive behavior). In order to minimize any stress that could influence our results, the mice were habituated for several days before testing. During habituation they were moved to new empty cages as well as enclosed in the wire corrals, simultaneously being habituated to being lifted and handled.

Given the influence of sex on social behavior, it is possible that the sex of the stimulus mouse could affect the behavior of the focal mice in social investigation tests. In order to investigate such possible influence, two different setups of stimulus animals were used: in paper I, both GDX and intact females were used as stimulus mice. In paper II, both GDX females and intact males were used for the same purpose.

Gonadectomy surgery

In order to generate GDX mice, the animals were sedated and an incision was made via the abdomen to remove the gonads. All efforts were made to prevent the animals from suffering during and after this procedure. Once the surgery was finished the animals were allowed to recover for 4 weeks before any experiments, also allowing the hormonal levels to decrease and stabilize properly before any social testing [149].

BEHAVIORAL TESTS

Due to their social nature, mice are suitable model animals for studying social behaviors. The fact that they prefer to be with other mice, and that they are more interested in novel things (in contrast to already encountered objects/individuals), they are widely used in different paradigms. Some of these paradigms have been used in this thesis and are presented below.

Social discrimination test (paper I and II)

The social recognition paradigm [150] is used to investigate if the test animal has an intact social memory, as displayed through a preference for novel individuals. The test consists of two sessions: a sample session and a choice session, with an interval of 30 minutes (corresponding to short term memory). During the sample session the test mouse is allowed to investigate a stimulus mouse contained in a wire corral for 5 minutes. After the interval pause, the choice session starts, during which the test mice are presented with both the familiar mouse from the sample session and a novel mouse, each enclosed in their own separate corral. As mice are social they tend to spend time with the new conspecific compared to the already encountered one, and this is defined as intact social memory.

The three-chambered test (paper I and II)

The three-chambered apparatus is developed to test for sociability [151] in mice. The testing arena consists of a box made of plastic with three different chambers. The focal mouse is first habituated to the new area where it can move freely between the three chambers. This is followed by leading the mouse into the middle chamber, and the doors to the other two chambers are subsequently closed. A stimulus mouse contained in a corral is placed in one of the side chambers, while an empty corral is placed in the other chamber. The sociability test is initiated when the doors between the chambers are removed. The focal

mouse is then allowed to explore the stimulus mouse or the empty corral for 10 minutes. Normally, mice are more interested in novel con-specifics, and consequently spend more time in the chamber with a con-specific. This is interpreted as sociability.

Novel object recognition test (paper I and II)

In order to verify the presence of intact object memory in our focal mice, the novel object test was conducted. This test has a similar setup to the social recognition test, comprised of a sampling session, where the test mouse is presented with two similar objects (instead of a mouse), followed by a 30 min inter-trial interval. Subsequently, a choice session, where it is presented with one of the sample objects and a novel object. Normally, mice investigate the novel object more than the familiar one, and this is interpreted as intact object recognition.

Odor habituation test (paper I and II)

To rule out the possibility that the mice tested had a reduced sense of smell, which could influence the results from the social behavior tests, an odor habituation test was performed. The test mouse was placed in a cage and allowed to acclimatize to the new cage containing a clean cotton tip 30 min prior to testing. The test comprises the exposure to different odors in a sequence; each odor was presented on cotton tips with durations of 2 min x 3 repeats: water, non-social odor number 1, non-social odor number 2, followed by a social odor, with a 1 min inter-trial interval. The odors presented were cinnamon, lemon and a social scent gained from rubbing the swab on the bottom of a female mouse cage. As in the social and object recognition tests, the predicted result is decreased interest in an odor with each subsequent presentation, and renewed interest with the introduction of a new smell for the first time.

Locomotion test (paper I and II)

The locomotion test was used to ensure normal motor function in the mice and to investigate if general activity level influenced results from the social tests. The test was performed in an open field box with the light turned off (in order to avoid an anxiety response). Each mouse was allowed to explore the dark box for 40 minutes, and its movement was recorded via horizontal photocells.

Experimental conditions for behavioral tests

All animals used in the behavioral experiments (both test animals and stimulus animals) were trained to be handled and moved to different rooms to reduce potential stress during the tests. This habituation procedure was performed for approximately 20 minutes, five days before the experiments.

Before all behavioral tests were commenced, the animals were transported to the testing room and were allowed to acclimatize to the new surroundings in their home cage for approximately one hour in order to reduce any stress. The testing room was kept free from strong smells and sounds, and the light level was 20 lux measured above the test cages. In between every test the mouse cage and the corrals were cleaned with ethanol and water. All mice were held in a conventional animal facility with a 12 h light/12 h dark cycle and were given *ad libitum* access to food and water. The experiments conducted were approved by the Ethics Committee of Animal experiments, Gothenburg.

MOLECULAR BIOLOGY TECHNIQUES

Genotyping

One of the most common methods of performing genotype analysis is the PCR technique where a selected DNA sequence is amplified to a large number of copies. In order to specifically amplify the sequence of interest, specific DNA primers are added together with a heat-activated DNA polymerase and free nucleotides.

A DNA primer is a single stranded DNA template, normally 10-20 base pairs, that binds to a complementary site in the genome from one direction. DNA primers are normally used in pairs, with the second primer used being complementary to the other strand at some distance from the first primer, generating a strand of DNA between the two primer sites. The PCR reaction is run for a number of cycles, normally between 30-40 cycles, where for each cycle the DNA strand between the primers are duplicated, hence creating many copies in order to detect the DNA product.

To confirm the genotype of the genetically modified mice in paper II and III, specific primers for *Cre*, *AR* and *Sry* were used.

Gene expression (paper II)

Gene expression analysis is used to find out to what extent a gene is active (expressed) in a specific tissue. This is done by measuring mRNA levels. To investigate how the mRNA expression levels in amygdala and hypothalamus differ between females, males and males AR^{NesDel}, gene expression analyses of 48 genes (43 genes of interest and 5 control genes) were performed in paper II.

Amygdala and hypothalamus from mice at the age of 4-6 months were dissected separately and the mRNA was extracted and purified. Furthermore, to run a quantitative PCR (qPCR; a method identifying quantities of mRNA), DNA is required. In order to retrieve DNA from the extracted mRNA, the mRNA needs to be transformed into complementary DNA (cDNA). To transcribe the mRNA, a reverse transcriptase PCR is performed, which makes copies from the mRNA sequence. In the qPCR reaction, two allele specific probes are added with an attached fluorescent reporter dye and a quencher dye to each side of the probe, respectively. Detection signals are only obtained after hybridization to the complementary sequence followed by the release of the quencher from the probe. The qPCR reaction involves several PCR cycles, making copies exponentially from the previous copies. When the gene reaches the exponential phase a threshold is set that is called the C_T value, which is the number of cycles required for the fluorescent signal to cross a given value threshold. The more starting product present in the sample, the quicker the threshold is reached (a lower C_T value), and the more expressed the gene of interest is. This value is further used for calculating differences in expression. Since the samples from different animals might not contain the same amount of cells (hence mRNA) it is important to use housekeeping genes to normalize between animals and runs.

