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Prenatal androgen influences on the brain: A review, critique, and illustration of research on congenital adrenal hyperplasia

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Abstract

Sex hormones, especially androgens, contribute to sex and gender differences in the brain and behavior. Organizational effects are particularly important because they are thought to be permanent, reflecting hormone exposure during sensitive periods of development. In human beings, they are often studied with natural experiments in which sex hormones are dissociated from other biopsychosocial aspects of development, such as genes and experiences. Indeed, the greatest evidence for organizational effects on sex differences in human behavior comes from studies of females with congenital adrenal hyperplasia (CAH), who have heightened prenatal androgen exposure, female-typical rearing, and masculinized toy play, activity and career interests, spatial skills, and some personal characteristics. Interestingly, however, neuroimaging studies of females with CAH have revealed few neural mechanisms underlying these hormone-behavior links, with the exception of emotion processing – instead showing reduced gray matter volumes and reduced white matter integrity most consistent with other disease-related processes. The goals of this narrative review are to: 1) describe methods for studying prenatal androgen influences, while offering a brief overview of behavioral outcomes; 2) provide a critical methodological review of neuroimaging research on females with CAH; 3) present an illustrative analysis that overcomes methodological limitations of previous work, focusing on person-specific neural reward networks (and their associations with sensation seeking) in women with CAH and their unaffected sisters in order to inform future research questions and approaches that are most likely to reveal organizational hormone effects on brain structure and function.

Significance Statement

Sex and gender differences – and reasons for them – are hotly debated scientifically and socially. Strong evidence suggests that sex hormones (especially androgens) present in early development contribute to differences in human behavior, but research on early androgen influences on the brain has yielded relatively few insights. In this narrative review, the authors consider methodological explanations for this in the context of congenital adrenal hyperplasia, a genetic condition in which females are exposed to male-typical hormones in utero. The authors also present a pilot data set and person-specific brain network analysis to illustrate how to move this area of inquiry forward.

The brain mediates gendered behaviors, that is, behaviors that differ – on average – between boys and men and girls and women. Although sex differences in the human brain are rarely analogs of sex or gender[1] differences in behavior, differences in regional brain volumes and patterns of functional activation are wholly expected – and borne out by data (Beltz et al., 2020; Cahill, 2017; Liu et al., 2020; Ruigrok et al., 2014). Some of the largest sex differences concern overall brain and gray matter volumes, regional brain volumes (especially in the amygdala, hippocampi, and commissures), activation during spatial, language, and emotion processing tasks, and trajectories of neural development and aging.

Despite consistent evidence of neural sex differences, key questions surround their origins. Sex hormones, such as androgens and estrogens, have long been of interest because of their organizational (i.e., permanent due to exposure during a sensitive period) and activational (i.e., transitory) effects on the brain and behavior in experimental animal models (McCarthy et al., 2018; Wallen, 2005). Even in people, there is converging evidence for neural sex differences in response to rises in pubertal androgens and estrogens (Goddings et al., 2019; Herting & Sowell, 2017), and for ovarian hormone influences on brain structure and function across the lifespan (Beltz & Moser, 2020).

Prenatal androgen influences on the development of neural sex differences are of particular interest because they have organizational effects on human behavior; that is, in utero exposure to androgens not only virilizes reproductive anatomy, but also permanently masculinizes behavior (reviewed in Berenbaum & Beltz, 2011, 2016). The neural underpinnings of these effects, however, have largely eluded researchers. Although there are conceptual explanations as to why prenatal androgens may affect behavior but be difficult to detect in the brain (e.g., compensation and equifinality; Beltz et al., 2020; De Vries, 2004), methodological explanations are also likely.

[1] Although it is common for "sex" to be used to refer to aspects of behavior with some biological influence in males and females, and for "gender" to be used to refer to aspects of behavior with some sociocultural influence in men and women, these stringent distinctions presume causes of behavior are known. Thus, in this paper, "sex" is used to refer to categories of male and female, and "gender" is used to refer to determinations about males and females linked to, for instance, roles and self-expressions (Blakemore et al., 2009). There is no presumed value linked to these terms.

