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D. W. Pfaff
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Multiple Origins of Sex Differences in Brain

Neuroendocrine Functions and their Pathologies

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Editors

Multiple Origins of Sex Differences in Brain

Neuroendocrine Functions and their Pathologies

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Foreword

Multiple Origins of Sex Differences in Brain. Neuroendocrine Functions and their Pathologies

In theoretical terms, sex differences in brains and behaviors of laboratory animals offer the possibility of fascinating scientific studies on a range of molecular phenomena such as genomic imprinting, DNA methylation, chromatin protein modification, non-coding DNA, potentially resulting in important neuroanatomical and neurochemical sex differences in the brain. Such sex differences could arise consequent to exposures to testosterone early in development, or to other effects deriving from the Y chromosome. However, this general subject has been treated with much hyperbole. Historically, sex differences were assumed to be present where they did not really exist, e.g. with respect to mathematics, executive leadership, etc. etc. Under what circumstances do we really care about sex differences in brain and behavior? These circumstances concern human maladies whose diagnoses are much different between boys and girls, or between women and men. Prominent examples discussed in this volume include autism, attention deficit hyperactivity disorders and congenital adrenal hyperplasia. In fact, infant boys are more susceptible than infant girls to a variety of disorders that arise early in development. This volume then ends with a consideration of effects of estrogenic hormones on the injured brain, and their roles as protective agents.

This volume contains the proceedings of the XIth Colloque Médecine et Recherche of Fondation Ipsen held in Paris, on December 3, 2011 which bring together clinicians and basic scientists working in endocrinology and neuroscience.

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Contents

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Hormone-Dependent Chromatin Modifications Related to Sexually Differentiated Behaviors

Khatuna Gagnidze and Donald W. Pfaff

Abstract Behaviors associated with reproduction depend on brain mechanisms which are the most extremely differentiated between the sexes. This article will describe those brain mechanisms, with an emphasis on sexually differentiated regulation of gene expression in the neurons of the hypothalamus. In turn, hormone-dependent transcriptional activation is regulated by changes in chromatin, histone proteins the tails of which are subject to several forms of chemical modification. We will describe specific examples of histone tail acetylation and methylation as they influence reproductive behavior and sex differences in the brain. Finally, we must ask: under what circumstances are sex differences in brain and behavior most important? Thinking that sexually differentiated maladies answer that question, we will propose a 3-hit theory of the large sex difference in autism: (1) genetic predispositions, followed by (2) androgenic hormone effects on CNS arousal systems and limbic forebrain, in young males; followed in turn by (3) deleterious effects of early stress. These three types of mechanisms yield the 'extreme male brain' of the autistic boy.

Introduction

Public attitudes toward sex differences in human behaviors are changing rapidly. Formerly thought to pervade large domains of cognitive activity, sex differences are now understood to be limited to those behaviors closely connected to the physiology of reproduction (Pfaff 2011). Now is the time to focus on detailed neurogenetic and neurochemical mechanisms for those behaviors that truly are sexually differentiated, both in humans and in laboratory animals.

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When it comes to the brain, sexual dimorphism is exhibited at several different levels: structural, behavioral and in the susceptibility to disease/injury. These sex differences are orchestrated under the combinatorial influences of genetic factors, environmental factors and steroid hormones during the critical developmental time period. In the course of fetal development, two temporally separated events take place that lead to sexual differentiation of an embryo: the first is fetal gonad development in males, initiated under the influence of *SRY*, the sex-determining gene on the Y-chromosome. SRY, which is a transcription factor, acts as a switch to initiate a cascade of gene expression that leads to differentiation of the genital ridges into testis (Koopman et al. 1990). The testes, in turn, start producing testosterone, which by means of its two metabolites, dihydrotestosterone and estradiol, respectively, drives the formation of male sexual organs and masculinizes the brain (Weisz and Ward 1980; Wilson et al. 1980; Rhoda et al. 1984; McCarthy 2008). In contrast, genetic females that lack Y chromosomes and the developmental trigger produced by the *SRY* gene product develop ovaries and female sexual characteristics (Sinclair et al. 1990). Due to quiescent ovaries that do not start producing estrogens until long after birth, brains of developing females are also devoid of hormonal influence. Precisely this imbalance of hormonal milieus in the brains of males and females during the critical period of development is believed to be the cause of sex differences in physiology and behavior exhibited in adulthood.

In their seminal study, Phoenix and colleagues (1959) proposed two different modes of action by which steroid hormones influence brain and behavior. During a perinatal sensitive period (beginning at approximately embryonic day E18 in rodents), testosterone produced by male testis freely enters the brain and permanently "organizes" male neural circuits in such a way that, in adulthood, they become responsive to "activating" actions of gonadal steroids and exhibit male-typical behaviors. In females, the lack of hormonal effect during this period leads to formation of a female-typical brain that is capable of producing an ovulatory surge of the gonadotropin luteinizing hormone (LH) (Homma et al. 2009) as well as female-specific behaviors under the appropriate hormonal influence in adulthood. The time window during which developing brains are most sensitive to organizational effects of hormones is very short and in rodents lasts only until a few days after birth, when testosterone levels start to decrease (Rhoda et al. 1984; Palacios-Pru et al. 1998). However, the molecular, cellular and anatomical changes that take place during this time period affect the social and reproductive fate of an animal for the remainder of its life.

An important development in the study of hormonal effects of brain differentiation was the observation that neonatal estradiol could masculinize the rodent brain to the same extent as testosterone (Feder and Whalen 1965; Booth 1977; McEwen et al. 1977). Later, it was unequivocally demonstrated that in neurons testosterone is locally converted into estrogen by p450 aromatase and, in fact, estrogen signaling through its nuclear receptors is the critical factor for brain masculinization (Reddy et al. 1974; Patchev et al. 2004). The subsequent discovery that sexually dimorphic brain regions express high levels of P450 aromatase, an enzyme that converts testosterone to estradiol, led to the aromatization hypothesis, which reconciled

the ability of estrogens to masculinize the brain with the fact that testosterone production from male testis was the developmental trigger for this process (Reddy et al. 1974; MacLusky and Naftolin 1981; Wagner and Morrell 1996). Studies demonstrating high levels of estrogen receptors (ERs) concentrated in brain regions that exhibit sexually dimorphic morphology or foster sexually dimorphic behaviors provided additional support for the aromatase hypothesis (Pfaff and Keiner 1973; Shughrue et al. 1997). Furthermore, behavioral analysis of mice with targeted genetic ablations of ERs (discussed below) firmly established that estradiol, locally aromatized from testosterone and signaling through ERs, is the main determining factor that leads to sexual differentiation of brain and behavior.

Behaviors

In rodents, effects of perinatal hormonal exposure are most pronounced with respect to behaviors that serve reproductive functions. These include proceptive and receptive behaviors in female rodents as well as appetitive and copulatory behaviors for male rodents (reviewed in Gagnidze et al. 2010). In addition, the aggressive and territorial behaviors typically displayed by males, and maternal behavior, characteristic of females, have also been shown to depend on organizing effects of hormones (Bridges and Zarrow 1973; Wu et al. 2009). A number of experimental manipulations have been employed to dissect out the developmental hormonal effects on complex behaviors. For example, it has been demonstrated that males castrated neonatally display lordosis behavior in adulthood if primed with estrogen and progesterone, and this behavior could be reversed with androgen or estrogen treatment concomitant with castration (Phoenix et al. 1959; Beach et al. 1969; Baum 1979; Morris et al. 2004). Treatment of neonatal females with either of these hormones can induce expression of male-typical sex behaviors in adulthood as well as displays of aggression towards intruders and some aspects of territorial behavior characteristic of male mice (Phoenix et al. 1959; Beach et al. 1969; Baum et al. 1982; Morris et al. 2004; Wu et al. 2009). Disrupting androgen conversion into estrogen by blocking aromatase activity in males within the first few postnatal days or deletion of the aromatase gene in mice also blocks the masculinizing effect of androgens on partner preference and results in abrogation of sexual dimorphism, thereby confirming that estrogens comprise the critical signal that permanently organizes the dimorphic nervous system (Simon and Whalen 1987; Bakker et al. 2002a, b; McCarthy et al. 2009).

Mice that lack either of the two isoforms of ER, ERα or ERβ, as a result of targeted gene deletion provided certain important information about the development of sexually dimorphic behaviors. These studies demonstrated that ERα is absolutely essential for reproduction and the expression of sexual behavior in response to estrogen; however, disruption of ERβ does not have an obvious effect (Ogawa et al. 1996, 1999). Although the interpretation of behavioral phenotypes of ERα knockout mice is complicated by the fact that we cannot dissociate

"organizational" developmental effects from "activational" adult-generated effects, comparison of sexual behaviors of male ERα and ERβ knockout (KO) mice led to one important finding. While ERα-deficient male mice display marked deficits in copulatory behaviors, such as intromission and ejaculation (Ogawa et al. 1998), the lack of functional ERβ did not seem to impair normal expression of male sexual behavior, suggesting that ERα-mediated signaling is important for the "masculinization" of this type of behaviors (Ogawa et al. 1999; Kudwa et al. 2005). On the other hand, when castrated male ERβ KO mice were primed with female hormones and tested for female sexual behavior, their lordosis quotient score was double that of wild type (WT) mice (Kudwa et al. 2005) indicating that ERβ activation is necessary for the "defeminization" of sexual behavior. Use of selective pharmacological compounds yielded similar results: treatment of neonatal female mice with an ERβ selective agonist led to significantly impaired lordosis behavior, but selective activation of ERα did not produce similar effects (Kudwa et al. 2006). Taken together, these data support the hypothesis that masculinization and defeminization are separate neural processes that depend on signaling by different ERs.

Neuroanatomy

In addition to sex differences evident in physiological and behavioral outputs produced by male and female brains, certain structural dimorphisms within preoptic and hypothalamic regions have been described. It is probably not incidental that these are precisely the areas that are involved in the execution of sexually dimorphic behaviors: the preoptic area (POA), which controls expression of male sexual behavior and female maternal behavior (Larsson and Heimer 1964; Numan et al. 1985); the ventromedial hypothalamic nucleus (VMH), which is critical for the display of female sexual behavior and aggression in males (Pfaff and Sakuma 1979a, b; Pfaff 1999; Lin et al. 2011); the bed nucleus of the stria terminalis (BNST), which is involved in the production of male copulatory behavior (Emery and Sachs 1976); and the anteroventral periventricular nucleus (AVPV), which is essential for the ovulatory surge of LH (Terasawa et al. 1980; Clarkson and Herbison 2006). However, despite an obvious initial assumption that structural dimorphisms of these brain regions may support the sex differences in behavior, such direct structure-function relationship has yet to be proven.

Although estrogen is the foremost developmental factor that drives the formation of all sexually dimorophic brain structures, its effects vary widely depending on brain region and sex of the animal. For example, the sexually dimorphic nucleus of the POA (SDN-POA) is five to seven times larger in male than in female rats, an effect that is due to sex differences in neuron numbers (Gorski et al. 1978, 1980). The VMH is also larger in male than female rats, only as a result of differences in the volume of neuropils and the number of synapses (Matsumoto and Arai 1986a, b; Madeira et al. 2001). Yet another structurally dimorphic hypothalamic nucleus, AVPV, is larger in females than in males, and the difference is revealed not only in the number and

density of cells but also in their chemical characteristics: females have more dopaminergic neurons (Simerly et al. 1985) and about 10 times more kisspeptin-expressing neurons (Clarkson and Herbison 2006; Kauffman et al. 2007). These kisspeptin-1-containing neurons are estrogen-responsive cells that project to and stimulate GnRH neurons, leading to a preovulatory LH surge (Terasawa et al. 1980; Clarkson and Herbison 2006; Homma et al. 2009). Thus the higher number of kisspeptin neurons in the female AVPV may have direct physiological consequences and explain the sex difference in the induction of LH surges.

Experimental manipulations of perinatal testosterone and estrogen levels have been shown to reverse structural sex differences in males and females. For instance, castration of neonatal male rats reduces the size of the SDN-POA (Gorski et al. 1978), and neonatal treatment of female rats with testosterone propionate (TP) or estradiol increases the size of the SDN-POA to that of normal male rats (Gorski et al. 1978; Dohler et al. 1984; Patchev et al. 2004). Interestingly, the same exposure to steroid hormones during the critical time period is responsible for the development of the opposite morphology of the AVPV, which is larger in females than males. Treatment of neonatal female rats with androgens or estrogens and, conversely, castration of neonatal male rats eliminate these sex differences as well (Ito et al. 1986; Davis et al. 1996; Patchev et al. 2004).

One possible mechanistic clue to the differential effects of estrogen on the development of the POA and AVPV came from studies using pharmacological compounds with selective binding affinity for two ER isoforms, ERα and ERβ. Treatment of neonatal female rats with ERα agonist mimicked testosterone or estradiol effects on SDN-POA development; however, ERβ agonist failed to do so. In contrast, both ERα and ERβ selective agonists were effective in the differentiation of AVPV (Patchev et al. 2004). Together, these data provide compelling evidence that estrogen signaling through ERs underlies the organizational effects of this hormone on sexually dimorphic brain and behavior.

Human Studies

It is widely believed that a hormonal imbalance during fetal development affects sexual differentiation of the human brain as well. During human development, the two critical periods are mid-pregnancy and the first three months after birth, when testosterone surges are produced and result in higher hormone levels in males than in females (van de Beek et al. 2009). These fetal and neonatal peaks of testosterone, much like in animal models, are thought to shape the development of structures and circuits in a male brain for the rest of his life (Bao and Swaab 2011). Although experimental data in humans do not exist for obvious ethical reasons, behavioral assessment of individuals with certain pathological conditions, such as congenital adrenal hyperplasia (CAH), provides some evidence for the organizing effects of steroid hormones. CAH is a genetic disorder resulting from mutations affecting components of biochemical pathways that produce cortisol (White and Speiser

2000). As a result, adrenal glands secrete large amounts of androgen during prenatal development that potentially can lead to "masculinizations" of affected females (Hines 2011). In support of this hypothesis, girls with CAH exhibited playmate and play style preferences typical of boys, scored more masculine than control girls on all scales measuring gender-related behaviors and were found to be more aggressive and active when compared to their unaffected sisters (Nordenström et al. 2002; Pasterski et al. 2007, 2011). Together these data point to the critical role of testosterone levels during pregnancy with regard to the development of such sex differences in behaviors. Although some critics question the validity of these behavioral measures as a proof of prenatal hormonal effects, studies done in non-human primates ward off some of the concerns that sex-typed toy preference may be the result of socialization history rather than hard-wired sex difference. Two studies have reported sex-typical toy preferences in vervet monkeys and rhesus monkeys that mirror the behaviors seen in children (Alexander and Hines 2002; Hassett et al. 2008), demonstrating that sex-typed toy preferences are most likely based on sex differences in perception and can arise independent of the social and cognitive processes involved in gender development.

Studies examining structural sex differences in humans employ postmortem examinations as well as in vivo imaging and report dimorphisms in measures such as volume, cell density or shape of various structures or cortical areas (reviewed in Cahill 2006). Another interesting observation is that some of these sex differences are age-dependent. For instance, total brain volume is larger in males than in females, and this dimorphism is evident from a very young age, even when corrected against differences in body size (Swaab and Hofman 1984; Hines 2011). In addition, significant differences between sexes have been described in white-gray matter composition in the cerebrum (Allen et al. 2003), as well as in the sizes of various structures such as the amygdala and the hippocampus (Goldstein et al. 2001). Although the involvement of sex steroids in the development of these sex differences has not been directly tested, the authors emphasized that there was a significantly greater magnitude of adult sexual dimorphism among brain areas with developmentally high levels of sex steroid hormone receptors than among other regions. Moreover, despite the overwhelming number of differences found in the cytoarchitecture of male and female brains, few of these structural dimorphisms have been linked to behavioral sex differences.

Nevertheless, there are some examples suggesting that structural differences found in the brains of males and females can affect certain behaviors, such as sexual preference and gender identity. Incidentally, these include structures that are analogous to regions in animal brains found to be sexually dimorphic. One of them is the interstitial nucleus of the anterior hypothalamus, INAH-3, which is thought to be the human homolog of the rodent SDN-POA. Similar to rodent nucleus, human INAH-3 has been reported to be larger in males than in females; however, it is also smaller (i.e., more female-typical) in homosexual than heterosexual men (Allen et al. 1989; LeVay 1991; Byne et al. 2000; Garcia-Falgueras and Swaab 2008). Although it is well established that the sex difference in SDN-POA volume in other mammals results from early testosterone exposure, this possibility

in regard to INAH-3 volume in humans has not yet been directly investigated. Another sexually dimorphic structure found in humans as well as in animals is BNST, which has been linked to gender identity. This conclusion was drawn based on a report that the central subregion of this nucleus (BNSTc) is smaller in women and in male-to-female transsexuals than in non-transsexual men (Zhou et al. 1995), and the size of the BNSTc was not influenced by sex hormones in adulthood and was independent of sexual orientation. Thus, authors speculated that gender-identity problems may develop as a result of a disturbed hormonal milieu during fetal brain development and cite these results as evidence for their hypothesis.

While sex differences between men and women are pronounced in certain specific brain structures and in reproductive behavioral outcomes, a more important aspect of sexual dimorphism is the susceptibility to disease or injury. Vast sex differences in the prevalence of certain developmental, psychiatric and neurodegenerative diseases have been described (reviewed in Bao and Swaab 2011). For example Rett syndrome, fatigue syndrome, anorexia and bulimia nervosa, depression and anxiety disorders are mostly found in women. The prevalence of dyslexia, attention deficit hyperactivity disorder (ADHD), autism, Tourette syndrome, and substance abuse is overwhelmingly higher in men. Moreover, sex differences are prominent in the symptomatology and the course of certain diseases. For instance, schizophrenia affects men 2.7 times more often than women, and men also have a tendency to have a more severe form of this disorder. Furthermore, females have more favorable outcomes after traumatic brain injury or subarachnoid hemorrhage (Niewada et al. 2005; Riecher-Rössler and Kulkarni 2011).

Some of these sex differences are the result of X chromosome-linked genetic mutations, as in the case of Rett syndrome (Matijevic et al. 2009). Others are most likely due to differences in circulating sex hormone levels (activating effects). A neuroprotective role attributed to estrogens may explain the better outcome seen after traumatic brain injury and vulnerability to neurodegeneration following the menopause in women (Brann et al. 2007). However, in the cases of certain developmental disorders, such as autism spectrum disorders and Tourette syndrome, the combination of genetic factors and environmental influences and developmental effects of hormonal exposure have been proposed as plausible explanations for the etiology and sex differences in prevalence (Leckman 1995; Pfaff et al. 2011; Baron-Cohen et al. 2011).

Taken together, these data provide compelling evidence that understanding the mechanisms involved in sex differences in brain structure and function are of utmost importance for human health and disease.

Molecular Mechanisms

Estrogens mediate their effects mainly through binding to their cognate receptors, ERα and ERβ, which belong to a large family of nuclear receptor transcription factors. Unliganded estrogen receptors are sequestered in the cytoplasm by specific chaperone proteins (Beato and Klug 2000). Upon ligand binding, ERs undergo activating conformational change, homodimerize and translocate to the nucleus,

where they bind to hormone responsive elements (such as estrogen responsive element; ERE) within the regulatory regions of target genes (Kumar and Chambon 1988; Beato and Sanchez-Pacheco 1996; Kuntz and Shapiro 1997). Depending on the cell and promoter contexts, the DNA-bound receptors induce either expression or repression of the downstream target genes. These diverse effects of ERs on transcription are mainly due to transcriptional co-regulators, large protein complexes that physically associate with the receptors and mediate the interaction between nuclear receptors and the transcriptional machinery (Onate et al. 1995; McKenna et al. 1999a). Large numbers of nuclear receptor co-factors also have an ability to modify chromatin by inducing covalent modifications of DNA or associated histone proteins and, as a result, lead to more accessible or restrictive confirmation of target sequences (Onate et al. 1995; Bannister and Kouzarides 1996; McKenna et al. 1999a).

The basic structural unit of chromatin is the nucleosome, which comprises 147 base pairs of DNA wrapped around a core of eight histones (two H2A, H2B, H3, and H4 histones; Allis 2007). The N-terminal tails of core histones protrude from the nucleosomes and are subject to a wide range of post-translational modifications of specific amino acid side chains, including acetylation, phosphorylation, methylation, ubiquitination, ADP-ribosylation and sumolation (Kouzarides 2007). These various modifications of histone proteins have differential effects on the transcriptional activity of sequences with which they are associated. Acetylation of lysine residues generally leads to a more relaxed confirmation of chromatin and promotes transcription (Utley et al. 1998; Turner 2000). Methylation of lysine or arginine residues appears to have multiple, sometimes opposing effects on gene expression based on modified residue. For example, methylation of H3K9 and H3K27 residues is mostly associated with repressed chromatin and gene silencing, whereas methylation of H3K4 often leads to permissive chromatin structure (Goll and Bestor 2002). According to the hypothesis put forward by Allis and colleagues, specific sequences and combinations of histone modifications establish the so-called "histone code," which in turn determines the transcriptional profile of target genes associated with it (Strahl and Allis 2000).

In addition to histone proteins, the DNA sequence itself can undergo covalent modification in the form of methylation of cytosine residue within CpG dinucleotide sequences. While histone modifications can lead to either gene transcription or gene repression, DNA methylation generally results in transcriptional repression (Klose and Bird 2006). It is important to note that neither histone modifications nor DNA methylation occur in isolation as a singular event. In turn, specific environmental factors or stimuli can induce sequences and/or combinations of these changes that ultimately will alter the transcriptional profile of target genes (Allis 2007). Such chromatin modifications that do not modify DNA sequence per se but nonetheless can lead to long-term alterations in transcription are collectively referred to as "epigenetic" regulation. Recently, we and others have proposed that it is precisely by employing epigenetic regulatory mechanisms during critical developmental periods that estrogens are able to orchestrate long-lasting sex- and brain region-specific changes in neural circuits that foster sexually dimorphic behaviors (Fig. 1; Gagnidze et al. 2010; Nugent and McCarthy 2011).

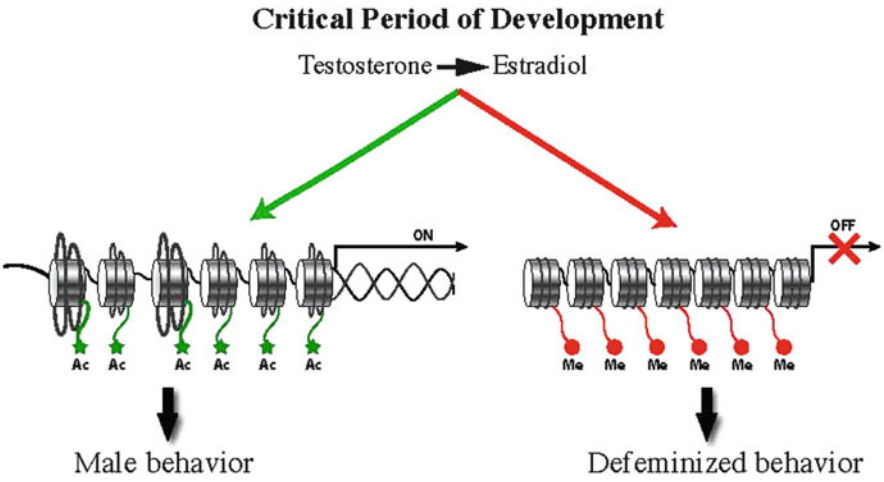

Fig. 1 Hypothesis about the role of histone modifications in the formation of sexually dimorphic brain and behaviors. We hypothesize that brain exposure to gonadal hormones during a critical developmental time period will lead to dimorphic histone modifications in the nerve cells. Association of acetylated histones with the promoters of genes important for the expression of male sexual behavior will promote transcription of these genes and lead to "masculinization" of the behavior. At the same time, methylation of histones associated with the promoters of genes that foster female-type behaviors will result in their suppression and "defeminization" of behaviors (Adapted from Gagnidze et al. 2010) *Ac* acetyl; *Me* methyl

This type of epigenetic regulation can work on different levels. It can target the promoters of ER genes and alter their expression profile in a sex-dependent manner or, conversely, ERs can employ distinct histone and DNA modifying co-regulators to induce sex-specific epigenetic changes in the brains of males and females. These two scenarios are not mutually exclusive and ultimately both will lead to dimorphic expression of hormone-responsive genes. Recent advances in our understanding of the importance and versatility of epigenetic regulatory mechanisms in the development and normal physiological functions of the nervous system spurred investigations into the role of these processes in the development of the sexually dimorphic brain.

Evidence for Epigenetic Regulation

Previously it has been shown that ERα expression within the developing POA and VMH is sexually dimorphic, with females exhibiting higher levels than males (DonCarlos and Handa 1994; Kühnemann et al. 1995; Yokosuka et al. 1997). It has also been demonstrated that gene expression of ERα and ERβ is autoregulated by estradiol-induced activation of the receptors themselves, suggesting that lower levels of ER in neonatal male brains may be due to hormone-induced down-regulation of the receptor (Lauber et al. 1991a, b; Kühnemann et al. 1995; Stoica

et al. 2003). Accordingly, when examined, the methylation pattern of the ERα gene promoter in the POA of neonatal rats was found to be sexually dimorphic. Specifically, males exhibited higher levels of methylation than females within the 5′ flanking region of ERα exon 1b promoter region (Kurian et al. 2010). The higher methylation level of the ERα promoter was correlated with lower expression levels of ERα mRNA within the developing POA, similar to what was previously reported. Importantly, treatment with estradiol increased methylation and decreased ERα expression in neonatal females, suggesting that sex differences in hormone levels may lead to sex differences in ERα expression by employing epigenetic regulation of DNA methylation.

Transfer of a methyl group to cytosine residue is catalyzed by DNA methyltransferases (DNMTs), and fluctuations in their expression levels can potentially affect the methylation status of DNA (Klose and Bird 2006; Miller and Sweatt 2007). In line with this hypothesis, it was found that neonatal females express significantly more DNMT3a mRNA and protein in the amygdala, and it was negatively regulated by both estradiol and DHS treatment (Kolodkin and Auger 2011). No sex differences were detected in the levels of DNMT3 in POA and MBH. This finding suggests that steroid hormone exposures may regulate lasting differences in gene expression and in amygdala function by altering the expression of the epigenetic factor, DNMT3a, in a region-specific manner.

Methylated cytosine residues within CpG dinucleotides provide binding sites for regulatory proteins that subsequently lead to transcriptional repression (Bogdanović and Veenstra 2009). One such binding protein is MeCP2, which has an important role in nervous system development, highlighted by the fact that its mutation leads to the neurodevelopmental disorder Rett Syndrome and is implicated in the etiology of autism spectrum disorders (Gonzales and LaSalle 2010). Both of these conditions are characterized by strong gender bias; thus the observation that expression of MeCP is sexually dimorphic in neonatal rat brains is of potential significance. Male rats express markedly less MeCP2 mRNA and protein than females within the amygdala, as well as in the VMH on postnatal day 1 (PD1); however, these sex differences disappear at PD10 (Kurian et al. 2008). In contrast, no sex differences were detected in MeCP2 levels within POA on PD1, but by PD10 males had more MeCP2 mRNA than females in this brain region. Although no direct link was reported between neonatal hormone levels and MeCP2 expression in this study, the temporal pattern of sex difference of MeCP2 expression during the steroid-sensitive period of brain development suggests that MeCP2 may participate in sexual differentiation of the rat brain. In fact, evidence exists that, in cortex, MeCP2 is recruited to a ERα promoter and regulates its expression during development, raising the possibility that it may serve a similar function in sexually dimorphic regions as well (Westberry et al. 2010). In addition, the region-specific sex differences of DNMT3 and MeCP2 expression described in these studies provide an interesting example of how epigenetic regulatory mechanisms may affect the spatio-temporal patterns of gene expression necessary for dimorphic brain development.

As mentioned above, DNA methylation and histone modifications often occur in concert to regulate gene expression. Evidence suggests that such combinatorial

regulation may be involved in sexually dimorphic expression of ERα. Indeed, during the critical period for sexual differentiation, histones associated with ERα gene promoters in the mPOA were differentially acetylated between the sexes (Matsuda et al. 2011). These differences were age-dependent and, by the age of PD3, were lower in males than in females. The same pattern was observed for histones associated with aromatase gene promoters. Further investigation revealed that the differences in acetylated histone levels were due to differences in the levels of histone deacetylases (HDAC) 2 and 4 present at the promoters of ERα and aromatase genes. To examine the importance of these epigenetic processes at the behavioral level, the authors used pharmacological inhibitors of HDAC activity as well as antisense-mediated knockdowns of HDAC2 and 4 during the critical period of development, both of which resulted in a significant deficit in male sexual behavior in adulthood.

In addition, sex differences have been found in global levels of acetylated (H3K9/14Ac) and methylated (H3K9Me3) histone H3 in the cortex and hippocampus (Tsai et al. 2009). Interestingly, H3 acetylation in these brain regions was regulated by prenatal exposure to testosterone; however, H3 methylation was dimorphic regardless of hormone levels. Hormonal regulation of these processes is most likely induced by the association of ligand-bound ERs with various transcriptional coregulators that can catalyze a range of covalent histone modifications on the promoters of target genes (McKenna et al. 1999b). To date, a large number of cofactors involved in ER-mediated transactivation have been identified, including SWI/SNF complexes that alter the spatial organization of nucleosomes in an ATP-dependent manner (Belandia et al. 2002): histone acetyltransferases (HAT), such as members of the p160 subfamily (steroid-receptor coactivators; Onate et al. 1995; Spencer et al. 1997), or the integrator complexes p300/CBP and p/CAF (Bannister and Kouzarides 1996; McKenna et al. 1999b) and histone methyltransferases (HMT), such as CARM1 or PRMT (Klinge 2000; McKenna and O'Malley 2002; Belandia and Parker 2003). In support of our hypothesis, it has been shown that nuclear receptor coactivators SRC and CPB, as well as the corepressor NCoR, exhibit sexually dimorphic patterns of expression (Auger et al. 2000, 2002; Jessen et al. 2010). Specifically, on PD0, males expressed higher levels of CBP within the VMH, medial POA, and arcuate nucleus, and neonatal CBP antisense-ODN treatment interfered with the defeminizing, but not the masculinizing, actions of testosterone on female sexual behavior (Auger et al. 2002). Notably, reducing levels of SRC-1, another ER cofactor with HAT activity in neonatal rats, also blocked the defeminizing effect of testosterone (Auger et al. 2000), indicating (albeit indirectly) an important role for histone acetylation in this developmental process.

We have recently obtained preliminary evidence that supports the hypothesis that estrogen-induced signaling through ERs promotes sexual differentiation of brain regions by inducing distinct combinations of histone modifications. Two histone marks — H3 acetylation that promotes transcription and H3K9methylation that is mostly associated with repressive chromatin — were examined in the extracts of POA and VMH tissues in neonatal (PD0) male and female mice by SDS-PAGE and Western blot. We found sex- and region-specific patterns of

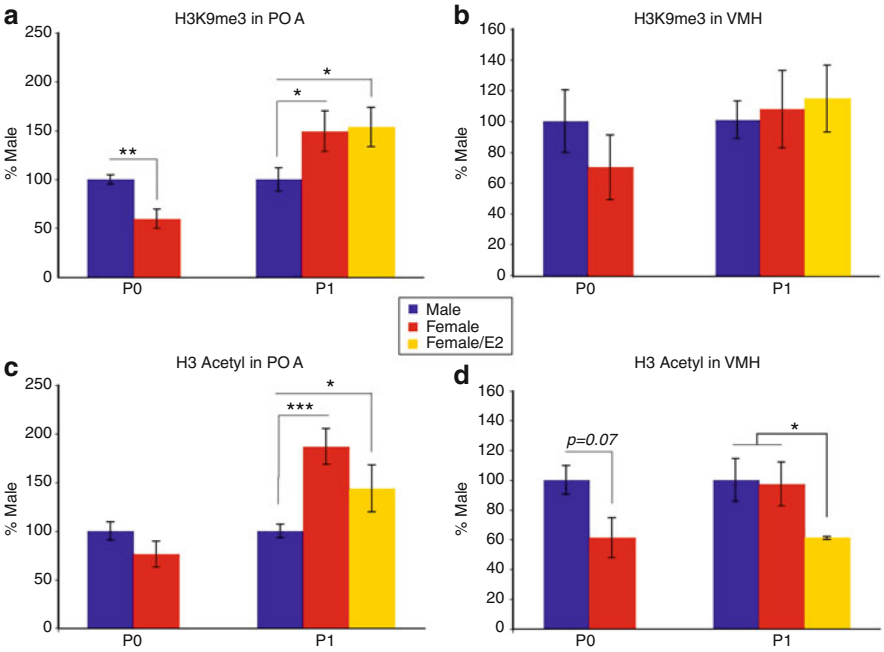

Fig. 2 Sex differences in the levels of histone H3 modifications are region-specific and are regulated by estradiol. (1) Overall, on PD0, both H3Acetylation and H3K9me3 marks were more abundant in male brain compared to female. Levels of trimethylated H3K9 were higher in newborn male POA (**a**) and VMH (**b**), although in the latter the difference failed to reach significance. Sex difference in the levels of acetylated H3 was only detected in the VMH (**d**) and only a similar trend was seen in POA (**c**). (2) On PD1, the amount of H3Acetyl and H3K9me marks in male and female VMH leveled (**b** and **d**). In contrast, in POA, females displayed significantly higher H3Acetyl and H3K9me3 marks compared to males (**a** and **c**). We hypothesize that such drastic and dynamic changes in the histone modifications within the first postnatal day reflect the complex "organizational" processes presented by hormonal and environmental stimuli. (3) Treatment of newborn females with E2 (5 μg in 10 μl oil, s.c) for 24 h on PD0 confirmed that H3Acetylation, but not H3K9me3, is regulated by hormones. In both, POA (**c**) and VMH (**d**), estradiol treatment resulted in a decrease in H3Acetyl levels; however, only in POA did this change reflect "masculinization" of the mark. These data lead us to speculate that H3Acetylation in POA may be involved in the transcriptional regulation of hormone-responsive genes important for the formation of the male-typical neural circuit

histone modifications (Fig. 2). Males exhibited significantly higher levels of H3K9 methylation levels in POA and showed a similar trend in VMH (Fig. 2a, b). In a microarray study, we previously reported that a large number of transcripts displayed lower expression levels in neonatal male POA compared to female (Gagnidze et al. 2010). Thus the high abundance of the H3K9me3 repressive mark in male POA compared to female at this time point correlates well with sex differences in transcriptional activity detected in this brain region. Conversely H3 acetylation was higher in the neonatal male VMH, but the difference was hardly detectable in POA (Fig. 2c, d). These data are in conflict with reports by Tsai et al.

(2009), who did not detect sex differences in these modifications in POA/hypothalamus. The discrepancy can be reasonably explained by differences in experimental design between the two studies. We analyzed two brain regions separately and found that the histone modification pattern differed (was almost the opposite) between them. Tsai and colleagues chose to pool POA and hypothalamic tissue and analyzed them as a single sample, which most likely resulted in the masking of the effect. As mentioned earlier, these two brain regions support the expression of sexually dimorphic behaviors: POA is important for male sexual behavior and VMH fosters female sexual behavior. Thus perinatal estrogen has to employ distinct signaling and regulatory mechanisms to "organize" these circuits to support differential functions. We believe that sex- and region-dependent differences in acetylated and methylated histone levels in these two brain areas reflect such regulatory mechanisms.

To determine whether these histone modifications are regulated by neonatal estrogen exposure, newborn female pups were treated with a single dose of estradiol on PD0. Twenty-four hours later, pups were sacrificed, brains removed and POA and VMH tissue were analyzed for levels of the same histone modifications, H3 acetylation and H3K9 trimethylation. Estradiol-treated females were compared to oil-treated females and males. Two interesting observations were made in this study. We found that, in VMH, sex differences in histone marks between control oil-treated female and male brains disappeared on PD1 (Fig. 2b, d). Moreover, in POA, the sex difference was reversed and oil-treated females exhibited significantly higher levels of histone H3 acetylation and H3K9 methylation compared to males (Fig. 2a, c). These data indicate that histone modifications are dynamically regulated during this critical developmental period. Considering the abundance of intrinsic and environmental stimuli that the newborn animal is exposed to on the first day of birth, all of which potentially can converge and influence epigenetic processes, these changes in the global levels of histone modifications within the first postnatal day is not surprising. In concert with the observations of Tsai et al. (2009), we found that the H3 acetylation mark was regulated by hormonal exposure and was significantly lower in the VMH of estradiol-treated females compared to oil-treated females and males (Fig. 2d). A similar trend was evident in POA, where estradiol treatment resulted in a slight decrease in H3 acetylation in female brains to the levels in between those of oil-treated females and males (Fig. 2c). In contrast, H3K9 methylation seemed to be insensitive to hormone levels in both POA and VMH (Fig. 2a, b). These data, although preliminary, clearly reflect the complex epigenetic processes that take place during the critical period of brain sexual differentiation.

Outlook

The causation of permanent changes in brain and behavior by the early actions of sex hormones in the brain brings mechanisms into play that often are subsumed under the phrase "epigenetic changes." Three classes of mechanisms can easily be

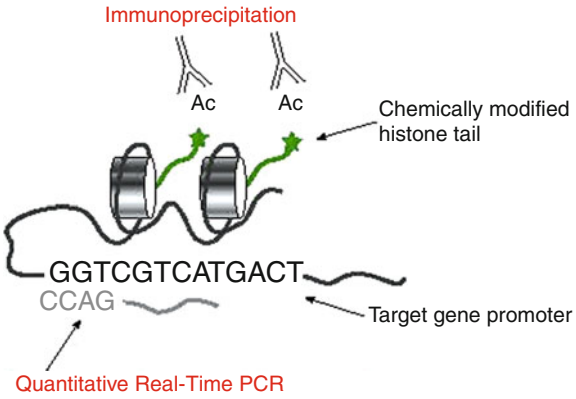

Fig. 3 Chromatin immunoprecipitation (ChIP). Antibodies that recognize specific modifications on histone tails are used to pull down the histone-DNA complex. The gene associated with active or repressive chromatin is identified and quantified by qPCR amplification with gene-specific primers. *Ac* acetyl (Adapted from Gagnidze et al. 2010)

envisioned: (1) DNA methylation; (2) effects of non-coding DNA, such as retrotransposons; and (3) the chemical modification of histone tails. While our chapter has focused on the latter of these mechanisms, histone chemistry, we have limited our treatment so far to global histone modifications in specific brain regions. New research will focus on histone changes over specific gene promoters using chromatin immunoprecipitation (ChIP) technology (Fig. 3). We hope that these detailed explorations of sexually differentiated mechanisms in the animal brain will aid in the understanding of sexually differentiated maladies of human behavior. One example of such a malady is autism, which is much more prevalent in boys than in girls (Pfaff et al. 2011) and represents a spectrum of behavioral disorders of such current importance that new research is urgently required.

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Importance of Genomic Imprinting in the Evolution and Development of the Maternal Brain

Barry E. Keverne

Abstract It was the French reproductive biologist, Alfred Jost (1970), who proposed that mammalian sexual differentiation is biased in a female direction and masculine characteristics are imposed on an essentially female life plan. The reproductive success of mammals places a considerable burden of time and energy on the matriline, with some 95 % of female adult life committed to pregnancy, lactation and maternal care. Viviparity has thus provided a major selection pressure on the matriline in the evolution of these events and with particular emphasis on the placenta and hypothalamus. Increased maternal feeding, maternal care, suspension of fertility and sexual behaviour, parturition and milk provision are all integral to hypothalamic function and have evolved under the influence of the placental hormones to meet the demands of the developing infant (Keverne 2006). Viviparity has also introduced a new dimension to evolutionary genetics in providing the co-existence and continuity for three generations of matrilineal genomes (i.e., mother, developing offspring and developing oocytes) in one individual (Keverne 2011). Also unique to the mammalian matriline has been the evolution of epigenetic marks (imprint control regions) which are heritable and undergo reprogramming to regulate gene expression according to parent of origin. This imprinting of autosomal genes (genomic imprinting) plays a significant role in mammalian development, particularly development of the placenta and hypothalamus (Keverne 2009). Indeed, a number of imprinted genes are co-expressed in the placenta and hypothalamus and are important for the co-adapted functioning of these structures. Such transgenerational co-adaptation ensures the foetal hypothalamus is genetically and epigenetically programmed for ensuring optimal maternal care and nurturing (Broad and Keverne 2011). In this way the foetus not only controls its own destiny via the placenta but also that of the next generation via the developing hypothalamus.