The housekeeping genes, or control genes, encode proteins responsible for maintenance and metabolism in every cell and are therefore assumed to be stably expressed. The most stable pair out of five housekeeping genes was used as controls throughout the calculations. To calculate for differences between groups the $2^{-\Delta\Delta C_T}$ method [152] was used by first calculating the $\Delta\Delta C_T$, where $\Delta C_T = (\text{target gene in treated group} - \text{control gene in treated group}) - (\text{target gene in control group} - \text{control gene in the control group})$. Additionally, the fold change was calculated using the $2^{-\Delta\Delta C_T}$ method from the $\Delta\Delta C_T$ values.

ELISA (paper I)

To measure the testosterone levels in the blood from the test mice in paper I, testosterone was quantified using an enzyme-linked immunosorbent assay (ELISA). First, the blood samples were collected from the mice and centrifuged at 4500 x g for 10 minutes in order to separate the plasma and for further analysis using an ELISA assay. This method detects a specific antigen in the sample by using a plate with 96 wells coated with an antiserum (capture antibody) reacting with the antigen in the samples when added to the plate. This is followed by the addition of a secondary antibody which has an enzyme linked to it, and the secondary antibody binds to the antigen. Subsequently, a substrate is added to the plate and converts the enzyme to a detectable form.

A standard curve is created and analyzed on the same plate as the samples. The standard curve is prepared by making serial dilutions of one known concentration of the analyte across a range of concentrations near the expected unknown concentration. The samples should be in the range of the standard curve to be detected in an accurate way. The concentration of unknown samples is determined by interpolation of the absorbance values, and the concentration from the standard curve. The absorbance is directly proportional to the antigen concentration in the plasma sample.

Proteomic analyses

To examine the differences in protein expression between P8 males, females and AR^{NesDel}, two methods measuring proteins were used. The first technique was an explorative method that quantifies a large amount of proteins in the samples. The second method was a targeted analysis of the most interesting proteins discovered using the first technique.

Global quantitative proteomic analyses using iTRAQ (paper III)

In paper III we explored protein expression differences between neonatal males, females and AR^{NesDel} males in amygdala and hypothalamus, by using the method isobaric Tag for Relative and Absolute Quantification (iTRAQ). This is a mass spectrometry (MS) method using both liquid and gas chromatography to identify and quantify the most abundant proteins. A reference sample was used throughout the analysis. This consisted of a mix of ♂AR^{fl^{ox}}, ♂NesCre^{+/-}, ♀AR^{fl^{ox}+/-} and ♂AR^{NesDel} and was used within and between the runs (that was performed in a 4-plex; 3 samples and 1 reference). From the reference sample and our sample, a ratio was calculated in order to compare the groups, calculated as (AR^{NesDel} male samples/reference sample)/(AR^{fl^{ox}} samples/reference sample).

The samples and the reference pool were homogenized in lysis buffer followed by iTRAQ reagent labeling into nine 4-plexed sets. The iTRAQ sets were fractionated by Strong Cation Exchange Chromatography. Peptides were then separated in an MS that were performed at m/z range 400-1800 and the 10 most abundant peptides were selected simultaneous for MS/MS-fragmentation for identification and quantification. MS-raw data for each iTRAQ-set were merged for relative quantification and identification. For quantification, the ratios of the iTRAQ-reporter ion intensities in MS/MS spectra were used. Only unique peptides were considered for quantitation (Figure 3).

Targeted quantitative proteomic analyses using Parallel Reaction Monitoring (PRM), (paper III)

The proteins that varied between females and males in our iTRAQ analysis were further selected. Out of these, seven proteins involved in synaptic connectivity and displaying a fold change above 1.1 or below 0.9 were selected and analyzed in a targeted quantitative proteomic analysis called Parallel Reaction Monitoring. This method is used to achieve a more specific quantitative measure of a limited number of pre-defined proteins.

The selected peptides were unique to the seven different proteins and contained a heavy lysine (¹³C6, ¹⁵N2) or arginine (¹³C6, ¹⁵N4). Initially calibration curves were prepared by digestion of a representative tissue extract together with the labeled peptide. The homogenized amygdala/hypothalamus samples from the groups investigated were digested with trypsin as above, except that the labeled peptides were added together with the trypsin. The samples were spiked with the labeled peptides resulting in a concentration close to the one determined for the endogenous peptide in the sample analyzed in the dilution series.

The PRM analyses were performed which allows the samples to be analyzed at the same time. Peptides were separated as above. The most intense fragments of the corresponding pair of endogenous peptides were selected to be used for quantification of MARCKS. To determine the concentration of the endogenous peptide from the concentration of the labeled peptide the ratio to standard (endogenous/labeled peptide) × (concentration labeled peptide) was used (Figure 3)

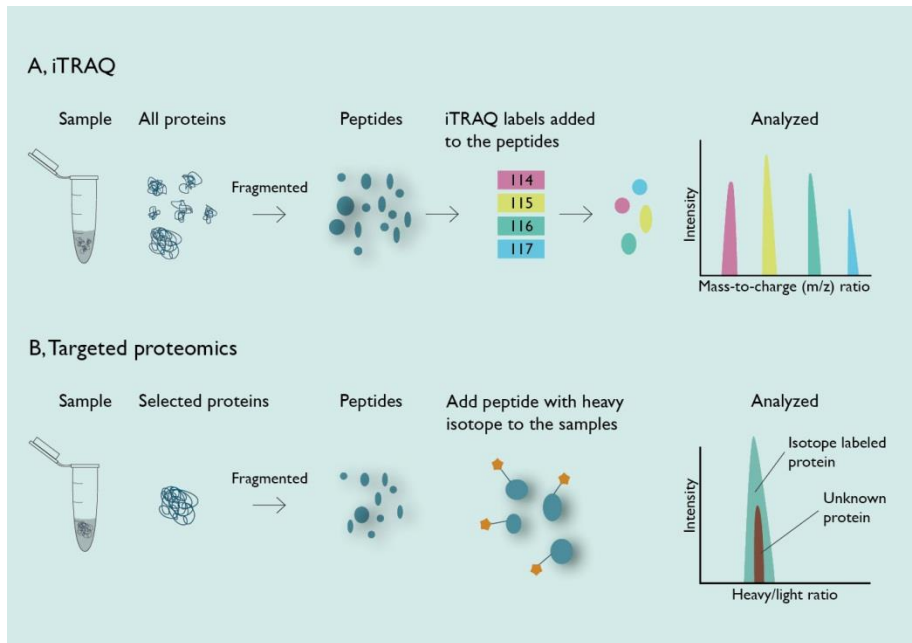


Figure 3. Proteomic techniques used for measuring protein expression in amygdala/hypothalamus of AR^{NesDel} male, AR^{floxy} male, $AR^{flox+/-}$ females and $NesCre^{+/-}$ males. (A) The iTRAQ technique quantifies the most abundant proteins in the sample. (B) Targeted proteomic is a targeted protein quantification techniques of pre-selected proteins.

HUMAN POPULATIONS

MultiEmo (paper IV)

The MultiEmo is a human population that includes 490 individuals (181 men and 309 females) from Stockholm, Sweden. From these individuals both DNA and behavioral data were collected. Participants included for analyses in this study were fluent in Swedish, Caucasian, and had no past or present psychiatric disorders or substance abuse. Ethnicity was ascertained using the birth country of their parents and grand-parents as a proxy variable.