The overarching aim of this narrative review is to explicate these methodological explanations. We accomplish this in three steps. First, we discuss methods for studying prenatal androgen exposure in human beings, summarizing the size and consistency of behavioral effects. Second, we review the neuroimaging literature on prenatal androgen influences on brain structure and function, focusing on findings as well as limitations of samples, study designs and analyses, and assumptions of homogeneity and neural localization. Finally, we illustrate ways to overcome these limitations in future work using pilot empirical data and a novel neuroimaging analysis.

Methods for Studying Human Neural and Behavioral Effects of Prenatal Androgens

It is, of course, neither ethical nor feasible to manipulate prenatal hormones in people, but there are excellent methods to examine their effects on later behavior (and the brain). We describe three types of studies below, and provide a summary of evidence from them.

Natural Experiments. Most evidence about effects of prenatal hormones comes from people with a difference/disorder in sex development (DSD) that results in prenatal hormone levels discordant with assigned sex. Of particular value are congenital adrenal hyperplasia due to 21-hydroxylase deficiency (CAH) and complete androgen insensitivity syndrome (CAIS). Females with CAH have a typical female karyotype (46,XX) but a genetic defect that leads to high levels of adrenal androgens beginning early in gestation; they are usually diagnosed and treated shortly after birth, so have low female-typical postnatal androgens and are reared as girls (Merke & Auchus, 2020). Individuals with CAIS have a typical male karyotype (46,XY) and normal testes, but have defective androgen receptors that prevent them from responding to androgens, so they have female-typical physical development and are reared as girls.

Females with CAH and CAIS represent different opportunities to examine effects of androgen influences during early development. If androgens affect the brain and behavior in human beings, as they do in other species, then: (a) females with CAH should be more male-typed and less female-typed than a comparison group of females without CAH, and (b) females with CAIS should be similar to typical females. Females with CAIS also provide an opportunity to examine whether the Y-chromosome plays a role in masculinizing human neural and behavioral development, as when there are differences between females with CAIS and typical females. It is important to note that females with CAH and CAIS could behaviorally differ from

comparison groups for reasons beyond prenatal androgens and due to combined biological and sociocultural influences. For example, females with CAH have masculinized genitals that are usually modified by surgery and have deficient cortisol without treatment, so are treated with glucocorticoids throughout their life to prevent androgen excess after diagnosis; thus, similarities between females and males with CAH (the latter have deficient cortisol production but not prenatal androgen excess) reflect disease processes unrelated to androgens. Females with CAIS have female-typical rearing and postnatal hormone levels in addition to female-typical prenatal androgen (see Blakemore et al., 2009). Therefore, it is important to obtain converging evidence.

Direct Assessment of Early Hormones. Some work seeking convergence in typical individuals has involved direct measurement of androgens during several early sensitive periods. The method used most often involves assessment of hormones from amniotic fluid, with those hormones related to behavior at later ages (Finegan et al., 1989). There are several limitations: Hormones are not obtained from the fetus; hormones are sampled only once and the time of sampling varies across participants; and amniocentesis is performed selectively to detect concerns about fetal anomalies. There are also studies linking hormones in umbilical cord blood to behavior at later ages (e.g., Whitehouse et al., 2012), but this is not particularly informative because cord blood contains hormones from both the mother and the neonate, and the neonatal period is not likely a sensitive period for hormone effects.

Indirect Indicators of Prenatal Hormones. The limitations of natural experiments and direct assessments of early hormones have led to the search for indirect indicators of prenatal hormone exposure. The most intriguing method involves studying opposite-sex twins; the rationale is based on animal studies showing altered sex-typed behavior associated with uterine position near opposite-sex littermates (Tapp et al., 2011). Effects are strongest for animals gestating between two opposite-sex littermates, limiting applicability to people. Moreover, behavioral studies of opposite-sex twins need to control for effects of sharing a postnatal environment.