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Introduction

The history of genomic imprinting started when reproductive biologists tried to produce parthenogenetic mammalian embryos (Surani et al. 1984). There was some success but development was poor, with major defects in the extra-embryonic membranes that form the placenta and with lethality of the embryo at the 25-somite stage of development. Similar studies were undertaken for androgenetic embryos developed from two copies of the paternal genome. Diploid XY androgenetic embryos likewise failed to complete normal development and again the primary defects were in trophectoderm and the developing placenta. It was concluded that some form of imprinting of the genome occurred and both a male and female genome was essential for full-term development. Some 7 years were to pass before any imprinted gene was identified, but in the meantime, the creation of androgenetic and parthenogenetic chimeras was accomplished that survived both pregnancy and birth (Allen et al. 1995). This advance enabled a detailed investigation of how these disomic cells containing two copies of either the maternal or paternal genome contributed to development of the brain. A clear and distinct patterning for the distribution of these cells in the brain emerged (Keverne et al. 1996a). At birth, cells that are disomic for the paternal genome (i.e., both allelic copies are from the father) contributed substantially to those parts of the brain that are important for primary motivated behaviour (hypothalamus, pre-optic area, bed nucleus of the stria terminals and septum). By contrast, parthenogenetic cells (i.e., both allelic copies are maternal) were excluded from these mediobasal forebrain areas but selectively accumulated in those regions where androgenetic cells are excluded, namely the neocortex and striatum. Furthermore, growth of the forebrain of parthenogenetic chimeras is enhanced by this increased gene dosage from maternally expressed genes whereas the brains of the androgenetic chimeras were smaller, both in absolute measurements and especially relative to body weight. Neurological studies of brain function in human Prader-Willi syndrome (Horsthemke et al. 2003) and Angelman's syndrome, where a restricted region of the chromosome is uniparentally disomic for paternal or maternal imprinted genes, are congruent with these experimental findings (Saitoh et al. 2005).

Allometric scaling across different mammalian species of those distinct parts of the brain to which maternally or paternally disomic cells differentially contribute reveals that a remodelling of the brain has occurred during mammalian evolution (Keverne et al. 1996b). A certain amount of caution has to be exercised when interpreting these findings. Although we now know that many maternally expressed genes are important for neocortical development (Ferron et al. 2011) and paternally expressed genes are important for hypothalamic development (Keverne 2009), the reduced size of the neocortex with paternal disomies may be due to a failure of these cells to thrive and survive when they reach the developing cortex.

The discovery of the first imprinted genes, the maternally expressed Igf2-receptor and subsequently its interacting ligand, paternally expressed Igf2, led to the theory of mother-infant genomic conflict (Haig 1992). Haig theorised that the optimal strategy for the mammalian embryo was to extract maximal resources from the mother, with

paternal loci of imprinted genes being selected to secure this strategy. This was a plausible theory of genomic imprinting based on paternally expressed Igf2, which promotes foetal growth, and its maternally expressed receptor Igf2r, which, being a null receptor, restricts these growth-promoting effects. However, at that time point, there was still a great deal to be learned concerning the complex mechanisms that regulate and reprogramme genomic imprinting. Monoallelic silencing in the context of genomic imprinting is not a property of the gene per se but results from its positioning in the genome with respect to imprint control regions (ICRs) which, in turn, result in differentially methylated regions of DNA (Ferguson-Smith 2011). Moreover, there is a distinct non-equivalent bias towards matrilineal control over these genomic regions that epigenetically regulate these imprinted genes (Schultz et al. 2010). Successful viviparity has required extensive cooperative functioning of both the genotypes and phenotypes in the two generations and, since this functioning is dominated by the matrilineal genome in both generations and since sons benefit as much as daughters, conflict is difficult to reconcile with genomic imprinting.

Matrilineal Control Over Genomic Imprinting

The active control over genomic imprinting is firmly embedded in maternal DNA. There is a biased distribution of methylation-dependent ICRs towards the maternal germline, with the majority of imprinted gene clusters being dependent on maternal germline methylation (Bourc'his and Proudhon 2008). Only three imprinted loci have been reported to be controlled by paternal germline methylation (H19/Igf2, A19/Rasgrf, Gt12, Dlk1). Even here, the methylation of Igf2 and Rasgrf does not causally determine the imprint, since parent of origin expression does not occur without the binding of maternal CTCF to the maternal ICR on the maternal chromosome (Kurukuti et al. 2006). The H19 ICR is methylated in sperm, but it also attracts methylation even when inserted into non-imprinted loci, and in Drosophila it is capable of silencing a whole chromosome (Gebert et al. 2010). This finding supports the idea that H19 is sufficient to act autonomously for methylation and does not require spermatogenic resetting, but it does require maternal CTCF binding to prevent biallelic silencing (Matsuzaki et al. 2010). Moreover, the maternal germline not only determines paternally expressed genes by maternal epigenetic marks that attract methylation and silencing but also regulates maternally expressed genes by the production in cis of non-coding RNA itself silenced on the maternal allele by DNA methylation inherited from the oocyte (Ferguson-Smith 2011).

Genomic Imprinting and Placental Hypothalamic Co-adaptation

Mammalian viviparity requires the action and interaction of two genomes in one individual, thereby providing a template for intergenomic co-adaptive function. The placenta, as part of the foetal genome, exerts considerable influence on the

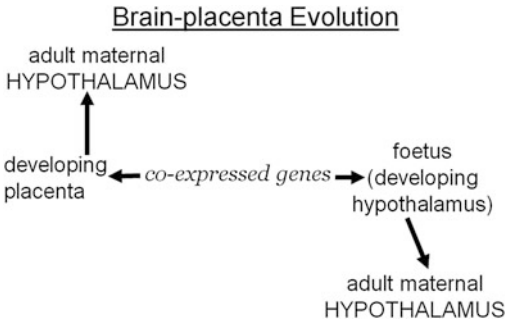

Fig. 1 Schema for co-adaptive evolution of brain and placenta. The success of the foetal genome is dependent on its placental interactions with the mother's hypothalamus. The developing placenta and foetal hypothalamus, through co-expressed genes, serve as a template for selection pressures to operate. Thus the success of this placenta-maternal interaction in determining good mothering will shape the success of the developing hypothalamus for good mothering in the next generation

maternal hypothalamus at the same time as the foetus itself develops a hypothalamus (Fig. 1). Hormones produced by the placenta, or their luteotrophic action in the ovary, influence the maternal brain, determining endocrine function and behaviour. Progesterone, in particular, suppresses maternal sexual behaviour and fertility, increases maternal food intake in anticipation of subsequent foetal demands, and promotes the synthesis of maternal hypothalamic oxytocin in anticipation of its requirements for parturition, maternal behaviour and milk ejection (Keverne 2006). In short, the foetal genome determines its own destiny via the placenta, hormonally regulating the maternal hypothalamus to serve the interests of the foetus which, at the same time, is developing its own hypothalamus. Conversely, maternal stress, glucocorticoids and insulin-like growth factors may induce changes in placental function which determine foetal programming in the context of hypertension and metabolic disease (Harris and Seckl 2011; Sferruzzi-Perri et al. 2011).

Those parts of the maternal brain which respond to placental hormones, namely the hypothalamus, undergo major development in the mouse during E11-16 (Forger 2005). This is also the period when placental vasculature becomes established and proliferation of the hormone-producing placental giant cells occurs. Many of the genes which change their expression over this developmental period (E11-12-13) are co-expressed in the placenta and hypothalamus (Fig. 2) and will demonstrate transcriptional synchrony across these co-adapted tissues. We have shown that genes which significantly change their co-expression in both hypothalamus and placenta increase from 9 % (days E11-12) of all gene expression changes to 47 % (days E12-13; Fig. 2a, b). Those genes whose expression significantly changes exclusively in the hypothalamus during this same period remain relatively stable from 37 % (days E11-12) to 36 % (days E12-13), whereas significant gene expression changes exclusively in the placenta decrease from 57 % (E11-12) to 16 % (E12-13; Fig. 2a, b; Broad and Keverne 2011). Thus a phase shift in changing

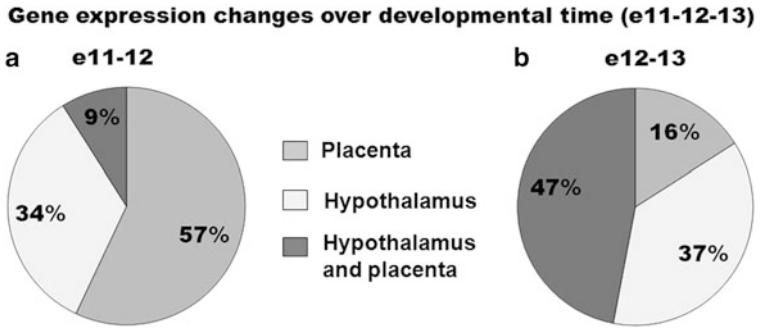

Fig. 2 Gene expression changes over developmental time. Notable increases for co-expression occur in those genes which change the expression over the period E11-12-13, (as meant?) thus making these the majority of genes that are active over E12-13

patterns of gene transcription over developmental time results in many genes that are common to, and synchronised for, expression in both the hypothalamus and placenta. Such co-expression would support this developmental period as potentially important for co-adaptive selection pressures to operate on the hypothalamus and placenta.

The maternal imprinting of specific genes, a unique epigenetic transcriptional regulatory mechanism that is restricted to viviparous mammals, has been thought to play an important role in the evolutionary development of placentation (Mess and Carter 2007; Renfree 2010). To date, 12 maternally imprinted genes have been shown to be developmentally expressed in both brain and placenta, although a recent study suggests this could be a considerable underestimate (Gregg et al. 2010). We have further considered how the transcriptional inactivation of the maternally imprinted gene (Peg3) influences the transcription of genes and co-expressed genes in hypothalamus and placenta over this same developmental time period. Peg3 was selected because it is co-actively expressed in the hypothalamus and placenta during this time period and its inactivation is known to impair both placental development (reduced placenta size and impaired development of endocrine giant cells and spongiotrophoblast; Hiby et al. 2001) and hypothalamic development (reduced cell numbers in the PVN, MPOA; Broad et al. 2009). This results in impaired maternal care, milk letdown, infant suckling, metabolism and adult reproduction (Curley et al. 2005). More importantly, when Peg3 transcription is inactivated in the hypothalamus of the pregnant mother carrying wild-type offspring, the functional phenotypic outcomes have remarkable similarities to those which occur when the same gene is selectively inactivated in the developing placenta and foetal hypothalamus in a wild-type mother (Fig. 3). These functional co-adaptations across two generations, mother and offspring, have clearly required genetic co-adaptation to occur between the developing hypothalamus and placenta across successive generations. Moreover, during development, Peg3 is restricted to expression in the basal part of the forebrain (future hypothalamus) as well as the spongiotrophoblast and giant cells of the placenta. Thus viviparity adds a new

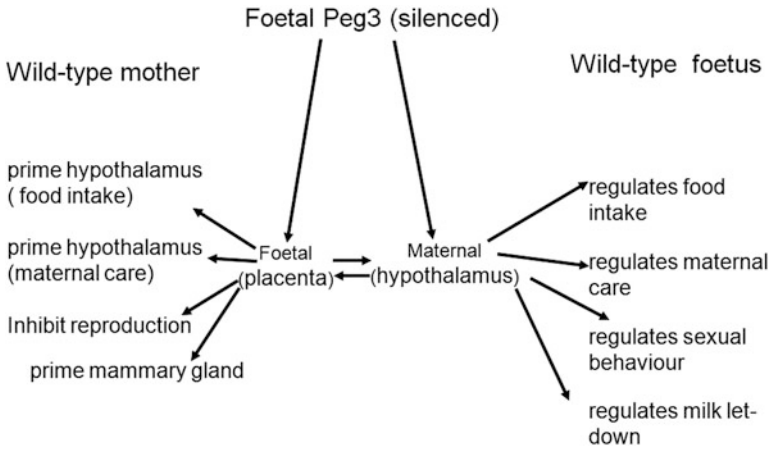

Fig. 3 Deletion of the Peg3 gene in the developing hypothalamus has remarkably similar functional consequences as deletion of this same gene in the foetal placenta. The latter results in the failure of the foetal placenta to communicate with adult maternal hypothalamus, while the former results from a failure of the foetal hypothalamus to develop an adequately responding adult maternal hypothalamus

environmental dimension of two genomes co-existing in one individual providing a template for co-adaptive selection pressures to operate, which is especially effective when this engages imprinted genes.

Between E11 and E12, Peg3 inactivation is particularly effective at targeting co-expressed genes (Fig. 4). Peg3 inactivation suppresses all of the co-expressed genes and induces co-expression of genes which are not usually co-expressed at this stage. The induced changes to co-expression by the Peg3 mutation are, however, not in synchrony; 90 % are up-regulated in the placenta, but in the hypothalamus they are all down-regulated. The next developmental period, E12-13, reveals a marked increase in hypothalamus and placental gene co-expression (up fourfold from E11-12; Fig. 4). Moreover, 59 % of these gene changes are synchronised for the direction of changed expression. At this same developmental time period, the inactivation of Peg3 suppresses 45 % of these co-expressed transcriptional changes and induces changes to a further 38 % of hypothalamus and placenta co-expressed genes (Fig. 4). Days E12-13 thus represent a period for major changes in transcriptional synchrony for genes co-expressed in the hypothalamus and placenta, and it is also the period when the Peg3 inactivation has maximal effects, not only suppressing many of these transcriptional changes but also inducing co-expression of new genes. Peg3 inactivation is thus particularly effective at desynchronising the expression of genes that are simultaneously co-expressed in hypothalamus and placenta, as well as disrupting the co-ordinated functional interactions between mother and foetus of this and the subsequent generation of wild-type offspring.

The brain and placenta are genetically co-regulated during developmentally important periods (E11-12-13) by sets of genes whose expression changes are

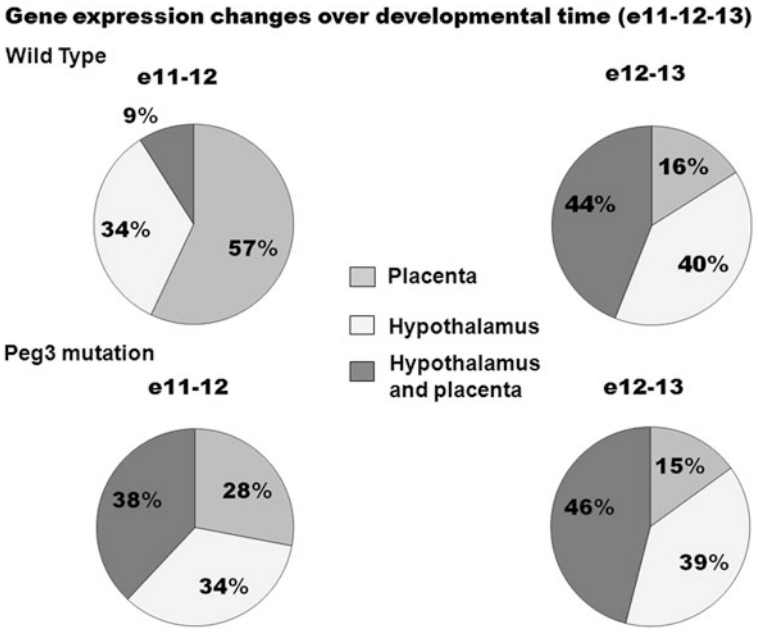

Fig. 4 Peg3 mutation is particularly effective at targeting co-expressed genes both for suppressing changes to co-expressed genes and inducing co-expression of new genes

synchronised in the placenta and hypothalamus. This co-expression is substantially disrupted by mutation to a maternally imprinted gene (Peg3), which also has significant developmental consequences for a range of phenotypes integral to successful growth and maternalism. An increase in atmospheric oxygen appears to have played a significant role in early mammalian placental evolution (Falkowski et al. 2005), and hypoxic conditions are a notable signal for Peg3 transcription in neurons (Yamaguchi et al. 2002) and in the developing placenta and for induction of vascularisation (Vaiman et al. 2005). Interestingly, although the mammalian Peg3 gene is structurally different from that of non-mammalian vertebrates, across mammalian lineages this gene and its promoter are highly conserved. The evolutionary significance of its imprinting must, therefore, be underpinned by its ability to regulate those large gene families (Prls, Psgs and Ceacams) which have themselves undergone multiple duplications and differential expansion across different mammalian species (Wildman 2011).

Evolution of functional co-adaptation between a mother's hypothalamus and the foetal placenta occurs developmentally at the level of the foetal genome but has the potential for further post-partum epigenetic modification through the mother's behavioural and endocrine responses to the offspring. In this way, offspring which receive "optimal in-utero" nourishment and improved maternal care will themselves develop a hypothalamus both genetically and epigenetically predisposed to optimal mothering (Keverne 2011).

A Reconsideration of SRY Evolution and Masculinisation of the Brain

The huge bias in the female's commitment of her time and resources to mammalian reproductive success has undoubtedly impacted on mammalian brain evolution in a major way. In terms of behaviour and neuroendocrine function during pregnancy and following parturition, mammalian brain development is dominated by the matrilineal genome, which has evolved under selection pressures that ensured successful pregnancy and maternal care. Integral to this has been the heritable matrilineal epigenetic marks which determine genomic imprinting, the co-adaptive development of hypothalamus and placenta and tight control over gene dosage. However, the question arises as to how the male brain becomes different, how maternalism is suppressed and how masculinisation is brought about? Males do not, of course, undergo pregnancy and parturition, but they nevertheless benefit from viviparity in the same way as females and have been subject to the same selection presence of a viviparous in-utero environment. Indeed the male brain is capable of maternal behaviour in monogamous mammals (Carter and Keverne 2002), and castration at birth enables paternal care in males in many species (Lonstein et al. 2002)—hence the importance for production of testosterone in male hypothalamic masculinisation. But is this the complete story?

An important evolutionary development in masculinisation of placental mammals has been the male SRY sex-determining gene, which activates Sox9 for testes development (Sekido and Lovell-Badge 2008; Wallis et al. 2008). SRY is not present in the earliest of mammals, the egg-laying monotremes, and is thought to have evolved in parallel with the placentation. Interestingly, SRY is a hybrid gene of DGCR8 (Di-George syndrome critical region 8) and the Sox3 HMG box, regulated by the transcription factor CP2 (TFCP2; Sato et al. 2010). SRY is an intronless gene which serves as a master switch for testes formation (Kashimada and Koopman 2010). It provides the trigger for sex determination by activating Sox9, which not only brings about testes formation but blocks the genetic pathways that lead to the differentiation of ovarian cells (Veitia 2010).

Sox3, an X-linked gene from which SRY evolved, is also required for, and integral to, the formation of the hypothalamus and pituitary (Rizzoti et al. 2004). DGCR8, which became inserted upstream of the ancestral Sox3 in SRY evolution, is important for regulating the expression of micro-RNAs processed by the DGCR8-Drosha (microprocessor) complex from the non-protein coding transcript, PolII, which is expressed exclusively in the placenta (Bortolin-Cavaille et al. 2009). Another member of the Sox gene family (Sox15), which has 75 % sequence in common with the HMG box of Sox 3, is also expressed in the placenta and plays an important role in the development of the placental giant cells that produce the hormones that regulate the adult maternal hypothalamus (Yamada et al. 2008). Are these relationships between the hybrid genes which constitute SRY, and also influence the developing hypothalamus and placenta, a coincidence? More likely they are telling us something about the evolution of mammalian hypothalamic

maternalism necessitating a critical timing for testes development to counteract such feminising events in males? Male sex determination is not new to mammals but the timing of this development is crucial, with SRY expression starting at E10.5 (mouse) in the developing testes. At E11.5, SRY induces the formation of foetal Leydig cells which produce high levels of testosterone (E15.5) without the need for luteinizing hormone (LH) stimulation (Barsoum and Yao 2010). The hypothalamus also undergoes its critical development (mouse) under the regulation of Sox3 between E11.5 and E16.5. Foetal Leydig cells are lost at birth and testosterone declines from pn1-30 until adult Leydig cells mature under the influence of LH at puberty (Chen et al. 2009).

Brain masculinisation in the mouse has long been established to be a consequence of testosterone aromatisation to oestrogen which epigenetically regulates sex-differences in neuronal composition of certain hypothalamic nuclei (BNST) and the pre-optic area (POA), both being larger in males, whereas the anteroventral periventricular nucleus (AVPN) is larger in females (McCarthy et al. 2009; Baum 2009a). However, the anatomical consequences of masculinisation are not evident until the post-natal period in the BNST, which relays pheromonal information to regions of the brain concerned with male sexual and female maternal behaviour. No sex differences were measureable on post-natal (pn) days 3, 5 and 7, but this nucleus does appear to contain significantly more neurons in the male between pn 9 and 11. Deletion of the pro-apoptotic gene, Bax, eliminated these BNST sexual dimorphisms by blocking activation of the p53 cell death pathway (Forger et al. 2004). However, this produced a significant increase in cell number in both sexes. Examining cell death, as opposed to preventing it by mutation, did provide a result which showed more cell death in the female BNST at pn 6 (Gotsiridze et al. 2007). AVPV neurons contain the peptide kisspeptin, an important regulator of GnRH in the onset of puberty, and also show post-natal sex differences which appear at post-natal day 12 (Semaan et al. 2010). The sexually dimorphic populations of dopamine neurons in this nucleus are reduced in the male post-natally by aromatisation of testosterone to oestrogen, which activates caspase-induced cell death of these dopamine neurons.

Thus there appear to be two phases in the masculinisation of the mouse hypothalamus: a pre-natal proliferative phase and a post-natal reorganizational apoptotic cell-death phase in certain hypothalamic nuclei. In the mouse, this cell death phase is particularly directed at the neural pathways concerned with processing pheromonal stimuli (Baum 2009b). Moreover, this pathway is integral to engaging appropriate sexually dimorphic behaviour, including mating and aggression in males (Stowers et al. 2002) and nursing and maternal aggression in females (Hasen and Gammie 2009). The vomeronasal sensory input is also integral to female reproduction, primarily by the AVPV release of kisspeptin and its activation of puberty in the female and by the induction of oestrous in the female in the presence of male pheromones. The imprinted Peg3 gene is expressed in all the neural relays of the olfactory system and increases fourfold in the AOB of the mother when exposed to pups (Canavan et al. 2001). In the 4- and 6-day post-partum mouse brain, inactivating Peg3 increased caspase3 induced apoptosis in

these neural relays, ameliorating any neural sex difference (Broad et al. 2009). However, the action of Peg3 is complex, interacting with the pro-apoptotic P53 and the counterbalanced anti-apoptotic p73 which has an intense signal in the VNO neurons, the AOB and its neural relays to the MPOA (Pozniak et al. 2000). Thus Peg3 may ensure a balanced regulation of apoptosis in the post-natally developing pheromonal pathways that play an integral sensory part in female maternal and male sexual behaviour.

Concluding Comments

This account has focussed on the complexity in development of the maternal brain, with special emphasis on its evolutionary origins in mammals. These events have been dependent on evolutionary changes associated with viviparity and the biased contribution of the matriline at the genetic, developmental and functional levels. Mammalian brain evolution has progressed considerably since the early appearance of viviparity some 40 Ma ago, reaching a pinnacle of complexity in the hominoids. Nevertheless, fundamental to this evolution in all mammals has been the contribution of matrilineal genomic imprinting and maternalism. To suggest, as many texts do, that 'female' is the default state fails to take into account how the matriline has actually provided the driving force for mammalian brain evolution. Indeed, both male and female mammalian brains have essential feminine components when viewed in the context of co-adaptive development of the placenta and hypothalamus. It has to be remembered that the male, too, has a placenta that communicates with its mother's hypothalamus while at the same time co-adaptively developing its own hypothalamus in utero. The male evolutionary response has been to construct the SRY gene as a master conscriptional switch for Sox9 expression and male gonadal development. SRY is a hybrid gene constructed from Sox3, which is itself important for hypothalamic development, and DGCR8, which is important for placental development. Thus the timing of male gonadal development has been a critical intervention by SRY for activating masculinisation of the maternal potential that is present in all mammalian brains. The development of secondary sexual characteristics can tolerate more variance in its onset and tends to follow development of the neocortex at puberty.

Thus, far from being a default state, maternalism of the mammalian brain has played an integral part in its evolution in both males and females. By far the most expansive growth of the mammalian brain has occurred in neocortical evolution, which mainly develops post-natally. This post-natal developmental period coincides with the close relationship that all mammals experience with their mothers, and subsequently with the social group in the case of large brain primates. Indeed, living in a social group has been hypothesised to be the important selection pressure in primate brain evolution (Curley and Keverne 2005). Mechanistically, the oxytocinergic and endorphin neurotransmitter systems that underpin and determine maternal care also provide the neural mechanisms called into action and

underpinning social reward (Broad et al. 2006). Again there is a matrilineal bias for the evolution of neural mechanisms that underpin social reward, but in the case of the neocortex the evolutionary emphasis is less dependent on genetic hardwiring. Here the brain's interconnections are epigenetically sculptured according to the social environment in which they develop and survive (Keverne 2011).

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Genomic Imprinting in the Adult and Developing Brain

Catherine Dulac and Gregg Christopher

Abstract Genomic imprinting results in the preferential expression of the paternally or maternally inherited allele of certain genes. Although originally studied in the context of early embryonic development, imprinted genes are also highly expressed in the adult and developing brain and have been implicated in key steps of brain development, cortical plasticity, and social and motivated behavior. Furthermore, defects in imprinted loci have been associated with various mental illnesses, including autism, psychosis, and mental retardation, generating an enormous interest in this mode of epigenetic regulation. We recently developed a genome-wide experimental strategy that led to the identification of over 1,000 new imprinted loci and large numbers of imprinted clusters in the adult and developing brain. Strikingly, the repertoires of imprinted genes in the developing brain and adult male and female cortex differ, suggesting a complex regulation of imprinting that has direct implications for the understanding of male and female brain development and function, and of impairment leading to mental illnesses.

Introduction

Imprinted genes of placental mammals are preferentially expressed from the maternal or the paternal allele as a result of inherited epigenetic modifications (Hore et al. 2007). This monoallelic expression makes these loci especially vulnerable to mutations and deregulation, and they often contribute to diseases and disorders

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(Davies et al. 2001; Jirtle and Skinner 2007). The first imprinted genes discovered were *Igf2* (DeChiara et al. 1991) and *Igf2r* (Barlow et al. 1991), which are paternally and maternally expressed, respectively, and have opposing effects on embryonic growth. Since these landmark papers, the vast majority of imprinting studies have focused on embryonic growth and development (Reik and Walter 2001; Fowden et al. 2006). In rodents and humans, nearly 100 imprinted genes have been identified, which are often organized in clusters in the genome (Hore et al. 2007). A bioinformatic approach estimated 600 imprinted genes in the mouse genome (Luedi et al. 2005), although this study failed to predict any imprinted genes on the X chromosome that are now known to exist (Davies et al. 2005; Raefski and O'Neill 2005). Remarkably, the imprinting status of a given gene can be both temporally and spatially regulated. For example, the paternally expressed gene *Ube3a* was found to only be imprinted in the olfactory bulb, hippocampus and cerebellum (Albrecht et al. 1997). Further, *Commd1* is biallelicaly expressed in the embryonic brain but maternally expressed in the adult brain (Wang et al. 2004), and, *Igf2* is biallelic in the embryonic human brain but, in the adult, is monoallelic in the globus pallidus and hypothalamus and biallelic in the pons (Pham et al. 1998). An imprinted allele may also be either completely or only partially silenced (Khatib 2007). Thus, imprinting status must be assessed quantitatively and in a temporally and regionally specific manner.

Genomic Imprinting and Brain Function

Strikingly, many imprinted genes have been found to be expressed in the brain, where they serve unknown functions (Wilkinson et al. 2007). A handful of studies have demonstrated roles for imprinted genes in the regulation of a variety of brain functions and behavior controls (Lefebvre et al. 1998; Li et al. 1999; Skuse 1999; Haig 2000; Isles et al. 2002, 2006; Roulin and Hager 2003; Curley et al. 2004; Plagge et al. 2005; Broad et al. 2006; Kozlov et al. 2007; Swaney et al. 2007). Clinical studies of patients with neurological disorders related to imprinting, such as Angelman Syndrome, Prader-Willi Syndrome and Turner Syndrome, have also demonstrated clear roles for imprinted genes in human social behaviors and cognitive functions (Isles et al. 2006). To further assess the relevance of genomic imprinting to gene expression in the brain, we mapped the expression of 85 known imprinted genes in 118 adult brain regions using the Allen Brain Atlas (Gregg et al. 2010a). This study revealed that regions regulating motivated behaviors, such as the nucleus accumbens (NAc; Carelli 2002; Pecina et al. 2006), periaqueductal gray (PAG; Seymour et al. 2007) and medial prefrontal cortex (mPFC; Carelli 2002; Wallis 2007) are major hotspots for imprinted gene expression.

This finding led us to propose that neural circuits governing social, motivational, cognitive and homeostatic brain functions are primary targets for genomic imprinting.

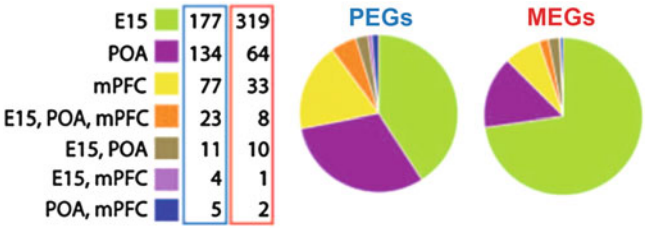

Fig. 1 Maternally (*MEG*) and paternally (*PEG*) expressed genes discovered using Solexa sequencing in the E15 brain, adult POA and mPFC

Genome-Wide Identification of Parental Effect in Transcription

We have carried out a large-scale analysis of genomic imprinting in the embryonic day 15 (E15) brain, adult preoptic area (POA) and medial prefrontal cortex (mPFC; Gregg et al. 2010a, b). We have used a novel approach based on Solexa sequencing that involves sequencing the entire transcriptome of brain regions isolated from male and female F1 hybrid mice generated through initial and reciprocal mating of the two distantly related mouse strains, C57BL/6J and CASTEiJ. This approach allows us to assess maternal versus paternal allele-specific expression in a genome-wide and highly quantitative fashion using single nucleotide polymorphisms (SNPs). These results, which have recently been published (Gregg et al. 2010a, b), uncovered parental expression bias at over 1000 loci not previously known to be imprinted.

We have thereby discovered that imprinting regulates a large number of genes expressed in the CNS. Importantly and very unexpectedly, we discovered extraordinary diversity in the imprintomes of different brain regions. Of the imprinted genes identified, the vast majority is specifically imprinted in the target tissue of interest whereas they are actually expressed in all brain regions analyzed (Fig. 1). This important finding reveals that the repertoire of imprinted genes varies from brain region to brain region and likely neuronal cell types, and at various developmental stages. Strikingly, a large number of genes displaying parent-of-origin expression bias in the developing brain and adult cortex appear particularly relevant to cortical maturation and/or show interesting associations with brain disorders. For example we have identified a large number of novel, paternally expressed transcripts in the Prader-Willi-Angelman Syndrome (PWS-AS) locus that may give rise to numerous regulatory noncoding RNAs (Fig. 2).

The PWS-AS locus is known to contain multiple imprinted loci, such as the small nucleolar RNA HBII- 52, which regulates serotonin receptor 2c splicing, and they are thought to influence social and motivated behaviors. Furthermore, we have found several genes associated with the processes of oxydoreduction, which may be very relevant to cortical development and, more specifically, to parvalbumin-positive cells, as this population of interneurons has indeed been described as being among the most metabolically active neurons due to their fast-spiking behavior (Buzsaki

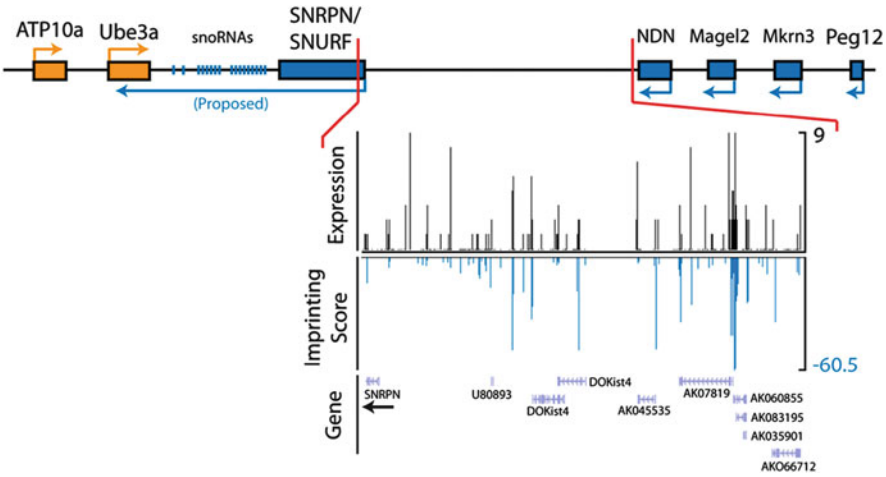

Fig. 2 Paternally expressed noncoding RNAs identified in the PWS-AS imprinted gene cluster between the SNURF/SNRPN and NDN loci. *Black peaks* show levels of transcription, whereas *blue peaks* show paternal expression from individual SNP sites; no maternal expression was observed

et al. 2007), whereas redox dysregulation has been specifically implicated in neurodevelopment defect and schizophrenia (Do et al. 2009). Moreover, Gtl2 and Nap1l5, two genes specifically enriched in parvalbumin-positive interneurons (Plessy et al. 2008), were found to be robustly imprinted in our data. Finally our data uncovered Argonaute 2 and Bcl2l1 as being robustly imprinted in the developing brain, pointing to miRNA processing and apoptosis, two processes directly relevant to cortical development, as affected by genomic imprinting (Gregg et al. 2010a). We have also uncovered evidence for imprinted X-inactivation in the CNS, such that the maternal X chromosome is preferentially expressed in the cortex (Gregg et al. 2010b). This finding was confirmed both with our sequencing approach and with an analysis using transgenic mice with an X-linked *egfp* reporter transgene. In cortical regions, a significant maternal bias was observed, such that 30 % more cells expressed the Xm than the Xp in the mPFC and the sensory and the piriform CTX. In contrast, analysis of multiple hypothalamic regions failed to reveal any significant difference in the number of cells expressing the Xm versus the Xp. Thus, our results uncover a major maternal influence on daughter's cortical function that may have direct implications for the sex-specific prevalence of many mental illnesses.

Conclusion and Perspective

1. A surprisingly high number of genes (>1,000) display parent-of-origin allele expression bias in the adult and developing mouse brain, suggesting that genomic imprinting is a major mode of epigenetic regulation in the brain.

2. The repertoires of imprinted genes in the adult and developing brain are distinct, with a preferential contribution of maternally expressed genes in the developing brain and of paternally expressed genes in the adult brain. These results suggest that the maternal and paternal genomes are not functionally equivalent for neuronal development and adult function, although the mechanistic consequences of this differential parental contribution at the molecular and cellular levels are unknown. There are, however, clear implications for the emergence of brain disorders.
3. A number of imprinted genes display sex-specific, parent-of-origin expression bias and may thus contribute to the sexually biased nature of many brain disorders, including autism, anxiety, depression, eating disorders and psychosis.
4. The repertoires of imprinted genes in the adult mPFC and adult POA are distinct from each other and from the E15 brain, uncovering a striking regulation of parental expression bias that may contribute to the molecular control of brain development and be affected in various mental illnesses. A remarkable example of this phenomenon is the impairment in the maternal expression of the Ube3A gene in humans, which causes Angelman syndrome, a neurodevelopmental disorder characterized by mental retardation, ataxia, and autistic features. Ube3A has recently been shown to regulate synapse development (Greer et al. 2010), and the monoallelic maternal expression of Ube3A during the critical period of visual cortex development in the mouse plays a key role in experience-dependent synaptic plasticity of visual cortical circuits (Sato and Stryker 2010).

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The Synapse: Differences Between Men and Women

Javier DeFelipe and Lidia Alonso-Nanclares

Abstract For many years, scientists have searched for structural variations between men's and women's brains to explain psychological studies showing that, overall, the sexes think and act differently. In general, while men on average have larger brains and some brain regions are structured or shaped differently, the effect of these differences is not well understood in terms of behavior or brain function. An additional problem is that there is a virtual lack of knowledge at the level of the synaptic organization of the human brain because the ultrastructural preservation of post-mortem human brain tissue is usually rather poor and it is generally unsuitable for detailed quantitative analysis. However, the examination of specimens removed during the course of neurosurgery in patients with tumors or intractable epilepsy represents an excellent opportunity to study human brain ultrastructure, partly because the resected tissue can be immediately immersed in the fixative so that post-mortem factors are mainly eliminated. Undoubtedly, this is why the quality of the electron microscope images of this human biopsy material is comparable to that obtained in experimental animals. Here, we will deal mainly with the finding from our laboratory—using fresh brain tissue removed during brain surgery of epileptic patients—that there are significant differences between men and women in terms of synaptic density in all cortical layers of the temporal neocortex. These differences may represent a microanatomical substrate contributing to the functional gender differences in brain activity.

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Introduction

Differences between females and males is a topic of general interest in many different areas (Cahill 2006; Jazin and Cahill 2010; Semaan and Kauffman 2010) including art, as reflected in the painting *Man and Woman* by Fernando Botero (2001) shown in Fig. 1. Indeed, scientists have been studying male and female brains for years, looking for variations that may explain these differences.

It is clear that men and women display different capacities in certain cognitive functions that are unrelated to differences in the general level of intelligence. The most consistently reported differences relate to spatial and language abilities, and while men excel in mental rotation and spatial perception, women perform better in verbal memory tasks, verbal fluency tasks and in the speed of articulation (Linn and Petersen 1985; Kimura 2000). These differences are thought to be a consequence of not only the influence of sex hormones on brain organization during development but also genetic factors (De Vries 2004; Bocklandt and Vilain 2007; Cosgrove et al. 2007; Davies and Wilkinson 2006; for a recent review, see Hines 2011). Since higher brain functions are directly related to the activity of the neocortex, many studies aimed at identifying possible structural correlations for cognitive gender differences have focused on the cerebral cortex, using a variety of anatomical and brain imaging techniques. Here, we will deal with these topics, focusing on our findings (Alonso-Nanclares et al. 2008) using stereological and correlative light and electron microscopy methods to examine possible gender differences at the synaptic level in the human cerebral cortex.

Sexual Dimorphism of the Human Brain

At the macroscopic level, sexual dimorphism has been reported in the cortical volume of the Wernicke and Broca areas (Harasty et al. 1997), as well as in the frontal and medial paralimbic cortices, amygdala and hippocampus (Allen et al. 2003; Amunts et al. 1999, 2007; Goldstein et al. 2001; Sowell et al. 2007), and in the thickness and density of the gray matter in the parietal lobes (for reviews, see Sowell et al. 2007; Luders and Toga 2010). At the microscopic level, there are two levels of analysis: (1) at an intermediate resolution (mesoscopic scale), using light microscopy, which allows us to observe cells, their processes and putative connections using specific markers; (2) at an ultrastructural resolution (nanoscopic scale), which can only be studied using electron microscopy (EM) and serves to map true synaptic contacts.