DNA collections and genotyping (paper IV)

In order to genotype the SNPs in the human populations in paper IV and V, the competitive allele-specific polymerase chain reaction (KASP) genotyping system was used, with fluorescence resonant energy transfer (FRET) quencher cassette oligos. DNA was extracted from saliva samples using OraGene DNA self-collection kit. The DNA was then genotyped for ten SNPs covering *ESR1* (rs1999805, rs2504063, rs488133, rs2071454, rs2234693, rs722208, rs3020314,

rs2273206, rs2747648, rs1062577), four in *ESR2* (rs1271572, rs1256030, rs928554 and rs4986938), and one in *AR* (rs6152) (Figure 6). The SNPs selection in paper IV was based on earlier studies showing these SNPs to be functional or associated with disorders/disease, alternatively that they had shown an association with ALTs [144].

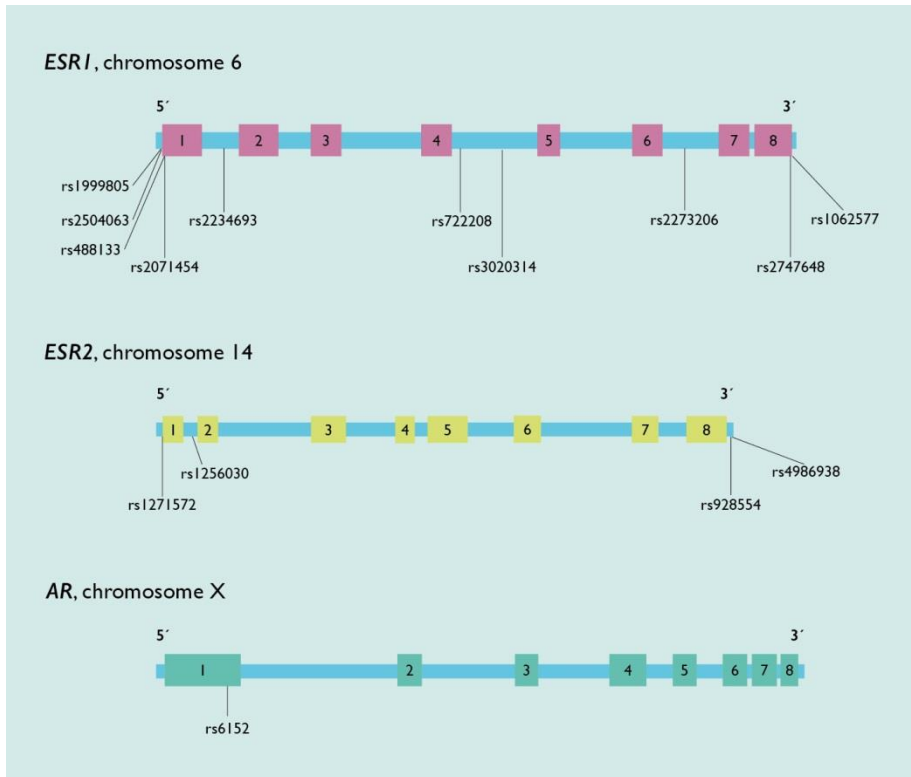


Figure 6, Positions of single nucleotide polymorphisms in *ESR1*, *ESR2* and *AR*. Exons presented as boxes and introns as blue lines.

Face and voice memory test

The memory task for recognizing the identity of faces and voices was conducted individually using *MediaLab* software [153]. The face stimuli were photos of young and middle-aged male and female adults portraying various emotions (Figure 4). The vocalizations consisted of various human sounds (e.g., crying, laughter, shrieks) and non-linguistic interjections (e.g., “ah”, “hm”, “oh”). twenty-four stimuli, expressing anger, disgust, fear, happiness, neutrality and sadness, were shown at encoding (4 stimuli/expression) for each presentation modality (faces only, voices only, and face-voice combinations). The face and

voice of the multimodal face-voice combination were matched in terms of gender and expression. At both encoding and recognition, all participants were presented with the stimuli categories in the order: faces, voices and at last face-voice combinations. The order of stimuli was randomized across subjects within each task category.

At encoding, the participants were unaware of the later recognitions session, and they were instructed to judge the emotional expression of the faces, voices, or face-voice combinations. Participants were allowed to repeat the playback of voice stimuli as many times as needed. The average time required for the encoding task was between, in average, 3 min/task. In the recognition memory task, the participants were presented with the 24 items from the encoding task interspersed with 24 previously unencountered stimuli. For each stimulus, the participants had to choose between: “yes, remember”, “yes, know” or “no”. To measure the recognition accuracy, following Snodgrass and Corvin (1988) [154], the discrimination index (d') was calculated as the overall hit rate (collapsed across both remember and know responses) corrected for false alarm, hence $d' = Z(\text{hit rate}) - Z(\text{false alarm rate})$.

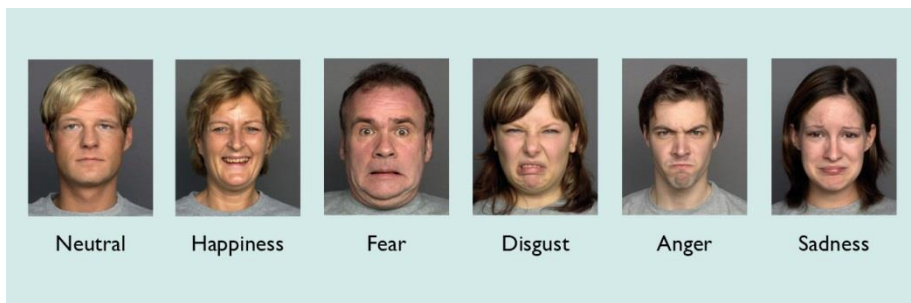


Figure 4, Examples of faces used in the face recognition task

CATSS (paper V)

The Child and Adolescent Twin Study of Sweden (CATSS) is an ongoing study, targeting all twins born in Sweden since 1992. The data is collected by the Swedish Twin Registry. The parents of all twins at the age of 9 or 12 years old are asked to participate in the CATSS study. If the parents approve they will take part in an interview: the Autism-Tics, AD/HD, and other Comorbidities inventory (A-TAC)[45]. The A-TAC covers a wide variety of neurodevelopmental problems, including but not limited to autism spectrum disorders (ASD), attention deficit hyperactivity disorder (ADHD), tic disorders,

developmental coordination disorder and learning disorder. Saliva is also collected from participants who agree to submit a sample of their children's DNA.

In paper V, two subsets of individuals from CATSS were used. The total sample (n=12 426) was divided into two subsets: a discovery sample (n=1 771) and a replication sample (n= 10 655) (Figure 5).