Multiple markers of prenatal androgen exposure have also been suggested. Most work is focused on finger lengths, especially the relative ratio of the second to fourth finger (2D:4D ratio). Digit ratio has been only modestly associated (in studies of females with CAH and CAIS) with prenatal androgen exposure, making it a poor reflection of *individual differences* in androgen exposure, necessitating extreme caution when linking digit ratio to behavior

(Berenbaum et al., 2009). Other work is focused on indicators that are potentially promising, but have not yet been validated as markers of prenatal androgen exposure (e.g., otoacoustic emissions; Wisniewski et al., 2014) or on biological measurements that are likely confounded by postnatal androgen exposure (e.g., anogenital distance; Dean & Sharpe, 2013).

Summary of Evidence for Human Behavioral Effects of Prenatal Androgens. Evidence for behavioral effects of prenatal androgens comes primarily from females with CAH, with converging evidence from other DSD and typical samples (reviewed in Berenbaum & Beltz, 2011, 2016; Blakemore et al., 2009; Peckins & Beltz, 2020). There are complexities, but in sum, androgens masculinize some behaviors more than others, emphasizing the multidimensional nature and causes of gender; they influence personal characteristics (e.g., interests) the most, and gender identity and cognitions the least, with social relationships in between and reflecting the combined effects of personal characteristics and identity. In terms of effect size (with small effects explaining ~1% variation in behavior, medium effects ~9%, and large effects ~25%; Cohen, 1988), prenatal androgens have large effects on activities and interests (e.g., increased childhood play with boys' toys, adolescent time in male-typed sports, and adult masculine hobbies), moderate effects on increased spatial skills, aggression, and sensation-seeking as well as decreased interest in babies and heterosexual orientation (e.g., increased non-heterosexual attractions), and small effects on binary gender identity.

Prenatal Androgen Effects on Brain Structure and Function

Neuroimaging studies have begun to reveal organizational effects of prenatal androgens on the human brain. Magnetic resonance imaging (MRI) and positron emission tomography studies that utilize natural experiments hold considerable promise owing to their statistical power (e.g., effects are larger in natural experiments than in direct and indirect early hormone assessments). Thus, neuroimaging studies of individuals with CAH and CAIS are reviewed below in order to identify prenatal androgen effects on brain structure and function; the review is not comprehensive in that many early studies (with especially small samples) and studies using other methods are not reviewed comprehensively (see Beltz et al., 2020), but are mentioned when relevant. Methodological critiques are provided in order to contextualize findings and identify opportunities for future research.

Brain Structure. The earliest neuroimaging studies of CAH focused on white matter abnormalities and hyperintensities, or small white matter lesions. Results showed that 27% to 45% of individuals with CAH – both males and females – evidenced these abnormalities, which are uncommon in comparatively healthy samples (Bergamaschi et al., 2006; Nass et al., 1997; Sinforiani et al., 1994). Samples were small (*N*=15 to *N*=39), with wide age ranges (3 to 36 years), but there were no trends linked to sex or age, and no changes in an 11-year follow-up (Bergamaschi et al., 2006). Given the abnormalities in both sexes, it is most likely that they do not reflect effects of prenatal androgens, but rather, aspects of the disease or its glucocorticoid treatment.

Recent studies of white matter in individuals with CAH involve fractional anisotropy (FA), a measure of the diffusion of water molecules in the brain; high values reflect directional (anisotropic) diffusion in strong white matter tracts. Consistent with early work on hyperintensities, reduced FA was seen in a sample of 19 females with CAH compared to 19 control women aged 18 to 50 years in regions that included the hippocampus and several prominent tracts, such as the longitudinal and uncinate fasciculi (Webb et al., 2018). Although males were not studied, some indicators of white matter integrity were negatively associated with glucocorticoid dose in women with CAH, suggesting that aspects of the disease – apart from prenatal androgens – were responsible for effects. This finding was not replicated (van't Westeinde et al., 2020): Although reduced FA was found in 16 males and 27 females with CAH versus 43 controls, results did not withstand corrections for overall brain volume, essential in studies of sex-related brain structure, as men have an approximately 10% larger brain volume than women (reviewed in Beltz et al., 2020). Together, these studies likely indicate that disease processes reduce FA in individuals CAH, but effects may be small or dependent upon brain volume.