At the mesoscopic scale, sex differences have been reported in cortical cytoarchitecture. Differences have been found in the density of neurons (Haug 1987; Witelson et al. 1995; Pakkenberg and Gundersen 1997; Rabinowicz et al. 1999; Stark et al. 2007) and in the complexity of the dendritic arbors of the pyramidal cells, as well as in the density of dendritic spines in several cortical areas (Jacobs et al. 1993).

Nevertheless, as we will discuss in the next section, the functional significance of these differences remains unknown because no generally valid equation relates neuronal number or morphology to behavioral complexity (Pakkenberg and Gundersen 1997; Jacobs et al. 1993).

Brain Size and Intellectual Capabilities

The marked increase in human brain size during evolution, its relationship with higher brain functions, and the large differences in intellectual abilities between individuals provoked studies in the nineteenth and early twentieth centuries to determine whether the brains of people with higher intellectual abilities could be distinguished from those with ordinary minds, based on anatomical brain features (sizes or shapes). The significance of the differences in brain size is not clear in our species. For example, the English poet Lord Byron (1788–1824) seems to have had a great brain, as demonstrated not only by the quality of his writings but also by the size of his enormous brain, weighing 2,238 kg. Oliver Cromwell (1599–1658), protector of the Republic of England, also had a brain that weighed between 2,233 and 2,330 kg, whereas the French writer Anatole France (1844–1924), who won the Nobel Prize for literature in 1921, had a brain that weighed only 1,100 kg (DeFelipe 2011). As far as we know, the smallest brain reported in a normal person is the case of Daniel Lyons, who died in 1907 at the age of 41. Daniel was a person with no special features, with a normal body weight and of normal intelligence, although his brain weighed no more than 680 g (Wilder 1911). Thus, it appears that a difference of almost 50 % of brain mass, with its billions of neurons and synapses, may have no functional significance in terms of intelligence. If it is not brain size that determines whether a person is adept at music, painting or literature, then it is probably the individual pattern of connections. In other words, both the quantitative and qualitative characteristics of connections are likely to influence intelligence, including the number of connections between particular functional groups of neurons, their molecular and physiological characteristics, etc., which in turn depend on genetic background and on the influence of the environment (DeFelipe 2006).

Microanatomical Sex Differences of the Neocortex at the Ultrastructural Level

It is important to bear in mind that connectivity at the light microscope level is in general rather imprecise. Indeed, axonal boutons are embedded in a complex neuropil adjacent to several possible synaptic targets. Thus, the presence of a labeled terminal in close apposition to a given neuronal element can only be considered a putative synaptic contact. Thus, one of the requirements for

understanding how neuronal circuits contribute to the functional organization of the cerebral cortex is to achieve a detailed analysis of neuronal connectivity at the ultrastructural level. However, the difficulties encountered when attempting to apply microanatomical techniques to study the human brain explain why most studies of the structure of the neocortex have been performed at the light microscopic level. When performing ultrastructural studies, the main problems are related to the lack of suitable human brain tissue to study synaptic circuitry, for which the only source of control tissue might be autopsy material (i.e., from individuals who did not suffer brain pathologies or psychiatric illness). Unfortunately, the ultrastructural preservation of post-mortem human brain tissue is usually rather poor, and it is generally unsuitable for the detailed quantitative analysis that can be performed on biopsy material. Indeed, this is one of the main reasons for the paucity of data regarding the synaptic circuitry in the normal human brain.

The analysis of specimens removed during the course of neurosurgery in patients with tumors or intractable epilepsy represents an excellent opportunity to study human brain material. Furthermore, it is inevitable that surgical excisions pass through cortical regions that are normal. Hence, this material can be exploited to analyze various ultrastructural aspects of the neocortex in detail, helping us to better understand the microorganization of the human cerebral cortex that would otherwise be impossible to define. This resected tissue facilitates the study of the human brain at the electron microscope level, in part because this type of tissue can be immediately immersed in the fixative and post-mortem factors are mainly non-existent. Undoubtedly, this is why the quality of the immunocytochemical staining at both light and electron microscopy levels in human biopsy material has been shown to be comparable to that obtained in experimental animals (e.g., del Río and DeFelipe 1994).

Hence, using correlative light and electron microscopy coupled to stereological techniques, we showed for the first time that there is significant sexual dimorphism in the density of synapses in all cortical layers of the human temporal neocortex (Alonso-Nanclares et al. 2008). These differences may represent a microanatomical substrate that contributes to gender functional differences in brain activity. What follows is a summary of the results of this study.

Cytoarchitecture of the Temporal Neocortex from Men and Women

We analyzed the thickness and neuronal density in layers I, II, IIIA, IIIB, IV, V and VI, of 100-μm Nissl-stained sections from human postoperative brain tissue obtained from eight patients suffering pharmaco-resistant mesial temporal lobe epilepsy secondary to hippocampal alterations (Fig. 1). Tissue was obtained from four women—26, 31, 31 and 41 years of age—and from four men—24, 27, 32 and 36 years of age. Video-EEG monitoring of bilateral foramen ovale electrodes was indicative of left mesial temporal lobe epilepsy in all patients. Furthermore, during surgery, the epileptogenic regions were identified through subdural recordings with

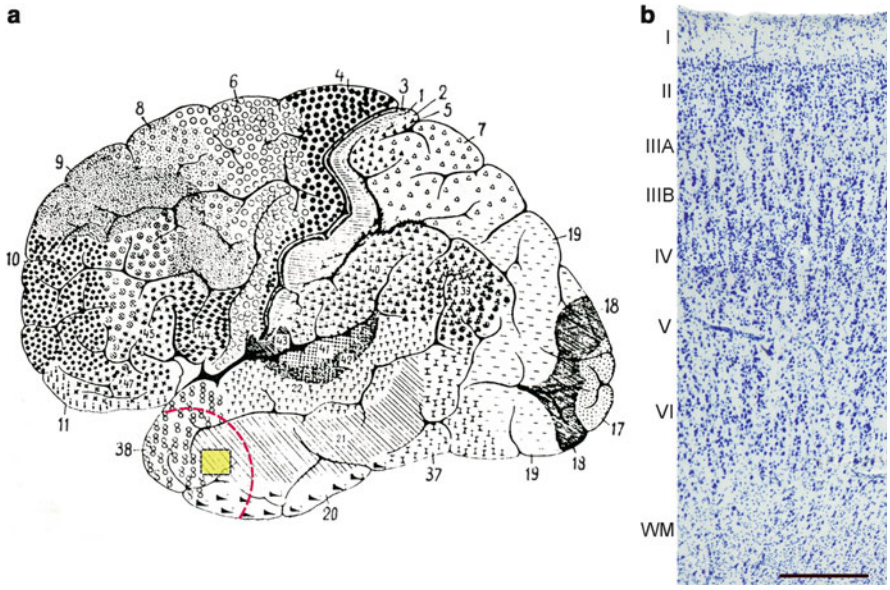

Fig. 1 (**a**) Brodman's map of the human brain to illustrate the specific part of the temporal lobe corresponding to area 21 (*in yellow*), which we examined (Alonso-Nanclares et al. 2008). *Red dotted line* indicates the surgical excision line. (**b**) Low-power photomicrograph of a 100 μm-thick section stained with toluidine *blue* to identify the cortical layers (*WM* white matter)

a 20-electrode grid (lateral neocortex) and a four-electrode strip (uncus and parahippocampal gyrus). Intraoperative electrocorticographic recordings revealed spiking activity localized in the mesial structures, while the lateral neocortex of all these patients displayed normal activity; no spikes, sharp waves or slow activities were observed during intraoperative electrocorticography. All of the patients were right-handed and they had normal intelligence quotients (IQ).

Neuronal density was estimated using optical dissectors, as described by West and Gundersen (1990; see also Williams and Rakic 1988). No significant differences were found between men and women in regard to neuronal density (Fig. 2; Table 1). However, other reports have shown greater neuronal density in the posterior temporal neocortex of women when compared to men (Witelson et al. 1995). The discrepancy between the results of Witelson et al. (1995) and our present results may be attributed to the cytoarchitectonic differences of the regions examined. We examined the anterior part of the middle temporal gyrus, corresponding to area 21 of Brodman (Fig. 1), whereas Witelson et al. (1995) analyzed the superficial surface of the posterior part of the superior temporal gyrus, also denominated the TA1 area (von Economo and Koskinas 1925) or area 22 by Brodman (1909).

Furthermore, the cell body (glia and neurons), neuropil and blood vessel volume fractions (V_v) were examined in each of these cortical layers in 2-μm thick semithin sections stained with toluidine blue by applying the Cavalieri principle (Fig. 3a).

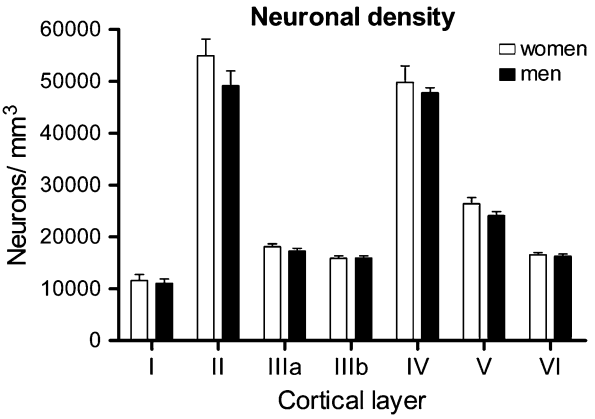

Fig. 2 Neuronal densities (mean ± s.e.m.) in each cortical layer, demonstrating that there are no significant differences between men and women

Table 1 Number of neurons per mm^3 (mean ± SEM) and the percentage of synapses per layer

	Neuronal density		Percentage of synapses			
			Women		Men	
Layer	Women	Men	% AS	% SS	% AS	% SS
I	11,558 ± 1,200	11,034 ± 850	76	24	72	28
II	54,915 ± 3,200	49,127 ± 2,900	80	20	83	17
IIIa	18,112 ± 510	17,279 ± 560	85	15	92	8
IIIb	15,869 ± 430	15,907 ± 220	84	16	86	14
IV	49,754 ± 3,200	47,758 ± 970	86	14	89	11
V	26,393 ± 1,200	24,070 ± 830	88	12	92	8
VI	16,520 ± 430	16,291 ± 380	88	12	91	9

AS asymmetric synapses, *SS* symmetric synapses

Again, no significant differences were found. In summary, no cytoarchitectonic differences were observed in the tissue obtained from men and women (Fig. 3b).

Ultrastructural Analysis

A variety of synaptic relationships has been observed, including dendro-dendritic, somato-somatic, somato-dendritic, dendro-somatic, dendro-axonic and somato-axonic synapses (Peters et al. 1991). In addition, neurons are not only connected by chemical synapses but may also be coupled electrically and through gap junctions (Bennett 2000), which permit bidirectional transmission. The plasma membranes of adjacent neurons are separated by a gap of about 2 nm, although they contain small channels (gap junctions) that connect the cytoplasm of the adjoining neurons, permitting the diffusion of small molecules and the flow of electric current (Bennett and Zukin 2004; Hormuzdi et al. 2004). Furthermore, the

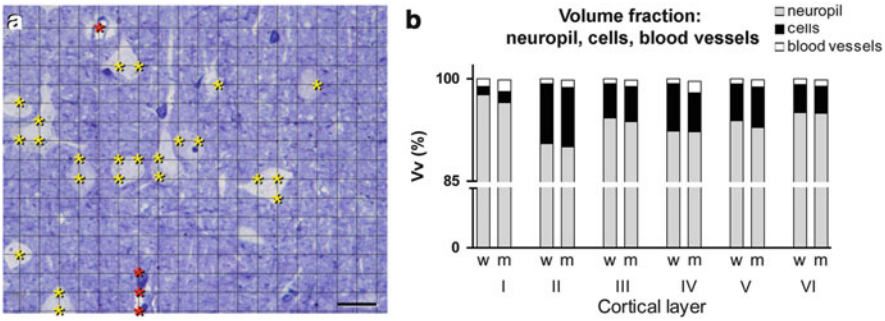

Fig. 3 (**a**) Photomicrograph of a semithin section (2 μm) stained with toluidine *blue*, from cortical layer III, to illustrate the Cavalieri method used to estimate the V_v of cells, blood vessels and neuropil. Scale bar 20 μm. In this example, the total area of the image is 30,400 μm^2. A grid of small intersections is displayed overlying the tissue. Each grid point has an associated area of $10 \times 10 = 100\ \mu m^2$. *Yellow asterisks* indicate the intersections in the grid that lie within cells ($n = 20$, $23 \times 100 = 2{,}300\ \mu m^2$). *Red asterisks* indicate the intersections in the grid that lie within blood vessels ($n = 4$, $4 \times 100 = 400\ \mu m^2$). The V_v of cells, blood vessels and neuropil was estimated with the following formulae: $V_{v\ cells} = 7.5\ \%$, $V_{v\ blood\ vessels} = 1\ \%$, $V_{v\ neuropil} = 100-(7.5 + 1) = 91.5\ \%$. (**b**) Comparison of the V_v between men and women, calculated for the neuropil, cell bodies (including those from glia and neurons) and blood vessels in each cortical layer. Note that the neuropil represents between 90 % and 98 % of the volume, for which no significant differences were found between men and women

transmitter released at synaptic or non-synaptic sites may diffuse and act on other synaptic contacts, or on extrasynaptic receptors (Fuxe et al. 2007). In certain cases, rather than being simply a non-specific phenomenon, this neurotransmitter spill-over may represent an intermediate situation between conventional point-to-point synapses and volume transmission (Merchán-Pérez et al. 2009).

Finally, not only are glial cells key components of the nervous system because of their numerous structural, metabolic and protective functions but it has also been proposed that astrocytes are involved in information processing through their bidirectional signaling with neurons (Perea and Araque 2010; Halassa and Haydon 2010; Heneka et al. 2010; Hamilton and Attwell 2010). Nevertheless, chemical axo-dendritic synapses are by far the most common type of synapse (followed by axo-somatic synapses). Other types of synapses are not found in all regions of the nervous system and, when present, they are usually only established between specific types of neurons. It can be concluded that there are two main morphological types of chemical synapses in the cerebral cortex, Gray's type I and type II synapses (Gray 1959); they correspond to the asymmetric and symmetric types of Colonnier, respectively (Colonnier 1968; see also, Colonnier 1981; Peters et al. 1991; Peters and Palay 1996). In general, asymmetric synapses are considered to be excitatory (glutamatergic) and symmetric synapses inhibitory (GABAergic). Moreover, asymmetric synapses are much more abundant (75–95 % of all neocortical synapses) than symmetric synapses, but there are cortical area and layer differences as well as between-species differences (5–25 %: for review, see DeFelipe et al. 2007). Thus,

in general, the examination of possible differences in the density and proportion of excitatory and inhibitory synapses among cortical areas, genders or species is extraordinarily important in terms of function.

For these reasons, we studied the morphology and density of synapses in each cortical layer. We found that the ultrastructure of the neuropil in women was indistinguishable from that in men (Fig. 4a, b). The synapses were classified into three categories: asymmetric, symmetric and uncharacterized (Tables 1 and 2).

In the case of asymmetric and symmetric types, the synaptic cleft could be visualized and synapses were identified based on the morphology of the postsynaptic density. Asymmetric synapses had a prominent postsynaptic density whereas symmetric synapses had a thin postsynaptic density (Fig. 4c, d; Gray 1959; Colonnier 1968, 1981; for review, see Peters et al. 1991; Peters and Palay 1996). In the uncharacterized synapses, the synaptic cleft could not be visualized, due to the oblique plane of section. Synaptic density per unit area (N_A) was estimated from electron microscopy samples of the neuropil from each cortical layer (for a detailed description, see DeFelipe et al. 1999). In this study, uncharacterized synapses were included in the final estimate of the total synaptic density. Furthermore, uncharacterized synapses were included as asymmetric and symmetric types, according to the frequency of both types of synapses. Therefore, the proportion of each type of synapse in this study is an estimate of the real ratio (see DeFelipe et al. 2007). When the mean cross-sectional lengths of asymmetric, symmetric and uncharacterized synapses were analyzed, no significant differences were found between men and women in any layer (Table 2).

Gender Differences in Synaptic Density of Temporal Neocortex

Synapses were quantified in the neuropil (i.e., avoiding the neuronal and glial somata, blood vessels, large dendrites and myelinated axons; DeFelipe et al. 1999), and we found that men had a higher synaptic density in all layers (Fig. 5). The smallest difference in density was found in layer II, in which the synaptic density was 18 % higher in men than in women (Fig. 5), whereas the greatest difference was found in layer V, where the synaptic density in men was 52 % higher than in women (representing an additional 678 million synapses in men). Considering all layers, men also had a significantly higher average synaptic density of $12.9 \times 10^8/mm^3$, compared to $8.6 \times 10^8/mm^3$ in women. Thus, there was a 33 % difference in synaptic density between men and women. Nevertheless, when considering all layers together, the proportion of asymmetric and symmetric synapses (Tables 1 and 2) was similar in men and women, 86 % and 14 % in men and 84 % and 16 % in women, respectively.

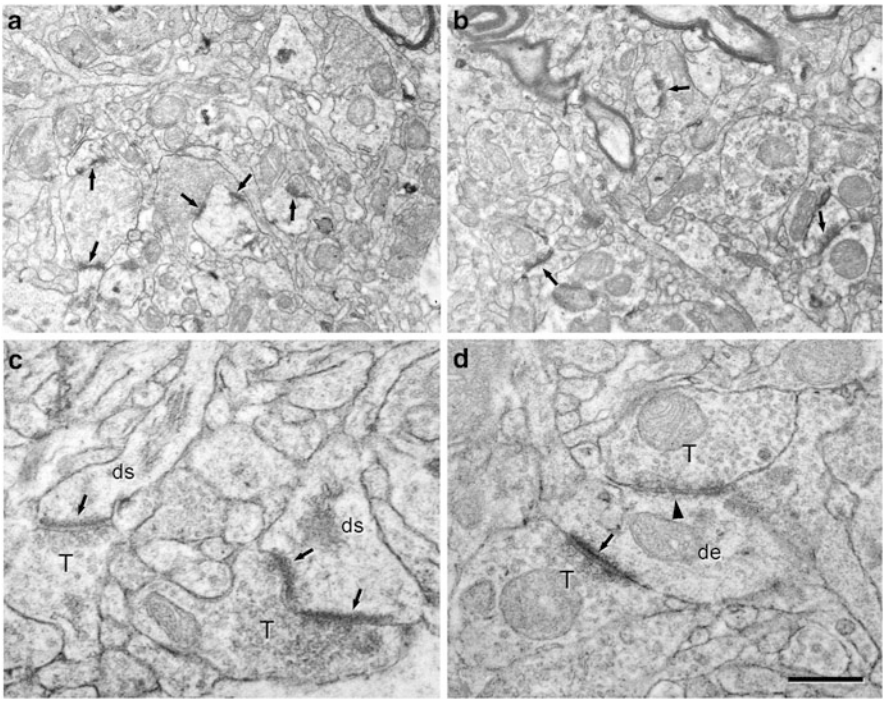

Fig. 4 Electron micrographs illustrating the ultrastructure of the human temporal neocortex. (**a**, **b**) Low-power electron micrographs showing the neuropil from layer IIIb of the temporal neocortex from a woman (**a**) and a man (**b**). Some synapses are indicated by *arrows*. (**c**, **d**) High-power electron micrographs showing the two major morphological types of synapses in the neuropil. Asymmetric synapses (*arrows*) had a prominent postsynaptic density, whereas symmetric synapses (*arrowhead*) had a thin postsynaptic density. *de* dendritic shaft, *ds* dendritic spines, *T* axon terminals. Scale bar (in **d**): 0.9 μm in **a**, **b** and 0.4 μm in **c**, **d**

Caveats

It was certainly a striking finding that, despite the well-known anatomical and functional interindividual variability in the brain (e.g., Uylings et al. 2005; Caspers et al. 2006), we consistently observed a lower synaptic density in women in all cortical layers of the temporal neocortex. Since we examined relatively few cases (four women and four men), we consider that these differences must be very robust in the general population. Nevertheless, we would caution the reader that the main limitation in this kind of study is that we have virtually no data about the synaptic density in biopsy samples of the strictly normal human neocortex. Indeed, it is well known that synaptic reorganization occurs in the epileptic brain, although these changes occur in regions with neuronal loss and gliosis, such as the sclerotic hippocampus (e.g., Houser 1999; Arellano et al. 2004) or the peritumoral or dysplastic cortex (e.g., Alonso-Nanclares and DeFelipe 2005; Alonso-Nanclares et al. 2005). The eight biopsies used in the present study can be considered to be

Table 2 Accumulated data from all cortical layers. Data of all synapses including asymmetric, symmetric and uncharacterized synapses

	Women	Men
Mean cross-sectional length of asymmetric synapses (mean ± SD, in μm)	0.30 ± 0.09	0.30 ± 0.08
Mean cross-sectional length of symmetric synapses (mean ± SD, in μm)	0.21 ± 0.10	0.20 ± 0.09
Mean cross-sectional length of all synapses (mean ± SD, in μm)	0.29 ± 0.06	0.27 ± 0.05
Mean no. of asymmetric synapses ($\times 10^8$/mm^3, mean ± SD)	3.17 ± 2.08	4.23 ± 2.59
Mean no. of symmetric synapses ($\times 10^8$/mm^3, mean ± SD)	1.06 ± 1.84	1.42 ± 2.00
Mean no. of all types of synapses ($\times 10^8$/mm^3, mean ± SD)	7.17 ± 3.29	10.61 ± 4.97
Percentage of asymmetric synapses	86	84
Percentage of symmetric synapses	14	16
No. of neurons/mm^3 (mean ± SD)	27,589 ± 16,854	25,924 ± 15,110
Vv cell bodies (glia and neurons)	5.3	5.2
Vv blood vessels	0.8	1.2
Vv neuropil	93.9	93.3

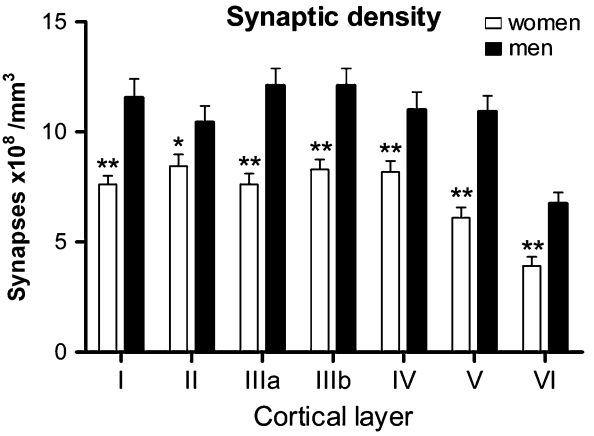

Fig. 5 Comparison of synaptic density (mean ± s.e.m.) between men and women in each cortical layer.*$p < 0.05$, **$p < 0.01$

close to normal conditions for the following reasons: first, the epileptic activity was clearly of mesial origin; second, the whole neocortex in all of these patients displayed non-spiking activity and; third, they presented normal cytoarchitectonic and ultrastructural characteristics. In addition, while we cannot rule out that synaptic changes may also occur in the neocortex, there is no reason to believe that the differences in synaptic density observed between men and women were due to the epileptic condition, given that all of the subjects were epileptic. Thus, it is likely that these differences are truly due to gender differences.

Significance of Sexual Differences in Synaptic Density

Importantly, no differences in cytoarchitecture were observed. More specifically, no significant differences were found between men and women regarding the thickness of the gray matter, the volume fraction of cortical elements (neuropil, cells and blood vessels) and neurons per volume. As a consequence, the number of synapses in each layer was greater in men than in women, and thus, in this particular region of the neocortex, the general connectivity in men appeared to be more extensive than in women. Accordingly, gender appears to influence synaptic connectivity and this phenomenon is regulated independently of other cytoarchitectonic features.

If we consider the columnar organization of the input connections, the differences in connectivity between neighboring neurons, and the combinations of the interlaminar connections of both pyramidal and non-pyramidal neurons, it is clear that neurons in different layers do not process the same information (Rockland and Ichinohe 2004; DeFelipe 2005). Furthermore, pyramidal neurons located in different layers project to different cortical and subcortical nuclei (Jones 1984; Lund 1988; White 1989). Hence, it is likely that the differences in synaptic density between men and women observed in all cortical layers represent a microanatomical substrate for sex differences in the fine-tuning of several functions. The larger number of synaptic connections in men does not necessarily mean that all cortical circuits in this region are more complex than in women. Rather, specific circuits may be more complex in the male brain. The temporal lobe is a complex, associative and multi-integrative cortical region (Brodman 1909; for a review, see Olson et al. 2007). Therefore, the functional consequences of the differences in synaptic circuitry observed here are particularly difficult, if not impossible, to correlate with specific functions related to men or women. Indeed, it could simply mean that men's brains are just more redundant. In addition, we have the tendency to think that a higher complexity (i.e., higher synaptic density) is always related to a greater functional capability or greater performance. However, in this case, "less may be more," since the temporal cortex is involved in language processing and, overall, women perform better on language tasks; fewer synapses in women may represent increasing specialization of the temporal cortex for language processing.

Furthermore, pyramidal neurons in the human prefrontal, temporal and visual cortex display clear differences in their number of dendritic spines (Jacobs et al. 2001; Elston et al. 2001). For example, the basal dendritic arbors of layer III pyramidal cells in the prefrontal cortex are considerably more spinous (average total number of dendritic spines; 15,138) than those in the temporal lobe (12,700) and in the occipital lobe (human, 2,417; Elston et al. 2001). Since the vast majority of dendritic spines establish synaptic contacts (Arellano et al. 2007), the total number of dendritic spines is practically equivalent to the total number of excitatory glutamatergic synaptic inputs of the pyramidal cells. Thus, there are clear differences in total numbers of synaptic inputs to pyramidal cells in these cortical areas. This of course does not imply that the degree of functional performance of

prefrontal cortex, temporal cortex and visual cortex differs. Rather, it means that cortical microorganization differs between different cortical areas and that it is related to their respective functional specializations. These observations underscore the point that a higher synaptic density is not necessarily related to greater functional capability.

Interestingly, a recent study on synaptic density carried out in the monkey prefrontal cortex seems to indicate that there are no differences between males and females (Peters et al. 2008). However, many studies have shown variations between species and cortical areas in terms of density, proportion and types of neurons, as well as in the density of synapses (e.g., DeFelipe et al. 2007). Thus, whether these gender differences are unique to the human cerebral cortex or if similar conditions arise in monkeys and great apes should be specifically analyzed in each species and cortical area. Finally, and in line with this consideration, we would advise the reader to exercise caution in extrapolating the present data to the whole brain. Indeed, it was reported that the anterior commissure, which connects several regions of the frontal and temporal lobes, is 12 % larger in women than in men, suggesting that women would have more commissural associative connections (Allen and Gorski 1991). Further work will be necessary to examine whether synaptic density is similar or different in other cortical areas.

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Gonadal Hormone Influences on Human Neurobehavioral Development: Outcomes and Mechanisms

Melissa Hines

Abstract Testosterone exposure during early development has enduring influences on mammalian behavior, increasing male-typical characteristics and decreasing female-typical characteristics. Research in non-human mammals indicates that testosterone also influences development of the mammalian brain, affecting programmed cell death, anatomical connectivity and neurochemical specification, and these neural changes, which occur during early development, are thought to explain the subsequent behavioral changes. The strongest evidence linking prenatal testosterone exposure to human behavioral sexual differentiation has come from studies of children's sex-typed play. There also is substantial evidence linking early testosterone exposure to sexual orientation and to core gender identity and some evidence linking such hormone exposure to physically aggressive behavior and to empathy. However, for most, perhaps all, human behaviors that show sex differences, other factors, including socialization, also play a role, and the magnitude of this role appears to vary across behavioral outcomes. In addition, in contrast to other species, the acquisition of sex-typical behavior in humans involves social-cognitive mechanisms related to gender identification. This chapter will suggest that these social-cognitive mechanisms could be involved in the developmental cascade of processes linking early testosterone exposure to sexual differentiation of human behavior.

Introduction

Thousands of experiments in non-human species indicate that exposure to testosterone prenatally or neonatally has enduring influences on behavior, increasing male-typical characteristics and decreasing female-typical characteristics (Arnold

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2009; Goy and McEwen 1980). Testosterone also influences the development of the mammalian brain, affecting programmed cell death, anatomical connectivity and neurochemical specification, and these neural changes, set in place during early development, are thought to underlie the subsequent behavioral changes (Arnold 2009; Goy and McEwen 1980; McCarthy et al. 2009). This chapter will describe evidence that testosterone has similar effects on human neurobehavioral development to those documented in other mammals. In addition, it will attempt to integrate a unique aspect of human gender development—cognitive understanding of gender—along with these hormonal influences and processes of socialization into our understanding of human gender development. Finally, it will suggest that the impact of hormones on the developing human brain could include secondary influences on gender identification and processes related to cognitive understanding of gender, and that these alterations in cognitive processes could represent an additional mechanism by which the early hormone environment alters human gender-related behavior.

Human Gender Development

Human gender development begins at conception, when the 23rd pair of chromosomes is determined as either XX or XY. Genetic information on these chromosomes then causes the primordial gonads, which are initially identical in males and females, to develop as testes or as ovaries (Wilson et al. 1981). By week 8 of human gestation, the testes in the XY fetus are producing testosterone, resulting in higher levels of testosterone in male than in female fetuses. Although testosterone appears to be elevated in male compared to female fetuses throughout gestation, the sex difference appears to be particularly pronounced from about gestational week 8 to about gestational week 24 (Abramovich and Rowe 1973; Abramovich 1974; Carson et al. 1982; Nagamani et al. 1979; Reyes et al. 1973; Robinson et al. 1977; Rodeck et al. 1985).

The prenatal elevation of testicular hormones in male fetuses causes the external genitalia to develop as penis and scrotum, whereas, in the absence of testicular hormones, they develop in the female pattern – clitoris and labia (Wilson et al. 1981). Testosterone, and its five alpha reduced metabolite, act through androgen receptors on the external genitalia to promote male-typical development of these tissues. A somewhat different mechanism produces male-typical development of the internal genitalia (Wilson et al. 1981). In this case, both male and female fetuses begin with two sets of structures: Müllerian ducts and Wolffian ducts. Testosterone causes the Wolffian ducts to develop, and a separate testicular hormone, Müllerian Inhibiting Factor, causes the Müllerian ducts to regress in the male fetus. The female fetus has no testes and so no Müllerian hormone; as a consequence, the Müllerian ducts persist. In addition, because the female fetus has no testicular testosterone, the Wolffian ducts are not stimulated, and they regress. Thus, slightly different processes lead to sexual differentiation of the internal and external genitalia.

There is extensive evidence that processes similar to those that promote male-typical development of the external genitalia also promote male-typical development of the brain and of behavior. Treatment of female animals with testosterone during early development causes masculinization of the external genitalia, as well as masculinization of regions of the brain that have the appropriate receptors and masculinization of behavior. The mechanisms involved in this gonadal steroid induction of neural virilization include influences on programmed cell death, neurite outgrowth, anatomical connectivity, and neurochemical specification (Arnold 2009; Goy and McEwen 1980; McCarthy et al. 2009). These virilizing effects of testosterone and its metabolites on brain development have been documented particularly extensively in the hypothalamic and limbic regions.

A growing body of research suggests that testosterone influences human neural and behavioral development in a manner similar to that documented in experimental studies of other species (Hines 2011). Much of this evidence has come from studies of girls and women exposed to high levels of testosterone and other androgens prenatally, because they have the autosomal, recessive disorder of sex development, congenital adrenal hyperplasia (CAH; Cohen-Bendahan et al. 2005; Hines 2004, 2009). CAH usually involves a deficiency in the enzyme, 21 hydroxylase, and a consequent inability to produce cortisol (New 1998). Because the pathway to cortisol is disabled, hormones that would normally be used for this purpose are shunted into the androgen pathway, producing high levels of testosterone and other androgens, beginning early in gestation (New 1998; Pang et al. 1980; Wudy et al. 1999). For affected girls, this high level of androgen exposure typically results in partial masculinization of the external genitalia (fused labia, penile enlargement) at birth, and the diagnosis is usually made soon thereafter. The diagnosis of CAH is generally followed by female sex assignment and rearing and lifelong treatment with corticosteroids to regulate hormone levels postnatally. Girls and women with CAH have female internal genitalia and reproductive potential.

Androgen and the Development of the Human Brain and Human Behavior

The masculinizing influences of elevated androgens during early life in girls with CAH are not limited to the external genitalia. Girls with CAH show increased male-typical toy, playmate and activity preferences and decreased interest in toys and activities usually preferred by girls. This outcome has been reported in numerous studies from different research groups, in different countries and using a range of assessment tools (Berenbaum and Hines 1992; Dittmann et al. 1990; Ehrhardt et al. 1968; Ehrhardt and Baker 1974; Hines et al. 2004; Nordenstrom et al. 2002; Pasterski et al. 2005, 2011; Slijper 1984; Zucker et al. 1996). It has been suggested that the increased male-typical behavior in girls with CAH might not reflect a masculinizing effect of androgen on the developing brain and behavior but rather

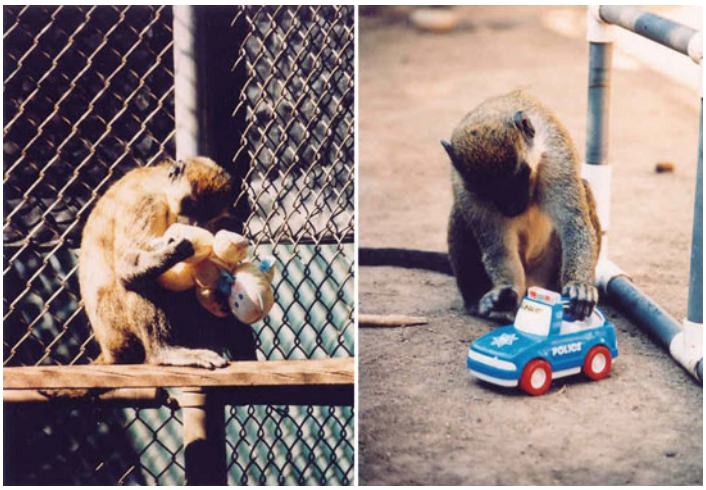

Fig. 1 Sex-typical toy play in female and male vervet monkeys resembles that of children. *Left*: A female vervet with a doll. *Right*: A male vervet with a toy car (Reprinted with permission from Alexander and Hines 2002)

consequences of other aspects of the CAH disorder, or even processes set into motion by the knowledge of genital virilization at birth (Fausto-Sterling 1992; Jordan-Young 2010). It is therefore important to see if normal variability in testosterone exposure prenatally relates to normal variability in sex-typed behavior in children with no disorder and no genital virilization. For childhood toy and activity interests, this evidence exists. For instance, testosterone measured in maternal blood during pregnancy (Hines et al. 2002) or in amniotic fluid (Auyeung et al. 2009) has been linked to male-typical childhood play behavior. In addition, there is evidence that non-human primates show sex-typed toy interests similar to those seen in children (Alexander and Hines 2002; Hassett et al. 2008), suggesting an inborn, probably hormonal, contribution to this behavior (Fig. 1).

This evidence of prenatal androgenic influences on children's toy preferences and the observation of sex-typed object preferences in non-human primates has led to a reconceptualization of the origins of children's sex-typed toy preferences. These preferences were widely assumed to result from socialization, intended to provide rehearsal for the sex-typed social roles of women and men. The existence of inborn influences has led to investigations of the object features that might make certain toys more or less interesting to brains exposed prenatally to different amounts of testosterone. Girls' and boys' toys differ in shape, with girls' toys tending to be rounded and boys' toys tending to be angular. They also differ in color, with girls' toys tending to be pink whereas boys' toys are not and may be any number of stronger colors, including blue. Sex differences in toy preferences have been documented in infants as young as 12–24 months of age (Campbell et al. 2000; Jadva et al. 2010; Serbin et al. 2001), before sex differences in preferences for pink or rounded shapes emerge (Jadva et al. 2010; Lo Bue and De Loache 2011),

Table 1 Human psychological and behavioral sex differences: evidence of relationship to prenatal androgen exposure

Characteristic	Altered in females with CAH?	Related to normal variability?
Juvenile play interests	YES	yes
Sexual orientation	YES	insufficient information
Core gender identity	YES	insufficient information
Physical aggression	yes	insufficient information
Empathy	yes	yes

YES independent replication exists, *yes* a study or studies from one research group exist, *maybe* results from different research groups are contradictory, *no* the existing evidence does not support an association, *insufficient information* the relevant studies are not available

suggesting that the toy preferences do not result from the color or shape preferences. An alternative suggestion is that the toys that boys like tend to be those that can be moved through space and that prenatal androgen exposure increases interest in watching things move in space (Alexander and Hines 2002; Alexander 2003; Hines 2004), perhaps by altering the development of visual or motor systems (Alexander 2003; Hines 2004, 2011).

Although the strongest evidence linking prenatal testosterone exposure to human behavior comes from studies of children's sex-typed play, there is also evidence linking early testosterone concentrations to other behaviors (Cohen-Bendahan et al. 2005; Hines 2009, 2011; Table 1). For some outcomes, like core gender identity or the sense of self as male or female, the evidence comes from a number of studies from several independent researchers investigating CAH (Dessens et al. 2005; Meyer-Bahlburg et al. 1996; Zucker et al. 1996), as well as research on other disorders of sex development that involve atypical androgen exposure during early development (Cohen-Kettenis 2005; Meyer-Bahlburg 2005), but not, at least as yet, from studies of normal variability. The situation is similar for sexual orientation, in regard to which several independent studies of women with CAH have reported reduced heterosexual orientation (Dittmann et al. 1992; Frisén et al. 2009; Hines et al. 2004; Meyer-Bahlburg et al. 2008; Money et al. 1984), but evidence of a link to normal variability in testosterone is lacking. For other characteristics, such as empathy and tendencies to physically aggressive behavior, there is evidence from one or more studies of individuals with CAH (Berenbaum and Resnick 1997; Mathews et al. 2009; Pasterski et al. 2007) and from studies of normal variability in testosterone, or hormonal treatment (Chapman et al. 2006; Reinisch 1981) but, as yet, limited or no consistent independent replication of either of these types of finding.

In addition, although there is evidence supporting a role for the early hormone environment in the development of human gender-related behavior, other factors, including socialization and cognitive developmental processes, also play a role, and the magnitude of this role appears to vary across behavioral outcomes (Hines 2004). In addition, the development of human sex-typical behavior involves mechanisms

related to cognitive understanding of gender—mechanisms that are unlikely to operate in other species. The next section of this chapter describes the socialization and cognitive developmental processes that are thought to influence the development of gender-related behavior in human beings.

Socialization and Cognitive Developmental Influences on Human Gender Development

After birth, the human infant encounters processes of socialization, and these are thought to influence the development of gender-related behavior. For instance, parents decorate rooms differently and dress children differently, depending on their gender (Maccoby and Jacklin 1974; Rheingold and Cook 1975). Parents also are more likely to respond positively to their children's gender-typical behavior than to their gender-atypical behavior; boys, in particular, are discouraged from engaging in play with girls' toys or girls' activities (Fagot 1978; Lytton and Romney 1991; Pasterski et al. 2005). Children also sex segregate, spending much of their time with other children of the same sex (Maccoby and Jacklin 1987; Maccoby 1998), and children, like parents, encourage girl-typical play in girls and boy-typical play in boys (Fagot and Patterson 1969; Fagot 1977), as do teachers (Fagot and Patterson 1969; Fagot 1977) and strangers (Seavey et al. 1975; Stern and Karraker 1989). Perhaps as a consequence, children's behavior becomes more sex stereotyped as they progress through early and middle childhood (Golombok et al. 2008; Maccoby 1998). Very little direct evidence is available as to whether or not the widespread encouragement of sex-typical play increases children's subsequent sex-typical behavior. One study has found that the amount of parental encouragement of sex-typical toy play correlates positively with the sex-typical toy choices of their typically developing children (Pasterski et al. 2005), but in general this question has not been studied. Perhaps investigations directly examining the effect of reinforcement of sex-typical play on subsequent behavior are lacking because of the extensive evidence documenting the effects of reinforcement on behavior in general.