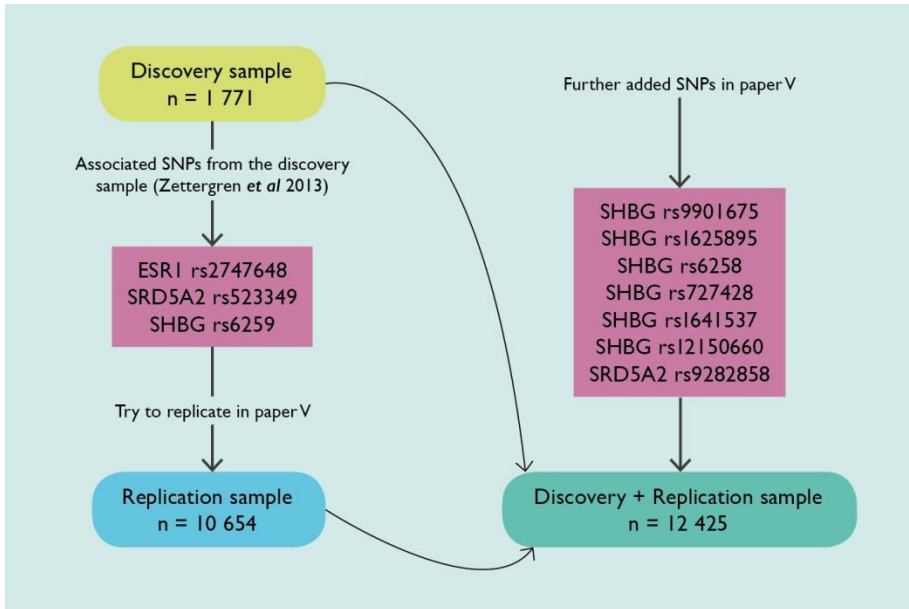


Figure 5, The SNPs analyzed in the CATSS population containing the discovery, replication and the combined population (discovery + replication).

A-TAC and ALTs

To diagnose a child with ASD, three subdomains needs to be fulfilled based on the Diagnostic and Statistical Manual of Mental Disorders (DSM). The subdomains are social interaction impairments and communication problems, as well as restricted and repetitive behaviour. If a child investigated for a possible ASD diagnosis does not fulfill the criteria for an ASD diagnosis they may still display ALTs, i.e. traits or impairments that fall under one of the classical ASD symptom domains, but in themselves are not sufficient to make a diagnosis. These impairments are assumed to be present in the general population as a continuum of general ability.

The parental interview A-TAC is a validated instrument to screen for neurodevelopmental disorders and coexisting disorders in epidemiological

studies. This questionnaire contains 96 questions, and 17 of these are related to ALTs and can be analyzed as a total ALT score, but also subdivided into three sub-scores corresponding to the ASD symptom domains; language impairments (6 questions), social interaction impairments (6 questions) and restricted and repetitive behavior (5 questions). The questions can be answered as “Yes” (scored as 1 point), “Yes, to some extent” (0,5 points) or “No” (0 points), yielding a maximum score of 17.

DNA collection (paper V)

The DNA from the CATSS population was collected through saliva samples, and was further extracted using Oragene Self Collection kit (Oragen). Twin zygosity was measured using a panel of 47 SNPs [155]. The genotype technique used in paper V was the same as described above (paper IV)

In paper V, different amounts of SNPs were genotyped in each subpopulation (Figure 5). Some SNPs were selected in order to replicate earlier findings [144], while others were added due to the fact that these have been found to influence circulating levels of androgens or SHBG [156-158], or alternatively because SNPs in *SRD5A2* have been shown to influence enzyme activity [159]. The added SNPs were *SHBG* rs9901675, *SHBG* rs6258, *SHBG* rs727428, *SHBG* rs1625895, and *SRD5A2* rs9282858.

Genotyping

It is a PCR reaction similar to the genotyping described above but it is generated in several PCR steps. Two allele specific forward primers (one primer is specific for one nucleotide in the SNP) are used in the first round of the PCR and in the second PCR step one reverse primer is added. The primers are also labeled with a tail sequence where a fluorescent molecule can bind and be detected to give the individuals genotype at that specific nucleotide position.

Genetic association studies

Each person carry two alleles at each locus of their DNA, the combination of which make up the genotype. A combination of alleles is called a haplotype. In association studies it is common to compare a polymorphism's genotype frequencies between cases and controls or to compare the mean values of a quantitative trait between genotypes. A significant association does not necessarily mean that the investigated polymorphism is the cause of the increased risk in the group of cases, since several neighboring polymorphisms may display high linkage disequilibrium (LD) with the investigated one. Two

loci are in LD when two of the alleles occur together on a haplotype more often than what would be expected by chance. To quantify the LD between SNPs, two correlation measures are used, the D' measure that basically measures the extent to which the two alleles occur together more than random, and the r^2 which measures absolute LD and also takes the allele frequencies into consideration, meaning that only when r^2 is equal to its max value ($=1$) do two specific alleles *always* occur together on a haplotype.

Different models may be applied in a genetic association test. An additive or co-dominant model assumes that the trait under investigation increase in an additive fashion as the number of alleles associated with it increases. A dominant model assumes that one copy of the risk allele is sufficient for the trait measure to be elevated. Finally, a recessive model assumes that two copies of the risk allele are necessary to increase the trait.

Hardy-Weinberg equilibrium

When performing association studies the genotype distribution needs to be assessed for Hardy-Weinberg equilibrium (HWE). A population that displays random mating is in HWE, *i.e.* the state in which the proportions of genotypes in the population depends only on the allele frequencies. The HWE principle declares that allele frequencies in a population will remain constant from generation to generation in the absence of evolutionary influences such as e.g. mate choice, mutations or genetic drift. The HWE test measures whether there is a significant deviation from HWE genotype proportions in the population. While a deviation from HWE may be biologically interesting in some cases, it typically raises suspicion that some error in the data is present, possibly due to faulty genotyping.

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STATISTICAL ANALYSES

Behavioral testing

To analyze differences between groups in the behavioral tests in paper I and II, a linear mixed model using the “Proc mixed” procedure in SAS 9.3 (SAS institute, Inc., Cary, NC) was used. To calculate the social memory score in paper I and II, a one sample t-test, with a test value of 0.5, was calculated using SPSS (IBM SPSS Statistics for Windows, Version 19.0, IBM Corp., USA). A p -value below 0.05 was considered statistically significant

Association studies

In paper IV a linear regression in SPSS was used to calculate the association between the response variable (dependent) and two explanatory/independent variables, followed by a Bonferroni test to correct for multiple testing. In paper V the statistic associations between SNPs and continuous measures of ALTs, including the modules described above, were investigated using a linear mixed effect model in the “Proc mixed” procedure of SAS 9.3 (SAS institute, Inc., Cary, NC). This model made it possible to adjust for the dependent nature of the twin observations. Mean scores and standard deviations, as well as standard errors presented, were calculated using the “proc means” procedure in SAS 9.3.

Gene expression

For calculating differences in gene expression in paper II between female, males and AR^{NesDel}, the linear mixed model “proc mixed” was applied to the ΔCT values from the gene expression data to calculate the effect of sex and AR

knockout. The fold change is presented as up or down regulated relative to the male group. Only association values below 0.05 are discussed in paper II and no correction for multiple testing was performed.

Proteomics

In paper III, the Welch's t -test was used to calculate differences in protein expression between females and males and males and AR^{NesDel}. Because of the explorative design in this study, a nominal p -value level of ≤ 0.05 was used. For all proteins with a p -value of ≤ 0.05 in the t -test, the fold change (mean male ratio versus mean female ratio, or mean male ratio versus mean AR^{NesDel} male ratio) was calculated. Proteins with missing expression values for more than three samples were not included in the analyses. Furthermore, the variance (SD/mean) in percent was calculated for each protein, to make sure that this would not exceed the percent of up- or down-regulation for a specific protein.