Several studies concern brain volume in individuals with CAH. Some results suggest that males and females with CAH have reduced overall brain volumes compared to controls (Herting et al., 2020; van't Westeinde et al., 2020). There has been particular interest in examining effects of prenatal androgens on brain regions that typically show sex differences, such as the amygdala and hippocampus (see Beltz et al., 2020). Although studies reported reduced volumes in CAH in both regions, even after correcting for overall brain volume (Herting et al., 2020; Merke et al., 2003), the reductions were generally present in both males and females, so are unlikely due to

prenatal androgens. Congruent with this, data from individuals without DSD failed to support links between testosterone in amniotic fluid and amygdala and hippocampal volumes (Lombardo et al., 2012). Interestingly, studies in CAH utilized samples varying in age from 6 to 18 years, further complicating interpretation because the amygdala and hippocampus change across childhood and adolescence, reaching peak volumes at different ages in girls and boys (see Beltz et al., 2020). Recent evidence also suggests smaller volumes in prefrontal regions and reduced thickness in prefrontal, parietal, and occipital areas in individuals with CAH compared to controls (Herting et al., 2020; van't Westeinde et al., 2020). These findings again were observed in both sexes, and so, are consistent with disease-related causes. In fact, volume decreases in individuals with CAH were related to increased body mass index, a side effect of prolonged glucocorticoid treatment (Herting et al., 2020).

Importantly, a study of 16 women with CAIS (compared to 32 control women and 32 control men) provides some insight into prenatal androgen effects on brain structure without glucocorticoid confounds (Savic et al., 2017). Compared to men, both women with CAIS and controls had reduced FA in portions of the longitudinal and uncinate fasciculi, thicker parietal and occipital cortices, and increased hippocampal volumes, implicating prenatal androgens in these regions. Women with CAIS and control men differed from control women in some ways, though, implicating the Y chromosome; they had larger overall brain volumes, thinner motor cortices, and smaller caudate volumes.

Brain Function. Research utilizing natural experiments to examine prenatal androgen effects on brain activation is relatively sparse. In CAH, the focus has been on responses to and memory of negative emotions in two studies with overlapping samples, but there is not behavioral evidence for prenatal androgen effects on emotion processing (see Berenbaum & Beltz, 2011). In CAIS, studies have focused on behaviors that show prenatal androgen effects, including sexual behavior and spatial skills.

In both studies of emotion-linked brain function in people with CAH, findings were more pronounced in females than in males, suggesting a prenatal androgen effect. The sample included adolescents (mean age 13.5 years) with CAH (n=14) and controls (n=14-22). Compared to control girls, girls with CAH showed greater bilateral amygdala activation when viewing negative (fearful and angry) faces than when viewing neutral faces; amygdala activation in girls with CAH was not statistically different from that of control boys (Ernst et al., 2007). This is

broadly consistent with work showing that amniotic testosterone is positively linked to male-typed right lateralization of emotion processing in a typical sample (Grimshaw et al., 1995). Again compared to control girls, girls with CAH had reduced amygdala, hippocampus, and anterior cingulate activation during an emotional memory task (i.e., when remembering fearful faces), whereas boys with CAH showed greater activation than control boys in the same regions (Mazzone et al., 2011). These studies suggest prenatal androgens might have particular organizational influences on amygdala function, but this interpretation is complicated. For instance, both males and females with CAH had reduced negative appraisals of fearful and angry emotions and remembered fewer fearful faces compared to controls (Ernst et al., 2007; Mazzone et al., 2011); this makes it unclear whether the prenatal androgen-influenced emotion substrates generalize to typical samples. Moreover, emotion processing develops behaviorally and neurally across adolescence (Blakemore & Mills, 2014) in ways associated with puberty (reviewed in Goddings et al., 2019); thus, findings likely reflect a complex interplay between organizational effects of prenatal androgens, adolescent brain development, and effects of pubertal hormones.