Humans are perhaps unique among animals in having a cognitive understanding of their own gender. There is no evidence, for instance, that individuals of any other species sort the world into males and females and know the group to which they themselves belong, much less that they have an identity related to their belonging to that group.

Children can accurately sort pictures of males and females and can add their picture to the correct pile by about the age of two (Slaby and Frey 1975; Stagnor and Ruble 1987). In subsequent years, they come to understand that their gender will not change over time and that their gender will not change if they change their appearance or the activities in which they engage (Slaby and Frey 1975; Stagnor and Ruble 1987). This developing gender understanding is thought to contribute to children's gender-related behavior, beginning with the first stage of understanding at about age 2. As evidence of this, children's behavior becomes increasingly

gender typed across the ages when their gender understanding is increasing (Golombok et al. 2008; Maccoby 1998). In addition, the sophistication of children's gender understanding has been found to relate to some aspects of their gender-typed behavior (Leinbach and Fagot 1986), and children who have gender identity disorder, and engage in extensive cross-gendered behavior including sex-atypical play, show delayed gender understanding (Zucker et al. 1999).

Boys and girls also engage in processes related to gender identification that contribute to their acquisition of sex-typical behavior. For instance, once they understand that they are male or female, they preferentially model the behavior of others of their own sex and prefer items that have been labelled as for their own sex. For example, if shown men consistently choosing items such as bananas, and women consistently choosing items such as apples, or vice versa, they are later more likely to show a preference for the items that they saw chosen by individuals of their own sex (Perry and Bussey 1979; Masters et al. 1979). Similarly, children respond to labels that tell them that certain toys or activities are "for girls" or "for boys." For example, if told that white balloons are for girls and green balloons are for boys, or vice versa, they are later more likely to indicate a preference for the balloons of the color that they were told was for their own sex (Masters et al. 1979).

Cascading Effects of Early Androgen Exposure on Cognitive Understanding of Gender and on Other Aspects of Gender Development

Women exposed to high levels of androgens prenatally, because they have CAH, are at increased risk of dissatisfaction with the female sex of assignment and of gender dysphoria, and gender change. Although most girls with CAH develop a female gender identity, about 2–5 % of individuals with CAH, who were raised as girls, choose to live as men in adulthood (Dessens et al. 2005; Meyer-Bahlburg et al. 1996; Zucker et al. 1996). In addition, even among women with CAH who identify as female, this identification is not as strong as it is in women without CAH (Hines et al. 2004). Other disorders of sex development that involve androgen abnormality in either XX or XY individuals also are associated with an increased likelihood of gender dysphoria and change from the assigned sex (Cohen-Kettenis 2005; Meyer-Bahlburg 2005). In childhood as well, girls with CAH, or girls exposed to high levels of androgens prenatally or other disorders of sex development, are at increased risk of gender identity problems (Slijper et al. 1998) and are more likely than other girls are to express satisfaction with being a girl (Ehrhardt et al. 1968) and to say that they might not have chosen to be a girl if given the choice (Ehrhardt and Baker 1974).

This reduced gender identification could contribute to increased male-typical behavior in girls with CAH. This may also be the case for girls exposed to relatively high levels of androgens for other reasons, including genetic variability in androgen

production or sensitivity within the normal range. How might these cognitive processes influence the acquisition of sex-typed behavior? Sex-typed toy preferences are apparent before the age of 2 (Campbell et al. 2000; Jadva et al. 2010; Serbin et al. 2001), before the age at which children can be shown to have even an initial understanding that they are a girl or a boy. In addition, the evidence of sex-typed toy preferences in non-human primates (Alexander and Hines 2002; Hassett et al. 2008), similar to those seen in children, argues for some inborn component to these preferences. It is possible that the development of a range of gendered behaviors starts with prenatal hormone influences on neural systems regulating object preferences, as evidenced in sex-typed toy preferences. Later, when children have come to understand that the world includes males and females, and that they are male or female, they will also have learned that they like the toys that other children of their own, or of the other sex, typically like, and this may further influence gender-typical or -atypical behavior. For instance, if a girl has cross-gendered toy interests because she was exposed to relatively high levels of testosterone prenatally, she may anticipate that she would like other toys and activities that are for boys and so seek these out. In addition, her interest in, engagement with, and enjoyment of boys' toys and activities could reduce her identification with and enjoyment of the female role, which could in turn lead to reduced modeling of individuals of the female sex and reduced interest in objects and activities that are labeled as for girls, accompanied by increased modeling of males and increased interest in objects and activities that are labeled as for boys. These mechanisms could then lead to further changes in behavior, including changes in behaviors other than object (e.g., toy) interests. Thus, social-cognitive mechanisms, such as gender identification and responses to models and labels related to gender, may be part of the cascade of processes involved in the influence of testosterone on sexual differentiation of human behavior, and, indeed, may be unique to human beings. Thus, human beings may share one mechanism of neurobehavioral sexual differentiation with other species, a mechanism involving prenatal actions of testosterone and its metabolites on basic processes of neural development. In addition, these effects may be compounded in humans by an additional mechanism set into motion by these prenatal hormone effects but involving the uniquely human cognitive understanding of gender and the acquisition of sex-typed behavior through processes such as modelling of sex-typical behavior and responses to labels as to what is and is not associated with one's own sex.

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Congenital Adrenal Hyperplasia: Neuroendocrine, Behavioral and Cognitive Implications

Phyllis W. Speiser

Abstract Congenital adrenal hyperplasia (CAH) is a group of disorders that disrupt the balance of adrenal cortical steroid hormone biosynthesis. Production of different classes of hormones is perturbed depending on the particular enzymatic defect. The most common form of CAH is steroid 21-hydroxylase deficiency, a recessively inherited trait that impedes the stepwise conversion of cholesterol to cortisol, the main human glucocorticoid, and in most cases, to aldosterone, the main mineralocorticoid. Absence of negative cortisol feedback to the hypothalamus and pituitary glands leads to over-secretion of corticotrophin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH). These altered servo-control mechanisms drive excess secretion of adrenal sex hormones that do not require 21-hydroxylation. These are primarily androgen precursors and progestins, but secondarily estrogens, as well. The most visible effect is virilization of genetic females beginning prenatally in the first trimester, leading to genital ambiguity at birth. Thus, this form of CAH and other congenital virilizing disorders represent a human model for prenatal sex hormone effects not only on the reproductive organs but also on the brain. This chapter addresses what is known about behavior in CAH females in terms of gender identity, gender behavior, sexual orientation, career choices, and parenting. Discrete brain structural and cognitive changes are also described. Finally, the effects of prenatal glucocorticoid exposure will be summarized. In brief, affected girls generally have a core female gender identity but show more male-typical play and tomboyishness compared with sibs (Berenbaum et al. 2000; Meyer-Bahlburg et al. 2004a, b). Most patients display heterosexual orientation, but same sex attraction and sexual activity are reported

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more often than in the general population (Frisen et al. 2009; Meyer-Bahlburg et al. 2008). Career choice is skewed toward male-dominant occupations in severely affected women. Thus, in contrast to animal models of prenatal androgen exposure, human behavior is not dictated solely by hormones.

Introduction

Human inborn errors of metabolism provide a unique view to understanding the physiologic importance of hormones. This discussion will center specifically on a disorder of steroid biosynthesis, congenital adrenal hyperplasia (CAH). The most common form of CAH is steroid 21-hydroxylase deficiency, a recessively inherited trait present in its classic, severe form in ~1:15,000 births worldwide. This enzyme deficiency impedes the stepwise conversion of cholesterol to cortisol, the main human glucocorticoid. In most cases, the conversion of cholesterol to aldosterone, the main mineralocorticoid, is also blocked. Absence of negative cortisol feedback to the hypothalamus and pituitary glands leads to over-secretion of corticotrophin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH), and these trophic hormones induce hyperplasia of the adrenal cortex in a futile effort to stimulate cortisol production. In the process, the altered servo-control mechanism drives excess secretion of adrenal sex hormones that do not require 21-hydroxylation. These are primarily adrenal androgen precursors, such as androstenedione (an immediate precursor of testosterone and estrone), progestins and, secondarily, other estrogens. The most visible effect is caused by androgen excess, i.e., virilization of genetic females beginning prenatally in the first trimester and culminating in genital ambiguity at birth. In extreme cases, a virilized girl might appear to have a phallus with empty scrotal sacs but more commonly manifests as an enlarged clitoris with conjoined vagina and urethra. Although the females' external genitals are atypical, the internal reproductive organs are normal in both males and females affected with CAH. Of particular interest to participants in this conference are the potential effects of sex hormones on the brain. In contrast to the stark effects on female external genitalia, neurocognitive and behavioral effects are less apparent. Complicating the clinical picture in CAH patients of both sexes are the potentially lethal consequences of cortisol and aldosterone deficiencies, i.e., sodium wasting, dehydration, failure to thrive, and shock. Also, adrenal medullary structure and function are disrupted in the setting of glucocorticoid deficiency, and CAH patients have subtle catecholamine deficits in the face of stress (Merke et al. 2000).

The severity of the disease is directly correlated with the type of mutation in the gene encoding the active steroid 21-hydroxylase enzyme, CYP21A2. Whereas severe salt-wasting (SW) cases have null mutations or deletions ablating enzyme activity, milder mutations are associated with disease of intermediate or mild severity, the so-called simple virilizing (SV) and nonclassic (NC) forms of CAH. Girls with SV-CAH have lesser degrees of androgen excess, lower grades of genital ambiguity, and no associated SW crises. In the era prior to newborn screening, boys

with SW-CAH often died before they received medical attention, but SV-CAH boys with normal sodium conservation suffered inappropriately rapid somatic growth and pseudo-puberty in early childhood. Children with NC-CAH usually have no genital ambiguity but instead have early onset of pubic hair, and adolescent females show a constellation of signs resembling polycystic ovarian syndrome: hirsutism, irregular menses, and acne. Statural growth is slightly compromised in CAH patients (height deficit for SW > SV > NC), due to the effect of adrenal-derived estrogens in causing premature fusion of the bone growth plates.

Within the past decade, all states in the USA and many developed countries worldwide have instituted newborn screening for the classic severe forms of 21-hydroxylase deficiency CAH. The effects of screening programs have been earlier diagnosis and treatment, reduced morbidity and mortality, and fewer errors in sex assignment.

One can differentiate among the various enzyme defects by the clinical presentation coupled with a cosyntropin (ACTH) stimulation test to examine serum hormones' precursor to product ratios, via urinary steroid profiling, and/or by selective genetic screening. There are unique phenotypes and genotypes associated with each enzyme deficiency (Krone et al. 2007).

The goal of medical treatment for 21-hydroxylase deficiency is to reset the imbalance of adrenal cortical hormones by supplying exogenous glucocorticoids to partially suppress CRH, ACTH and excess adrenal sex steroids while replacing cortisol in a near-physiologic manner. Programmed infusion pumps to mimic diurnal cortisol patterns are uncommon in clinical practice. Instead, patients usually receive two or three daily doses of hydrocortisone. Alternative therapies may include prednisone or dexamethasone in older adolescent or adults. Adjunctive treatment with salt-retaining mineralocorticoid is recommended in classic CAH patients. Infants and children require supplemental sodium, whereas older individuals consume sufficient salt in their diet. Due to the difficulties inherent in achieving a perfect balance between overtreatment and undertreatment, patients inevitably experience periodic hormone fluctuations.

Whether and when to perform surgery to modify genital appearance in affected females is somewhat controversial due to the lack of evidence supporting intervention or non-intervention and to the strong emotions evoked by these discussions (Braga and Pippi Salle 2009). In summary, the efficacy analysis for surgical treatment of virilizing CAH is a work in progress and should be separated from that of other less well-characterized disorders of sex development. With more recent refinements in urologic surgical technology, both cosmetic and physiologic outcomes seem better (Braga et al. 2006). Most young women with CAH can engage in heterosexual intercourse and achieve pregnancy, if desired. Although fecundity rates are lower that those of the general population (Casteras et al. 2009), these women can bear healthy children. Currently, an international group of Endocrine Societies supports surgical intervention for severely affected girls in early infancy in experienced centers (Speiser et al. 2010).

CAH: A Model for Sex Hormone Effects on Behavior

Gender identity, defined as the sense of self as male, female, intersex or neither, is most often established in early childhood, generally by about age 2. The majority of classic CAH females, despite having had prenatal androgen exposure, self-identify as girls, regardless of their sex of rearing. Limited sampling suggests that so-called tomboys (hormonal and genetic females who engage in male play and behavior patterns) are more apt to identify as males than CAH girls (Berenbaum and Bailey 2003). Only about 5 % of CAH patients express gender dysphoria, and rarely do CAH patients undertake (female to male) sex changes. Interestingly, these cases are not necessarily found among girls or women with severe genital virilization. There have also been reports of females with Prader 5 severe genital virilization raised as males who identify as female (Dessens et al. 2005). Taken together, these data imply that androgen exposure is neither necessary nor sufficient to produce cross-gender identity. The preponderance of evidence supports female sex of rearing for all 46, XX CAH patients.

Gender role behaviors as exemplified by childhood play styles provide insight into androgen effects on human behavior, albeit molded by the cultural and social milieu. Observational studies show that CAH girls, similar to all boys, prefer playmates with a masculine style of play (Pasterski et al. 2011) and boys' typical toys (Berenbaum and Hines 1992). Aggressive behavior is more prominent in the CAH girls than among controls (Pasterski et al. 2011). CAH females' preferences for male activities and occupations persist into adolescence and adult life (Frisen et al. 2009). There are data to suggest that the degree of masculinity correlates with the genotype and with their degree of genital ambiguity, i.e., being most prominent in those with null mutations or deletions in the active 21-hydroxylase gene (CYP21A2; Hall et al. 2004).

From early adolescence, female CAH patients exhibit low interest in infants and nurturing behavior (Mathews et al. 2009). Indeed, CAH females are more interested in objects than in people (Beltz et al. 2011). Males affected with CAH show the same low level of interest in infants, and they display less dominant behavior compared with unaffected male controls (Mathews et al. 2009).

As discussed, although core gender identity is usually female, gender role behaviors, such as play styles and nurturing behaviors, in younger female CAH patients often tend to align with those of normal males. After puberty, sexual orientation assessed in interviews or questionnaire-based surveys by at least 10 groups of investigators is skewed to same-sex or bisexual fantasies and liaisons, mainly in SW, severely affected CAH women (reviewed in Hines 2011). An unexpected finding in one study was that women with mild or nonclassic CAH, a condition not associated with clinically obvious signs of prenatal virilization, reported a higher rate of non-exclusive heterosexuality (Meyer-Bahlburg et al. 2008). It should still be emphasized that most CAH patients remain heterosexual in orientation and activity. Due to inexpert surgical repair of severe genital ambiguity, many older CAH women had vaginal stenosis and/or clitoral damage. Thus,

for many such patients, peno-vaginal intercourse was painful or just not feasible. Some of these women reported they were essentially either asexual or anorgasmic. About 70 % of the CYP21A2 null genotype patients (i.e., severely affected) reported no sexual partners (Frisen et al. 2009).

Factors affecting gender role behavior and sexual orientation are manifold in the CAH population. These include, but are not necessarily limited to, prenatal and postnatal hormone perturbations, atypical genital appearance and the effects of surgical intervention, disruption of parental-child bonding, poor psychological coping strategies, and the effects of living with a chronic illness. Unlike experimental animal models of discrete periods of prenatal hormone excess or deficiency, human CAH patients continue to experience hormone fluctuations. These take place beginning with adrenal steroid synthesis at about 7–8 weeks of gestation and continue through term. Interruption of fetal adrenal sex hormone production may be induced by medication administration to the mother, as in the case of transplacental passage of dexamethasone to avoid female genital virilization (Nimkarn and New 2010). Alternatively, if timely diagnosis is not made and treatment is delayed, as occurred routinely before the era of newborn screening, postnatal androgen exposure continues. Moreover, variable levels of both endogenous and exogenous therapeutic glucocorticoids during critical periods may influence neuropsychiatric development. Finally, an additional overlay to these hormonal changes is that patients experiencing shock due to the combination of inadequate glucocorticoid, mineralocorticoid and catecholamine reserves may suffer brain damage affecting cognition and behavior.

CAH and Brain Structure

Because of all the variables in the hormonal milieu described above, it is rather difficult to sort out cause and effect in identifying differences between CAH patients and controls with respect to brain structure. Limited data have been collected to show slightly, but significantly, lower amygdala size in children of both sexes who are affected with CAH compared to controls (Merke et al. 2003). Further studies with functional MRI indicated that CAH females' amygdala, hippocampus and anterior cingulate response patterns to facial emotion were attenuated compared to control females and resembled those of control males. Conversely, CAH males showed enhanced activity in these regions compared to control males (Ernst et al. 2007; Mazzone et al. 2011). It has been suggested that prenatal glucocorticoid deficiency, sex steroid excess, or some combination of these alters development of the amygdala, a structure involved in processing emotions.

CAH and Cognition

Intelligence is normal among CAH patients (Berenbaum et al. 2010). The data suggesting either superior or inferior intelligence in this population are confounded by methodologic flaws. Notably, low IQ has been correlated with frequent SW crises (Berenbaum 2001). Girls and women with CAH perform better than control females and close to males' performance on visual-spatial targeting tasks. In contrast, females with CAH did not out-perform unaffected females on mental rotation tasks. Males, especially those with severe CAH, show impaired performance on mazes (Mueller et al. 2008) and mental rotation tasks (Hines et al. 2003).

Prenatal Glucocorticoid Treatment Glucocorticoid Treatment of CAH: Brain Effects

Suppression of fetal adrenal androgens is feasible by administering dexamethasone to the mother. This is the only one of the glucocorticoids that avoids inactivation by placental 11 beta hydroxysteroid dehydrogenase. The aims of prenatal dexamethasone treatment in a pregnancy known to be at risk for CAH are to reduce female genital virilization, to avoid reconstructive genital surgery, and to avoid the associated emotional distress of having a girl with severe CAH. An international Task Force appointed by The Endocrine Society has recommended that prenatal dexamethasone be considered experimental, in part due to concerns about potential adverse side effects on the developing brain (Speiser et al. 2010). Many of these concerns derive from animal models of prenatal glucocorticoid exposure.

Sheep have reduced brain weight (Huang et al. 1999) and myelin formation (Moss et al. 2005) following betamethasone treatment in mid-to-late gestation. Rhesus monkeys show disrupted hippocampal neurons as a consequence of high dose prenatal dexamethasone exposure (Uno et al. 1994). No structural data are available in prenatally treated CAH patients; however, mild behavior and cognitive alterations have been reported.

Questionnaires administered to a small group of mothers of dexamethasone-treated CAH children showed more shyness and inhibition (Trautman et al. 1995). A larger questionnaire survey of US mothers of 174 children prenatally treated and 313 untreated control children found no differences between treated and untreated groups with respect to nine social/developmental scales (Meyer-Bahlburg et al. 2004a, b). A Swedish cohort subjected to standardized neuropsychological tests by a clinical psychologist showed no differences in intelligence, handedness, or long-term memory compared with matched controls. However, CAH-unaffected children prenatally treated for brief periods had poorer verbal working memory, rated lower on self-perception of scholastic competence, and had increased self-rated social anxiety at age 8–18 (Hirvikoski et al. 2007). This same Swedish group surveyed parents of school age children following prenatal dexamethasone and

there seemed to be no differences in psychopathology, behavioral problems, or adaptive functioning (Hirvikoski et al. 2008). Interestingly however, prenatally treated unaffected boys showed marginally greater gender neutral role behaviors compared to controls (Hirvikoski et al. 2011). Clearly, observations regarding the impact of prenatal glucorticoids on the central nervous system depend on developmental timing of treatment, total dose, age at the time of investigation, method of inquiry (questionnaire versus direct testing), type of brain perturbation being sought (structural abnormality versus functional impairment), and on the domain being tested.

Summary

The most salient effects of excessive prenatal exposure to adrenal androgens in children with CAH are apparent in females. Girls display typical female gender identity but more often display masculine and aggressive types of play compared with unaffected sisters, and as women they frequently choose male-typical jobs. At maturity, female CAH patients may have same-sex fantasies and homosexual orientation, yet most are heterosexual. It is interesting to note that true gender dysphoria is rare in this population. Child bearing is possible; however, fecundity rates are below those observed in the general population. There is some evidence to suggest both structural and functional brain differences among CAH patients. Finally, prenatal dexamethasone treatment to prevent female genital ambiguity is not sanctioned outside of formal clinical trials, due to the potential for unanticipated adverse effects of this potent glucocorticoid given at a critical time in organogenesis, with particular concerns about perturbations in the development of the central nervous system.

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Genetic Variation Within Serotonin Genes, Hormones, and Aggression

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Abstract Inter-individual differences in aggressive behavior and related mental illnesses have important genetic as well as environmental underpinnings. Twin and adoption studies have shown that genetic factors contribute to approximately 50 % of vulnerability to aggression. Across-species aggressive behavior is more common in males than females, reflecting the different evolutionary roles of the two genders and pointing to the different effects on brain and behavior of the neuro-endocrinological process involved in sex differentiation. Indeed, a correlation between testosterone levels and aggression has been reported in studies on humans and other species. Multiple pieces of evidence support the role of genetic variation within genes belonging to the serotonin system, such as monoamine oxidase A (MAOA), HTR1B, HTR3B, SLC6A4, and HTR2B, in determining vulnerability to aggression. Recent studies also indicate the existence of a complex interaction between genes, hormones, and stress. MAOA is an X-linked gene encoding the monoamine oxidase A, a mitochondrial enzyme that metabolizes monoamine neurotransmitters including norepinephrine, dopamine, and serotonin. MAOA knockout mice have higher levels of these neurotransmitters and manifest increased aggressive behavior and stress reactivity. In humans, a rare stop codon variant leading to a complete MAOA deficiency has been reported in a single Dutch family in which eight males were affected by a syndrome characterized by borderline mental retardation and severe aggressive behavior. More recently, a common polymorphism influencing MAOA transcription (MAOA–LPR) has been found in

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the human MAOA promoter. At this locus, the allele associated with low MAOA activity (e.g., increased monoamines level) has been associated with increased risk of developing Conduct Disorder and Antisocial Personality Disorder among subjects exposed to severe childhood trauma. In addition, a powerful MAOA-LPR × endocrine interaction has been described in a sample of Finnish male violent offenders. In this study, a strong correlation between testosterone and aggressive behavior was found only among carriers of the low MAOA activity allele. More recently, a rare stop codon variant (Q20*) within the HTR2B serotonin receptor gene has been associated with psychiatric diseases marked by severe impulsivity and aggression. Similar to what has been observed for MAOA, the impact of Q20* was not in itself sufficient in determining aggressive behavior, but male sex, testosterone level, and exposure to alcohol had important roles.

Introduction

Aggression refers to any behavior that is intended to harm someone physically or psychologically. Aggression may occur in a wide array of conditions, ranging from episodic reactions that can be considered within the 'normal' range to more generalized and pervasive manifestations that occur in the context of psychiatric diseases such as Conduct Disorder (CD), Antisocial Personality Disorder (APD), Borderline Personality Disorder (BPD), and Intermittent Explosive Disorder (IED). These disorders are quite common, with lifetime prevalences in the U.S. of 3.6 % for APD, 1.1 % for CD (Compton et al. 2005), 5.9 % for BPD (Grant et al. 2008) and 7.3 % for IED (Kessler et al. 2006). Aggressive behavior is also common in patients suffering from other mental illnesses such as internalizing disorders, addiction and dementia (Heinz et al. 2011).

Aggression includes multiple heterogeneous conditions in terms of both clinical phenomenology and neurobiology. The multifaceted nature of aggression has led to different classifications that represent attempts to group together forms of aggression that are etiologically and phenomenologically similar. For example, aggression can be classified according to the type of aggression (e.g., physical vs verbal), the motivation (hostile, in which the goal is aggression per se, vs. instrumental, in which aggressive behavior is motivated by a specific reason), or the target of aggression (e.g., self-directed vs other-directed). The most widely used, and probably useful, classification distinguishes two main types of aggression: proactive and reactive. Proactive aggression is pre-meditated and is conceptualized as an instrumental form of aggression, with positive expectancies about the outcomes of aggression, lack of empathy, and remorse. Criminal behaviors, in which violence is well planned in advance and is clearly goal-directed, fall within this category. This type of aggression is linked to psychopathy, whose central feature is a lack of emotional sensitivity (Raine et al. 2006). In contrast, reactive or impulsive aggression develops in response to a real or perceived threat and is associated with high emotional arousal, impulsivity, and negative emotions such as anger or fear (Barratt

and Felthous 2003; Siever 2008). Of note, the aggressive response to an imminent threat can be considered as an adaptive defensive response and, as such, part of the normal range of human behavior. Pathologic reactive aggression is characterized by a low threshold for activation of aggressive responses to external stimuli without adequate reflection about the aversive consequences of the behavior and with a response that is not proportionate to the stimulus (Siever 2008).

The neurobiological mechanisms underlying reactive aggression are only beginning to be understood. Central to this type of aggression is an increased emotional sensitivity that is believed to result from the unbalanced activations of sub-cortical and cortical regions regulating emotional reaction and controlling behavioral response (Barratt and Felthous 2003; Siever 2008). Indeed, multiple neuromaging studies indicate that this type of aggressions is associated with hyperactivity of limbic areas that mediate response to emotional stimuli, such as the amygdala and the insula, and hypo-function of regions such as the prefrontal cortex and anterior cingulated cortex that are involved in behavioral control and in predicting expectancies of reward and punishment (Heinz et al. 2011). In this chapter, we will mainly refer to reactive or impulsive aggression.

Heritability of Aggression

The etiological determinants of human aggression are clearly multi-factorial, emerging from cultural, religious, political, socioeconomic, and psychological factors. A number of pieces of evidence has shown that genetic factors also play a role. According to large twin and adoption studies, the heritability estimate for aggression among adults is approximately 50 % (Miles and Carey 1997; Rhee and Waldman 2002). Similar to other behavioral phenotypes, the relative importance of genetic and environmental factors changes over time, with genetic factors becoming more important moving from childhood into adulthood (Tuvblad et al. 2009). Environmental risk also plays a major role in aggression and antisocial behavior. The environmental factors that are involved include exposure to childhood abuse or neglect (Caspi et al. 2002), peer influences, poor parenting (Rodriguez and Tucker 2011), use of alcohol and illicit drug use (Heinz et al. 2011), traumatic brain injuries, low socio-economic status and low education level (Mendes et al. 2009). Protective factors are also important in reducing risk of aggressive/impulsive behavior; these include high social support and good parenting care.

Although the neurobiological mechanisms underlying aggressive behavior remain mainly unknown, multiple pieces of evidence support the role of neurosteroids, especially testosterone, of the neurotransmitter serotonin, and of synergetic interactions between these two systems in the genesis of severe aggressive and impulsive behavior.

Testosterone and Aggression

Aggression is to some extent an evolutionarily advantageous trait and an important predictor of success in the competition for survival and reproduction. Across-species aggressive behavior is more common in males than females, reflecting the different evolutionary roles of the two genders. IED is 1.7-fold more common in men than women (Kessler et al. 2006). According to the NESARC epidemiological study, which included 34,653 individuals across the U.S., APD and antisocial behavioral syndrome are 2.8 and 1.8 times more common in men than women respectively (Fazel and Danesh 2002).

Gender differences found in aggressive behavior and related mental illnesses point to the different effects on brain and behavior of the neuro-endocrinological processes involved in sex differentiation. The effect of testosterone on the brain during neurodevelopment is crucial to shaping the organization of the brain into a 'male-like pattern.' Given the higher frequency of aggressive behavior in males than females, it is not surprising that multiple studies on both animals and humans have explored the relationship between aggression and testosterone level.

Studies in animal models fairly consistently show that higher endogenous and administered levels of testosterone predict aggressive behavior (Beeman 1947a, b; Archer 1988). Among primates, Mazur (1985) showed that testosterone concentration varied after a competitive male-male interaction and was important in establishing dominance and hierarchy. Indeed, after a competition, winners experienced an increase in testosterone, whereas losers experienced a decrease. Similarly, among birds, male-male aggressive interactions stimulate the production of androgens (Wingfield et al. 1990). In animal models, the administration of exogenous testosterone increased aggressive behavior (Gleason et al. 2009).

Studies exploring the correlation between aggression and testosterone in humans have reported mixed results. In a study conducted in a sample of criminal alcoholics, levels of testosterone were higher among impulsive and violent offenders as compared to non-impulsive, non-violent offenders and healthy controls (Virkkunen et al. 1994). These data might suggest that high testosterone levels could be a specific endocrinological correlate for reactive-impulsive aggression. A positive correlation between testosterone and irritability and verbal aggression has been found in a sample of patients affected by APD or BPD (Gustavsson et al. 2003). In contrast, other studies failed to find a correlation (Coccaro et al. 2007). A meta-analysis based on 45 independent studies encompassing 9,760 individuals provided support for an overall weak positive correlation ($r = 0.14$; Book et al. 2001).

Interpretation of the relationship between testosterone and aggression is complicated in humans by measurement errors, heterogeneity across studies, differences in exposure to environmental factors, genetic differences (as will be discussed later), and fluctuations in hormone levels in response to environmental conditions and circadian rhythm (Mazur 1985; Wingfield et al. 1990; Oliveira 2009). Indeed, dynamic fluctuations in testosterone concentrations appear to be more correlated to aggressive behavior than do baseline levels (Hermans et al. 2008). An experimental

paradigm that allowed evaluation of aggressive behavior in humans under controlled conditions within laboratory settings showed that the circulating concentration of testosterone was strictly situation-related (Wingfield et al. 1990), and a correlation between aggressive behavior and testosterone has been found by some studies (Berman et al. 1993) but not others. Also, studies based on the administration of exogenous testosterone among humans appear overall to be inconclusive, with a mix of positive and negative findings (Kouri et al. 1995; Yates et al. 1999; Pope et al. 2000).

Interestingly, neuroimaging studies indicate the relevance of testosterone in modulating neuronal circuits involved in aggression and behavioral control (Mehta and Beer 2010; Volman et al. 2011; van Wingen et al. 2009). For instance, endogenous testosterone levels were negatively correlated with task-elicited activation in the orbital frontal cortex (Mehta and Beer 2010) and influenced the connectivity between the pre-frontal cortex and the amygdala (van Wingen et al. 2009). Also, testosterone administration increased amygdala response to negative stimuli (van Wingen et al. 2009). These findings support the notion that testosterone might play an important role in influencing the unbalanced activation of the limbic and cortical areas that characterizes reactive aggression. Evidence suggests that the impact of testosterone in aggressive behavior could be to some extent elicited through modulation of the serotonergic system (Birger et al. 2003). Indeed, testosterone has been shown to modulate the expression of multiple serotonin-related genes, including the serotonin receptor 2A, the serotonin transporter, and monoamine oxidase A (MAOA; Birger et al. 2003; Ou et al. 2006). As will be discussed further, an interaction between genetic variation within MAOA and the serotonin receptor 2B gene and testosterone level has been described in severe forms of impulsive aggression.

Genes and the Serotonin System

Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter that is biochemically derived from L-tryptophan (Fig. 1). Within the CNS, serotonergic neurones are localized in the brain stem within the raphe nuclei that project diffusely to a variety of brain regions, including cortex, amygdala, and hippocampus.

Serotonin is a key regulator of emotional response, and multiple lines of evidence support the involvement of a dysfunction of the serotonin system in the genesis of aggression (Takahashi et al. 2011). Indeed, serotonin depletion is associated with increased aggressive behavior in individuals who already show high levels of aggression or hostility (Salomon et al. 1994). Criminal alcoholics with APD display low serotonin turnover rates [measured via the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the cerebrospinal fluid] and increased aggressive behavior (Virkkunen et al. 1994). Pharmacological manipulation of the serotonin system appears to have an effect on aggression. Antagonists of 5-HT2A receptors reduce impulsivity in animal models (Winstanley et al. 2004), and

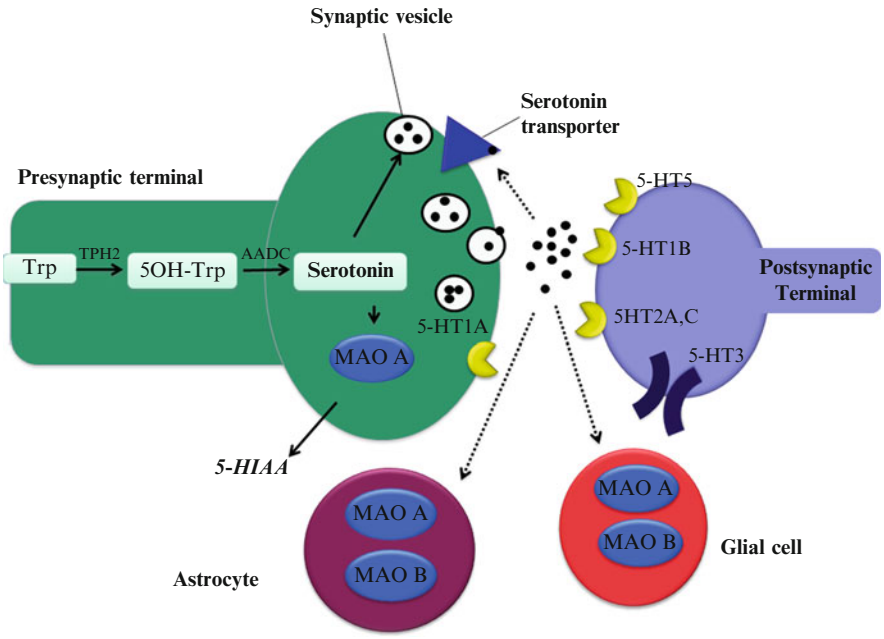

Fig. 1 Serotonin, or 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter biochemically derived from L-tryptophan by the action of two enzymes, tryptophan hydroxylase (TPH) and amino acid decarboxylase (AADC). There are two types of TPH, namely TPH1 and 2; the latter is the main form found in the CNS. Serotonin is stored in vesicles and then released in the synaptic space. Here serotonin activates 5-HT receptors that include at least 14 pre- or postsynaptic receptor subtypes. Serotonin is then removed from the synaptic space by the serotonin transporter located in the presynaptic neuron, and it can be re-stored in vesicles or it can be metabolized by the monoamine oxidase A (MAOA) enzyme in is 5-hydroxyindolacetic acid (5- HIAA). MAOA is also expressed in astrocytes and glial cells

atypical neuroleptics with prominent 5-HT2A antagonism have anti-aggressive efficacy (Krakowski et al. 2006). Agonists, on the other hand, reduce impulsivity at the 5-HT2C receptor (Bubar and Cunningham 2006), suggesting that the two receptor subtypes may have complementary roles in the regulation of aggression. Finally, genetic variation within genes belonging to the serotonin systems, such MAOA; (Caspi et al. 2003), the serotonin transporter (SLC6A; Sugden et al. 2010), the serotonin receptor 3B (HTR3B; Ducci et al. 2009), the serotonin receptor 1B (Lappalainen et al. 1998), and the serotonin receptor 2B (HTR2; Bevilacqua et al. 2010), have been shown to moderate vulnerability to aggressive behavior and related mental illnesses. Recent studies also indicate the existence of a complex interaction between genes, hormones, and stress.

In this chapter we will focus on two genes, namely MAOA and HTR2B, that are currently the best-known candidate susceptibility genes for human aggression.

MAOA

MAOA and monoamine oxidase B (MAOB) are mitochondrial enzymes that metabolize monoamine neurotransmitters such as norepinephrine, dopamine and serotonin. MAOA and MAOB are encoded by two genes that are tail to tail located in the same chromosome region (Xp11.23–Xp11.4). MAOA and MAOB genes have similar intron-exon structure and are thought to derive from an ancient chromosome duplication (Grimsby et al. 1991). In line with this idea, MAOA and MAOB enzymes share 70 % similarity in amino acid sequence (Bach et al. 1988).

Both enzymes degrade monoamines but with a different substrate preference: MAOB has higher affinity for dopamine (Stenstrom et al. 1987; Lakshmana et al. 1998), phenylethylamine (Fowler and Oreland 1981; Arai et al. 1986) and benzylamine (White and Glassman 1977), whereas MAOA displays higher affinity for norepinephrine and serotonin. Further, both enzymes are expressed in the central nervous system (CNS) but with different distributions: MAOB is preferentially expressed on serotonergic (Thorpe et al. 1987) and histaminergic (Westlund et al. 1988) neurones, whereas MAOA is mainly expressed in noradrenergic neurons (Thorpe et al. 1987; Arai et al. 1997). Both genes are expressed in glia (Westlund et al. 1988).

Several lines of evidence from studies on both humans and animals have shown that a deficiency in MAOA is associated with impulsive aggressive behavior.

MAOA knockout (KO) mice have higher levels of serotonin, noradrenalin, and, to lesser extent, dopamine and manifest increased offensive aggressive behavior, increased stress reactivity, and a dramatic reduction in defensive and fear-related behaviors in the presence of predator-related cues, suggesting a general inability to appropriately assess contextual risk and attune defensive and emotional responses to the environment (Godar et al. 2011). Interestingly, behavioral abnormalities of MAOA KO mice are partially reversed by serotonin synthesis inhibitors, indicating that they are mainly secondary to increased levels of serotonin (Cases et al. 1995; Fig. 2a). In humans, a rare mutation leading to a total suppression of MAOA activity, similar to the KO mouse, has been described by Brunner in a Dutch family in which 14 males were affected by a syndrome characterized by abnormal monoamine levels, borderline mental retardation and impulsive behavior, including impulsive aggression, arson, attempted rape, fighting, voyeurism, and exhibitionism. The cause was a stop-codon variant in the eighth exon of MAOA, leading to complete and selective deficiency of MAOA activity. Within the Dutch pedigree, the mutation had an X-linked pattern of transmission from unaffected mothers carrying the stop-codon (see Fig. 2b).

More recently, a common MAOA polymorphism influencing MAOA transcription was discovered (Sabol et al. 1998). This locus, termed the MAOA-linked polymorphic region (MAOA–LPR), is a variable number tandem repeat (VNTR) located approximately 1.2 kb upstream from the MAOA start codon and within the gene's transcriptional control region (Sabol et al. 1998; Deckert et al. 1999). Alleles at this VNTR differ in the number of copies (2, 3, 3.5, 4, or 5) of a 30-bp repeat motif, with the 3 and 4 repeat alleles being by far the most common. Alleles with

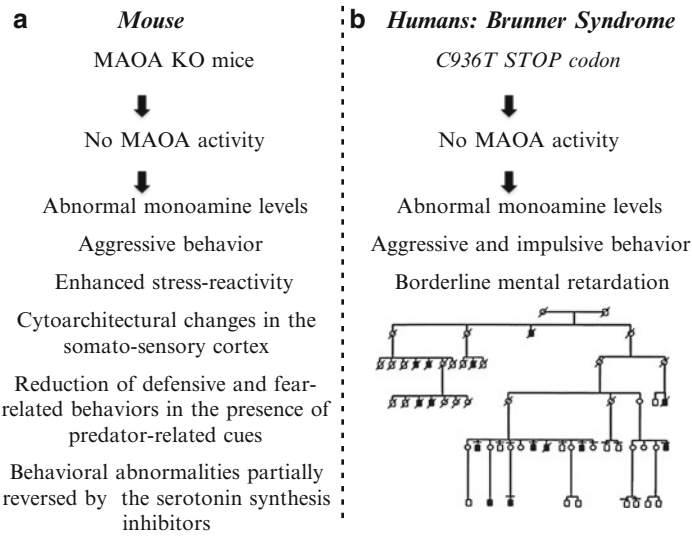

Fig. 2 Phenotypic consequences of a complete deficiency of MAOA in mice (**a**) and in humans (**b**). MAOA knockout (KO) mice display abnormal monoamine levels, increased offensive aggressive behavior, stress reactivity, and inability to appropriately assess contextual risk. In humans, a rare mutation leading to a total suppression of MAOA activity has been described by Brunner in a Dutch family, including 14 males displaying abnormal monoamine levels, borderline mental retardation and impulsive behavior

3.5 and 4 repeats appear to be transcribed more efficiently than alleles with three copies (Sabol et al. 1998; Deckert et al. 1999). However, it remains unclear whether the 5 repeat allele is associated with high (Deckert et al. 1999) or low (Sabol et al. 1998) MAOA activity. In vivo studies conducted in humans, using PET, confirmed that genotype at the MAOA–LPR influences serotonin function measured as binding serotonin 1A receptor (Mickey et al. 2008; Fig. 3).