RESULTS AND DISCUSSION

HOW DO THE SEX STEROIDS INFLUENCE SOCIABILITY, SOCIAL RECOGNITION AND EXPRESSION OF RELATED GENES IN MICE? (PAPER I AND II)

Social behaviors, including social memory and sociability, are dependent on the ability to encode and recognize social information from conspecifics. Consequently, individuals with social disabilities such as ASD often experience difficulties in their daily life. As previous studies show that other sexually dimorphic social behaviors, like aggression and sexual behavior are influenced by sex steroids, the aim of paper I was to elucidate how gonadal hormones and sex modulate sociability and social recognition in mice. We initially validated the social discrimination test in males and investigated if social recognition was dependent on gonadal status of the female stimulus mice. Furthermore, social recognition and sociability were investigated in females and males, with or without gonads. In order to evaluate if differences in social tests may be caused by related functions, object recognition memory, locomotion and olfaction was tested on the same mice.

RESULTS AND DISCUSSION PAPER I

The results show that both male groups as well as intact females displayed social recognition whereas the GDX females, in line with previous studies [99], displayed no or diminished social recognition abilities. Based on these findings and similar results [101] it could be speculated that female mice are better at encoding social information compared to males since they display intact social memory although they investigate the stimulus mice for shorter duration than the males. This assumption is further supported by studies comparing social memory between men and women [160, 161].

Further, we did not see any influence of the gonadal status of the stimulus females on social recognition in males. In the three-chambered test investigating sociability, all four groups showed sociability and all groups spent

more time sniffing the stimulus mouse compared to sniffing empty corrals. The results clearly show that intact male mice have higher social investigatory persistence compared to intact females, GDX females and GDX males. Since GDX males showed similar investigation times as GDX females and intact females, the observed differences seem to be testes-dependent. Interestingly, the results from our experiments using the three-chambered test in mice investigating GDX females are perfectly in line with those of Thor (1980) using rats investigating juveniles in the home cage [89]. Furthermore, in the novel object recognition test intact males also displayed elevated investigatory behavior compared to the female groups, suggesting that the sex differences seen in social preference may be partly due to higher levels of novelty exploration in intact males than in females. Moreover, since females displayed elevated locomotor activity compared to males, the sexual dimorphism in exploration of novel conspecifics and objects could not be explained by differences in general locomotor activity. However, as the sex difference in object investigation was not seen in paper II this issue needs to be further evaluated in future studies.

In conclusion, our results suggest that intact male mice investigate conspecifics more than females do, and that these differences seem to depend upon circulating hormones released from the testis; obviously testosterone and its metabolite are likely candidates. As these results seem to contrast what is known from studies of social attention and ASD risk in humans, they should be taken into consideration when using the three-chambered apparatus as animal models of social deficits in ASD.

Results and discussion paper II

It is known that both ERs and ARs regulate aggression and sexual behavior and that ERs are crucial for social memory in mice, however, less is known concerning ARs role in social memory. The aim of paper II was therefore to investigate how sex and ARs modulate social recognition, measured with the social discrimination procedure, and sociability, measured with the three-chambered apparatus test. To this end, we compared males, females and AR^{NesDel} male mice in these paradigms. Furthermore, we evaluated to what extent the sex of the stimulus animal modulates social investigation and recognition in the social discrimination test. Finally, in order to reveal molecular mechanisms involved in the effects of sex and androgens on social behavior we compared the

expression levels of genes of probable importance for social memory and sociability between the three groups of animals.

Our results showed that, in contrast to female and male siblings, AR^{NesDel} males lacked social memory when presented to male conspecifics, while all three groups displayed social preference and social memory when presented to female stimulus animals (Figure 7). In addition, an AR-independent sexual dimorphism was seen in relation to social investigation of female conspecifics whereas all three groups showed similar social interest toward male conspecifics. Object memory and olfaction were not affected in the AR^{NesDel} males.

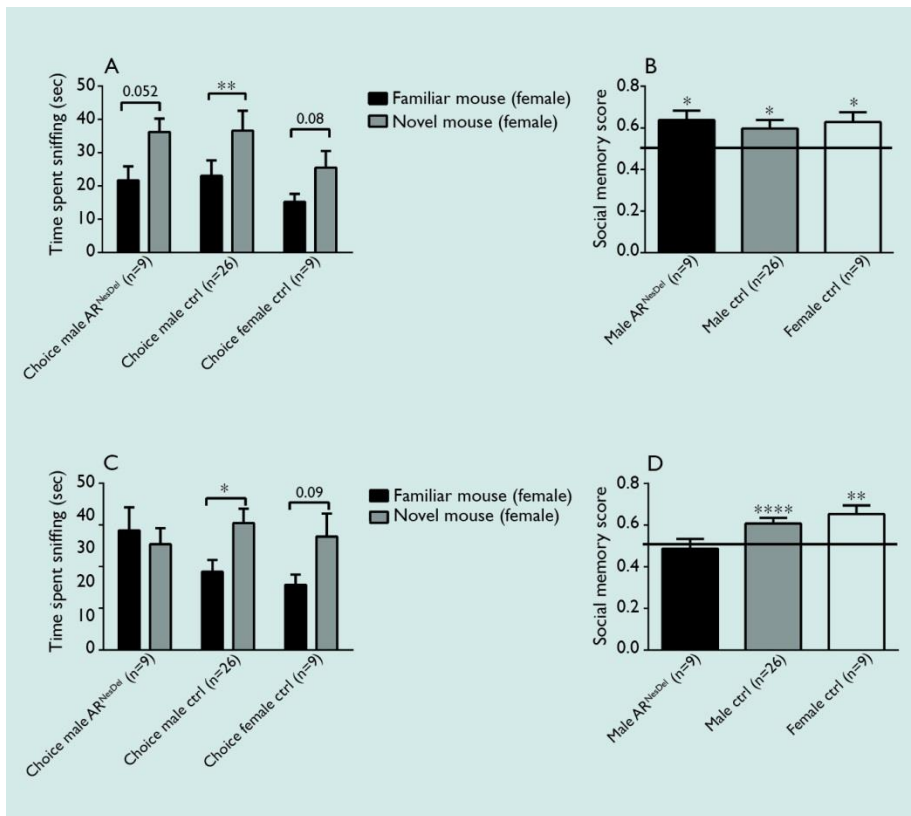


Figure 7, Social investigation and social recognition measured in the social discriminating paradigm in AR^{NesDel} males, male controls (wildtype, AR^{flox/y}, NesCre^{+/-}), and female controls (AR^{flox/+}). (A) Social recognition of female stimulus animals. (B) Social memory score when presented to female stimulus animals. (C) Social recognition of male stimulus animals. (D) Social memory score when presented to male stimulus animals. Bar represent mean \pm SEM, * < 0.05, ** < 0.01 and **** < 0.0001 (for within-group comparison).

Our study of genes relevant for social behavior suggested overall expression differences between the three groups for the following genes: *Cd38* and *Oxtr*

from the oxytocin group, *Cyp19a1* and *Esr1* from the sex steroid group, *Ucn3* and *Crh* from the stress regulation group, and *Gtf2i* from the social disorder group. The finding of an AR dependent sex difference in *Oxtr* expression in hypothalamus has, to our knowledge, not been presented before. We find it highly interesting since previous studies, mainly in humans (reviewed in [162]), suggest that testosterone and oxytocin have opposite effects on social behaviors. Hence, our finding that *Oxtr* expression is inhibited by AR may provide one possible mechanism for such an inverse relationship. Our findings of sex differences in the expression of *Ucn3* and *Cd38* are new and should be evaluated in future studies. Additionally, the finding of a sex difference in the expression of *Gtf2i* is also a novel finding and interesting since this gene is located in the chromosomal deletion causing Williams syndrome, characterized by a hypersocial personality.