Studies of sexual behavior and spatial skills in adults with CAIS also provide evidence for prenatal androgen effects on brain activation. In one study, control men had greater amygdala activation during the viewing of sexual images than did control women or women with CAIS (N=39), with both groups of women providing similar arousal ratings after viewing the images (Hamann et al., 2014), consistent with sex differences in this task (Hamann et al., 2004). In another study, control men (n=30) outperformed (i.e., had faster reaction times) and had greater parietal activation during a mental rotation task than did both control women (n=29) and women with CAIS (n=21) (van Hemmen et al., 2016), consistent with a relatively large literature on sex differences in spatial skills (reviewed in Beltz et al., 2020). Although both studies align with expectations, male-typed socialization or activational hormone influences could account for the effects because women with CAIS do not have prenatal or circulating androgen exposure, often receive estrogen treatment, and are generally reared female and identify as women. Thus, CAIS is not as strong an experiment of prenatal androgen effects on the brain and behavior as is CAH; it is a comparatively better test of Y-chromosome effects.

Methodological Critiques. It is important to acknowledge both the innovation of neuroimaging work utilizing natural experiments and to consider its limitations in order to advance understanding of prenatal androgen effects on brain structure and function. For instance,

most studies are small, with samples ranging from 14 to 39 individuals with CAH or CAIS. This is understandable because the diseases are rare, but small samples inflate the likelihood of bias and false positives (Button et al., 2013; David et al., 2018). Given the rarity of the diseases, most researchers report on multiple measures in the same participants (Ernst et al., 2007; Mazzone et al., 2011) or focus their resources on studying females with CAH, who provide the best test of prenatal androgen effects (e.g., Webb et al., 2018). An improvement would be to compare females with CAH to their unaffected sisters; the dependent design provides some control for genes and shared environment, increasing statistical power (see Berenbaum, 2018).

Sample characteristics are important to consider in neuroimaging studies utilizing natural experiments. Owing to the rarity of the diseases, age ranges are often large. For instance, ages ranged from 3 to 36 years in a structural study of individuals with CAH (Nass et al., 1997), and CAIS women were on average 38 years old compared to 28-year-old control women in a functional study (Hamann et al., 2014). This is usually addressed by covarying age in analyses, but a single linear variable is unlikely to equate children and adults (Miller & Chapman, 2001). Even when age ranges are similar, development must be considered, as seen in the emotion processing studies of adolescents with CAH (Ernst et al., 2007; Mazzone et al., 2011); emotion undergoes neural reorganization with puberty, arguably muddying effects of prenatal androgens. There are other key sample characteristics that often go unaddressed in research with clinical samples, such as race, ethnicity, and socioeconomic status (Gatzke-Kopp, 2016).

Experimental design and data analyses also impact inferences. Regarding design, if the goal of a study is to detect neural mechanisms underlying prenatal androgen influences on behavior, then functional data should be collected while individuals are engaging in that behavior in the scanner; in other words, opportunities to detect prenatal androgen influences on brain function are maximized by examining behaviors that evidence prenatal androgen effects. This has been done in studies of CAIS on sexual behavior and spatial skills (Hamann et al., 2014; van Hemmen et al., 2016). In functional studies of CAH, however, the focus has been on emotion (Ernst et al., 2007; Mazzone et al., 2011), which shows a sex difference but not a clear pattern of prenatal androgen effects. Work in CAH on activities and interests, which show large effects, is needed (as are novel tasks to assess this in the scanner).

Regarding analyses, studies of brain structure in CAH and CAIS must consider adjustments for overall brain volume due to its robust sex difference. If adjustments are not

made, it is unclear whether effects seen in females with CAH (e.g., regarding amygdala volumes) are unique or merely reflect brain size (see Beltz et al., 2020). This has fortunately been done in recent studies in individuals with CAH, with some studies reporting results both with and without overall brain volume corrections, which altered inferences in some cases (e.g., van't Westeinde et al., 2020), but not others (e.g., Merke et al., 2003).