Interaction Between MAOA and Environmental Risk Factors

Several studies on both humans and animals have shown that MAOA is an important modulator of stress resiliency. Low MAOA activity (and the consequent increase in monoamine level) is associated with increased risk of developing aggressive/antisocial behavior in response to environmental stressors.

In a longitudinally studied cohort of boys, Caspi et al. (2002) found that MAOA–LPR influenced the risk of developing antisocial behavior in participants exposed to childhood maltreatment (Fig. 4a). In this study, boys with the low activity genotype (in red) who were exposed to severe childhood maltreatment were more likely to develop antisocial problems later in life than boys with the high activity genotype (in black). A meta-analysis including almost 1,000 participants

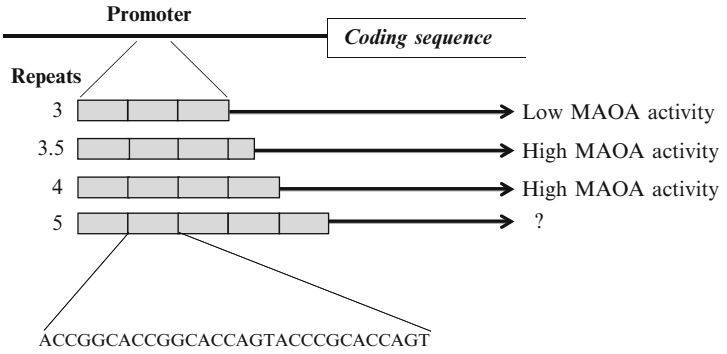

Fig. 3 The MAOA-linked polymorphic region [MAOA–LPR]. MAOA–LPR is a variable number tandem repeat (VNTR) located approximately 1.2 kb upstream from the MAOA start codon. Alleles at this VNTR differ in the number of copies (2, 3, 3.5, 4, or 5) of a 30-bp repeat motif, with the 3 and 4 repeat alleles being by far the most common. Alleles with 3.5 and 4 repeats appear to be transcribed more efficiently than alleles with three copies. It remains unclear whether the 5 repeat allele is associated with high (Sabol et al. 1998; Deckert et al. 1999) or low MAOA activity

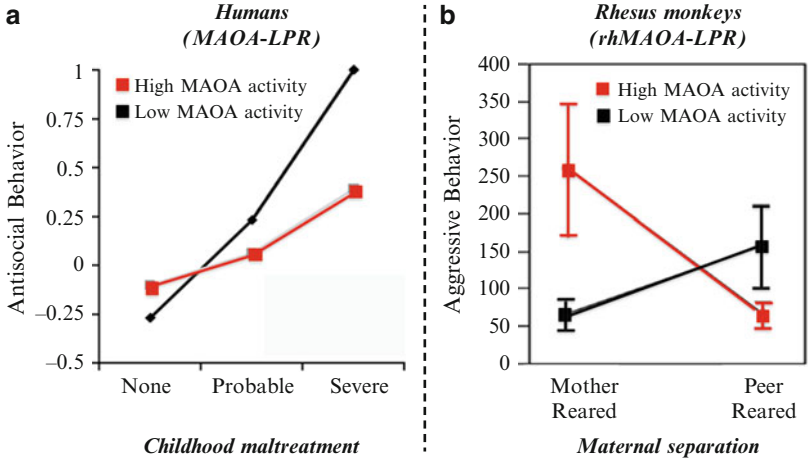

Fig. 4 Interaction between MAOA genotype and environmental factors in humans (**a**) and in Rhesus monkeys (**b**). In humans, boys with the low activity genotype (in *red*) at MAOA–LPR and who were also exposed to severe childhood maltreatment were more likely to develop antisocial problems later in life than boys with the high activity genotype (in *black*). In Rhesus monkeys, the impact of maternal separation on aggressive behavior is contingent on rhMAOA-LPR

revealed a significant pooled interactive effect between MAOA and childhood adversity (Kim-Cohen et al. 2006). Alcohol and drug abuse are also an important risk factors for antisocial behavior and can trigger aggressive behavior with effect thats appear to be to some extent modulated by genotype. A significant interaction between MAOA and heavy drinking or risk for impulsive violent crimes has been reported in a sample of Finnish male alcoholic offenders with high rates of APD and BPD (Tikkanen et al. 2010, 2011).

Interactions between environmental and genetic factors have also been studied in animal models. These are powerful because of the ability to control stress exposures and many other confounding variables. In this regard, nonhuman primates are especially useful because of their genetic similarity to humans as well as similarities in complex social structures and behaviors (Barr 2011). In the Rhesus macaque (*Macaca mulatta*), early life stress exposure, particularly early separation from the mother, leads to behavioral problems including increased alcohol consumption, higher impulsive aggression, incompetent social behavior, serotonin dysfunction, and increased behavioral and endocrine responsivity to stress (for review, see Barr 2011). An orthologous VNTR polymorphism is also found in the promoter region of MAOA in the rhesus macaque (rhMAOA-LPR). Similar to what is seen in humans, the lower activity allele predicts aggressive behavior in these animals, and the association is contingent on maternal separation (Newman et al. 2005; see Fig. 4b).

Most of the genetic studies conducted so far on MAOA are on males. The functional effects of MAOA-LPR are more difficult to predict in female subjects than in male subjects. As this gene maps on the X chromosome, males are hemizygotes, whereas females have two copies of the gene. Further, among female subjects, one copy of each gene located on the X chromosome is usually inactivated through a mechanism that can be either random or selective. To date, studies exploring the inactivation status of MAOA gene on the X chromosome have reported conflicting findings and it is not clear whether this gene escape the inactivation (Carrel and Willard 2005). Studies exploring the MAOA-childhood trauma interaction in women have reported mixed results. Some studies did not report any interactive effect (Eley et al. 2004; Huang et al. 2004; Widom and Brzustowicz 2006). Other studies, similar to what has been described for men, found an increased risk of impulsive/aggressive behavior among carriers of the low activity allele (Ducci et al. 2008). Finally, other studies found an increased risk of antisocial behavior among women carrying the high, rather than low, activity allele (Sjoberg et al. 2007). Discrepancies between studies conducted in females might be linked to some extent to a lack of power, due to the low rates of disorders characterized by aggression and impulsivity among women. Indeed, a MAOA-maltreatment interaction has been described in a Native American sample of women with extremely high rates of externalizing psychopathologies (antisocial behavior/addiction; Ducci et al. 2008). Within particular socio-cultural contexts, such as occur in certain American Indian tribes, rates of violence and cumulative trauma are very high, contributing to the high vulnerability in both sexes to psychiatric disorders, including externalizing disorders, and possibly increasing the power of detecting MAOA-environment interactive effects in women. In this Native American population, almost 50 % of women were exposed to sexual abuse in infancy, and rates of alcoholism and APD were 50 % and 13 %, respectively. By comparison, prevalences of alcoholism and APD among U.S. epidemiological samples of women are 8 % and 2 %. In this study, similar to what was observed among males, the effect of childhood sexual abuse on the risk of developing alcoholism and APD was influenced by MAOA–LPR genotype (Widom and

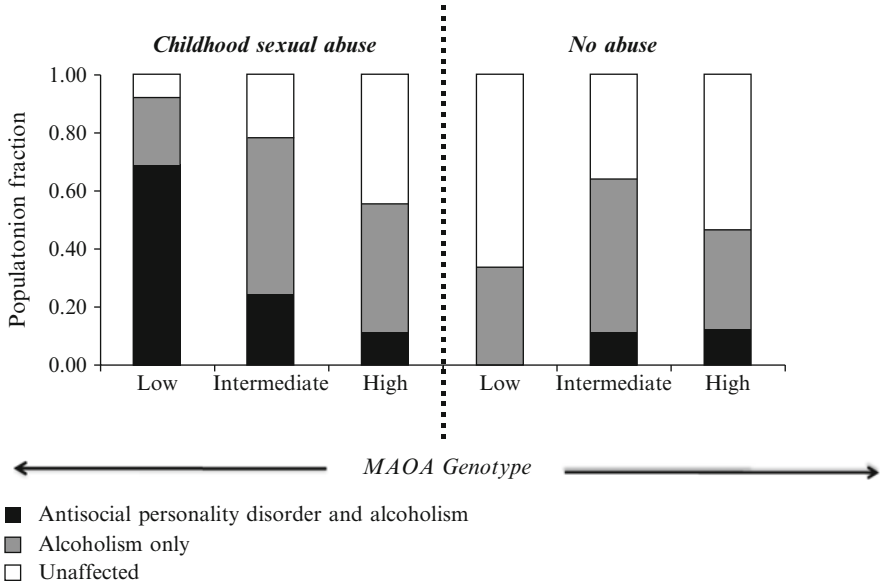

Fig. 5 Sexually abused women homozygous for the low activity MAOA–LPR allele display high rates of alcoholism with co-morbid antisocial personality disorder as compared to woman who are homozygous for the high activity allele. Heterozygous women display an intermediate risk pattern. No association between MAOA genotype and alcoholism and antisocial personality disorder is observed among women not exposed to sexual abuse

Brzustowicz 2006). Sexually abused women homozygous for the low-activity MAOA–LPR allele had high rates of alcoholism with co-morbid APD (Fig. 5) and antisocial symptoms (Fig. 6) as compared to women homozygous for the high activity allele. Heterozygous women displayed an intermediate risk pattern. In contrast, in the absence of childhood sexual abuse, there was no relationship between MAOA genotype and these disorders.

A significant interaction between MAOA-LPR and early trauma has been reported by another study conducted on woman. In this study, MAOA-LPR genotype appeared to influence not only the psychopathological consequence of childhood adversity but also the protective effect of positive experience. In this study, high perceived parental care mitigated the effect of MAOA genotype and childhood adversity on impulsivity score (Kinnally et al. 2009).

Impact of MAOA Genotype on Neuronal Circuits Moderating Emotional Response and Behavioral Control

Genetic variation within MAOA appears to predispose individuals to aggression through modulation of brain areas that mediate the response to emotionally evocative stimuli, such as the amygdala and the pre-frontal cortex (Meyer-Lindenberg et al. 2006; Alia-Klein et al. 2009) (Fig. 7).

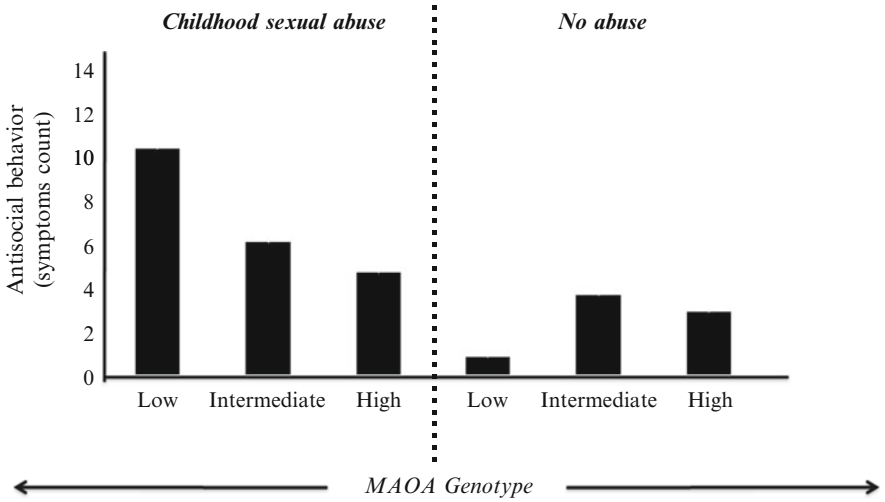

Fig. 6 Sexually abused women homozygous for the low-activity MAOA–LPR allele had high rates of antisocial symptoms as compared to woman who are homozygous for the high activity allele. Heterozygous women display an intermediate pattern. No association between MAOA genotype and antisocial symptoms is observed among women not exposed to sexual abuse

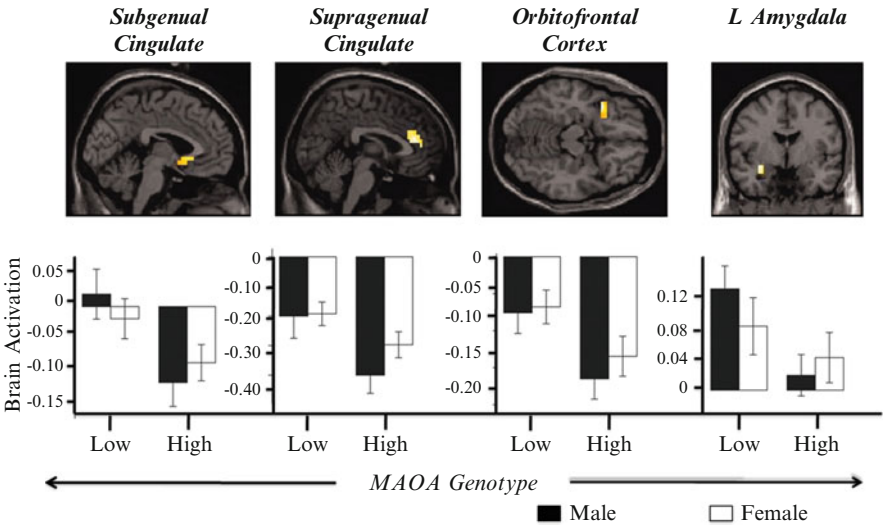

Fig. 7 MAOA-LPR predicts differential fMRI activations to angry and fearful faces in limbic and paralimbic regions. Both male (in *black*) and female (in *white*) carriers of the low activity MAOA-LPR genotype showed significantly increased activity in left amygdala and decreased response of subgenual and supragenual ventral cingulate cortex and left lateral orbitofrontal cortex compared to carriers of the high activity allele (Adapted from Meyer-Lindenberg et al. 2006)

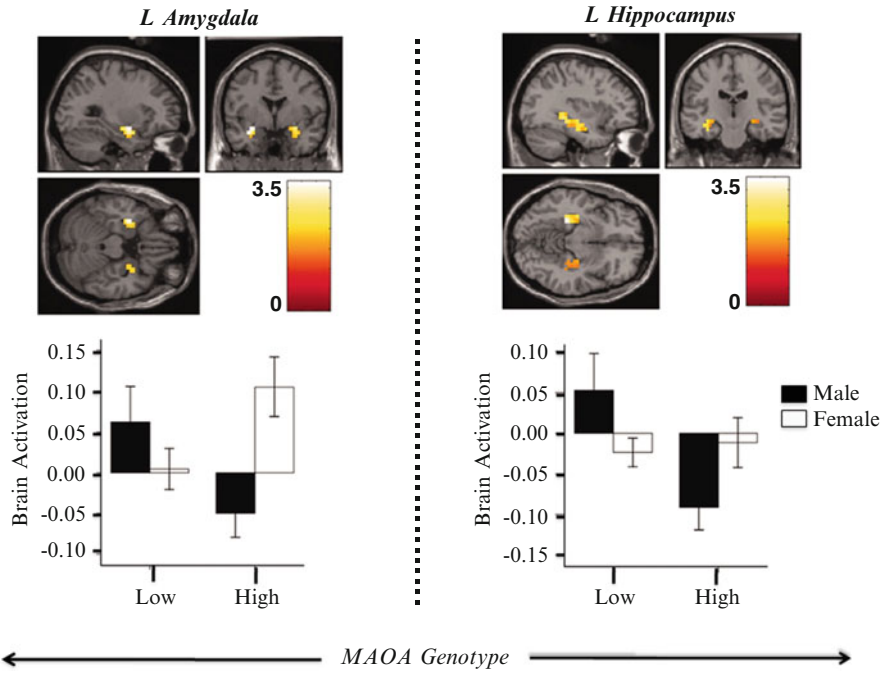

Fig. 8 MAOA-LPR moderates hippocampus and amygdala activity during the retrieval of negatively valenced emotional material. In men (in *black*) carrying the low activity MAOA–LPR allele there is a hyperactivation of the hippocampus and amygdala as compared to carriers of the high activity allele. These differences are not observed for women (in *white*)

Analysis of fMRI data during perceptual matching of angry and fearful faces showed that the activation of brain areas was strongly modulated by genotype at the MAOA-LPR (Fig. 7): both men (in black) and women (in white) carriers of the low activity MAOA-LPR genotype showed significantly increased activity in the left amygdala and decreased response in the subgenual and supragenual ventral cingulate cortex and left lateral orbitofrontal cortex, as compared to carriers of the high activity allele (Meyer-Lindenberg et al. 2006; for review, see also Heinz et al. 2011)

Genetic influences in brain areas, such as the hippocampus, involved in the encoding, retrieval, and extinction of negative emotional information could explain the MAOA-childhood trauma interaction. Indeed, MAOA-LPR has been shown to influence activity in the hippocampus, a key brain area involved in memory and processing of emotion (Fig. 8). In men (in black) carrying the low activity MAOA–LPR allele, the hippocampus and amygdala hyperactivate during the retrieval of negatively valenced emotional material but not during the retrieval of neutral material (Fig. 8; Meyer-Lindenberg et al. 2006). Therefore, the increased sensitivity to adverse experiences of carriers of the low activity MAOA allele might be due to their stronger activation of memory circuits by negative stimuli and their consequent impairment in extinguishing adverse memories and conditioned fears. However, these differences in hippocampus and amygdala are not observed in women (in white).

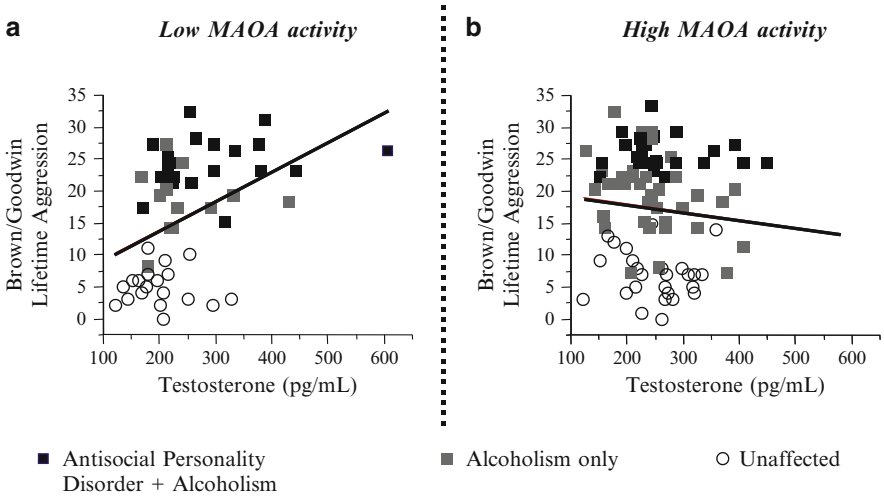

Fig. 9 Non-additive interaction of MAOA-LPR and testosterone predicts antisocial behavior. A positive correlation between endogenous testosterone level and aggression is found among carriers of the low activity allele at the MAOA-LPR (**a**) but not among carriers of the high activity allele (**b**; Sjoberg et al. 2008)

Interaction Between MAOA and Testosterone

Testosterone and the serotonin system appear to have a synergistic effect in modulating aggression. Indeed, low MAOA activity and an increased testosterone level have both been reported to lead to severe impulsive aggression (Sjoberg et al. 2008). As shown above, in males (females have much lower testosterone levels), there is a moderate correlation between testosterone level and lifetime aggression. This relationship appears to be moderated by genotype at MAOA-LPR. In a sample of Finnish criminal alcoholics, a positive correlation between testosterone level and aggression score was found among carriers of the low activity MAOA allele (Fig. 9a) but not among carriers of the high activity allele (Fig. 9b).

Similarly, an association between testosterone level and risk for alcoholism and of alcoholism in co-morbidity with antisocial behavior was found among carriers of the low activity MAOA allele but not among carriers of the high activity allele (Fig. 10).

The mechanism of the interaction between MAOA and testosterone could be related to a synergistic effect of testosterone and the MAOA-low activity allele on the neuronal circuits moderating aggression. Indeed, both low MAOA activity and increased testosterone level appear to lead to hyperactivity of the amygdale and reduced activity of the prefrontal cortex during exposure to emotionally evocative stimuli. Alternatively, the mechanism of interaction could be related to molecular mechanisms, because androgens influence MAOA expression through response elements located within the MAOA promoter next to the MAOA-LPR locus (Ou et al. 2006).

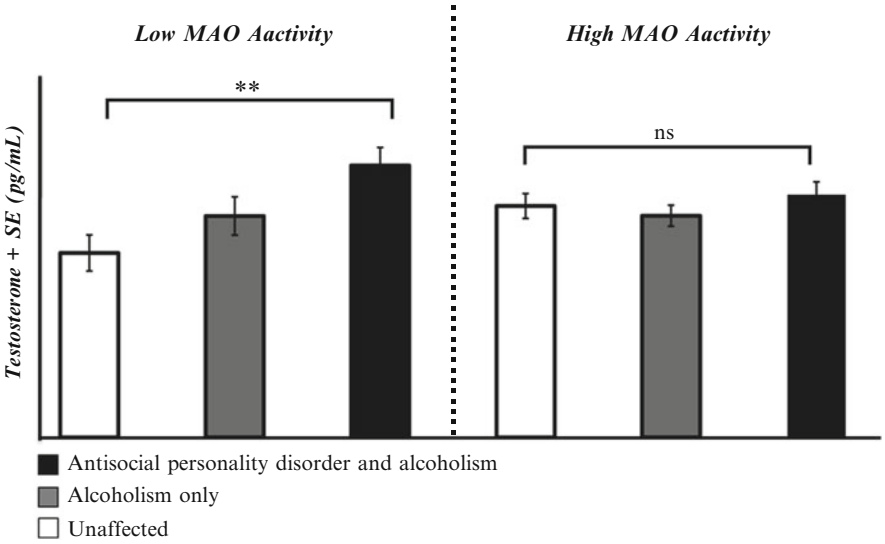

Fig. 10 Mean endogenous testosterone levels are compared between controls (in *white*), patients affected by alcoholism without antisocial personality (in *gray*) disorder and alcoholics with antisocial personality disorder (in *black*). Results are shown stratified by genotype at the MAOA-LPR

The Serotonin Receptor B (HTR2B)

Similarly to what has been observed for MAOA, genetic variation within HTR2B has been linked to severe reactive/impulsive aggression with an effect that appears to be modulated by stress, alcohol consumption, and hormones.

The HTR2B gene, located on chromosome 2 (2q36.3-q37.1), encodes for the serotonin 2B receptor, a G protein-coupled receptor. Serotonin 2B receptors are widely expressed in the human brain (Choi and Maroteaux 1996; Schmuck et al. 1996; Bonaventure et al. 2002; Doly et al. 2008; Bevilacqua et al. 2010). During development, the 5-HT2B receptors are expressed in heart and neural crest-derived cells at the earliest stages (E9) of embryonic life (Choi and Maroteaux 1996). Activation of the 5-HT2B receptor enhances cell proliferation, increases cell migration and reduces apoptosis (Gaspar et al. 2003). The function of the 5-HT2B receptor in the adult brain is mainly unknown, and few studies have directly measured the effects of 5-HT2B receptor activation. A preferential 5-HT2B agonist increased food consumption, reduced grooming, and was anxiolytic in a social interaction model when microinjected into the medial amygdala (Kennett et al. 1998). 5-HT2B receptors are also involved in sleep regulation (Popa et al. 2005) and neonatal respiratory rhythms (Gunther et al. 2006). The 5-HT2B receptor has been proposed to have a role in the regulation of food intake (De Vry and Schreiber 2000), and recently it was reported that 5-HT regulates

appetite, possibly via 5-HT2B receptors on hypothalamic neurons (Yadav et al. 2009).

Both HTR2B mRNA and protein are expressed in mouse raphe neurons that also express the serotonin transporter (Launay et al. 2006). Launay and colleagues observed that the 5-HT2B receptor modulates serotonin transport by promoting phosphorylation of the serotonin transporter (resulting in the attenuation of 5-HT transport), representing a presynaptic effect of this receptor. Activation of presynaptic 5-HT2B receptors has also been shown to be a limiting step in the serotonin transporter-dependent release of serotonin by dexfenfluramine, an anorectic drug, now banned, that was used for treatment of obesity (Banas et al. 2011). Activation of presynaptic HTR2B receptors also appears to play a role in the development of the serotonin syndrome (Diaz and Maroteaux 2011).

A functional stop codon in the HTR2B gene was recently identified via sequencing of Finnish male violent offenders and controls (Bevilacqua et al. 2010). The case group was comprised of individuals who underwent psychiatric evaluation for the extreme nature of their crimes (homicides, batteries, assaults, arsons) at the time of their initial incarceration. The variant was enriched in individuals with a history of impulsive, non-premeditated violence. In the cases identified, who were all heterozygous for the HTR2B stop codon, the variant seemed to play a role in releasing impulsive aggression under conditions in which it is known that impulse control is impaired, such as alcohol intoxication. The stop codon also co-segregated with antisocial personality disorder in eight informative families (Bevilacqua et al. 2010). In an epidemiologic sample, Finnish males carriers of the stop codon variant performed less well at the "digit forward and backward" task, a neuropsychological test assessing working memory that accesses frontal lobe function.

The HTR2B stop codon led to variable nonsense-mediated decay of the RNA but blocked expression of the receptor protein. Western blot analyses in lymphoblastoid cell lines from heterozygous carriers of the 5-HT2B receptor stop codon revealed that Htr2b protein expression was halved as compared to those from homozygous non-carriers.

The HTR2B stop codon appears to be restricted to one founder population. It is less limited in its distribution (being present in at least 100,000 people) than the MAOA stop codon found by Brunner and colleagues in one Dutch family, but it has only been observed in individuals of Finnish ancestry (Bevilacqua et al. 2010)

Htr2b knockout mice offered the opportunity to test the predictive validity of the behavioral effects of the HTR2B stop codon. 5-$HT_{2B}{}^{-/-}$ mice displayed behaviors consistent with impulsivity, namely impaired delay discounting, high novelty seeking and high reactivity to novelty (Bevilacqua et al. 2010). Further supporting the link between serotonin and testosterone in aggression, a significant increase in plasma testosterone levels was observed in $Htr2b^{-/-}$ male mice. An increase in CSF testosterone levels was also observed in violent offenders carrying the stop codon, raising the possibility of an interaction between the HTR2B stop codon and testosterone contributing to impulsive behaviors, as previously described for MAOA in this same Finnish population (Sjoberg et al. 2008).

Although the HTR2B stop codon is associated and co-segregates with disorders characterized by impulsivity and can contribute to severe violent behavior, the presence of the genetic variant is necessary but not sufficient. The vast majority of carriers are apparently normal and, at the population level, the stop codon has little predictive power. Male sex, testosterone level, the decision to drink alcohol and probably other factors such as stress exposure play important roles, and more studies are needed to understand these interactions and to confirm the effect of the gene in impulsivity.

Conclusions

Although most of the genetic determinants of aggression remain to be discovered, here we have focused on two genes, namely MAOA and HTR2B, for which the neurobiological mechanisms translating gene effect into complex behavior, such as aggression, have been partially understood. For these two loci, the functional variants mediating genetic risk have been discovered and findings in humans and animal models converge in a remarkable way. In addition, the use of imaging-genetic techniques have elucidated, at least for MAOA, the brain areas that mediate the effects of genes on aggressive behavior, i.e., the amygdala and prefrontal cortex. For both MAOA and HTR2B, the effect on aggression is modulated by a complex interaction between genes, environmental exposure, and endocrinological systems. The recent development of new, powerful sequencing technologies, promises that more genetic factors modulating aggression/impulsivity are likely to be discovered in the near future. As shown for MAOA and HTR2B, the integration of studies on animal and humans, the integration of neuro-imaging and genetic technologies, and the study of gene X environment and gene X hormone interactions will be fundamental to further elucidating the biological processes involved in aggressive behavior.

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Multiple Origins of Sex Differences in Attention Deficit Hyperactivity Disorder

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Abstract Attention deficit hyperactivity disorder (ADHD) is considered one of the most prevalent psychiatric disorders; by some criteria it affects about 10 % of school-aged children and nearly 5 % of adults. The current diagnostic criteria evolved from the core features (attention deficits and behavioral excesses) described over 100 years ago by Still (1902). More males than females are recognized and treated for ADHD, but the observed sex difference is smaller in adults than children. To address the topic of the multiple origins of sex differences in ADHD, we discuss some factors that influence ADHD-related sex differences: (1) secular trends that have recently increased the recognition and treatment of ADHD, (2) diagnostic approaches based on the evaluation of symptom severity (a categorical approach) compared to underlying behaviors (a dimensional approach), and (3) study designs that are longitudinal or cross-sectional.

Introduction

Attention deficit hyperactivity disorder (ADHD) is considered one of the most prevalent psychiatric disorders of childhood; it is present in both sexes but is more frequently recognized in boys. For example, a population survey in 2007–2008 of 4–17-year-old children in the USA (Visser et al. 2010) estimated that 10.3 % of boys and 4.0 % of

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girls had a current diagnosis of ADHD, yielding an observed sex ratio of 2.8:1. ADHD is also recognized in adults. For example, based on a nationally representative survey in 2001–2003 of 18–44-year-old adults in the USA (Kessler et al. 2006), 5.4 % of males and 3.2 % of females were estimated to have a current diagnosis of ADHD, yielding a lower observed sex ratio of 1.7:1.

The primary purpose of this chapter is to evaluate ADHD-related observed sex differences from multiple perspectives. First, we will describe a secular trend in the USA and elsewhere—the dramatic increases in recognition and treatment of ADHD over the past decade—that may have changed the sex ratios observed in clinical studies. Second, we will describe how evaluation of the full range of underlying behaviors (a dimensional approach) may provide a better understanding of the sex differences than the evaluation of symptom severity or psychopathology (a categorical approach). Third, we will discuss how longitudinal assessments of cases identified in childhood may provide a better understanding of age-related sex differences than cross-sectional assessment of different age groups, which may be confounded by age-related referral biases and maturational differences.

Brief Background

Even though the true population prevalence is thought to be similar in most countries, in clinical practice the rate of recognition and treatment (usually with stimulant medication)—the administrative prevalence—varies widely. Historically, stimulant medication has been used "…with a frighteningly different frequency in different places" (Taylor 1979). For example, in the pre-DSM III (American Psychiatric Association 1980) era, when the diagnosis and treatment were primarily in children, in the USA and UK the cross-national difference in treatment rate was greater than 50:1. With the general trend of increasing recognition and treatment in most countries, cross-national differences have narrowed over the past 30 years, but based on national supplies of stimulants in 2005 (Swanson and Volkow 2009), the estimated USA:UK cross-national difference was still large (13.4:1). Presumably, the cross-national differences are due to non-biological factors that affect referral and access to services, including regulatory restrictions on stimulant drugs and cultural factors that influence tolerance of the symptoms and stigma associated with the diagnosis. Some of these factors also may influence the ratio of males to females who are diagnosed with ADHD and treated for this disorder.

The current diagnostic criteria are based on 18 symptoms listed for ADHD in the Diagnostic and Statistical Manual, Edition IV (DSM-IV) of the American Psychiatric Association (APA 1994) and for Hyperkinetic Disorder (HKD) in the International Classification of Diseases, Revision 10 (ICD-10) of the World Health Organization (WHO 1993). In both manuals, these symptoms are grouped into domains of Inattention, Hyperactivity, and Impulsivity, although in slightly different ways (see Table 1). Some additions to this list have been proposed for DSM V (Kessler et al. 2010), including four additional symptoms of impulsivity that are listed in Table 1.

Table 1 Symptoms of ADHD in DSM-IV and HKD in IDC-10

Inattention (IN)	Hyperactivity (H)	Impulsivity (IMP)
Falls to attend to details	Fidgets with hands or feet	
Difficulty sustaining attention	Leaves seat in classroom	
Does not seem to listen	Runs about or climbs	
Falls to finish	Difficulty playing quietly	
Difficulty organizing tasks	Motor excess ("on the go" in DSM-IV)	
Avoids sustained effort	Talks excessively (DSM-IV)	Talks excessively (ICD-10)
Loses things		Blurts out answers to questions
Distracted by extraneous stimuli		Difficulty waiting turn
Forgetful		Interrupts or intrudes on others
		Proposed DSM V additions: Acts without thinking Impatient (feeling restless, driving too fast, etc.) Uncomfortable doing things slowly Difficulty resisting temptations or opportunities

The ADHD/HKD symptoms evolved from the core features described over 100 years ago by Still (1902), which included the domain of cognitive or attention deficits now considered to represent deficits in executive function, behavioral inhibition, controlled processing, and delayed response, as well as the domain of excess motor activity. In a seminar commissioned to celebrate the centenary of the three-part publication by Still (1902) in The Lancet, Swanson et al. (1998) outlined several basic features of ADHD that still hold today: (1) "The ratio of boys to girls with ADHD/HKD is between 3:1 and 9:1 but this may decrease with age," (2) "although hyperactive/impulsive symptoms decrease with age the symptoms of inattention do not," (3) "DSM and ICD manuals recognize the same problem behaviors as the basis for diagnosis," and (4) "there are major difference in decision rules . . . that make HKD a subset of ADHD" (in ICD but not DSM, the full syndrome with symptoms from all three domains is required; the comorbid presence of conduct disorder is a separate disorder; the presence of internalizing disorders is an exclusion criterion; and the pervasiveness across parent and teacher is required).

As an introduction, a brief review will be provided to present some consensus views about sex differences in ADHD. An influential meta-analysis by Gaub and Carlson (1997) provided a critical review of sex differences in children with categorical diagnoses of the disorder in the pre-DSM-IV (APA 1994) era. They reviewed studies of ADHD [or its equivalent diagnosed according to the criteria provided by DSM-II (APA 1968), DSM-III (APA 1980), and DSM-III-R (APA 1987)] and noted an enduring characteristic relevant to the topic of this chapter: the observed male-to-female sex ratio was higher for clinic-based samples (6:1–9:1) than for population-based samples (2:1–3:1). Compared to boys, girls manifested

clear differences in several domains (“. . .lower levels of hyperactivity, fewer conduct disorder diagnoses, lower rates of other externalizing disorders”), but these differences depended on referral source. In general, girls were less impaired than boys only in the population-based samples, whereas among clinic-based samples “. . .girls and boys with ADHD showed similar levels of impairment (with the exception of inattention, for which a trend of greater severity among girls was found)” (p 1041). The authors speculated that, compared to girls, boys with ADHD had “. . .more disruptive behaviors within structured settings, leading to higher referral rates” (p 1042).

Two studies of ADHD cases diagnosed by DSM-III-R criteria were not reviewed by Gaub and Carlson (1997) since they were published after 1994. Biederman et al. (1996) evaluated a clinic-referred sample of 140 boys and Biederman et al. (1999) evaluated a clinic-referred sample of 140 girls. Biederman et al. (2002) reviewed the findings from these two studies and concluded that sex was associated with clinical presentation of ADHD “. . .largely due to the finding that girls with ADHD were less likely than boys with ADHD to have comorbid disruptive behavior problems, learning disabilities, and social dysfunction and that the rate of inattention was higher in girls with ADHD” (p 41). Several other analyses were performed on the rich sets of data from these two cohorts. Faraone et al. (2000) evaluated the familial dose hypothesis that girls need more familial risk to develop ADHD than boys. However, the percentage of relatives with ADHD in the girl cohort ADHD (18 % of female and 24 % of male relatives) was not significantly different than in the boy cohort (12 % of female and 20 % of male relatives), refuting this genetic account of lower prevalence of ADHD in girls than boys. Biederman et al. (2002) evaluated environmental risk factors, which were higher in the overall ADHD group than the control group but did not show a gender difference. Seidman et al. (2005) evaluated performance on a neuropsychological test battery, which revealed deficits relative to non-ADHD controls, but sex difference was not significant. Biederman et al. (2005) evaluated the non-referred siblings ($n = 577$), and in the affected males ($n = 73$) and females ($n = 25$) there were no sex differences in psychiatric diagnoses or clinical correlates of ADHD. Overall, Biederman et al. (2005) concluded that the differences in sex ratios “. . .in groups of subjects seen in clinical settings may be caused by referral biases” (p 1083).

Subsequent studies of children diagnosed by DSM-IV criteria addressed sex differences in subtypes of ADHD (Inattentive, Hyperactive/Impulsive, and Combined) that were not specified by DSM III-R criteria (see Lahey et al. 1994). In a clinic-referred sample, Lahey et al. (2005) provided basic information on the prevalence and stability of the subtypes. In 118 children, 98 were male (for a sex ratio of 4.9:1) and the percentages with the three DSM IV subtypes at 4–6 years of age were 70.3 % with the Combined, 19.5 % with the Hyperactive/Impulsive, and 10.2 % with the Inattentive subtype, but the percentages changed by 11–13 years of age to 33.3 % with the Combined, 1.9 % with the Hyperactive/Impulsive, and 35.2 % with the Inattentive subtype, and 35.2 % with no ADHD diagnosis. They proposed the Combined and Inattentive subtypes were stable but the Hyperactive/Impulsive subtype was not, and they recommended a gradient of severity of Hyperactive/Impulsive

symptoms as a diagnostic qualifier. In contrast, in a non-referred sample (e.g., DuPaul et al. 1998), the Inattentive subtype had the highest prevalence (70 %), with about 19 % with the Combined and 11 % with the Hyperactive/Impulsive subtypes. However, a general finding is that the presence of hyperactive/impulsive symptoms is greater in males than females (see Wolraich et al. 1996).

In referred samples with confirmed DSM-IV diagnoses of ADHD, neuropsychological studies have been consistent with the studies of DSM-III-R diagnoses. For example, in a neuropsychological study of 572 referred children and adolescents, Goth-Owens et al. (2010) evaluated the Combined and Inattentive subtypes using tests of processing speed (i.e., the Trailmaking and Stroop tests). They found ADHD versus control differences but they did not find sex differences. In a cohort of girls with ADHD (n = 82 with Combined type and 40 with Inattentive type), Hinshaw et al. (2007) documented a pattern of neuropsychological deficits compared to controls that persisted into adolescence but with little difference in performance across the subtypes.

The recognition of ADHD in adults emerged from follow-up studies of children identified in the pre-DSM III (APA 1980) era, which suggested that the condition persisted in some cases but not all cases. Faraone et al. (2006) reviewed the literature and concluded that, for the full criteria for ADHD, the rate of persistence was about 15 % at 25 years of age but, for relaxed criteria, it was higher (up to 65 %). However, ADHD in adults was considered rare in 1994, and it was not surveyed in the baseline National Comorbidity Survey that provided estimates of prevalence of adult psychiatric disorders based on DSM-IIIR criteria (see Kessler et al. 1994). By 2001, evidence supporting the concept of adult ADHD had accumulated, so ADHD based on DSM-IV criteria was surveyed in the National Comorbidity Survey replication (see Kessler et al. 2005a), which estimated a prevalence of 5.4 % in males and 3.2 % in females, for an observed sex ratio of 1.7 in this sample (see Kessler et al. 2006).