In conclusion, our results suggest that ARs are vital for recognition of males but not female conspecifics, while being dispensable for social investigation towards both sexes. In addition, novel object recognition and odor habituation were not regulated by ARs. In addition, the AR seems to regulate genes related to oxytocin, estrogen and Williams's syndrome.

DO SEX AND AR INFLUENCE THE EXPRESSION OF PROTEINS IN AMYGDALA AND HYPOTHALAMUS IN NEONATAL MICE? (PAPER III)

Amygdala and hypothalamus comprise nuclei essential for the regulation of social behaviors. Many of these areas are sexually dimorphic with respect to e.g. size, dendritic branching and spine density. To identify proteins of importance for the sexual differentiation of amygdala and hypothalamus we compared protein expression in newborn males and females using an explorative proteomics approach. The masculinizing and defeminizing effects of pre- and neonatal testosterone are crucial for the sexual differentiation of the brain. As these actions are mediated by the AR and the ERs, we included amygdala/hypothalamus samples from newborn (P8) AR^{NesDel} males to explore downstream protein targets for AR in the sexual differentiation process.

Results and discussion (paper III)

Firstly, we compared protein expression in amygdala/hypothalamus from neonatal males, females and AR^{NesDel} males using iTRAQ for unbiased proteomic analysis that allows quantification of proteins in multiple samples. Secondly, targeted proteomics methodology was used for absolute quantification of seven proteins selected for validation.

Our explorative proteomic approach allowed us to investigate differences in approximately 3000 of the most highly-abundant proteins in our dissected tissue. These proteins were to a high degree important for synaptic function. Few proteins displayed large differences between the three groups, but many proteins (>170) displayed small expression differences between the sexes (fold change of less than 1.34 or more than -1.31). About 40 proteins differed between AR^{NesDel} males and male controls, and only two of those proteins, Actin (involved in cell motility) and CutA (membrane anchoring protein) were differently expressed both when comparing females to males and males to AR^{NesDel}. Hence, the AR does not seem to influence protein expression to a great extent at this age. Due to these small effect sizes and the large number of tests, no finding would survive correction for multiple testing. Interestingly, however, the MARCKS protein was found to be differently expressed between the sexes showing a relatively high fold change (above 30%). This was found and verified by both proteomic techniques. MARCKS is a protein of known importance for synaptic transmission and dendritic branching suggesting that MARCKS may contribute to the sex differences of such features.

Our results are in line with previous analysis of gene and protein expression showing no or few large differences between female and male mice [163, 164] as well as between men and women [165]. One explanation for the few differently expressed proteins detected between AR^{NesDel} and control males could be that the AR expression in these areas is initiated as late as P7 [69, 166]. Hence, older AR^{NesDel} mice may exhibit many more protein alterations in amygdala/hypothalamus.

In conclusion, our results may suggest that the sexual differentiation of the brain is due to the actions of many interacting proteins. Furthermore, our finding of a sexually dimorphic expression of MARCKS in the brain during development warrants further investigation on its involvement in sexual differentiation of amygdala and hypothalamus.

ARE POLYMORPHISMS IN *AR*, *ESR1* OR *ERS2* IMPORTANT FOR SOCIAL MEMORY IN HUMANS? (PAPER IV)

Women display better social memory abilities than men, and sex steroids seem to play a role. In mice estrogen receptors are known to be crucial for social recognition in females. Our results from Paper II also indicate that ARs may modulate social memory in male mice. In order to understand if sex steroid receptors also may modulate social memory in humans the aim of paper IV was to understand if genetic variation in sex steroid receptor genes would explain variation in social memory in humans. To this end a sample of subjects assessed for performance in recognition memory of the identity of faces and voices displaying neutral and emotional expressions, was genotyped for several common polymorphisms in the genes encoding AR, ER α and ER β , i.e. *AR*, *ESR1* and *ESR2*.

Results and discussion paper IV

In the studied population, we demonstrated, specifically in women, significant associations between variations in *ESR1* and *ESR2* and recognition of identity through vocal sounds and faces, respectively. Out of the 10 investigated *ESR1* SNPs one (rs2504063) showed a significant association with recognition of identity through vocal sounds in women. This relationship was observed for both male and female vocal stimuli. Out of the four investigated *ESR2* SNPs three (rs1271572, rs1256030 and rs928554) displayed significant associations surviving correction for multiple testing with face recognition in women. The associations were not dependent on the sex of the stimuli subjects. The *AR* polymorphism was associated with overall social memory function but the association did not survive correction for multiple testing (Table 1).

Table 1, Associations of SNPs in ERS1, ESR2 and AR with the social memory task

Gene	SNP	MAF	Model	p-value	Measure	Means ± sd (n)		
						Major genotype	Minor genotype	
ESR1	rs1999805	0.42						
	rs2504063	0.42	Recessive	F:0.0001*	d'vocal	GG:1.0 ± 0.4 (n=110)	GA:1.0 ± 0.4 (n=152)	AA:1.3 ± 0.5 (n=49)
	rs488133	0.33						
	rs2071454	0.11						
	rs2234693	0.42						
	rs722208	0.31						
	rs3020314	0.35	Dominant	F:0.009	d'vocal	TT:1.0 ± 0.5	TC:1.1 ± 0.4	CC:1.1 ± 0.5
			Dominant	M:0.03		TT:1.2 ± 0.5	TC:1.0 ± 0.5	CC:1.0 ± 0.6
	rs2273206	0.14						
	rs2747648	0.03						
rs1062577	0.08							
ESR2	rs1271572	0.48	Dominant	F:0.00003*	d'face	GG:1.8 ± 0.8	GT:2.2 ± 0.6	TT:2.2 ± 0.7
	rs1256030	0.49	Dominant	F:0.0001*	d'face	TT:1.9 ± 0.8	TC:2.2 ± 0.6	CC:2.2 ± 0.7
			Dominant	M:0.03	d'multimodal	TT:1.5 ± 0.6	TC:1.2 ± 0.6	CC:1.2 ± 0.5
	rs928554	0.45	Dominant	F:0.00001*	d'face	AA:1.8 ± 0.8	AG:2.2 ± 0.6	GG:2.2 ± 0.6
			Dominant	M:0.04	d'multimodal	AA:1.4 ± 0.6	AG:1.2 ± 0.6	GG:1.2 ± 0.5
rs4986938	0.35							
AR	rs6152	0.14		M:0.01	d'all	G:1.4 ± 0.4	A:1.3 ± 0.5	

*Survives correction for multiple testing ($p < 0.00019$) uncorrected p-values displayed
MAF = minor allele frequency, F = female, M = male

Interestingly, the SNP rs1271572 has been shown to affect the expression of ESR2, and to be associated with various types of hormone-related cancers [167]. The consequences of this and the other associated polymorphisms for brain function remain unknown. The findings of specific associations between ESR1 and voice recognition and ESR2 and face recognition, respectively, are fascinating but not easily interpreted at this point.