Moreover, increased emphasis on neural integration (e.g., network connectivity) is needed in analyses of both brain structure and function in studies of CAH and CAIS. The extant literature largely focuses on *a priori* brain regions of interest (ROIs, such as the amygdala; e.g., Ernst et al., 2007; Herting et al., 2020; Mazzone et al., 2011; Merke et al., 2003) followed by full brain analyses to detect *a posteriori* regions in which effects may have also occurred. This localized approach may increase power (e.g., via small volume corrections), but it is an unrealistic representation of the brain and lags behind the network approach used in neuroscience broadly (Bassett & Sporns, 2017). Studies of individuals with CAH and CAIS are fortunately advancing in this regard. For example, functional connectivity during exposure to masculine and feminine pheromones did not differ in women with CAH compared to controls in one study (Ciumas et al., 2009), and another study revealed that women with and without CAIS had greater connectivity than control men in the default mode network, suggesting prenatal androgens might undergird sex differences in hallmark resting state networks (Savic et al., 2017).

Lastly, studies of prenatal androgen influences on brain structure and function must consider heterogeneity across people. Although not stated explicitly, comparisons of women with CAH or CAIS to control women and men assume homogeneity in order to average across all individuals in a group. This assumption is helpful because it facilitates comparisons and generalizations, but it is often violated, especially in clinical samples, such as individuals with DSD, who are often more variable than controls (e.g., Beltz et al., 2011; Beltz et al., 2016; Herting et al., 2020; Mazzone et al., 2011). Violations of the assumption of homogeneity result in statistical inaccuracies, including spurious connections in brain networks, faulty inferences, and failures to replicate (Gates et al., 2010; Molenaar, 2004; Poldrack et al., 2017). Instead, person-specific analyses are needed; they leverage heterogeneity and the time series nature of functional neuroimaging data to create connectivity networks that are accurate for individuals (Gates & Molenaar, 2012).

Illustration: Personalized Reward Processing Networks in Women with CAH

An illustration demonstrates how study designs and analyses can overcome some limitations of previous work. Our goal was to investigate whether patterns of neural integration underlying reward processing systematically differed in women with CAH compared to unaffected women, while accounting for individual-level neural heterogeneity. This study has four main strengths. First, despite the small sample, we maximized power by comparing women with CAH to their unaffected sisters, who served as controls. Second, we studied reward processing, which is related to behavioral sensation seeking and substance use, and shows a moderate sex difference. Neurally, it shows stronger striatal activation in a wider reward-like network for men than women (Spreckelmeyer et al., 2009). Behaviorally, it reflects organizational effects of prenatal androgens: Among opposite sex twins, women with male co-twins had higher sensation seeking than those with female co-twins (Resnick et al., 1993; Slutske et al., 2011); in a population-wide study, women with CAH had more substance use diagnoses compared to controls (Engberg et al., 2015). Third, neural integration was examined via connectivity analyses of 12 *a priori* ROIs. Fourth, heterogeneity was reflected in person-specific connectivity analyses (that can incorporate homogeneity).

Methods. Thirteen women with classic CAH and 7 unaffected sisters provided informed consent to participate in this behavioral and neuroimaging study. They were part of a parent longitudinal behavioral study (see Beltz et al., 2011; Berenbaum et al., 2012; Berenbaum & Snyder, 1995) and were recruited for neuroimaging if they were at least 18 years old and had a (preferably) same-sex sibling without CAH who also agreed to participate. Behavioral surveys included the valid and reliable Zuckerman Sensation Seeking Scale-V (Roberti et al., 2003; Zuckerman et al., 1978), which consists of 40 forced-choice items (e.g., 0="A sensible person avoids activities that are dangerous." versus 1="I sometimes like to do things that are a little frightening.") from which a mean score of sensation seeking was created; subscale scores (i.e., boredom susceptibility, disinhibition, experience seeking, and thrill and adventure seeking) were explored.