A retrospective account of childhood symptoms can be used to estimate the persistence of ADHD from childhood to adulthood. For example, for adults in the USA, Kessler et al. (2005b) estimated that 8.1 % of the adult population reported childhood symptoms of ADHD, which persisted into adulthood in 36.3 %. There was no significant sex difference in the persistence of ADHD: in males, 9.8 % reported childhood symptoms that persisted into adulthood in 39.7 %, and in females, 6.4 % reported childhood symptoms that persisted in 31.5 %. Lara et al. (2009) reported similar estimates for nine additional countries, with 3.2 % as the average prevalence of adult ADHD and persistent ADHD in 50.0 % of these cases, with no sex difference. Sánchez-Mora et al. (2011) evaluated 1,440 adults with persistent ADHD (defined by onset before age seven, life-long persistence, and current diagnosis) in the International Multicentre Persistent ADHD Genetic Collaboration (IMPACT) study and reported that 55.2 % were male and 44.8 % were female, yielding a sex ratio of 1.23:1 in this sample.

A simple comparison of the recognition rates for males and females in childhood and adulthood suggests the observed sex ratio may decrease with age. As pointed out earlier, in epidemiologic studies of children (Visser et al. 2010) and adults (Kessler et al. 2005a,b), 10.4 % of boys and 4.0 % of girls were reported to have

received a medical diagnosis of ADHD (a 2.6:1 sex ratio) while 5.4 % of men and 3.2 % of women met the criteria for a diagnosis of ADHD (a 1.7:1 sex ratio). A simple comparison of these reports may suggest that the prevalence in males decreases by 50 % from childhood to adulthood (10.3–5.4 %) whereas in females the prevalence remains about the same (4.0–3.2 %), resulting in a decrease in the observed sex ratio. However, these studies differ on multiple factors, such as when they were conducted (2001 for adults vs. 2007 for children) and methods of recognition of ADHD (parent report of clinical diagnosis in children versus direct assessment of randomly selected adults).

In summary, this brief background indicates that, in studies of ADHD children diagnosed both by DSM-III-R (before 1994) and DSM-IV criteria (after 1994), the observed sex ratios favor boys over girls. One consistent finding is that the sex ratio is much larger in the clinic-based samples than in population-based samples. However, in studies that evaluate rigorously diagnosed cases of boys and girls, sex differences in risk factors for ADHD or in performance on neuropsychological tests of attention typically were not significant.

Increasing Trends of Recognition and Treatment

In the pre-DSM III (APA 1980) era, the recognition and treatment rates were low and were considered to be below the estimates of population prevalence of ADHD. Dramatic increases in the recognition and treatment of children with ADHD occurred in the early 1990s in the USA, accompanied by increases in the supply and prescriptions of stimulant medications. These increases were interpreted as corrections for prior under-recognition and under-treatment (see Swanson et al. 1995).

We speculated there should be an asymptote for the increasing annual supply and number of prescriptions for stimulant medication, which would be logical for a condition that reflects psychopathology and thus would have a fixed and relatively low true prevalence in the population (e.g., for ADHD, 3–5 % as stated in DSM criteria). However, regular increases in the USA have continued for more than two decades, which we have documented (Swanson and Volkow 2009) based on records of national supplies and prescriptions. The supply records were obtained from the United Nations, which tracks the annual supply and consumption in terms of the statistical Defined Daily Dose (S-DDD) per 1,000 inhabitants in each county. The DDD for methylphenidate is set as 30 mg/day and for amphetamine as 15 mg/day. The prescription records were obtained from commercial services (e.g., VeriSpan Vector One®).

As shown in Fig. 1 (from Swanson and Volkow 2009), from 1996 to 2006 in the USA, prescriptions increased from about 15 million to about 30 million and the national supply increased from 5 DDD to 17 DDD. For the population of the USA—over 300 million total and 40 million children—the 2006 supply would have been sufficient to treat five million individuals with 30 mg/day for 365 days/year. The linear increases indicate that the expected asymptote had not occurred by 2006.

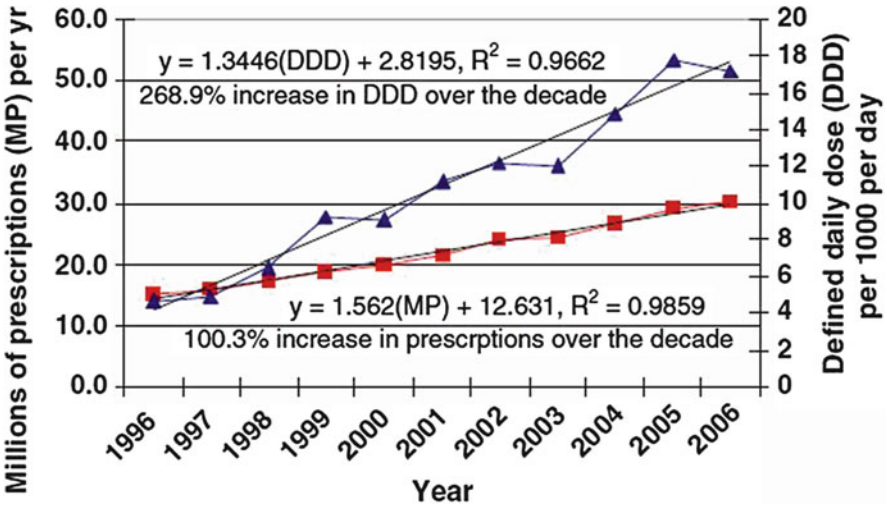

Fig. 1 A decade of increases in national supply and prescriptions for stimulants

In our prior report, we did not describe differences in prescription rates by sex, which is particularly relevant for the main topic of this chapter. Here we extend our prior report by presenting annual prescription rates by sex and age (in 10-years increments) from 2002 through 2009. As shown in Fig. 2, prescription rates were stable for children 10 years of age and younger, but they were increasing for the other ages. For adults, in 2002 the prescription rates were about the same for males and females, suggesting a sex ratio of about 1:1. However, since 2002, increases in prescriptions for adults in the USA were steeper for females than for males. As a result, for each age group over 20 years of age, by 2009 the annual rate of prescriptions for stimulants was greater for females than for males.

In summary, the sex difference in the prevalence of ADHD is well established in children, favoring boys over girls. However, the sex difference is much larger in clinic-referred samples than in population-based samples, due to dependence on factors other than symptom severity that influence referral for clinical treatment. Prescription records provide a measure of treatment rates, and the annual rate for children and the annual number of prescriptions for stimulant medication have remained constant since 2002, favoring males over females with a sex ratio about 2:75:1. In contrast, for adults the number of annual prescriptions has been increasing, and the increase has been greater for females than males. By 2009, there were from 15 % to 50 % more prescriptions for females depending on age.

Dimensional and Categorical Assessments of ADHD

The DSM approach is based on a categorical diagnosis of ADHD (i.e., positive when a patient is thought to have a disorder and negative otherwise). The gold standard for diagnosis of a child suspected of ADHD is a psychiatric interview with

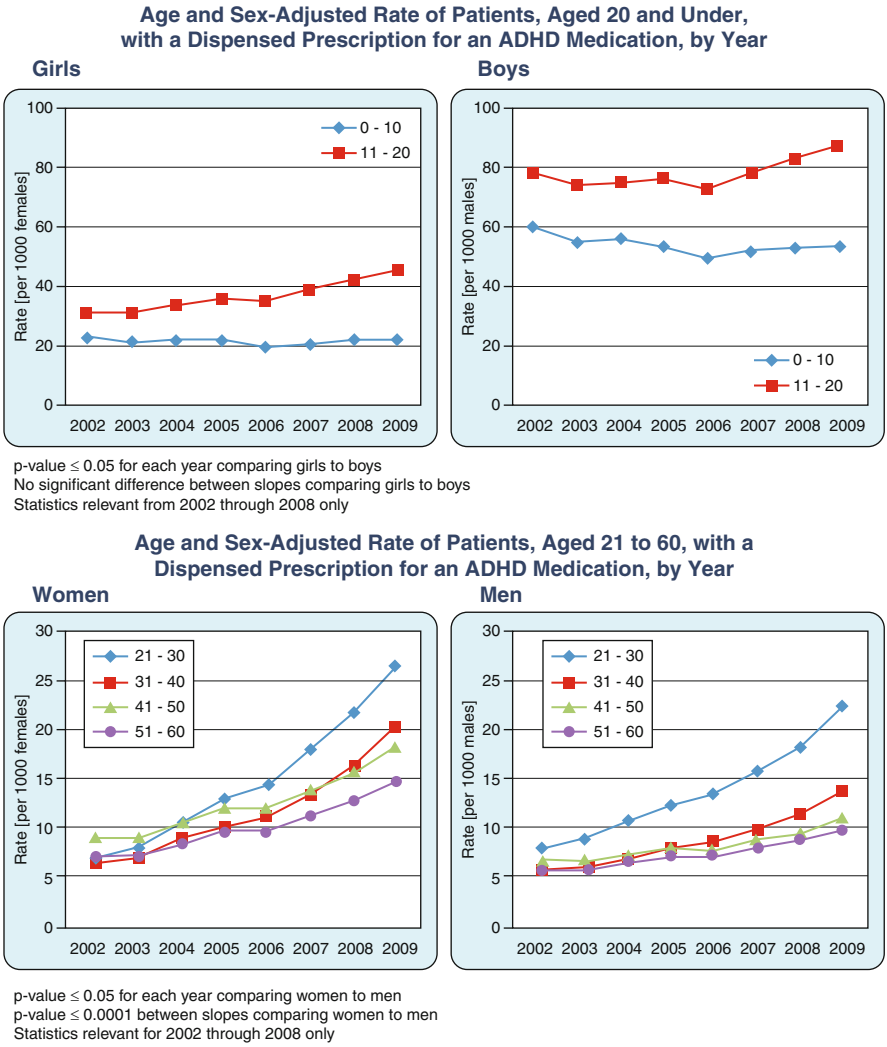

Fig. 2 Recent trends in prescriptions by age and sex (from Verispan, a healthcare-information company)

the parents by an experienced clinician who filters the information from the interview and makes qualitative judgments about symptom presence (for each item) and impairment (based on the overall symptom-related impact on functioning across multiple settings, usually the home and school).

In preparation for future changes in DSM V, the American Psychiatric Association proposed dimensional adjuncts to categorical diagnosis (see Helzer et al. 2008). Evaluation of symptom severity rather than symptom presence or absence provides one way to accomplish this (Kraemer 2007). Elsewhere, we have discussed the

proposed dimensional adjuncts in the revisions underway for to DSM V (see Swanson et al. 2007) and the concept of spectrum disorders applied to ADHD (see Swanson et al. 2011). Some of the key points will be summarized here.

There is a long and illustrious history of rating scales in the assessment of symptom severity associated with ADHD and its equivalent or related conditions (for an early example, see Cytryn et al. 1960). Eisenberg et al. (1962) described the prototype symptom checklist designed to quantify problem behavior of preschool children in a "therapeutic nursery program" in the Baltimore school system. For a range of problem behaviors, teachers were asked to rate children on a four-point scale of symptom severity (Very Much, Pretty Much, Just a Little, or Not at All). In this innovative early development of a new instrument for child psychiatry, ratings of children in regular classrooms were compared to ratings of children in the special classroom (with 65 % of the students placed there for "discipline problems"). Eisenberg et al. (1962) provided an insightful and still relevant discussion of sex differences: in regular classrooms, boys received higher ratings than girls on the symptom checklist, but in special classrooms there was no sex difference in these ratings. Conners et al. (1970) used this four-point scale (in the reversed order) to develop a Hyperactivity Index based on 10 items well before the modern symptom lists were published in DSM III for attention deficit disorder (APA 1980). Swanson et al. (1983) used this four-point symptom severity scale with the DSM III symptoms to develop the Swanson, Nolan and Pelham (SNAP III) rating scale and updated it with the DSM IV (APA 1994) symptoms to develop the SNAP IV (Swanson 1994). Several similar ADHD symptom rating scales were developed based on DSM IV symptoms and the four-point symptom severity scale, including the Vanderbilt Rating Scale (Wolraich et al. 1996), the DuPaul Rating Scale (DuPaul et al. 1998), the Disruptive Behavior Disorders Rating Scale (Molina et al. 1998), and the Conners Rating Scales (Conners 2008).

Collette et al. (2003) provided a thorough review of some of the psychometric properties of these rating scales, which all show about the same characteristics (e.g., adequate test-retest reliability, validity, etc.). However, another property of these rating scales imposes a serious limitation. In population-based samples (as opposed to clinic-based samples), the ratings of symptoms are not normally distributed. An example is provided by the SNAP-IV rating scale of ADHD symptoms (see Swanson et al. 2001), which uses the traditional four-point rating scale for symptom severity. The summary ratings for a population-based sample are highly skewed to the right, which is to be expected since—by definition—most individuals in the population do not manifest psychopathology, a defining feature of a symptom of a disorder.

The development of norms for symptom severity ratings presents some logical and statistical challenges. Typically, norms are based on ratings in a population sample, in which the absence of the behavior to be rated (calling for a response of "Not at All" on the four-point scale) is expected in most cases and the severity of the problem behavior (defined as a "symptom") is rare. Thus the ratings are not normally distributed in the population. Despite this, one approach is to use T-scores (a transformation of the z-score with a mean of 50 and a standard deviation of 10), with a statistically extreme score 1.5 SDs above the mean (i.e., a T-score of 65) as a cutoff. This approach assumes a normal distribution, and the skewed distribution of

symptom severity violates this assumption. The lack of a normal distribution is not unique to ADHD rating scales. For example, Achenbach and Rescorla (2001) addressed this problem in ratings of problem behavior from the widely used Child Behavior Check List. Due to skewness, they recommended rather torturous modifications of T-scores, including truncation of lower T-scores at 50 and interpolated (and expanded) assignment of higher T-scores above 70.

Similar problems have been recognized in discussion of norms for the ADHD rating scales, and a variety of solutions have been proposed. Transformation of the skewed distribution has been discussed by Conners (2008), who advised against transformation based on the observation that "the tendency to psychopathology may not be normal in shape." Instead of using T-scores to obtain theoretical percentiles based on the normal distribution, Conners (2008) recommended the use of empirical percentiles. Some others do not recommended the use of T-scores (see Power et al. 1998, for the DuPaul ADHD rating scale and Swanson et al. 2001 for the SNAP), but nonetheless means and standard deviations for population samples are presented. No method to adjust for the expected skewness of symptom severity ratings offers a good solution to this problem.

In recent discussions of underlying behavior of ADHD symptoms (Swanson et al. 2007) and of ADHD as a spectrum disorder (Swanson et al. 2011), we reviewed another way to address skewness of symptom severity ratings in the population. Swanson et al. (2001) proposed to avoid skewness in population-based ratings by redefining the ADHD items and the rating format to cover the full range of behavior in the population. The content remained the same for each of the 18 ADHD items, which had been used to construct the SNAP IV rating scale based on the standard four-point format for symptom severity (0 = Not at All, 1 = Just a Little, 2 = Quite a Bit, and 3 = Very Much). For the new rating scale, a format was developed with an anchor at 0, defined as "average" for the population. As summarized by Swanson et al. (2012), one side of the anchor covered the usual range of problems or weaknesses defined by how far it was judged to be below average (i.e., with symptom severity scored on the usual scale, +1, +2, and +3), but on the other side the ratings covered the opposite of symptom severity—the range of strengths (i.e., how far above average, with anti-symptoms scored −1, −2, and −3). The new rating scale was named the Strengths and Weaknesses of ADHD-symptoms and Normal-behavior (SWAN) rating scale.

As a result of the rewording and revised response options, the distribution of the summary score (the average rating per item) of the SWAN rating scales approximated the normal distribution in a population sample (see Fig. 3), which is in stark contrast to the distribution of the average rating per item of the SNAP rating scales that is based on the same content (defined by the 18 ADHD items) but uses the traditional four-point symptom severity scale (Swanson et al. 2001).

As shown in Fig. 3, the symptoms of ADHD are not present in most individuals in the population, so the modal response on the four-point scale of the SNAP is "Not at All," and the modal summary score is near 0, which creates the impression of a highly skewed distribution. The lack of skewness of the SWAN distribution (see Lakes et al. 2011; Kudo et al. 2012) suggests that skewness of the SNAP distribution may be a measurement artifact. On the SWAN, these non-affected

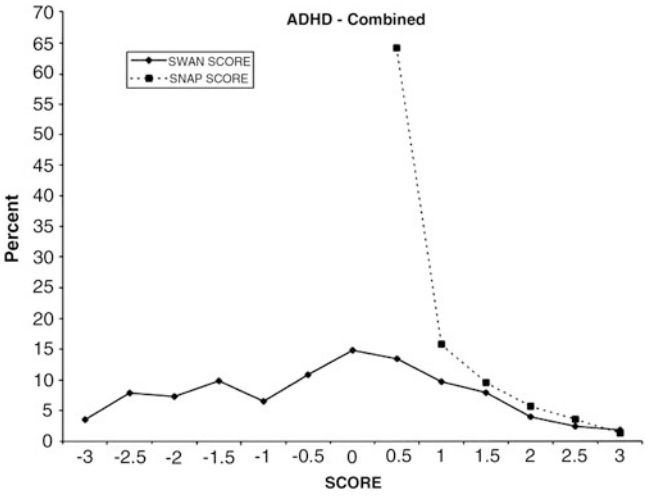

Fig. 3 Distribution of SNAP and SWAN in a population-based sample

individuals vary in placement on the dimension defined by the full range of behavior, which results in a distribution that appears to approximate the normal distribution. The non-skewed distribution of the SWAN in population samples made it attractive both for defining the extremes of the distribution for genetic studies (see Cornish et al. 2005) and for use in twin studies (see Hay et al. 2005 and Poderman et al. 2007).

One of the most definitive studies of the distributional characteristics of the SWAN was based on a population sample of adolescents in northern Finland (Smalley et al. 2007). This study used a two-stage process to diagnose ADHD. First, the SWAN was used to screen a population of 6,622 adolescents, and the overall (across males and females) 95th percentile cutoffs on the SWAN subtype scores (Inattentive, Hyperactive/Impulsive, or Combined) were used to identify individuals who exceeded a cutoff for any subtype. Based on the near-normal population distribution of the SWAN ratings (see Fig. 4), this percentile method identified slightly more than 5 % (530 individuals, consisting of 346 males and 184 females, or 8 % of the population). Based on this finding, the percentile method produced a sex ratio of 1.9:1. Using the same data from the SWAN from this sample, Lubke et al. (2007) applied factor mixture modeling (a combination of factor analysis and latent class analysis) and identified two dimensions that are moderately correlated and reflect liability for ADHD. These dimensions were similar in separate analyses of males and females and identified a small class of individuals at the extreme for both males (8.4 %) and females (6.8 %), yielding an observed sex ratio of 1.3:1.

Second, a psychiatric interview was conducted, and DSM IV criteria were applied to make diagnoses of current (adolescent), retrospective (childhood), and lifetime ADHD. The estimated sex ratio for these ADHD diagnoses were 5.7:1, 5.2:1, and

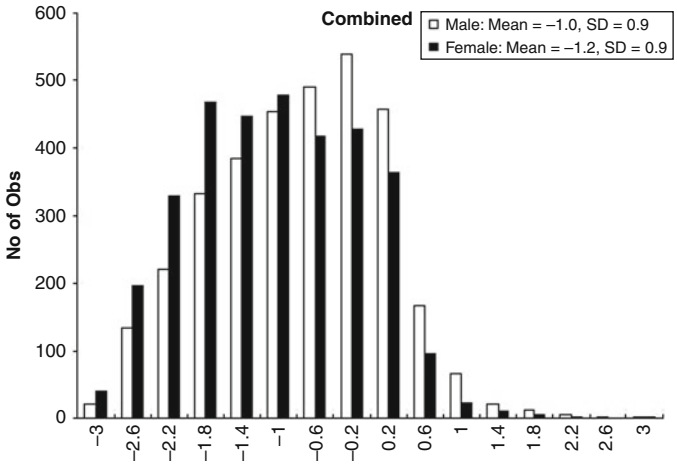

Fig. 4 Distribution of SWAN in a population-based sample of adolescents (From Smalley et al. 2007)

6:1:1, respectively. Therefore, the observed sex ratio for the categorical diagnosis was about 3 times higher than for the percentile method based on the SWAN.

In summary, in this population sample of adolescents from northern Finland, the observed sex ratio differed dramatically for ADHD defined by a categorical approach (based on qualitative differences in behavior to identify psychopathology in a clinical population) and a dimensional approach (based on quantitative differences in behavior in the population). In this example, the dimensional assessment method yielded a much lower observed sex ratio that the categorical assessment method.

Sex Differences in the Longitudinal MTA Study

The Multimodal Treatment study of ADHD (MTA) evaluated a referred sample (n = 579) of cases with confirmed diagnosis of DSM-IV ADHD-Combined Type (see MTA Group 1999a). The MTA sample was recruited from a variety of sources (i.e., clinics, advertisements, etc.), and 114 of the 579 cases were girls. The sex ratio of the MTA was 466:114 = 4.0 in favor of boys.

The MTA provides information on sex differences in response to treatment. The MTA was designed as a 14-month randomized clinical trial (RCT) of four assigned treatment conditions: Medication Management (Med), Behavior Modification (Beh), their Combination (Comb), and a Community Comparison (CC). The Med condition was based on the MTA medication algorithm that included an individualized double-blind, dose–response, titration phase to select the best dose for each individual (from 10 to 60 mg/day), followed by a 1-year maintenance phase with dose increases if effectiveness waned. The main finding (see MTA Group 1999a) at the end of the 14-month treatment phase of the RCT was a general

reduction in symptom severity (measured by parent and teacher ratings on the SNAP-IV ADHD scale). However, the reduction was greater for the randomly assigned conditions with stimulant medication provided by the MTA as a component of treatment (Med and Comb) than for those without medication provided (Beh and CC). The finding most relevant for this chapter is related to the moderator analysis of sex that was performed (MTA Group 1999b). In the 14-month RCT, the observed responses to treatment were not significantly different for girls and boys: on almost all (13 of the 14) core dependent variables, girls as well as boys had a larger reduction of symptom severity in the treatment conditions with stimulant medication as a component than in those that did not.

The observed sex ratio in the MTA sample when it was recruited in childhood (between the ages of 7.0 and 9.9 years) was 4.0:1, which was in line with expectations from the literature. In the publications describing the longitudinal follow-up at the 36-month (e.g., Swanson et al. 2007) and the 8-year (Molina et al. 2009) assessments, we did not report the sex ratio in persistence of the diagnosis that we observed at older ages, which is very relevant to this chapter. Studies using cross-sectional design suggest that the prevalence of persistent ADHD in adults is about the same in males and females (see Kessler et al. 2005), but this finding has not been confirmed in a longitudinal study. In the 10-year MTA follow-up, when the participants were between 17 and 20 years of age, we determined how many cases still met the same criteria used for diagnosis when they were between 7 and 10 years of age (i.e., the full DSM-IV criteria for ADHD Combined type). As expected from the literature (e.g., see Faraone et al. 2005), most cases no longer met the full criteria in adulthood but, for those who did (14 %), the persistence rate was higher for males (16 %) than for females (9 %). Thus, a lower persistence of ADHD symptoms in males than females does not account for the lower observed sex ratio in adults than in children. Instead, the convergence may be due to different underlying characteristics of ADHD that are dependent on the age when the disorder is recognized and diagnosed.

In our ongoing evaluation of a possible side effect in the MTA, stimulant-related growth suppression (see MTA Group 2004 and Swanson et al. 2007), we became aware of another sex difference. Since we used the same age range (7.0–9.9 years of age) as an entry criterion for both males and females, we created a large sex difference in physical maturation at the time of entry into the study. This is a consequence of a difference in the onset of the adolescent growth spurt, which in the general population is about 2 years earlier for girls than boys (see Tanner 1990). Therefore, for the same age range at baseline for boys and girls, a large sex difference in physical maturation would be expected. If the manifestation of ADHD depends on maturational level rather than age, then part of the difference in observed sex ratios in childhood and adolescence may be related to an expected difference in maturation for boys and girls and not to sex itself.

We used modern methods from auxology (see Bock et al. 2005) to document the onset of the adolescent growth spurt as an indicator of maturity. The rationale for this approach is summarized by Hauspie et al. (2004), who reviewed some fundamental characteristics of human growth. As they stated, “somatic growth from

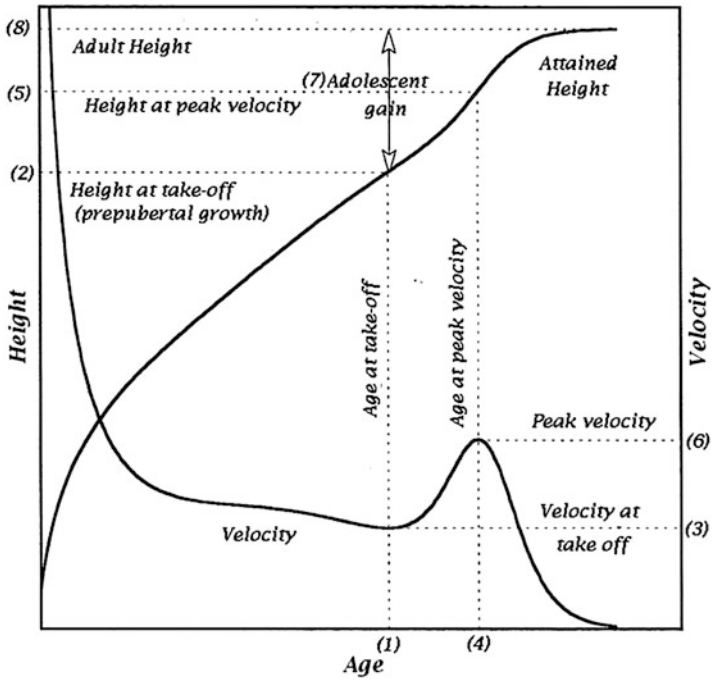

Fig. 5 Height and velocity curves for human growth with milestones

conception to adulthood consists of three phases, which are under the control of different hormonal systems": the infancy phase under the control of thyroid hormones, with a very rapid growth rate that declines sharply until about 2 years of age; the childhood phase under the control of growth hormones, with a gradually declining growth rate until reaching a minimum at the "take off" point of the adolescent growth spurt; and the pubertal phase under the control of sex hormones that determine the onset and the "peak" point of the adolescent growth spurt and also lead to closure of the epiphyses and the termination of growth. As shown in Fig. 5, milestones of the childhood and adolescent phases of growth are provided by the age, height, and velocity at (1) the take-off point of slowest growth in the childhood phase and (2) the peak point of fastest growth in the adolescent phase.

In the MTA, height measures collected at the baseline assessment (ages 7–10) and at 14 months (ages 8–11), 24 months (ages 9–12), 36 months (ages 10–13), 6 years (ages 13–16), 8 years (ages 15–18), and 10 years (ages 17–20) after baseline have been analyzed (see Bock, 2011). Height measures collected at, 12 years (ages 19–22), 14 years (ages 21–24), and 16 years (ages 23–26) after baseline have been collected and are in the process of being analyzed. We used a computer program (see Bock et al. 2005), the Auxological Analysis of Longitudinal Measurement of Human Stature (AUXAL), to obtain estimates of age at "take off" point (as well as other milestones shown in Fig. 5) for individuals in the MTA. Based on height

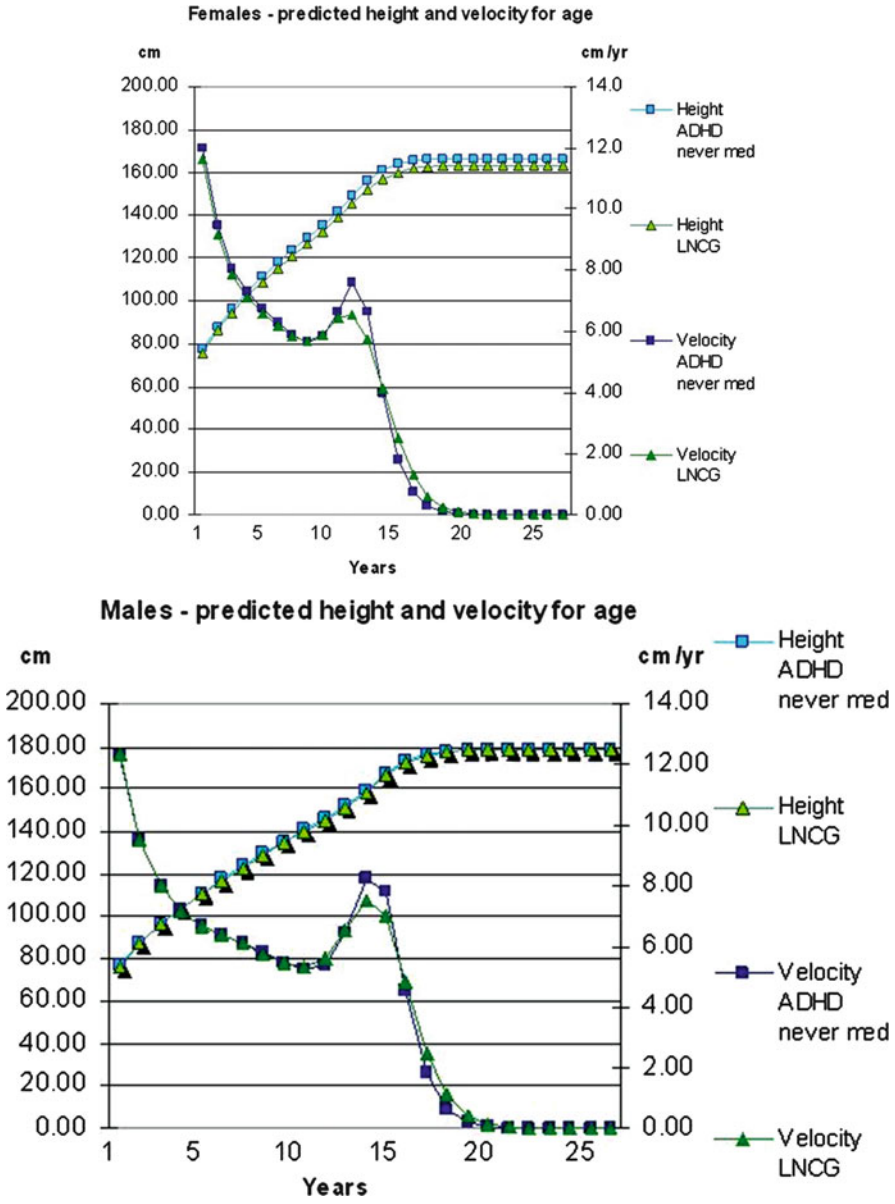

Fig. 6 Height and height velocity curves for ADHD (untreated) cases in the MTA and the local normative comparison group (LNCG) of classmates (adapted from Bock, 2011)

measures through the 10-year assessment (see Bock, 2011), there were sufficient longitudinal data to obtain estimates for 568 of the 579 cases, as well as for 288 classmates in the local normative control group (LNCG) of the MTA.

The group height and height velocity curves (see Fig. 6) from AUXAL reveal two findings that are relevant to this chapter: (1) compared to controls, ADHD children are not delayed in physical maturation as measured by the adolescent growth spurt, and (2) girls with ADHD transition from childhood to the adolescent growth spurt about 2 years earlier than boys with ADHD. Using the individual estimates of milestone for the cases in the MTA, we estimated that only 24 % of the boys but 90 % of the girls were already in or entered the adolescent growth spurt during the initial 14-month RCT phase of the MTA.

In summary, the entry criteria for the MTA accepted only one subtype of ADHD (ADHD-Combined Type), allowed only a restricted age range (7.0–9.9 years), and used multiple sources for ascertainment (clinic referrals, response to advertisement, etc.). Under these conditions, the sex ratio was 4:1, which is in line with expectations for a clinic-referred sample of school-aged children. Using height velocity to estimate the onset of the adolescent growth spurt, the aged-matched subgroups of boys and girls differed significantly (as expected) in terms of this sign of physical maturation. Over the developmental stages from childhood through adolescence and into adulthood, most cases no longer met the full criteria for ADHD Combined type. The persistence of ADHD was about the same for males and females, so the observed sex ratio was similar in the MTA follow-up in adulthood as in the initial assessment in childhood.

Conclusions

We have addressed the main topic of this symposium — multiple origins of sex differences — by discussing observed sex differences in ADHD from three perspectives: (1) a secular trend (which shows dramatic increases in recognition and treatment of ADHD over the past decade); (2) a dimensional rather than a categorical approach to assessment (which defines behavior underlying ADHD symptoms that is normally distributed in the population), and (3) a longitudinal study of ADHD cases (which offers an improvement over cross-sectional studies for evaluating the effects of maturational level as well as age on persistence of ADHD symptoms). As we have discussed here, these three factors may have contributed to decreases in the observed sex ratio that historically has favored males over females.

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Fetal Testosterone in Mind: Implications for Autism

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Abstract Autism Spectrum Conditions (ASC) are strongly biased towards males, with a male:female ratio of 4:1 for classic autism and over 10:1 for Asperger syndrome. The cause of the observed sex difference in ASC remains a topic of debate. The Extreme Male Brain (EMB) theory proposes that autism is an exaggeration of typical sex differences in empathizing and systemizing. Here we review evidence that levels of prenatal androgens (particularly testosterone) lead to masculinization of the brain and play a role in empathy, systemizing, and autistic traits. Evidence that elevated testosterone levels may be a risk factor for ASC is discussed.

Sex Biases in Clinical Conditions

A number of clinical conditions occur in males more than females, including autism, dyslexia, specific language impairment, attention-deficit hyperactivity disorder (ADHD), and early-onset persistent antisocial behavior (Rutter et al. 2003). This fact raises the question of whether there are sex-linked or sex-limiting factors involved in the etiology of conditions that do exhibit this male bias.

Autism and Asperger syndrome are referred to as Autism Spectrum Conditions (ASC)[1] and are considered to lie on the same continuum. Individuals with an ASC

[1] The American Psychiatric Association uses the term ASD for Autism Spectrum Disorders. We prefer the use of the term ASC, as those at the higher-functioning end of the autistic spectrum do not necessarily see themselves as having a 'disorder,' and the profile of strengths and difficulties in ASC can be conceptualized as atypical but not necessarily disordered. ASC remains a medical diagnosis, hence the use of the term 'condition,' which signals that such individuals need support. Use of the term ASC recognizes that the profile includes areas of strength (e.g., in attention to detail) as well as areas of difficulty.

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diagnosis are impaired in reciprocal social interaction and communication and show strongly repetitive behaviors and unusually narrow interests (APA 1994). The prevalence of ASC is estimated to be 1 % (Baird et al. 2006; Baron-Cohen et al. 2009). These conditions have a strong neurobiological and genetic component (Stodgell et al. 2001). The male to female ratio in ASC is estimated at 4:1 for classic autism (Chakrabarti and Fombonne 2005) and as high as 10:1 in Asperger syndrome (Gillberg et al. 2006).

The striking sex ratio in ASC might provide important clues to the etiology of the condition (Baron-Cohen 2002; Baron-Cohen et al. 2005). The specific factors (hormonal, genetic, or environmental) that are responsible for the higher male prevalence of ASC remain unclear. In this chapter we review evidence that sex steroid hormones and, in particular, prenatal exposure to testosterone, may be related to the development of autistic traits (Baron-Cohen et al. 2004).

ASC has been described as an extreme manifestation of certain sexually dimorphic traits [an "extreme male brain" (EMB); Baron-Cohen 2002; Baron-Cohen et al. 2005]. Hormones have a clear role in physical and behavioral sexual differentiation (Hines 2004). We begin by reviewing evidence that prenatal exposure to hormones affects the development of cognitive sex differences and traits associated with ASC.

Typical Sex Differences and ASC

Males and females show differences in neuroanatomy, cognition and behavior from an early age. In early development, female infants show a stronger preference for looking at social stimuli (faces) from 24 h after birth (Connellan et al. 2000). Girls also make more eye contact immediately after birth (Hittelman and Dickes 1979), at 12 months of age (Lutchmaya et al. 2002a) and at 2 and 4 years of age (Podrouzek and Furrow 1988). Studies examining play preferences point towards more interest in mechanical and constructional play in boys, demonstrated by a preference to play with toy vehicles or construction sets, whereas girls are more likely to choose to play with dolls or toy animals (Berenbaum and Hines 1992; Liss 1979; Servin et al. 1999; Smith and Daglish 1977). In addition, sex differences in spatial ability favoring males have been found in infants as young as 3–4 months of age (Quinn and Liben 2008) and 5 months of age (Moore and Johnson 2008) and remain consistent throughout development (Voyer et al. 1995).

The EMB theory of ASC was initially defined using psychometric evidence and proposes that individuals with ASC are impaired in empathy (the drive to identify another person's emotions and thoughts, and to respond to these with an appropriate emotion) while being average or even superior in systemizing (the drive to analyze, explore and construct a system). The Empathy Quotient (EQ) and Systemizing Quotient (SQ) were developed to examine these domains (Baron-Cohen et al. 2003; Baron-Cohen and Wheelwright 2004). Findings from the EQ in children and adults have shown that, on average, females score significantly higher than males

(Auyeung et al. 2009c; Baron-Cohen and Wheelwright 2004; Carroll and Chiew 2006; Wheelwright et al. 2006). Results from the SQ indicate that, on average, males score significantly higher than females (Auyeung et al. 2009c; Baron-Cohen et al. 2003; Carroll and Chiew 2006; Wheelwright et al. 2006). Individuals with ASC show an extreme of the typical male profile, with adults and children with ASC scoring lower than typical males on the EQ and higher than typical males on the SQ (Auyeung et al. 2009c; Baron-Cohen et al. 2003; Baron-Cohen and Wheelwright 2004; Wheelwright et al. 2006).

Using the EQ and SQ, 'brain types' can be calculated, where individuals are described as being 'balanced' (Type B), better at empathizing (Type E) or better at systemizing (Type S). 'Extreme' empathizing (Extreme E) or systemizing (Extreme S) types are also assigned where an individual shows a significant discrepancy in different directions (Goldenfeld et al. 2005; Wheelwright et al. 2006). Type S (S > E) is more common in males, whereas Type E (E > S) is more common in females. Extreme types are also found, and a large proportion of children (47.2 %) and adults (61.6 %) with ASC fall in the Extreme S (S>> E), compared to approximately 5 % of typical males and 1 % of typical females (Auyeung et al. 2009c; Wheelwright et al. 2006).

Individuals with ASC tend to show impairment in empathy-related tasks that normally give rise to female superiority, such as the 'Social Stories Questionnaire' (Lawson et al. 2004), the 'Reading the Mind in the Eyes' task (Baron-Cohen et al. 1997) and the recognition of 'faux pas' in short stories (Baron-Cohen et al. 1999). Adults with ASC score lower on the Friendship and Relationship Questionnaire, which assesses empathic styles of relationships (Baron-Cohen and Wheelwright 2003). Children with autism perform less well than controls on the 'Feshbach and Powell Audiovisual Test for Empathy,' a measure of empathy and emotional responsiveness (Yirmiya et al. 1992). Children with ASC also show delays in passing 'theory of mind' tests compared to typically developing children (Happe 1995).

In non-social tasks, individuals with ASC are superior to typical controls on tasks that involve systemizing (Lawson et al. 2004) and on certain visuo-spatial tasks that normally give rise to male superiority, such as figure disembedding (Falter et al. 2008; Jolliffe and Baron-Cohen 1997; Ropar and Mitchell 2001; Shah and Frith 1983), block design (Ropar and Mitchell 2001; Shah and Frith 1993) and mental rotation (Brosnan et al. 2009; Falter et al. 2008).

Toddlers with ASC spent significantly more time fixating on dynamic geometric images, whereas typically developing toddlers showed longer looking times at social stimuli. Further, if a toddler spent more than 69 % of his or her time fixating on geometric patterns, then the positive predictive value for accurately classifying that toddler as having an ASD was 100 % (Pierce et al. 2010). These findings suggest that these looking preferences can be found very early in life and can be used to differentiate toddlers with ASC from typically developing toddlers.