In conclusion, our study suggests that ERs may regulate social memory function in women. The role of AR for recognition of identity in men warrants further investigation.

ARE ESR1, SRD5A2 AND SHBG ASSOCIATED WITH AUTISTIC LIKE TRAITS? (PAPER V)

In a recent study our research group reported associations between ALTs and single nucleotide polymorphisms in the ESR1, the steroid-5-alpha-reductase, type 2 gene (SRD5A2) and SHBG in a discovery sample (n = 1 771) from the Child and Adolescent Twin Study in Sweden (CATSS). The first aim of the present study was to try to replicate these findings in an additional larger sample of individuals also available from the CATSS study (n = 10 654). The second

aim was to analyze additional SNPs of functional importance in *SHBG* and *SRD5A2* in the combined population (n = 12 425) (Figure 5).

Result and discussion paper V

Our previous findings of associations between functional SNPs in *ESR1* and *SRD5A2* and ALTs could not be replicated in the substantially larger replication sample of over 10 600 individuals. However, our results show that another SNP, rs6259, in *SHBG* may be of importance for language impairment problems in boys, and this is a replication of one of the findings from the discovery sample in the previous study. Furthermore, the rs6259 is known to affect protein function and has been found to be associated with several disorders and traits related to SHBG-levels such as polycystic ovary syndrome [168]. Interestingly, the rare genotype of this variant has been strongly associated with higher levels of circulating SHBG and testosterone levels in both females and males [156, 169]. Moreover, comparisons of serum proteins in individuals with ASD and controls showed alterations of SHBG levels in both sexes (decreased in females and increased in males) [170]. These results taken together with our findings, suggest that the rare *SHBG* genotype contribute to higher levels of SHBG and testosterone in boys, which may contribute to language problems.

In conclusion, our results show that functional genetic variation at the *SHBG* gene locus might be of importance for language impairments in boys. Furthermore, the results do not point toward a major role for the investigated SNPs in *ESR1* and *SRD5A2* in ALTs.

CONCLUDING REMARKS

SEX STEROIDS AND SOCIABILITY

In the human literature there is convincing evidence that girls already in early infancy are more socially attentive than boys and this is true throughout life. In line with these observations boys are more likely to be affected by disorders of social communication e.g. ASD. In order to understand the underlying mechanism of this variation between the sexes we established the three-chamber paradigm of sociability which is commonly used in mice studies investigating the function of genes contributing to ASD in patients. When comparing male and female mice in the sociability assay we observed that, in contrast to what would be expected from the human data, males investigated conspecifics more persistently than the females did. As emerging evidence indicate that testosterone, mainly by prenatal effects but also by effects on adults, may increase the risk of an ASD diagnosis, we compared GDX female and male mice with intact mice. Males lacking their testis investigated conspecifics with the same persistency as females with or without their ovaries, suggesting that hormones released from the testes fully explain the sex difference in social investigation. Although the results cannot be considered as unexpected they are rather interesting since they show that the testis-dependent sex difference in social investigation is very consistent between experiments using different arenas (home cage vs. the three-chamber apparatus), species (rats vs. mice) and stimulus animals (juveniles vs. adult OVX females) [8, 89]. Moreover, they indicate that rodents may not be the optimal choice of experimental animal model, when trying to elucidate the neural mechanism of the sexual dimorphism in social attention seen in human subjects. It may, for example, be more successful to use animals that primarily use their vision rather than olfactory cues for encoding and interpreting social stimuli.

In contrast to our results in mice the findings from the human genetic study (paper V) are more in line with the previous human literature. Here, we showed

a replicated association between a SHBG genotype contributing to elevated levels of serum testosterone and SHBG, and increased risk of autistic-like problems in boys.

In paper II, where we compared males, females and mice not expressing neural ARs, we again observed the same sex difference in social investigation but also that the ARs do not influence this dimorphism. On the other hand, since females without gonads investigate conspecifics as much as intact females, estrogens and hence the ERs do not seem to play an important role for social investigation in females. This is in sharp contrast with for example general locomotor activity, which is highly dependent on estrogens, and substantially decreased in GDX compared to intact females. Our results taken together with previous reports indicate that testosterone increases social investigation via the AR in females and via ERs in males.

SEX STEROIDS AND SOCIAL MEMORY

Convincing evidence show that estrogens are important for social memory in rodents. In line with our findings in paper 1, showing less social recognition in GDX females compared to intact females, several studies suggest that estrogens have a subtle facilitatory effects on short-term social memory in female rodents [99]. Furthermore, according to studies in both mice and rats pharmacological doses of estrogens exhibit pronounced effects on long-term social memory in females [102]. These effects are probably due to activational effects of ERs. It seems however reasonable that the total lack of social recognition seen in ERKO α females may be due to a combination of deficient organizational effects of prenatal estrogen, and insufficient activational effects of circulating estrogens via ER α in relevant neural circuits. Although less critical, ER β also plays a role for social recognition in female mice. In addition, estrogens exert acute effects on social recognition via the estrogen receptor GPER [171]. The underlying neural mechanisms for mediating the effects of estrogens via the various ERs are far from established, but medial amygdala and hippocampus seem to be involved in social recognition. Furthermore, as it is well-known that estrogens regulate the expression of oxytocin and its receptor, which are both known to be crucial for social recognition in mice, it has been suggested that oxytocin function may, at least partly, mediate estrogens stimulatory effects on social memory function.

As described above the estrogens and ERs seem to be mainly important for social memory in female rodents. In line with these findings, in paper IV we

revealed strong associations between polymorphisms in the genes encoding social memory and ER α and ER β , respectively, in adult women, suggesting that ERs are important for identity recognition also in humans. The importance of sex steroids for social recognition in males is less investigated. Male mice lacking the aromatase enzyme displayed social recognition deficiencies which were reversed by treatment with estradiol and dihydrotestosterone, mainly suggesting an activational role for estrogens on social recognition in males. Inconsistent with those findings the ER α was not crucial for short-term, but for long-term social recognition in studies using ERKO α male mice [98]. In paper II, we show that the AR may be crucial for short-term recognition of males but not female mice, indicating that androgens have the ability to modulate this ability in males. Interestingly in this context is that we, also in in paper II, showed that the AR regulates the expression of the oxytocin receptors in the hypothalamus. Considering the important role of oxytocin for social recognition in both male and female mice, oxytocin function may be relevant for the male recognition inability seen in mice lacking neural ARs. Intriguing studies reveals that progesterone via the progesterone receptor inhibits social recognition in males, an effect probably mediated by vasopressin [172, 173].

Regarding AR, a potential pitfall for our findings in AR^{NesDel} is that they display elevated testosterone and estrogen levels, which probably to a large extent is due to a deficient negative feedback system in AR^{NesDel} mice. On the other hand our gene expression analyses in paper II show that the knockout males have a decreased expression of the aromatase enzyme in both amygdala and hypothalamus compared to wildtype males. Thus, estrogens may be involved in the male recognition deficiency in AR^{NesDel} mice.