Details of neuroimaging data collection and MRI scan sequences, the reward processing task, and data preprocessing are contained in the Supplemental Materials. Briefly, blood oxygen-level dependent (BOLD) fMRI data were collected using an echo planar imaging sequence during a monetary incentive task adapted from prior research (Delgado et al., 2000) in which

participants won or lost money in each trial of a card guessing game with a fixed response pattern, such that each participant won $20 in the task and 10 *win* and 10 *loss* trials in each of 3 runs. Functional data were preprocessed in FSL (www.fmrib.ox.ac.uk/fsl) using a standard pipeline.

The primary goal of the study was to delineate connectivity among 12 *a priori* ROIs representing 3 networks important for reward processing (Demidenko et al., 2020; Haber & Behrens, 2014; Haber & Knutson, 2010): *regulatory*, *approach*, and *salience*. Names, abbreviations, and central coordinates (of 10mm diameter spheres) are in Table 1, with anatomical placement in Figure 1A. Rationale for ROI selection is in the Supplemental Materials.

A first-level general linear model was fit to each functional run using six motion regressors and derivatives as well as double gamma hemodynamic response function-convolved regressors indicating feedback during *win* and *loss* trials, and then the *win>loss* contrast was averaged across runs for each participant in a second-level fixed effects analysis. A higher-level mixed-effects analysis was then conducted to examine effects across participants. Results of the *win>loss* contrast are shown in Figure 1B. There was substantial activation in expected regions (Bartra et al., 2013; Delgado et al., 2000; Forbes et al., 2009), including the bilateral striatum, OFC, insula, DLPFC, and vmPFC, overlapping substantially with approach network ROIs.

Finally, person-specific connectivity analyses were conducted using group iterative multiple model estimation (GIMME; Gates & Molenaar, 2012). BOLD data were extracted from the preprocessed functional timeseries (concatenated across runs) of the 12 ROIs for each person and used in analyses. GIMME is a data-driven connectivity approach that outperforms other approaches (including seed-based approaches used in Ciumas et al., 2009; Savic et al., 2017) in large-scale simulations (Gates & Molenaar, 2012; Smith et al., 2011). Specifically, GIMME is a functional connectivity approach that creates sparse networks among ROIs with directed connections that are time-lagged or time-locked (contemporaneous). Following other applications (e.g., Beltz et al., 2018), connections apply to: (a) everyone in the sample (i.e., group-level reflecting homogeneity in at least 75% of the sample), (b) only those with CAH or unaffected controls (i.e., pre-defined subgroup-level reflecting potential prenatal androgen effects), or (c) a single person (i.e., individual-level reflecting heterogeneity). After estimating networks, the density of connections within the regulatory, approach, and salience systems was

calculated as a proportion of overall network connections (see Beltz & Gates, 2017). They were tested for group differences and links to sensation seeking scores.

Results. The person-specific GIMME networks (with group-, subgroup-, and individual-level connections) fit the data well, according to average fit indices: $\chi^2(158.7)=855.96$, $p<.001$, RMSEA=.096, SRMR=.041, CFI=.953, NNFI=.918. Results are in Figure 2 regulatory ROIs are blue, approach ROIs are green, and salience ROIs are red, with contemporaneous connections as solid lines, and lagged connections as dashed lines. Aside from autoregressive connections (i.e., lagged connections from each ROI to itself depicting stability), there were 13 group-level connections that applied to all individuals in the sample (black lines in Figures 2A and 2B), 13 CAH-specific connections (dark gray lines in Figure 2A), 22 control-specific connections (light gray lines in Figure 2B), and an average of 10.15 (*SD*=6.53) individual-level connections. Figures 2C and 2D show the final networks for a sister pair; all group and subgroup connections are estimated for each woman along with unique individual-level connections (red are positive, blue are negative, and width reflects magnitude).

Comparisons of network densities across groups (controlling for nonsignificant residual motion via framewise displacement), revealed that women with CAH had significantly greater density in the approach network than did unaffected controls, $F(1,17)=73.64$, $p<.001$ (green bars in Figure 2E). This is also seen in the sisters' networks: the woman with CAH (Figure 2C) had 38 network connections, with an approach density of .29, whereas her sister (Figure 2D) had 37 network connections with an approach density of .16. Interestingly, however, the correlations of approach network density with sensation seeking did not differ between women with CAH (r=.36) and unaffected control women (r=.43), who also reported similar levels of sensation seeking (CAH: *M*=.46, *SD*=.17; Control: *M*=.46, *SD*=.19). There were no consistent patterns across subscales.