In addition to the evidence at the behavioral level, characteristics of neurodevelopment in autism, such as larger overall brain volumes and greater growth of the amygdala during childhood, may also represent an exaggeration of typical sex differences in brain development (Baron-Cohen et al. 2005). Studies

using fMRI indicate that typical females show increased activity in the extrastriate cortex during the Embedded Figures Test and increased activity bilaterally in the inferior frontal cortex during the 'Reading the Mind in the Eyes' task. Parents of children with ASC also tend to show hyper-masculinization of brain activity, suggesting that hyper-masculinization may be part of the broader autism phenotype (Baron-Cohen et al. 2006). An extensive review of the EMB theory at both the behavioral and neural levels can be found elsewhere (Baron-Cohen et al. 2005, 2011).

Human Behavioral Sex Differences and Exposure to Hormones

Prenatal exposure to hormones has lasting effects on later behavior and development. Sexual differentiation of the mammalian brain occurs, at least in part, under the control of gonadal hormones, particularly androgens, during early development (De Vries and Simerly 2002; Ehrhardt and Meyer-Bahlburg 1981; Goy and McEwen 1980). Sex hormones also have an epigenetic role in changing gene expression throughout development and likely interact with sex chromosome effects on sexual differentiation (McCarthy and Arnold 2011; McCarthy et al. 2009).

There are thought to be two general types of hormonal effects: organizational and activational (Phoenix et al. 1959). Organizational effects are most likely to occur during early development when most neural structures are established, producing permanent changes in the brain (Phoenix et al. 1959), whereas activational effects are short term and are dependent on current hormone levels. Since neurodevelopmental conditions are typically persistent, with an early onset, any hormonal influence on neurodevelopmental conditions such as ASC is likely to be organizational in nature.

Organizational effects are thought to be maximal during sensitive/critical periods of development. These are hypothetical windows of time in which a tissue can be formed (Hines 2004). In animal models, the critical period for sexual differentiation of the brain occurs when differences in serum testosterone are highest between sexes (Smith and Hines 2000). Therefore, it is likely that this is an important period for sexual differentiation of the human brain as well. For typical human males, there is believed to be a surge in fetal testosterone (fT) levels at around weeks 8–24 of gestation (Baron-Cohen et al. 2004; Collaer and Hines 1995; Hines 2004; Smail et al. 1981), with a decline to barely detectable levels from the end of this period until birth. Any effects of fT on development are likely to take place during this period. For typical human females, fT levels are generally very low throughout pregnancy and childhood (Hines 2004). During the fT surge, male fetuses produce more than 2.5 times the levels observed in females (Beck-Peccoz et al. 1991).

The role of prenatal hormone levels in the development of later behavior in nonhuman mammals has been explored in experiments comparing castrated males, normal males, normal females, and females treated with androgens. Castrated males usually show feminized neural development, cognition and behavior,

whereas females treated with androgen show masculinized neural development, cognition and behavior, in a number of species (Arnold and Gorski 1984; Clark et al. 1996; Williams and Meck 1991).

The direct manipulation of fT levels is obviously not possible in humans for ethical reasons. The investigation of prenatal hormone exposure and its relation to development in humans has therefore been performed in naturally occurring, abnormal environments, such as in individuals with Congenital Adrenal Hyperplasia (CAH), a genetic disorder that causes excess adrenal androgen production prenatally in both males and females (New 1998). Girls with CAH show masculinization of performance in activities such as spatial orientation, visualization, targeting, personality, cognitive abilities and sexuality (Hampson et al. 1998; Hines et al. 2003; Resnick et al. 1986). Girls with CAH also exhibit more autistic traits, measured using the Autism Spectrum Quotient (AQ), than their unaffected sisters (Knickmeyer et al. 2006a).

The ratio between the length of the second and fourth digit (2D:4D) is sexually dimorphic, being lower in males than in females, and may be a useful proxy measure for fT production in humans during the first trimester of gestation (Manning et al. 1998). The sex difference in 2D:4D ratio is evident in fetuses between 9 and 40 weeks of gestation (Malas et al. 2006). The 2D:4D ratio is negatively associated with the ratio of fT to fetal estrogen (Lutchmaya et al. 2004). Lower (i.e., hyper-masculinized) digit ratios have been found in children with autism compared to typically developing children. This pattern was also found in the siblings and parents of children with autism, suggesting genetically based elevated fT levels in autism (Manning et al. 2001; Milne et al. 2006). If the 2D:4D ratio reflects prenatal exposure to testosterone, this evidence suggests children with ASC may have been exposed to higher than average levels of fT.

Due to the dangers of directly sampling fetal hormone levels, studies generally measure fT levels through amniocentesis samples that are obtained for clinical purposes. Amniocentesis is typically performed during a relatively narrow time window thought to coincide with the hypothesized critical period for human sexual differentiation (between approximately weeks 8 and 24 of gestation; Hines 2004). In early prenatal life, testosterone enters the amniotic fluid via diffusion through the fetal skin and later enters the fluid via fetal urination (Robinson et al. 1977). Males are exposed to testosterone from the fetal adrenals and testes. The female fetus is also exposed to androgens, but at lower levels. A number of studies have linked elevated levels of fT in the amniotic fluid with the masculinization of certain behaviors, beginning shortly after birth.

The Role of fT in Cognitive Sex Differences and Autistic Traits

The Cambridge Child Development Project is an ongoing, longitudinal study investigating the relationship between prenatal hormone levels and the development of behaviors relating to ASC (Baron-Cohen et al. 2004; Knickmeyer and

Baron-Cohen 2006). Mothers of participating children had all undergone amniocentesis for clinical reasons between 1996 and 2005 and gave birth to healthy singleton infants. These children have been tested postnatally at several time points and the findings of these studies are discussed next.

Eye Contact at 12 Months

Reduced eye contact is a characteristic common in children with autism (Lutchmaya et al. 2002a; Swettenham et al. 1998). The first study measured fT and estradiol levels in relation to eye contact in a sample of 70 typically developing, 12-month-old children (Lutchmaya et al. 2002a). Frequency and duration of eye contact were measured using videotaped sessions. Sex differences were found, with girls making significantly more eye contact than boys. The amount of eye contact varied with fT levels when the sexes were combined. Within the sexes, a relationship was only found for boys (Lutchmaya et al. 2002a). Results were taken to indicate that fT may play a role in shaping the neural mechanisms underlying social development (Lutchmaya et al. 2002a).

Vocabulary Size at 18–24 Months

In some subgroups within ASC, such as classic autism, vocabulary development is also delayed (Rutter 1978). Another study (of 87 children) focused on the relationship between vocabulary size in relation to fT and estradiol levels from amniocentesis. Vocabulary size was measured using the Communicative Development Inventory, a self-administered checklist of words for parents to complete (Hamilton et al. 2000). Girls have significantly larger vocabularies than boys at both time points (Lutchmaya et al. 2002b). fT inversely predicted the rate of vocabulary development in typically developing children between the ages of 18 and 24 months (Lutchmaya et al. 2002b). Within-sex analyses showed no significant relationships in boys or girls which the authors believe may have been due to the relatively small sample sizes. The significant findings in the combined sample suggest that fT may be involved in shaping the neural mechanisms underlying communicative development (Lutchmaya et al. 2002b).

Intentional Language at Four years

Thirty-eight children completed a ‘moving geometric shapes’ task at age 4, where they were asked to describe cartoons with two moving triangles whose interaction with each other suggested social relationships and psychological motivations

(Knickmeyer et al. 2006c). Girls used more mental and affective state terms to describe the cartoons. Girls also used more intentional propositions than males, and a negative relationship between fT levels and frequency of intentional propositions was observed when the sexes were combined, and in boys alone. These results are consistent with the EMB theory, since individuals with ASC show worse performance than typical males on a similar moving geometric shapes task (Klin 2000).

Restricted Interests and Social Relationships at Age Four

Individuals with ASC demonstrate more restricted interests as well as difficulties with social relationships (APA 1994). A follow-up study at 4 years of age used the Children's Communication Checklist (Bishop 1998). The quality of social relationships subscale demonstrated an association between higher fT levels and poorer quality of social relationships for both sexes combined but not individually. A lack of significant correlations within each sex may reflect the small sample size ($n = 58$). Levels of fT were also associated with more narrow interests when the sexes were combined, and in boys only (Knickmeyer et al. 2005). Sex differences were reported, with males scoring higher (i.e., having more narrow interests) than females (Knickmeyer et al. 2005).

Gender-Typical Play at Five to Nine Years

Girls with ASC have been shown to exhibit a more masculinized play style (Knickmeyer et al. 2007). At 6–9 years of age, higher fT levels predicted more male-typical scores on the Pre-School Activities Inventory, which is a standardized questionnaire measure of gender-typical play in both boys and girls. This relationship was significant in both boys and girls (Auyeung et al. 2009a).

Visuospatial Ability at Seven to Ten Years

The Embedded Figures Test (EFT) was used with this sample at 7–10 years and showed a clear advantage for boys, consistent with previous research showing superior male performance on this task (Nebot 1988). Individuals with ASC have been found to perform better on this task compared to controls figure disembedding (Falter et al. 2008; Jolliffe and Baron-Cohen 1997; Ropar and Mitchell 2001; Shah and Frith 1983). fT levels were found to be a significant predictor of EFT scores for all participants together and also when boys and girls were examined separately (Auyeung et al. 2012).

Mind Reading at Six to Nine years

Mind reading is the ability to put oneself into the mind of another person and infer what that person is thinking or feeling. It is also referred to as theory of mind (Leslie 1987) or mentalizing (Frith et al. 1991). The children's version of the 'Reading the Mind in the Eyes' test consists of 28 pictures of the eye region of the face, each depicting a mental state, including subtle emotions (Baron-Cohen et al. 2001). Results revealed a significant negative correlation with fT, suggesting that higher levels predicted worse mind-reading ability. Within-sex analyses revealed there was also a significant negative correlation between fT and the eyes test for both boys and the girls (Chapman et al. 2006).

Empathy and Systemizing at Six to Eight Years

Difficulties in the ability to empathize, and a strong drive to systemize, appear to be characteristic of ASC (Baron-Cohen 2002). Using the children's versions of the Systemizing Quotient (SQ-C) and Empathizing Quotient (EQ-C), boys scored higher than girls on the SQ-C, and levels of fT positively predicted SQ-C scores in boys and girls individually (Auyeung et al. 2006). Sex differences were observed in EQ-C scores, with girls scoring higher than boys. A significant negative correlation between fT levels and EQ-C was observed when the sexes were combined and within boys. For the EQ-C, a main effect of sex was found, but no main effect of fT. However, the effect of fT cannot be disregarded, since sex and fT are strongly correlated (Chapman et al. 2006).

Autistic Traits in Toddlers at 18–24 Months

A more direct approach of evaluating the links between autistic traits and fT has been conducted. The relationship between fT levels and autistic traits, measured using the Quantitative Checklist for Autism in Toddlers (Q-CHAT), was examined. The Q-CHAT revealed a significant sex difference in autistic traits, with boys scoring higher (indicating more autistic traits) than girls. Q-CHAT scores were predicted by fT levels only, with both Sex and the FT/Sex interaction excluded from the model (Auyeung et al. 2010).

Autistic Traits at Six to Nine Years of Age

At 6–9 years old, the effects of fT were directly evaluated against autistic traits as measured by the Childhood Autism Spectrum Test (CAST; Scott et al. 2002; Williams et al. 2005) and the Child Autism Spectrum Quotient (AQ-Child; Auyeung

et al. 2008). fT levels were positively associated with higher scores (indicating greater number of autistic traits) on the CAST as well as on the AQ-Child. For the AQ-Child, this relationship was seen within sex as well as when the sexes were combined, suggesting it is an effect of fT rather than an effect of sex. The relationship between CAST scores and fT was also seen within males, but not within females (Auyeung et al. 2009b). These findings are consistent with the notion that higher levels of fT may be associated with the development of autistic traits.

Children with ASC have not been included in the analyses, since a much larger sample in which to measure fetal hormone levels would be needed.

fT and the Brain

Recently, we have extended an investigation into how fT may affect brain development. Increased fT levels have been shown to affect brain morphology, showing a significant relationship with increased rightward asymmetry (e.g., Right > Left) of a posterior subsection of the callosum (Chura et al. 2010). We have also found that fT influences later cortical gray matter volume observed to be sexually dimorphic (Lombardo et al. 2012). Increases in fT predicted increased gray matter in the right temporo-parietal junction, and this brain region showed a Male > Female pattern of sexual dimorphism. Similarly, gray matter volumes in the planum temporale and posterior lateral orbitofrontal cortex are inversely related to fT levels and also show a Female > Male pattern of sexual dimorphism. Thus, fT predicts the development of gray matter in directions that are congruent with observed sexual dimorphism and is indicative of the organizational nature of its influence on sexually dimorphic brain development.

Implications for Autism

Results from these studies suggest that variations in fT levels are related to aspects of sexually dimorphic behavior and cognition in typically developing children. However, extrapolating these results to individuals with a formal diagnosis of ASC needs to be done with caution. The sample sizes of the current fT studies are too small to be able to test if fT levels are elevated in formally diagnosed cases of ASC, since these have a prevalence rate of about 1 % (Baird et al. 2006), and a sample size of thousands would be required. A large-scale UK-Danish collaboration is currently underway to increase sample sizes sufficiently to compare fT levels in cases of ASC versus controls.

Genetic influences may interact with prenatal hormone levels in the development of ASC. Evidence of a genetic link to sex steroid hormones and ASC comes from a study that shows that genes regulating sex steroids are associated with autistic traits, as measured by scores on the AQ, in a typical adult sample (Chakrabarti et al. 2009).

A parallel study also showed that genes regulating sex steroids are associated with a diagnosis of Asperger syndrome in a case–control sample (Chakrabarti et al. 2009). Other genes, such as the SRD5A1 and Androgen Receptor (AR) genes, have been associated with ASC (Henningsson et al. 2009; Hu et al. 2009).

Using a different line of evidence, a number of studies have found current androgen dysregulation in ASC or in their relatives, or androgen-related genes being associated with ASC. Androgen-related medical conditions such as polycystic ovary syndrome (PCOS), ovarian growths, and hirsutism occur with elevated rates in women with Asperger syndrome and in mothers of children with autism (Ingudomnukul et al. 2007). A subset of male adolescents with autism show hyperandrogeny, or elevated levels of androgens, and precocious puberty (Tordjman et al. 1997). Delayed menarche has also been found in females with ASC (Ingudomnukul et al. 2007; Knickmeyer et al. 2006b). Puberty timing reflects hormonal programming of the hypothalamic-pituitary-gonadal axis during gestation (Grumbach and Styne 1998).

In addition, left-handedness and ambidexterity are more common in typical males (Peters 1991) and individuals with autism (Gillberg 1983). Increased fT is implicated in left-handedness and asymmetric lateralization (Fein et al. 1985; McManus et al. 1992; Satz et al. 1985; Soper et al. 1986). The typical male brain is also heavier than the female brain, a difference that in animals is partly due to fT. Individuals with autism have even heavier brains than typical males (Hardan et al. 2001).

Conclusions

Research suggests that gender-typical behaviors may be affected by gonadal hormones, in particular fetal exposure to testosterone. The objective of the studies we have reviewed was to examine the link between fT levels (measured in amniotic fluid) and a series of sexually dimorphic behaviors. Results suggest that prenatal exposure to elevated fT levels enhances masculinization of certain behaviors. In addition, direct measurement of autistic traits was used to examine whether these measures of behavior are consistent with the EMB theory of autism. It was striking that, on all three measures (CAST, AQ-Child, and Q-CHAT), fT positively predicted a number of autistic traits.

In summary, fT levels have been found to be significantly related to some, but not all, male-typical traits, and these results lend support for a role for fT levels in the development of behaviors related to sex differences. Although higher levels of fT are unlikely to be the sole cause of autism, the studies reported here provide evidence for a role of fT in the development of autistic traits in typically developing children. This hypothesis remains to be tested in clinical samples. It is hoped that results from this series of studies may enable further understanding of the etiology of ASC and of typical variation in sexually dimorphic behavior.

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Sex Differences in HPA and HPG Axes Dysregulation in Major Depressive Disorder: The Role of Shared Brain Circuitry Between Hormones and Mood

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Abstract Major depressive disorder (MDD) is the fourth leading cause of disease burden worldwide, and women have approximately two times the risk of onset than men. Thus understanding the pathophysiology of MDD has widespread implications for attenuation and prevention of disease burden, particularly in women. MDD has been historically linked to adrenal and gonadal hormone dysregulation. This review argues the importance of applying prenatal stress models [i.e., fetal disruption of hypothalamic-pituitary-adrenal axis (HPA) circuitry] to understanding the fetal programming of sex differences in MDD. We review the literature on the important roles of HPA and HP-gonadal (HPG) hormones in understanding the comorbidity of MDD and endocrine dysregulation. We further review the literature on the fetal programming of MDD. Integrating

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these literatures and our current work, we argue the critical importance of investigating the disruption of the development of fetal HPA circuitry, during periods in which the sexual differentiation of the brain occurs, that we hypothesize place the male or female fetus at differential risks for MDD in adulthood. We believe that an understanding of the mechanisms involved in sex differences in the dysregulation of gonadal and adrenal hormones during *fetal* development in MDD will have etiologic implications and importance for the psychopharmacologic and hormonal treatment and prevention of MDD, particularly in women.

Clinical Evidence of Endocrine Disruption Related to Mood Disorders

Major depressive disorder (MDD) is the fourth leading cause of disease burden worldwide (Murray and Lopez 1997; Ustun et al. 2004), and the incidence of MDD in women is twice that of men (Kessler 2003; Kendler et al. 2006). Thus, understanding the pathophysiology of MDD has widespread implications for attenuation and prevention of disease burden (Ustun et al. 2004), particularly in women. Over 40 years of research implicates hormonal dysregulation in mood disorders, with the earliest reports citing elevated cortisol in patients with major depression (Board et al. 1956; Gibbons and McHugh 1962). While in subsequent years a number of hormonal systems have been demonstrated to be associated with depression [i.e., appetite-regulatory, thyroid, and growth hormones (Coplan et al. 2000; Brouwer et al. 2005; Kurt et al. 2007; Barim et al. 2009)], evidence overwhelmingly supports the involvement of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes (Plotsky et al. 1998; Young and Korszun 2002; Swaab et al. 2005) in the development of mood dysregulation. In particular, hormonal dysfunctions in women have been found to precede MDD onset (Nemeroff et al. 1984; Harlow et al. 2003), suggesting that hormonal abnormalities are important in female vulnerability to MDD. Despite the important findings stemming from this critical line of investigation, there is a dearth of literature on sex differences in HPA-/HPG-axis functioning. Further, a number of other confounds (illness state versus disorder trait, treatment and medication status, age, and single episode versus recurrent diagnosis of MDD) present challenges to elucidating the role of sex in the co-occurrence of hormonal dysregulation and mood disorders.

HPA Axis and MDD

There is a long history of work characterizing the HPA system as central to understanding the development of MDD (Nemeroff et al. 1984; Holsboer et al. 1987; Plotsky et al. 1998; Arborelius et al. 1999; Heim et al. 2002; Parker et al.

2003; Raison and Miller 2003; Barden 2004; Swaab et al. 2005; Antonijevic 2006). Depressive symptoms can occur in the context of either endogenously elevated cortisol (i.e., Cushing syndrome; Sonino et al. 1998) or exogenously administered corticosteroids (Kelly et al. 1980), and patients treated with corticosteroids can develop MDD (Ling et al. 1981). Animal and human studies demonstrated consistent HPA axis abnormalities associated with MDD, most notably elevated levels of cortisol in plasma, CSF, and 24-h urine, in addition to high CSF fluid corticotrophin releasing hormone (CRH) levels, blunted responses to CRH administration, and nonsupression of cortisol secretion upon dexamethasone suppression test (Carroll et al. 1976a, b, 1981; Jarrett et al. 1983; Nemeroff et al. 1984; Halbreich et al. 1985; Holsboer et al. 1985; Banki et al. 1987; Evans and Nemeroff 1987; Holsboer et al. 1987; Rubin et al. 1987; Nemeroff et al. 1991; Arborelius et al. 1999; Heim and Nemeroff 2001; Heim et al. 2001; Newport et al. 2003; Oquendo et al. 2003; Raison and Miller 2003; Barden 2004). HPA dysregulation has been related to, among other things, age (Nelson et al. 1984a, b; Bremmer et al. 2007), depression subtype (hypercortisolemia in atypical depression and normal cortisol levels in melancholia; Brouwer et al. 2005), and single versus recurrent episodes (Poor et al. 2004).

Several studies have examined the utility of HPA reactivity as an indicator of treatment response in MDD. For example, while the elevation in CRH has been shown to resolve with treatment (Nemeroff et al. 1991; De Bellis et al. 1993; Veith et al. 1993), some studies report an incomplete resolution to normal levels, suggesting that these HPA abnormalities may be part of the vulnerability to MDD (or a trait) and not only state-related. Although decreases following treatment in abnormally elevated pre-treatment cortisol levels have been widely reported (Gibbons and McHugh 1962; Carroll et al. 1976a, b), a recent meta-analysis found that cortisol levels did not change pre- versus post-treatment in over half of subjects with depression (McKay and Zakzanis 2010). An examination of subject characteristics related to changes in cortisol post-treatment revealed the greatest decreases in those with the melancholic subtype. Time of sample collection, inpatient versus outpatient setting, type of treatment or antidepressant, subject sex, and number of past episodes were not associated with cortisol changes following treatment, although the length of the current episode was negatively associated with change in cortisol levels (McKay and Zakzanis 2010). This finding supported the hypothesis that the nature of HPA axis dysregulation shifts dramatically from acute [overall hypersecretion of corticotrophin releasing hormone (CRH), adrenocorticotropin hormone (ACTH), and cortisol] to chronic (reduced ACTH and hypercortisolemia) phases of depression (Parker et al. 2003).

The issues of state versus trait (independent of treatment) and underlying vulnerability to relapse in depression have been examined sparingly, with findings initially pointing to hypercortisolemia as a state (not trait) feature of MDD. In remitted patients compared with controls, cortisol levels were reported to be similar (Trestman et al. 1993) or even decreased (Ahrens et al. 2008), although these studies were small (n/group ~20–30). However, a recent well-powered study examined morning and evening salivary cortisol levels in 308 controls, 579 individuals with remitted MDD, and 701 patients currently in an MDD episode

(Vreeburg et al. 2009). Results showed that remitted and current MDD subjects demonstrated significantly higher awakening cortisol levels compared to controls, providing compelling evidence that elevated cortisol is not specific to current state but persists following recovery and may therefore be a trait characteristic (Vreeburg et al. 2009). Although findings held when adjusted for sex (57–71 % female, depending on group) and other demographic variables, specific sex differences were not explored. When tracked longitudinally, baseline cortisol and dexamethasone suppression test abnormalities also predicted vulnerability to relapse, necessity for continued medication to sustain remission, and remission rate following hospitalization in MDD (O'Toole et al. 1997; Zobel et al. 1999; Appelhof et al. 2006; Ising et al. 2007).

Further evidence for the role of the adrenal cortex is suggested by work on dehydroepiandrosterone sulfate (DHEA-S) and MDD. DHEA-S, which is produced by the adrenal gland, is considered a weak androgen and has been significantly associated with MDD, depending on age and sex (Orentreich et al. 1984; Schmidt et al. 2002), illness severity, medication status, and time of sampling. In unmedicated MDD patients, DHEA-S had anti-glucocorticoid action in the brain (Young et al. 2002; van Broekhoven and Verkes 2003). In fact, studies demonstrated lower depressive symptoms and better memory function with increased levels of DHEA-S (Wolkowitz et al. 1999; van Broekhoven and Verkes 2003). A high cortisol:DHEA-S ratio, which is a functional indicator of hypercortisolemia (Gallagher and Young 2002), was significantly associated with MDD, emphasizing anti-glucocorticoid DHEA-S action (Young et al. 2002; van Broekhoven and Verkes 2003).

Despite significant advances in understanding the comorbidity of major depression and HPA axis dysregulation as evidenced above, there is a paucity of data on sex differences in the comorbidity. This is striking for three reasons. First, as mentioned previously, there are well-documented sex differences in MDD incidence and prevalence (Kessler 2003; Kendler et al. 2006). Second, substantial data support sex differences in HPA-HPG axes functioning during stress in healthy control populations (Kudielka and Kirschbaum 2005; Goldstein et al. 2010; Andreano et al. 2011) and in MDD women (Holsen et al. 2011). Finally, as discussed below, there are significant interactions of the HPA axis with the HPG axis, which we know is different between the sexes.

Among the few investigations reporting significant sex differences in HPA axis functioning in MDD, the direction of effects is mixed. Men, but not women, with MDD demonstrate abnormal ACTH pulsatility (Young et al. 2007a). Additionally, elevated cortisol has been documented in depressed men versus depressed women (Bremmer et al. 2007) and versus non-depressed men (Hinkelmann et al. 2011), but also in depressed women versus depressed men (Poor et al. 2004) versus non-depressed women (Young and Altemus 2004; Chopra et al. 2009). These conflicting reports on sex differences in cortisol levels may be related to timing of cortisol assessment across studies and/or genetic factors. Recent data suggest an interaction between sex and adrenoreceptor gene polymorphisms in HPA hyperactivity using a dexamethasone/CRH test pre- and post-treatment (Haefner et al. 2008). Specifically, increased ACTH and cortisol responses were seen in males (but not females)

homozygotic for the *alpha*(2)-adrenoreceptor (ADRA2A) gene and females (but not males) homozygotic for the *beta*(2)-adrenoreceptor (ADRB2) gene (Haefner et al. 2008). Collectively these findings offer initial evidence of sex differences in the role of HPA axis functioning in MDD pathophysiology.

Several reports have found no effect of sex on HPA dysfunction in MDD (Carroll et al. 1976a, b; Nelson et al. 1984a; Maes et al. 1987, 1989, 1994; Dahl et al. 1989; Deuschle et al. 1998; Brouwer et al. 2005; Rubin et al. 2006; Vreeburg et al. 2009). However, the majority of these studies cited above (including those reporting sex differences and those with null findings) did not initially design their studies to investigate sex differences but rather analyzed the data by sex post hoc. This approach is problematic, since potential confounding (uncontrolled in the initial designs) is typical. For example, the vast majority of studies of MDD oversample women (Maes et al. 1987; Brouwer et al. 2005; Rubin et al. 2006; Young et al. 2007a; Vreeburg et al. 2009; Hinkelmann et al. 2011). Some have matched on sex whereas some included women using oral contraceptives or estrogen-replacement therapy (Brouwer et al. 2005), which have been shown to affect plasma levels of cortisol (Kirschbaum et al. 1999). Further, only a few mention "matching for menstrual status" (Maes et al. 1987; Rubin et al. 2006), and those that do generally refer to including similar numbers of women who are pre- or post-menopausal rather than actually controlling for menstrual cycle phase (for example, conducting study visits only within certain phases such as early follicular or late luteal). These methodological confounds present significant challenges to understanding the inconsistencies in the literature on sex differences in HPA axis dysregulation and MDD comorbidity.

The importance of HPA axis abnormalities in MDD is underscored by human postmortem studies. One human postmortem study found a 25 % decrease in the density of glucocorticoid receptor (GR) mRNA in MDD compared with healthy brains in frontal cortex, dentate gyrus, and subiculum, suggesting a down-regulation of GRs affecting the negative feedback system of the HPA axis resulting in hypercortisolemia (Webster et al. 2002). CRH action on ACTH is potentiated by arginine vasopressin (AVP), which is co-expressed with CRH in some neurons of the paraventricular nucleus of the hypothalamus (PVN) and was enhanced in MDD (von Bardeleben et al. 1989; Muller and Holsboer 2006). A recent postmortem study reported increased AVP mRNA in the PVN and supraoptic nucleus in MDD, particularly with melancholic features (Meynen et al. 2006). This finding is consistent with an increased number of AVP-immunoreactive neurons in PVN (Purba et al. 1996), particularly those co-localizing with increased CRH in PVN in MDD (Raadsheer et al. 1994a, b). It is also consistent with studies of MDD reporting elevated AVP plasma levels (van Londen et al. 1997; van Amelsvoort et al. 2001; de Winter et al. 2003), positive correlations of plasma AVP with cortisol (De Bellis et al. 1994; Inder et al. 1997; de Winter et al. 2003), and increased ACTH and cortisol in MDD and controls with intravenous administration of AVP (Gispen-de Wied et al. 1992), which are findings that were not due to medication confounds (van Londen et al. 1997; van Amelsvoort et al. 2001; Meynen et al. 2006).

HPG Axis and MDD

The relationship between mood disturbances and gonadal hormones was initially recognized, in part, through developmental endocrine disorders such as polycystic ovarian syndrome (PCOS), which is associated with high levels of comorbid depression (Himelein and Thatcher 2006b). The mechanisms behind this comorbidity are not fully understood, as data do not support an association between depressive symptoms and androgen levels, infertility issues, or hirsutism (Keegan et al. 2003; Rasgon et al. 2003; McCook et al. 2005; Himelein and Thatcher 2006a). Further evidence is derived from the abundant literature relating women's reproductive system to mood fluctuations and depression (Payne 2003; Spinelli 2005; Payne et al. 2009). For example, pubertal onset (Angold and Costello 2006), late luteal menstrual cycle phase (Steiner 1992), chronic use of oral contraceptives (Young et al. 2007b), the postpartum period (Bloch et al. 2000; Brummelte and Galea 2010), and postmenopause (Graziottin and Serafini 2009) are all associated with vulnerability to MDD. However, the establishment of this relationship in women has not been accompanied by parallel examination of possible hormonal deficits linked to mood dysregulation in men at similar ages.

Human studies of MDD patients have found deficits in gonadal function (Rubinow and Schmidt 1996; Harlow et al. 2003), e.g., androgens (Baischer et al. 1995; Rubinow and Schmidt 1996; Schweiger et al. 1999; Seidman et al. 2001; Weiner et al. 2004) and estradiol (Young et al. 2000), and pituitary function, i.e., low follicle stimulating hormone (FSH; Daly et al. 2003). Women with persistent MDD had two times the risk of earlier perimenopausal transition, and those with a lifetime history of MDD had higher FSH and lower estradiol levels, suggesting an early decline in ovarian function (Young et al. 2000; Harlow et al. 2003). Further reports suggested a relationship between depressive symptom severity and estradiol levels (Baischer et al. 1995) and that ovarian dysfunction preceded the onset of MDD (Harlow et al. 2003). Abnormalities in luteinizing hormone (LH) levels and pulsatility in women with MDD have been consistently documented (Young et al. 2000; Meller et al. 2001; Harlow et al. 2003). LH pulse frequency and testosterone secretion in males with MDD were also lower (Schweiger et al. 1999), although conflicting reports suggested relatively normal HPG axis functioning in MDD males (Rubin et al. 1989).

HPA-HPG Interactions

There is some evidence that inhibition of HPG activity by stress and other factors may be linked to HPA activity (Halbreich and Kahn 2001). CRH inhibits gonadotropin releasing hormone (GnRH) and gonadotropin secretion in model animal studies (Nikolarakis et al. 1986; Olster and Ferin 1987). In fact, the low levels of estradiol seen in MDD premenopausal women may lead to decreased inhibitory

feedback of the HPA axis in the presence of increased HPA drive with unopposed progesterone. This may in turn account for elevated levels of cortisol in MDD women compared to MDD men or non-depressed women (Young and Altemus 2004). Although the mood disturbances in premenstrual syndrome do not reach the severity or duration of major depression, transient dysregulation of the HPA axis during the luteal phase has been noted in this population (Rabin et al. 1990; Roca et al. 2003), offering further support for the influence of gonadal steroid hormones on HPA functioning related to mood.

Further, in postmortem studies of MDD, CRH-producing neurons in PVN that co-localized with estrogen receptor alpha (ERα) were enhanced in MDD, again suggesting HPA-HPG interactions in MDD (Bao et al. 2005). In our recent functional imaging study in MDD women, gonadal hormone abnormalities (lower estradiol) were significantly associated with functional brain activity deficits in key regions in the stress response circuitry (e.g., amygdala and hippocampus; Holsen et al. 2011). We are currently testing the hypothesis that the vulnerability for these stress response circuitry deficits and endocrine abnormalities begins during fetal development.

Brain Circuitry Linking MDD, HPA and HPG

The comorbidity between depression and HPA-HPG-axis dysregulation is not surprising from a brain circuitry point of view, given that depression is a disorder that involves hypothalamic nuclei [such as paraventricular (PVN) and ventromedial (VMN)], central amygdala, hippocampus, subgenual anterior cingulate cortex (ACC), and medial and orbitofrontal cortex (mPFC OFC; Dougherty and Rauch 1997; Mayberg 1997; Drevets et al. 2002; Sheline et al. 2002; Rauch et al. 2003), regions that are dense in glucocorticoid and sex steroid hormone receptors (MacLusky et al. 1987; Clark et al. 1988; Handa et al. 1994; Kawata 1995; Tobet and Hanna 1997; Donahue et al. 2000; Östlund et al. 2003). The overlap between these circuitries has been historically noted from behavioral and endocrinological findings but, with the advent of magnetic resonance imaging (MRI) technology, there is a greater focus on the investigation of brain circuitry implicated in the regulation of mood and endocrine functioning. This technology allows for hypothesis-driven in vivo exploration of this shared circuitry.

HPA Axis Hormones Associated with Brain Activity

Over the past 5 years, there has been a rapid increase in studies examining the relationship between HPA hormones (more specifically endogenous and exogenous cortisol) and brain activity in subcortical and cortical stress response regions using a variety of functional MRI (fMRI) paradigms in healthy control subjects (generally comprising mixed-gender samples with age ranges between 18 and 35 years).

Amygdala and hippocampal activity in response to stimuli of high negative emotionality was positively associated with pre- versus post-scan (Root et al. 2009) and diurnal amplitude (Cunningham-Bussel et al. 2009) salivary cortisol. This relationship between hyperactivation in the amygdala and increased cortisol is supported by additional evidence suggesting that, when categorized by level of endogenous cortisol, individuals with high cortisol demonstrate greater amygdala activity than those with low cortisol levels (van Stegeren et al. 2007, 2008), an effect that is blocked by administration of a noradrenergic antagonist. Cushing syndrome (CS), which is associated with chronic hypercortisolemia, also appears to be associated with hyperactivity in arousal regions. Adolescents with CS compared with age- and gender-matched controls demonstrated increased activation of the amygdala and hippocampus during successful encoding of emotional faces, despite similar memory performance (Maheu et al. 2008). Further, adults with CS showed hyperactivation in the anterior hippocampus, medial frontal gyrus, ACC, caudate, and superior parietal lobule during identification of emotional facial expressions. Accuracy in CS patients was lower and correlated with brain activity, suggesting these differences could be partially explained by compensatory recruitment of these regions (Langenecker et al. 2012). However, in general, these findings point to a pattern of significantly enhanced activation in the presence of heightened endogenous cortisol levels in healthy controls and CS patients.

In cortical stress response circuitry regions, however, somewhat contrasting results emerge. For example, one study reported a negative correlation between ventromedial prefrontal cortical activation to negative (versus neutral) stimuli and pre- versus post-scan cortisol levels (Root et al. 2009). Further, decreased activations in the ACC and OFC were observed in individuals who demonstrated a significant increase in cortisol levels (i.e., "responders") during a psychosocial stress paradigm (negative feedback during arithmetic problems), as compared to cortisol non-responders (Pruessner et al. 2008). Interestingly, although several of these studies included sex as a covariate in the analyses, only one focused specifically on sex differences. They reported a lateralized pattern of activations in the frontal cortex in males [i.e., increased cerebral blood flow (CBF) in the right PFC and decreased in left OFC, which were associated with cortisol levels] and activations in the ventral striatum, putamen insula, and ACC (unrelated to cortisol level variation) in females (Wang et al. 2007). Taken together, these findings suggest potentially divergent roles of subcortical versus cortical arousal regions in response to stress and cortisol variation, which may be influenced by gonadal steroid hormones given substantial differences in activation patterns between men and women and the importance of HPA and HPG interactions.

Importantly, the literature on changes in endogenous cortisol levels associated with brain responses to emotional and stressful paradigms highlights the significant variability among even healthy individuals in the psychophysiological reaction to stress, as not all study subjects are ultimately classified as cortisol "responders" (Wust et al. 2000; Muehlhan et al. 2011). One methodological alternative to relying on natural variation in cortisol levels is to observe similar phenomena following administration of exogenous cortisol (i.e., hydrocortisone). In general, the

amygdala and hippocampus appear to be most sensitive to hydrocortisone, demonstrating significant decreases in activation in comparison to placebo (Lovallo et al. 2010). Striking sex differences in the neural response to hydrocortisone (versus placebo) during fear conditioning have been observed, with increased activation in the ACC, OFC, and mPFC in response to the conditioned (versus unconditioned) stimulus in females and decreases in these same regions in males (Stark et al. 2006; Merz et al. 2010).

HPG Axis Hormones Associated with Brain Activity

Although studies on the HPA hormone-brain relationships occasionally report controlling for menstrual cycle phase in women (Stark et al. 2006), gonadal hormone variation is the primary focus of functional neuroimaging studies of HPG hormone effects on brain activity. A number of investigations have provided evidence of estradiol and progesterone influences on brain activity during cognitive paradigms, including executive functioning (Berman et al. 1997), language processing (Fernandez et al. 2003), and verbal memory (Craig et al. 2008; Konrad et al. 2008) among others (Dietrich et al. 2001; de Leeuw et al. 2006). Here we focus on emotional paradigms, given the role of emotion dysregulation in mood disorders. Activation in stress response circuitry regions has been shown to be modulated across menstrual cycle in healthy women in response to negative arousal images, with greater activation in the anterior hypothalamus, amygdala, hippocampus, ACC, and OFC during the early follicular phase compared with late follicular/midcycle (Goldstein et al. 2005). Further, in healthy women, hyperactivity of the amygdala and hippocampus was present during late luteal compared with early follicular menstrual cycle phase (Andreano and Cahill 2010), with estradiol being negatively associated with amygdala activation (Andreano and Cahill 2010). Direct comparisons between males and females were consistent with these patterns, with greater hyperactivity in men than in women during their late follicular/midcycle compared to when they were in early follicular (Goldstein et al. 2010). Results suggested gonadal hormonal modulation of subcortical arousal by prefrontal circuitry (Goldstein et al. 2005, 2010).

Using paradigms that examine inhibitory control during cognitive and emotional processing, inhibitory responses to negative (versus neutral) emotional stimuli targets during the luteal phase (versus follicular) are associated with greater activation in the medial OFC (Protopopescu et al. 2005), ACC, DLPFC, and putamen (Amin et al. 2006). In contrast, increased OFC activity during follicular rather than luteal phase was observed in response to male faces judged on sexual desirability (Rupp et al. 2009), which may suggest a significant shift in OFC processing and decision-making related to positive and negative stimuli across the menstrual cycle. However, a study utilizing a reward paradigm recently demonstrated greater follicular than luteal phase activation to anticipation of positive reward delivery in the OFC (Dreher et al. 2007). These discrepancies might be related to differences in menstrual phase definition, with follicular phase defined as days 4–8 (Dreher et al.

2007), 8–12 (Protopopescu et al. 2005), or 10–12 after the start of menstruation (Rupp et al. 2009), and luteal phase defined as 19–23 days following the start of menstruation (Rupp et al. 2009), 6–10 days post LH surge (Dreher et al. 2007), or 1–5 days before menses onset (Protopopescu et al. 2005). Although the healthy control women in these samples had regular cycles, this variation in phase definition across studies could have significant effects on estradiol and progesterone levels, leading to substantial differences reported in these studies in brain activity across the menstrual cycle.