Girls and women are superior to boys and men with regards to face recognition memory. Interestingly, most studies show that this sex difference is limited to recognition of female faces; women show an own-gender preference in face recognition [109]. As the underlying neural mechanisms are not well understood, we investigated to what extent the sex or gonadal status of the stimulus animals affected social memory as measured in our studies using mice and humans. In paper I we did not reveal any major differences when investigating social recognition in males interacting with either intact or GDX females. In paper II the sex of the stimulus mice did not seem to influence social recognition abilities in either males or females. Furthermore, it may be speculated that hormonal variations between the sexes could be relevant for the

sex difference in social recognition in human. Noteworthy in this context are the associations between *ESR1* and *ESR2* and social recognition measures in women, seen in paper IV. These associations were not affected by the sex of the stimuli faces or voices. Interestingly however, in paper II we showed that male mice lacking ARs recognized female but not male conspecifics. This result is currently difficult to interpret, and needs to be confirmed and further explored in future studies. Intriguingly, mice lacking ARs in both brain and periphery display alterations in social interactions dependent on the sex of the stimulus animals, which are not seen in sibling controls [94]. In the light of our findings that oxytocin receptor expression in the hypothalamus may be altered in AR^{NesDel} males compared to sibling controls it is noteworthy that neuron-specific *Oxtr* knockouts [174] also display alterations in social interactions dependent on the sex of the stimulus animals. In conclusion, differences in hormonal function may be relevant for social memory, dependent on the sex of the conspecific.

MOLECULAR AND NEURAL MECHANISMS FOR THE EFFECTS OF SEX STEROIDS ON SOCIAL BEHAVIOR AND SEXUAL DIFFERENTIATION

Considering the pronounced effects of the sex steroids on sexual differentiation of the brain as well as on social behaviors studies of downstream targets of sex steroid receptors in relevant brain regions provide a great opportunity to reveal molecular mechanisms involved in these differences. Fortunately, it is rather straightforward to measure gene and protein expression from studies of selected candidate genes all the way to explorative proteomic or genomic-wide investigations. In paper II we used a low-density approach to measure expression of about 40 genes in adults and in paper III we use a high-density proteomics approach allowing us to investigate almost 3000 proteins in newborn mice. Although the great potential of these approaches our and recent studies have shown it hard to obtain robust findings that survive correction for multiple testing and replicate between studies. The main difficulty of large scale studies is the combination of large numbers of tests and small differences in gene and protein expression between groups. The few studies actually identifying genes differentially expressed between the sexes and affected by sex steroids have often conducted very comprehensive validation experiments of their initial findings using for example, *in situ* hybridization on brain sections. This is because the expression differences usually are restricted to specific neurons of certain brain

nuclei [63]. Hence our findings in paper 2 and 3 must be further validated by studying expression profiles in specific nuclei and neurons of hypothalamus and amygdala. Overall, future similar studies should attempt to investigate protein expression in specifically dissected brain nuclei in larger sets of samples.

Due to our interest in the neurobiology of ASD we evaluated the results from our proteomic results for ASD risk genes. Few such proteins were found to differ between the sexes in our proteomics study of newborn mice. Interestingly, this is well in line with a recent study of postmortem brains from ASD patients as well as from male and female control subjects [165]. When comparing the sexually dimorphic genes with the genes differentially expressed between patients and controls they found that few of the established ASD risk genes were sexually dimorphic instead they observed that genes expressed at higher levels in men tend to also be upregulated in brain from ASD patients. This suggests that it is not sex-differential regulation of ASD risk genes, but rather naturally occurring sexually dimorphic processes that modulate the impact of risk variants and contribute to the sex-skewed prevalence of ASD. These findings are highly interesting and provide an opening for future studies of the neurobiology of ASD.

SAMMANFATTNING PÅ SVENSKA

Sociala beteenden innefattar sexuella beteenden, aggressivitet, anknytningsbeteenden mellan barn och föräldrar eller mellan partners, samt sociabilitet och socialt minne. Våra beteenden styrs av hjärnan och det är känt att könshormonerna, östrogen och testosteron, påverkar områden i hjärnan som kontrollerar beteenden i både människa och djur. Dock är kunskapen om de exakta mekanismerna mindre känd.

Att agera på ett lämpligt sätt i sociala situationer är viktigt för interaktionen med andra. Det finns personer med syndrom som uppvisar svårigheter i sociala relationer t.ex. autism. Autism förekommer oftare hos pojkar än hos flickor. Då pojkar har högre nivåer av testosteron under fosterutvecklingen, utvecklas pojkars och flickors hjärna något olika vilket kan ge upphov till skillnader i beteende. Förhöjda testosteronnivåer under de kritiska perioderna i hjärnans utveckling har föreslagits vara en orsak till utvecklingen av autism.

För att förstå dessa mekanismer bättre undersökte vi om könshormoner påverkar socialt minne och sociabilitet i en musmodell (*arbete I*). Vi kunde bland annat se att hanmöss med normala testosteronnivåer hade mer påtagligt socialt intresse än hanar utan testosteron vilka undersökte andra möss i samma låga grad som honmöss. Det är känt att östrogenreceptorn är viktig för socialt minne, men mindre känd är androgenreceptorns inblandning i denna förmåga. Därför undersökte vi om denna receptor är involverad i sociabilitet och socialt minne i möss (*arbete II*). Resultaten visade att androgenreceptorn inte påverkar hanmössens intresse av andra möss, men däremot att de krävs för att hanar ska minnas andra hanar, men inte för att minnas honor.

Både östrogen- och androgenreceptorn är transkriptionsfaktorer vilka påverkar uttrycket av gener och därmed proteiner. Mindre känt är dock exakt vilka gener och proteiner som påverkas. I *arbete II* undersöktes uttrycket av ett antal gener som tidigare föreslagits vara involverade i sociabilitet och socialt minne hos honor, hanar och hos hanar utan androgenreceptorer i hjärnan. Ett antal gener skiljde mellan grupperna, bland annat skiljde uttrycket av två gener viktiga för neuropeptiden oxytocins funktion mellan könen, och androgenreceptorn tycktes orsaka denna skillnad. I *arbete III* undersökte vi ca 3000 proteiner i

hjärnregioner viktiga för könshormoners påverkan på sociala beteenden i 8 dagar gamla hon- och han möss eller hos hanar utan androgenreceptorer i hjärnan. Resultatet av denna studie visade endast små skillnader i proteinuttryck mellan könen, och att androgenreceptorn hade liten betydelse för mängden av dessa proteiner i hjärnan hos nyfödda möss.

Då östrogenreceptorerna är viktiga för socialt minne i mus och testosteron tros ge upphov till sociala svårigheter för personer med autism, undersökte vi om genetiska variationer som påverkar könshormonernas funktion var associerade med socialt minne (*arbete IV*), samt om de var förknippade med autism-liknande drag hos människa (*arbete V*). Vi kunde se att östrogenreceptorn var viktig för kvinnors sociala minne av både manliga och kvinnliga ansikte och röster. Vi kunde också se att variationer i könshormonernas transportprotein, SHBG, tycks påverka språkförmågan hos pojkar.

Sammanfattningsvis visar vi att testosteron påverkar hur länge hanmöss undersöker andra möss, att androgenreceptorer tycks viktiga för hanmöss minne av andra hanar, att variation i gener av betydelse för könshormoner var relaterade till socialt minne hos kvinnor och till språkproblem hos pojkar. Dessutom fann vi könsskillnad i uttrycket av ett antal gener samt några gener vars uttryck påverkades av androgenreceptorn. I en storskalig jämförelse av proteiner i hypotalamus och amygdala skiljde ett stort antal proteiner mellan könen men skillnaderna var överlag små.

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