Similar to literature (cited above) on exogenous HPA hormone administration and brain activity, a number of investigations have examined effects of exogenous gonadal hormone regulation of neural responses to emotional stimuli. Compared with placebo, progesterone administration (during early follicular phase when progesterone levels are naturally low) is related to increased amygdala reactivity to emotional face processing, increased amygdala-dorsal ACC connectivity, and decreased amygdala-fusiform gyrus connectivity (van Wingen et al. 2008b). Testosterone administration, contrastingly, increased hippocampus and inferior temporal gyrus activation during memory formation and retrieval of male faces in middle-aged women (van Wingen et al. 2008a). Compared with placebo, testosterone increased amygdala responsivity to levels equivalent to those observed in young women (van Wingen et al. 2009) and reduced functional connectivity between the amygdala and OFC (van Wingen et al. 2010). Thus, administration of exogenous gonadal hormones exerts significant influence on amygdala responsivity in general and coupling between the amygdala and other limbic and cortical regions during evaluation of emotionally salient cues.

Recent Findings in Mood Disorders

A few studies recently demonstrated compelling evidence of links between HPA-HPG hormone dysregulation and brain activity deficits in MDD. Hydrocortisone administration to currently depressed women resulted in increased hippocampal activation during encoding of neutral (versus negative or positive) words in comparison to healthy control women, a trend not observed during placebo (Abercrombie et al. 2011). Importantly, this relationship between exogenous cortisol administration and memory formation did not occur in depressed men, suggesting sex differences in the effect of cortisol on memory processing in depression (Abercrombie et al. 2011). Further, we showed that young women with MDD displayed hypoactivation in a number of regions involved in the stress response circuitry that were significantly associated with gonadal hormone deficits (Holsen et al. 2011), including decreased estradiol and increased progesterone levels in MDD women during late follicular/midcycle phase of the menstrual cycle. Finally, hypoactivation to positive stimuli in the nucleus accumbens and hyperactivations in the amygdala and lateral OFC in response to negative stimuli during the luteal phase (versus late follicular) were reported in women with

premenstrual dysphoric disorder compared with healthy controls (Protopopescu et al. 2008b). Findings from these initial studies indicate a complex interaction between HPA (cortisol) and HPG (progesterone, estradiol) dysregulation and brain activation during cognitive and emotional processing (respectively) in women with mood disorders, providing support for mechanisms implicating neuroendocrine systems associated with sex differences in depression.

Sexual Dimorphisms in Shared Mood and Endocrine Circuitry

Extant literature suggests that the circuitry shared between mood regulation and endocrine functioning also includes highly sexually dimorphic regions and therefore may help us understand sex differences in MDD. In vivo imaging and postmortem studies have demonstrated sex differences in brain volumes (or nuclei) of regions associated with MDD. In women, relative to cerebrum size, findings support greater relative volumes of hippocampus (Filipek et al. 1994; Giedd et al. 1996; Murphy et al. 1996; Goldstein et al. 2001), ACC (Paus et al. 1996; Goldstein et al. 2001) and OFC (Goldstein et al. 2001). In men, there are greater volumes (relative to cerebrum size) of the amygdala (Giedd et al. 1996; Goldstein et al. 2001), hypothalamus (Swaab and Fliers 1985; Allen et al. 1989; Goldstein et al. 2001), and paracingulate gyrus (Paus et al. 1996; Goldstein et al. 2001). Thus women tend to have relatively larger volumes of hippocampus, OFC, and ACG, whereas men have relatively larger amygdala and hypothalamic volumes. Recent findings offer additional evidence that regional brain volumes in women vary across the menstrual cycle, with hippocampal gray matter volume increased and dorsal basal ganglia gray matter volume decreased during follicular rather than luteal phase (Protopopescu et al. 2008a). Further, estradiol, progesterone, and testosterone levels in young adults explained 13 %, 13 %, and 2 % in the variation of superior parietal gyrus, medial temporal pole, and inferior frontal gyrus gray matter volume, respectively (Witte et al. 2010), suggesting significant associations between gonadal hormone levels and neuroanatomic variation in humans.

One potential factor involved in human sexual dimorphisms may be the role of gonadal hormones on brain development, as seen in particular in model animal work by collaborators Tobet and Handa (McEwen 1983; Simerly et al. 1990; Tobet et al. 1993, 2009; O'Keefe et al. 1995; Park et al. 1996; Tobet and Hanna 1997; Gorski 2000; Chung et al. 2006). Our findings in humans indirectly suggested that this factor might also, in part, contribute to understanding human sexual dimorphisms in adulthood (Goldstein et al. 2001). In animals, nuclei of the corticomedial amygdala, PVN, VMN, hippocampus, OFC, and ACG express high concentrations of gonadal and/or adrenal hormone receptors compared with other brain regions (Handa et al. 1994; Pacak et al. 1995; Koob 1999; Solum and Handa 2002; Tobet 2002; Östlund et al. 2003; Lund et al. 2004, 2006; Suzuki and Handa 2004). These brain regions have been implicated in MDD and HPA function. Our hypotheses are in part based on the premise, supported by our work on sex

differences in another disorder with fetal origins, schizophrenia (Goldstein et al. 2002), that normal sexual dimorphisms during fetal development in MDD may go awry in brain regions associated with MDD and HPA function and that mechanisms involved in understanding normal sexual dimorphisms, such as the roles of gonadal and adrenal hormones (in association with genes; Handa et al. 1994; Majdic and Tobet 2011), will contribute to understanding sex differences in MDD in adulthood.

Prenatal Stress Models of Understanding Sex Differences in MDD and Comorbid Endocrine Dysregulation

Preclinical and clinical studies have demonstrated lasting effects of prenatal adverse events on the HPA axis and noradrenergic stress systems (Takahashi et al. 1992; Weinstock et al. 1992; Vallee et al. 1997; Weinstock 1997). These include conditioned stress responses such as heightened glucocorticoid, norepinephrine, and autonomic response to novel stressors and altered dopaminergic, gamma aminobutyric acid (GABA)-ergic, and serotonergic function (Heim et al. 2000; Heim and Nemeroff 2001). Animal studies demonstrated the impact of prenatal stress on hypothalamic and hippocampal structure and function (Takahashi et al. 1992; Matsumoto and Arai 1997; Weinstock 1997), with lasting effects on the HPA axis in adult offspring by programming a "hyperactive" system that was vulnerable to adult depression, anxiety, and autonomic nervous system deficits among others (Weinstock et al. 1992; Henry et al. 1994; Barker 1995; Arborelius et al. 1999; Seckl 2001). Our current work has demonstrated that mid-to-late gestation is a particularly vulnerable time for the impact of prenatal events on sex-specific brain development (Tobet et al. 2009; Majdic and Tobet 2011) and development of the hormonal systems such as HPA (Celsi et al. 1998; Slotkin et al. 1998; Tronche et al. 1999; Sandau and Handa 2007; Zuloaga et al. 2011). Thus preclinical studies, including our own work, have demonstrated the vulnerability of the HPA system to adverse prenatal events with sex-specific effects on HPA function and affect.

Models for investigation of HPA compromise have included prenatal stress and infection during mid-to-late gestation that have demonstrated sex-specific effects in preclinical studies. From preclinical studies, sex effects (i.e., greater in females than males) include (1) greater glucocorticoid transfer across the placenta in female mice (Montano et al. 1993; Fameli et al. 1994); (2) greater immobility in standard tests associated with MDD phenotypic behavior (Alonso et al. 2000); (3) increased ACTH corticosterone and glucocorticoid receptor (GR) binding (Weinstock et al. 1992; McCormick et al. 1995; Regan et al. 2004); (4) increased corticosterone sensitivity (Rhodes and Rubin 1999); (5) greater susceptibility to changes following loss of $GABA_B$ receptor function (McClellan et al. 2010; Stratton et al. 2011); (6) greater susceptibility to cell death in the amygdala following developmental exposure to the glucocorticoid agonist dexamethasone (Zuloaga et al. 2011); and (7)

greater susceptibility to diet-induced hepatosteatosis and insulin growth factor (IGF)-1 deficits (Carbone et al. 2011). In humans, MDD females compared with MDD males show (1) increased GR and MR mRNA in temporal and PFC regions (Watzka et al. 2000); (2) higher levels of cortisol (Frederiksen et al. 1991; Heuser et al. 1994; Laughlin and Barrett-Connor 2000); and (3) decreased volume of hippocampus and increased amygdala (Vakili et al. 2000; Janssen et al. 2004; Weniger et al. 2006). Thus we have been testing the hypothesis that sex differences in the impact of adverse fetal HPA programming demonstrated in preclinical studies, contribute to sex differences in adult MDD.

Receptors responsible for the expression and/or regulation of expression of HPA hormones reside in brain regions implicated in MDD. Hypothalamic nuclei (such as PVN and ventromedial nucleus) and hippocampus are involved in the regulation of HPA hormones, as demonstrated in earlier work by Tobet and Handa (Tobet and Hanna 1997; Brown et al. 1999; Lund et al. 2006; Sandau and Handa 2006; Foradori and Handa 2008; McClellan et al. 2010; Stratton et al. 2011). They are dense in CRH and glucocorticoid receptors, vasopressin, GABA receptors, and sex steroid receptors (Keverne 1988; Handa et al. 1994; Pacak et al. 1995; Koob 1999; Tobet et al. 1999; Dellovade et al. 2001; Davis et al. 2002; Solum and Handa 2002; Tobet 2002; Östlund et al. 2003; Lund et al. 2004, 2006; Suzuki and Handa 2004; Weiser and Handa 2009). Studies have argued that the effects of prenatal stressors on the brain are mediated by neurotransmitter systems that interact with glucocorticoids and gonadal steroid receptors such as GABA and glutamate (Tobet et al. 1999; Seckl and Walker 2001; Owen et al. 2005; McClellan et al. 2008, 2010; Zuloaga et al. 2011), which we have demonstrated in our current program project [National Institute of Health Office for Research on Women's Health-National Institute of Mental Health P50 MH082679].

In Summary

MDD is a major public health problem with substantial economic, social, and disease burden worldwide. Women are approximately two times more likely than men to present with a lifetime history of MDD. Moreover, this sex difference starts in early adolescence and persists through the mid-50s. Thus, understanding the pathophysiology of this disorder, particularly for women, has important implications for attenuation of suffering worldwide. There is substantial literature supporting the notion that MDD (at least some forms) is a disorder whose vulnerability begins during fetal development. A number of potential pathways may connect adverse conditions arising during fetal development and sex differences in MDD in adulthood. We are currently investigating prenatal stress models that focus on disruption of the development of the fetal HPA circuitry—during periods in which the sexual differentiation of the brain occurs—that we hypothesize place the male or female fetus at differential risks for MDD in adulthood. Further, we have been testing the hypothesis that the fetal hormonal programming will be significantly associated with sex differences in brain activity

deficits in stress response circuitry and adult HPA and HPG deficits in MDD. Finally, the demonstration of altered neuroendocrine regulation in relation to sex differences in brain activity in MDD may contribute to understanding the higher prevalence of endocrine disorders in MDD than in the general population, thus promoting further inquiry into development of neuroendocrine treatment modalities. We believe that an understanding of the mechanisms involved in sex differences in the dysregulation of gonadal and adrenal hormones during fetal development in MDD will have etiologic implications and importance for the psychopharmacologic, hormonal and immunoregulatory treatment and prevention of MDD, particularly in women.

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Estrogens: Protective or Risk Factors in the Injured Brain?

Phyllis M. Wise, Candice M. Brown, and Jodi L. Downs

Abstract Many women spend over three decades of their lives in a postmenopausal state, during which time circulating estrogen concentrations are chronically low. Several studies have suggested that this hypoestrogenic state may make women more vulnerable to a variety of age- and hormone-related diseases. The lack of ovarian hormone production was thought to explain the fact that postmenopausal women are at higher risk for stroke, neurodegenerative diseases, heart disease, and osteoporosis compared to their male counterparts or compared to premenopausal women. Over the past two decades, numerous observational and retrospective studies demonstrated that estrogen therapy (ET), given to postmenopausal women, provided benefits against cardio- and cerebrovascular diseases. Despite these studies, several more recent clinical trials, including the Women's Health initiative (WHI), showed a negative impact of ET. In addition, some studies in animal models suggest that estrogens are not universally protective and can be deleterious under some circumstances, a finding that has spurred reconsideration of the use of universal ET in postmenopausal women. These contradictions make it even more important to continue to perform studies using different appropriate animal models to improve our understanding of the wide spectrum of estrogen's actions and to probe the mechanisms that underlie these seemingly divergent effects. We will discuss our findings that low, physiological concentrations of ET are protective against stroke injury when, but only when, administered immediately

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after estrogen withdrawal. We have utilized a permanent middle cerebral artery occlusion animal model of stroke in mice and rats, a model of cerebral ischemia that produces an infarct in the cortex and striatum. Our findings that mice exposed to a prolonged period of hypoestrogenicity are not protected against stroke injury lend strong support to the "timing hypothesis" of ET, which posits that the beneficial or detrimental effects of ET depend upon the length of time that a woman is postmenopausal before initiating ET. We have also found that estradiol (E2) exhibits profound anti-inflammatory actions in our stroke model through multiple mechanisms that include suppressing central and peripheral pro-inflammatory cytokines, decreasing expression of the enzyme inducible nitric oxide synthase, and promoting the integrity of the blood–brain barrier. Further studies have probed the role(s) of classical estrogen receptors α (ERα) and β (ERβ) in these mechanisms by using a combination of genetic (ER knockout mice) and pharmacological approaches. Our studies have uncovered critical roles for both ERα and ERβ as enhancers of neurogenesis and suppressors of neuroinflammation. Collectively, our work demonstrates that ET attenuates cell death, promotes neurogenesis, and alters the immune response through mechanisms that involve both ERα and ERβ.

Introduction

Menopause marks the end of ovarian hormone synthesis and secretion and the loss of reproductive capacity in women. Over the past century, the average life expectancy of women living in developed countries has increased to over 80 years, yet the age of the menopause has not increased at all and has remained at approximately 51 years of age. Thus, today, in the absence of estrogen therapy (ET), women may spend over 30 years of their lives in a chronic hypoestrogenic state. Several studies have suggested that this hypoestrogenic state may make women more vulnerable to a variety of age- and hormone-related diseases. For example, women are protected from stroke and other cardiovascular diseases until they reach menopause (Paganini-Hill 2001; Behl 2002; McCullough and Hurn 2003; Turgeon et al. 2004; Wise et al. 2005; Turgeon et al. 2006). It seemed logical that the lack of ovarian hormone production might explain the findings that postmenopausal women are at higher risk for stroke compared to their male counterparts. Since the incidence of stroke in women increases after menopause and the risk continues to rise with age (Mitka 2006), it has been thought that the gender difference is related to a combination of both the longer life expectancy of women and the protective roles of estrogens. Moreover, over the past 20 years, several observational and retrospective studies demonstrated that ET given to postmenopausal women provided benefits against cardio- and cerebrovascular diseases (Paganini-Hill 2001; Behl 2002; McCullough and Hurn 2003; Wise et al. 2005; Turgeon et al. 2004, 2006). In contrast, several clinical trials, including one by the WHI, reported a negative impact of ET and spurred reconsideration of its use around the world. To date, the literature suggests we must be careful to consider the context of hormone

treatment when interpreting the results (Turgeon et al. 2006). Studies in animal models reveal just how important it is to consider the form of estrogen that is used, the dose of the hormone and whether it is administered in a constant or cyclic manner, the age of the animal and previous hormonal environment of the animal, and whether other hormones are combined either simultaneously or sequentially. It is clear that estrogens, even in animal models, are not universally protective and can be deleterious under some circumstances. Several excellent recent reviews show the complexity of estrogen action and that this family of ovarian steroids can be deleterious or protective (Liu et al. 2010; Strom et al. 2010; Leon et al. 2011; Liu and McCullough 2011; Strom et al. 2011).

Together, our findings, in combination with the results of the WHI and its recent re-evaluation, emphasize the tremendous importance of strengthening the collaboration between basic science and clinical researchers to take maximum advantage of empirical and mechanism-based information and approaches to gain a deeper understanding of the diverse mechanisms of E2's protective actions. It becomes even more important to continue to perform studies using different appropriate animal models to deepen our understanding of the complexities of estrogen's actions and to probe the mechanisms that underlie these seemingly divergent and contradictory effects.

Why Are There so Many Discrepancies over the Actions of ET in Ischemic Stroke Injury?

Until recently, most researchers and clinicians accepted the proposition that estrogens were protective against stroke and a variety of other neurodegenerative conditions, including memory loss and Alzheimer's disease. In striking contrast, the results of the WHI reported that ET increases the risk for stroke, and it does not provide any beneficial effects on long-term stroke outcomes (Viscoli et al. 2001; Nordell et al. 2003; Bushnell 2006; Sohrabji and Bake 2006). The WHI consisted of a randomized, placebo-controlled clinical trial primarily designed to test whether ET is protective against coronary heart disease in postmenopausal women. In 2004, the WHI was terminated due to an increased risk of stroke in women receiving treatment (Mitka 2006). Since the initial publication of the results of the WHI, many other studies, using some of the same patient populations, have suggested that estrogens also do not protect against memory loss or cognitive decline in older women (Espeland et al. 2004; Shumaker et al. 2004).

It is important to remember that, in the WHI, the mean age of the subjects was 63 years, and the vast majority of them were postmenopausal for an average of 12 years prior to the initiation of any hormone treatment (McCullough and Hurn 2003; Mitka 2006). In striking contrast, observational studies that previously reported cardio- and cerebrovascular benefits of ET examined women averaging 51 years of age, many of whom initiated hormone therapy in their menopausal transition (Rossouw et al. 2002;

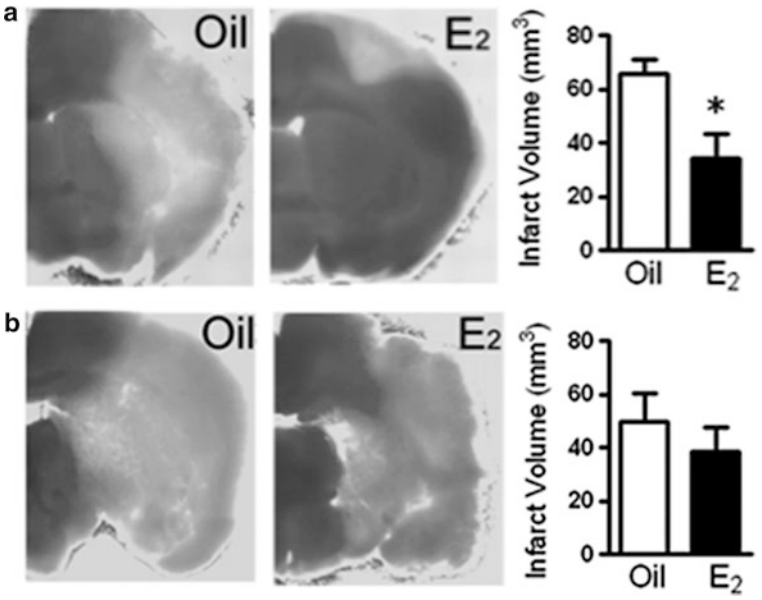

Fig. 1 Delayed E2 treatment after an extended period of hypoestrogenicity does not protect during ischemic injury. (**a**) C57BL/6 J mice (19 weeks) were ovariectomized and immediately implanted with capsules containing oil or E2 for 1 week. (**b**) C57BL/6 J mice (9 weeks) were ovariectomized and implanted with capsules containing either oil or E2 10 weeks later. Subsequently, at 20 weeks of age, both groups underwent permanent MCAO for 24 h. Brains were removed and sliced into 1-mm sections, and infarct volumes were quantified using TTC staining. Immediate E2 treatment (**a**) significantly reduced the total infarct volume ($p < 0.02$), whereas mice subjected to a prolonged period of hypoestrogenicity (**b**) were not protected against ischemic injury ($p = 0.58$). Data represent the mean ± SEM of 8–11 animals per group (Figure reprinted from Suzuki et al. 2007)

Wassertheil-Smoller et al. 2003; Bushnell 2006). More recent analyses of the WHI showed that if the women in the study were divided into two cohorts — a younger group, who had not been hypoestrogenic for a prolonged period, and an older group, who had been hypoestrogenic for longer than a decade — the negative effects of estrogen were only observed in the latter group (Harman et al. 2005). These findings spurred us to mimic the delayed initiation of ET used in the WHI to decipher the circumstances under which ET provided benefits against cerebral stroke. We investigated whether a prolonged period of hypoestrogenicity disrupted the ability of E2 to protect the brain against stroke injury. Our results demonstrate that E2 exerts profound neuroprotective actions only when administered immediately upon ovariectomy but not when administered after 10 weeks of hypoestrogenicity (Fig. 1; Suzuki et al. 2007). Therefore, our findings show that an extended period of hypoestrogenicity, similar to that experienced by the women included in the WHI, prevents E2 from protecting the brain against ischemia.

The work of Strom and colleagues (2010) further suggested that the dose and the temporal profile of elevated hormone treatment influenced the outcome.

They found that, when E2 replacement resulted in a spike of E2 in plasma, it did not protect against injury; but when hormone release was more constant and in a physiological range, it provided significant neuroprotection. These investigators also reported that hormone must be present both prior to and after, confirming our data (Dubal et al. 1998).

Additional factors determine E2's ability to protect the brain against stroke injury, such as the type of stroke that is experimentally induced: complete global cerebral ischemia, incomplete global ischemia, focal cerebral ischemia, or multifocal cerebral ischemia. Focal ischemia models have been used most frequently by basic scientists since ischemic stroke in humans usually results from a thrombotic or embolic occlusion in a major cerebral artery (Strom et al. 2011). In this model, insertion of a suture into the middle cerebral artery serves an "embolism" and can be used to temporarily block blood flow by withdrawing the suture after a given period, or to permanently block blood flow by allowing the suture to remain for the entire experimental period. Because withdrawal of the suture results in reperfusion injury, which confounds the injurious effect of occlusion itself, we have used permanent occlusion in all of our studies.

It is also important to consider that not all forms of estrogen act in a similar manner. The WHI used a pharmacological preparation with the trademark name Premarin, which is a conjugated equine estrogen (CEE), a cocktail of estrogens (and other hormones) derived from horses. Other estrogens or estrogen-like compounds such as soy, raloxifene, compounds, which preferentially bind to only one of the estrogen receptor subtypes, have all been tested in animal models with varying results. Simpkins and colleagues (Yang et al. 2000b, 2003, 2005) have used estrogen-like compounds that only have the aromatic A-ring of the estrogen molecule and have found that these compounds exert protective actions using totally estrogen receptor-independent mechanisms.

What has become clear from all of these studies is that the form of estrogen that is used, the dose of the hormone and whether it is administered in a constant or cyclic manner, the age of the animal or human subject and her previous hormonal environmental history, whether or not the subject has been hypoestrogenic for a prolonged period of time before initiation of ET, and whether other hormones are combined either simultaneously or sequentially all influence whether ET protects or exacerbates ischemic injury.

Neuroprotective Actions of Estrogen Against Stroke

Low, Physiological Levels of E2 Exert Profound Neuroprotective Actions

In all of our work, we have used an animal model of stroke that involves permanent occlusion of the middle cerebral artery through permanent placement of a suture into the middle cerebral artery, or middle cerebral artery occlusion (MCAO).

We have used this model to determine under what circumstances E2 protects against neuronal death and to decipher its mechanism(s) of action. We have found that, at low physiological doses (25 pg/ml serum; Dubal et al. 2001), E2 must be administered prior to the onset of injury, since acute administration of E2 at the time of injury does not reduce the extent of infarction (Dubal et al. 1998). This finding contrasts with the discoveries of Toung and colleagues (1998) that show that supraphysiological concentrations of E2 administered immediately before the onset of ischemic injury exert neuroprotection. In addition, pharmacological levels of E2 effectively protected against ischemic brain injury when administered as late as 6 h after the onset of injury (Yang et al. 2000a, 2003). The finding that acute, postischemic administration of E2 at supraphysiological doses exerts protective actions against ischemic injury may have strong clinical implications, especially if non-reproductive estrogen-like compounds can be designed, i.e., selective estrogen receptor modulators (SERMs), and used as the treatment as opposed to the preventive method. Together, our findings clearly demonstrate that administration of even basal levels of E2 profoundly protect the brain against stroke-like injury and that the mechanisms by which estrogens achieve their protective actions are diverse and complex (Nagayama et al. 1999; Bruce-Keller et al. 2000; Bryant et al. 2006; Vegeto et al. 2004; Ramaswamy et al. 2005). Strom and colleagues (2010) have recently shown that E2 must remain elevated after the stroke injury in order to protect. In addition, we found that E2 continues to exert protective effects in middle-aged rats (Dubal and Wise 2001), whereas others have found that E2 does not protect in even older rodents (Liu and McCullough 2011).

Permanent blockage of the middle cerebral artery leads to a severe constriction of blood flow and, therefore, deprivation of oxygen, resulting in metabolic imbalance in the cerebral cortex and striatum. This, in turn, leads to necrotic cell death within the first few hours following artery occlusion. E2 has no protective impact on this type of cell death. On the other hand, regions that surround the ischemic core (ischemic penumbra) undergo partial metabolic impairment and are salvageable by several different therapies. We have previously demonstrated that E2 exerts powerful neuroprotective actions in ischemic penumbra, where it protects neurons from delayed programmed cell death or apoptosis. Our data show that E2 attenuates the increase in TUNEL-positive cells as well as other markers of apoptosis, including caspase-3 activation and injury-induced DNA fragmentation (Fig. 2; Rau et al. 2003b).

ERα Mediates Estrogen's Neuroprotective Action

Two forms of nuclear estrogen receptor (ERα and ERβ) have been cloned and additional subtypes have been functionally characterized, but not isolated and cloned. After the discovery of ERβ in 1996, researchers have shown that, although ERα and ERβ share homologies in their structures and exhibit somewhat overlapping distributions throughout the body and brain, they exhibit very important differences

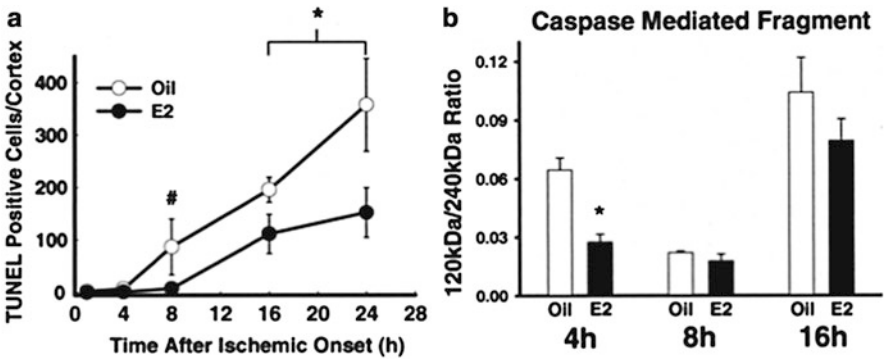

Fig. 2 E2 attenuates markers of apoptosis in the ischemic penumbra. (**a**) E2 treatment significantly decreased the number of TUNEL-positive cells in the ischemic cortex in the early ($p < 0.05$) and late ($p < 0.05$) stages of injury compared to oil-treated controls. Data represent the mean ± SEM of 8–10 animals per group. (**b**) E2 treatment attenuated the caspase-mediated (120 kDa) spectrin breakdown product in the early stage (4 h post-MCAO, $p < 0.05$) of ischemic injury. Data represent the mean ± SEM of 3–4 animals per group (Figure reprinted from Rau et al. 2003a)

in their functions. Interestingly, ERα is only transiently expressed in the fetal cerebral cortex, declines during the postnatal period and remains at very low levels throughout adulthood (Shughrue et al. 1990; Solum and Handa 2001; Prewitt and Wilson 2007). We have found that the presence of ERα after MCAO is essential for E2's neuroprotection against ischemia (Dubal et al. 1999, 2001). We used ERα (ERαKO) and ERβ (ERβKO) knockout mice and found that the presence of ERα, but not ERβ, is a prerequisite for the ability of E2 to exert protective action against stroke injury (Dubal et al. 2001); knocking out ERα blocks E2's ability to reduce the extent of infarction. In marked contrast, E2 continued to exert powerful protective action against injury-induced neuronal death in ERβ-null mice (Fig. 3; Dubal et al. 2001). Thus, the injured brain seems to provide signals that stimulate the re-expression of ERα, which mediates the ability of E2 to protect against neuronal apoptosis and possibly reinitiate differentiation of the injured brain.

Mechanisms of Estrogen's Neuroprotective Action

E2 Suppresses Programmed Cell Death

Our laboratory has investigated potential mechanisms that underlie E2's neuroprotective action and identified multiple downstream targets of its action in neuroprotection. We and others have demonstrated that E2 modulates the expression of multiple genes in ischemic brains, including those that influence the balance between cell death and cell survival. We have shown that E2 prevents injury-induced down regulation of Bcl-2, but does not influence the expression of other

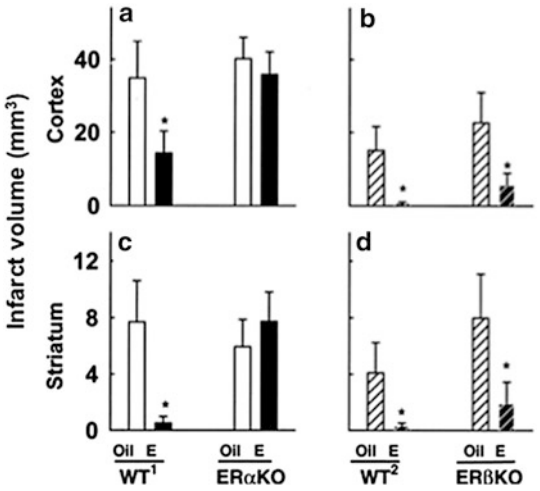

Fig. 3 ERα mediates the neuroprotective effects of E2 during ischemic injury. ERαKO and ERβKO mice, along with their wild type (WT) controls (WT^1 and WT^2, respectively), were ovariectomized and treated with oil or E2. Following 24 h of MCAO, quantification of infarct volumes revealed that E2 failed to protect in the (**a**) cortex and (**c**) striatum of E2-treated ERαKO mice compared to WT^1 controls. In contrast, E2 significantly reduced infarct volumes in the (**b**) cortex and (**d**) striatum of ERβKO mice, similar to their WT^2 counterparts. Data represent the mean ± SEM of 8–13 animals per group, (*, $p < 0.05$) (Figure reprinted from Dubal et al. 2001)

members of Bcl-2 family genes (Dubal et al. 1999). We also examined the effects of stroke injury and E2 on immediate early genes (IEGs), since many of them are steroid-modulated genes that change their expression patterns in response to injury and mediate programmed cell death (Dubal et al. 1999; Rau et al. 2003a). We observed that the levels of c-fos, fosB, c-jun, and junB increase following ischemic injury and that E2 specifically attenuates injury-induced upregulation of c-fos mRNA and protein (Rau et al. 2003a).

E2 Stimulates Neurogenesis in the Face of Injury

One of the most remarkable discoveries in modern neuroscience is that the adult brain is highly plastic and continues to generate new neurons under both normal and neurodegenerative conditions. Studies have suggested that adult neurogenesis is restricted predominantly to two major forebrain regions: the dentate gyrus of the hippocampus and the subventricular zone (SVZ) lining the lateral ventricle (Alvarez-Buylla and Garcia-Verdugo 2002; Taupin and Gage 2002). Under normal conditions, neural stem cells born in the SVZ differentiate into neuroblasts and migrate through the rostral migratory stream to the olfactory bulb, where they become functional interneurons (Lois and Alvarez-Buylla 1994). However, studies have shown that, after injury, neurogenesis may be stimulated and the anatomical

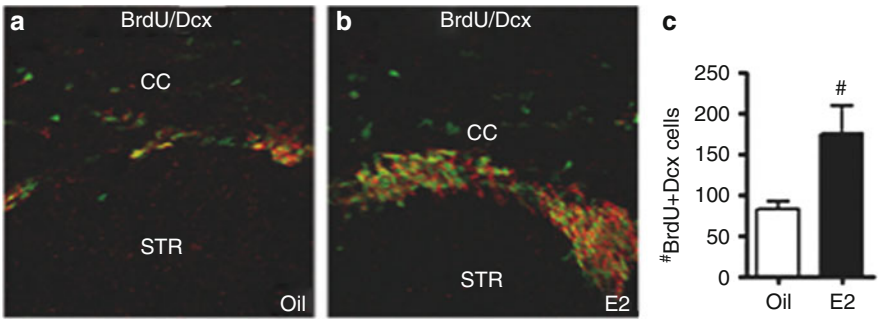

Fig. 4 E2 enhances neurogenesis in the subventricular zone (SVZ) 96 h after ischemic injury. Confocal photomicrographs of BrdU-labeled cells (*green*) dual-labeled with doublecortin (Dcx, *red*), an early neuronal marker, in the ipsilateral SVZ of (**a**) oil-treated and (**b**) E2-treated mice 96 h following MCAO. (**c**) E2 significantly increased the number of BrdU/Dcx dual-labeled cells in the SVZ ($p = 0.0008$, n = 6–7 mice). CC = corpus callosum; STR = striatum (Figure reprinted from Suzuki et al. 2007)

destiny of these new neurons may be very different than under normal circumstances. Recently our laboratory investigated whether E2 stimulates generation of newborn neurons in the SVZ under ischemic conditions. We have found that the same low, physiological levels of E2 replacement that we have used in our earlier studies, which profoundly protect neurons against ischemia, increase the number of newborn neurons in the SVZ after stroke injury (Fig. 4). At 96 h after MCAO, the newborn neurons do not appear to have differentiated into mature neurons, since they only express doublecortin, a marker of immature neurons, and not NeuN, a marker of mature neurons. In addition, the proliferative action of E2 was confined to neuronal precursors and did not stimulate proliferation of astrocytes or microglia (Suzuki et al. 2005). It is important to emphasize that we have discovered a dose of E2 that does not enhance neurogenesis in the SVZ in the absence of injury when uncontrolled proliferation is undesirable. We have begun to decipher some of the mechanisms that underlie E2's ability to influence neurogenesis in the face of a neurodegenerative stimulus. It appears that the presence of both ERα and ERβ is essential for E2 to exert is neurogenic actions, since neither ERα nor ERβ knockout mice exhibited any E2-induced neurogenesis. Future studies will examine changes in gene expression that may mediate E2's stimulation of neurogenesis.

Role of Inflammation and the Immune Response in Injury and E2-Mediated Neuroprotection

One prominent feature that follows stroke injury is a massive, complex inflammatory response. This response includes a cascade of changes in expression of pro- and anti-inflammatory factors that, in turn, strongly contribute to the extent of cell death and ischemic brain injury. Extensive evidence also suggests that post-ischemic

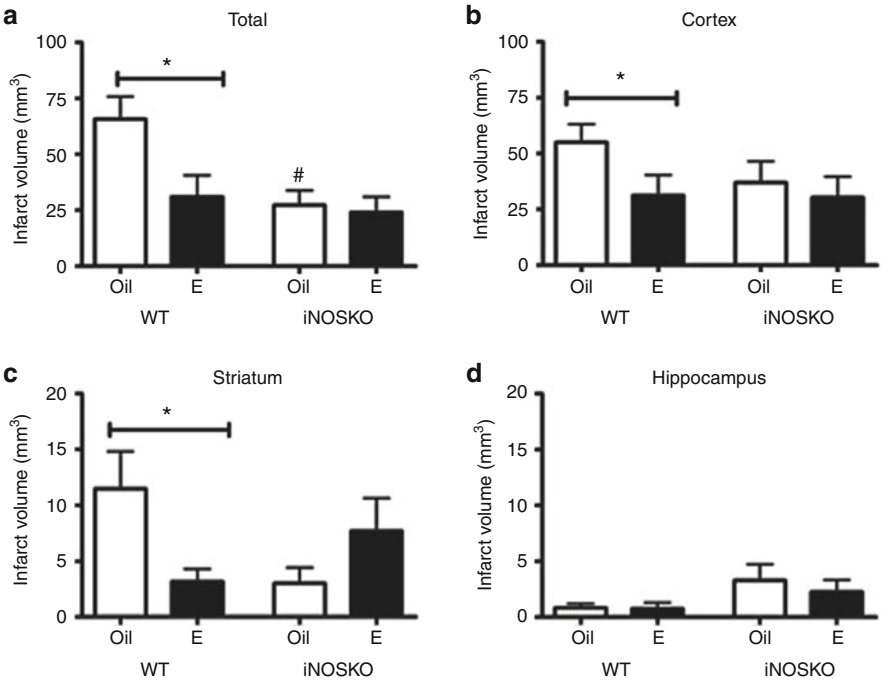

Fig. 5 Female iNOSKO mice are as equally protected following MCAO as E2-treated WT mice. (**a**) Total, (**b**) cortical, (**c**) striatal and (**d**) hippocampal infract volumes were quantified in ovariectomized WT and iNOSKO female mice treated with oil or E2. E2 reduced infarct volume in cortex (**b**) and striatum (**c**) of WT mice (* $p < 0.05$), but there was no further suppression of infarct volume in E2-treated iNOSKO mice compared to their WT counterparts. Infarct volumes of iNOSKO oil-treated mice were also smaller compared to WT oil-treated mice (# $p < 0.05$). Data represent mean ± SEM of 8–14 mice per group (Figure reprinted from Brown et al. 2008)

inflammation strongly exacerbates the extent of cell death in the ischemic penumbra, and E2 may protect the ischemic brain by exerting anti-inflammatory actions (Czlonkowska et al. 2006; Miller and Duckles 2008; Vegeto et al. 2008). We found that E2 attenuated microglial activation and suppressed CNS production of the pro-inflammatory cytokines, interleukin −6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1/CCL2), 24 h after ischemic injury. Concurrently, E2 also enhanced the production of vascular endothelial growth factor (VEGF), an important growth factor that promotes the integrity of the BBB (Suzuki et al. 2009).

To address whether additional anti-inflammatory mechanisms were employed by E2 during stroke, we examined interactions between E2 and inducible nitric oxide synthase (iNOS) in wild type (WT) and iNOS knockout (iNOSKO) female mice 24 h following MCAO. The induction of iNOS plays a critical role in the pathophysiology of stroke by increasing the production of nitric oxide, which contributes to oxidative and nitrosative stress in the ischemic core and penumbra (del Zoppo et al. 2000). We found that ischemic injury is attenuated in iNOSKO compared to WT mice but not protected further in the presence of E2 (Fig. 5; Brown et al. 2008). We also found that E2 suppressed iNOS gene expression, suggesting that E2 protects by down regulating

a critical component in stroke's pro-inflammatory gene cascade (Brown et al. 2008). To determine whether E2's interactions with iNOS also differentially suppressed cytokine production, we measured peripheral cytokines. We found that macrophage inflammatory protein 1-α (MIP1α/CCL3), a chemokine, is suppressed by E2 in WT mice, but E2 did not suppress MIP1α/CCL3 levels further in the iNOSKO mouse (Suzuki et al. 2009). Collectively, our findings in the iNOSKO mouse demonstrate that many of the anti-inflammatory properties exerted by E2 during stroke are partially dependent on the presence of iNOS. Future studies will address whether E2 also modulates other components of the iNOS pathway in ischemic injury.

Summary

We have discussed our findings that low, physiological concentrations of ET are protective against stroke injury when, but only when, administered immediately after estrogen withdrawal. Utilizing a permanent MCAO animal model of stroke in mice and rats, we reported that treatment with basal levels of E2, initiated immediately after ovariectomy to maintain basal levels of E2, exerts profound protective effects in the cortex. We have reported that this is due to suppression of apoptosis and enhanced neurogenesis. The mechanisms that underlie these protective actions include enhanced expression of genes that prevent apoptosis and enhance cell survival, stimulation of the birth of new neurons, and suppression of the inflammatory response. Our studies have uncovered critical roles for both ERα and ERβ in suppressing cell death, enhancing neurogenesis, and suppressing inflammation. Finally, we have shown that the timing of E2 therapy determines whether or not it exerts neuroprotective actions. When initiation of E2 administration is delayed, none of the protective actions were observed.

Together with the work of numerous other laboratories, studies clearly demonstrate that administration of even basal levels of E2 profoundly protects the brain against stroke-like injury and that the mechanisms by which estrogens achieve their protective actions are diverse and complex.

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Index

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