Interpretation. Although these findings are preliminary, they illustrate the neural similarities (black connections) and differences (gray connections) between women with CAH and their unaffected sisters, as well as individual differences. Both women with CAH and their sisters had integrated salience networks (in red) that interfaced with the regulatory network (in blue), but women with CAH had greater integration of the approach network (in green) than their sisters. This greater integration was not consequential for sensation seeking behavior, as there were similar links in both groups, potentially suggesting that prenatal androgens influence

processing of reward-related stimuli, but that other neural systems compensate; for instance, approach density may interact with amygdala activation or white matter integrity to predict sensation seeking behavior. Moreover, individual differences were reflected in the person-specific networks, as each woman in the sample had some unique connections that differed from the full sample, her subgroup, and her sister (e.g., Figures 2C and D).

Combined with the strengths of the pilot study design (e.g., sisters completing an androgen-linked MRI task), these findings were uniquely revealed using GIMME, a novel approach in the study of prenatal androgen influences on brain function. GIMME is more accurate than other connectivity approaches (Gates & Molenaar, 2012; Smith et al., 2011), especially for heterogeneous clinical samples, because it creates a unique network of time-lagged and time-locked connections for each person that includes information about the full sample and their subgroup (e.g., all women with CAH). GIMME goes beyond mere comparisons of average activation in brain regions that neglect the integrated, network properties of brain activity and fail to represent variation among individuals in a group (Bassett & Sporns, 2017; Molenaar, 2004). Thus, these pilot analyses illustrate the enormous scientific potential of wedding natural experiments of prenatal androgen influences with state-of-the-art brain analytics: Group similarities and differences can be detected in accurate person-specific brain networks, which hold promise for personalized medicine (Fröhlich et al., 2018).

The pilot study and GIMME approach are certainly not without limitations. Beyond the small sample size, comparisons to men (with and without CAH) were not available to help parse prenatal androgen effects from potential disease characteristics. Moreover, GIMME relies on *a priori* ROI selection (see Table 1), so it is possible that key brain regions that are functional targets of organizational hormone effects or that mediate sex differences in the neural processing of reward were missed. Nonetheless, the empirical data provide a useful illustration of ways in which research on prenatal androgen effects on brain function can be advanced.

Conclusion

Natural experiments, especially studies of women with CAH, have significant potential to reveal organizational effects of prenatal androgens on the brain bases of behavioral sex and gender differences. A narrative review of the neuroimaging literature in individuals with CAH, though relatively sparse, reveals some consistent findings. Structural studies indicate that there are white matter and gray matter reductions in both males and females with CAH, providing

more evidence for disease than prenatal androgen effects on brain structure. Functional studies suggest prenatal androgens affect brain activation underlying emotion, but these studies also highlight the limitations of existing neuroimaging research on CAH, including small samples, age effects, task selection, and emphases on functional localization and sample homogeneity. Our pilot study illustrates ways to overcome some of these limitations to advance research on the neuroendocrine bases of sex differences. Examining functional brain networks underlying reward processing, which shows sex differences and links to prenatal androgens, we found that the person-specific networks of women with CAH had greater density among approach-related regions than did those of their unaffected sisters. We hope this encourages future work utilizing natural experiments to uncover prenatal androgen effects on the human brain through careful study design and sophisticated analytics.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

Author Contributions

All authors had full access to all the illustrative data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Conceptualization*, *Methodology*, and *Resources*: A.M.B., S.J.W., and S.A.B.; *Formal Analysis* and *Visualization*: A.M.B. and M.D.; *Writing - Original Draft*, A.M.B., M.D., and S.A.B.; *Writing - Review & Editing*, A.M.B., M.D., S.J.W., and S.A.B.; *Funding Acquisition*, S.J.W. and S.A.B.

Figure Captions

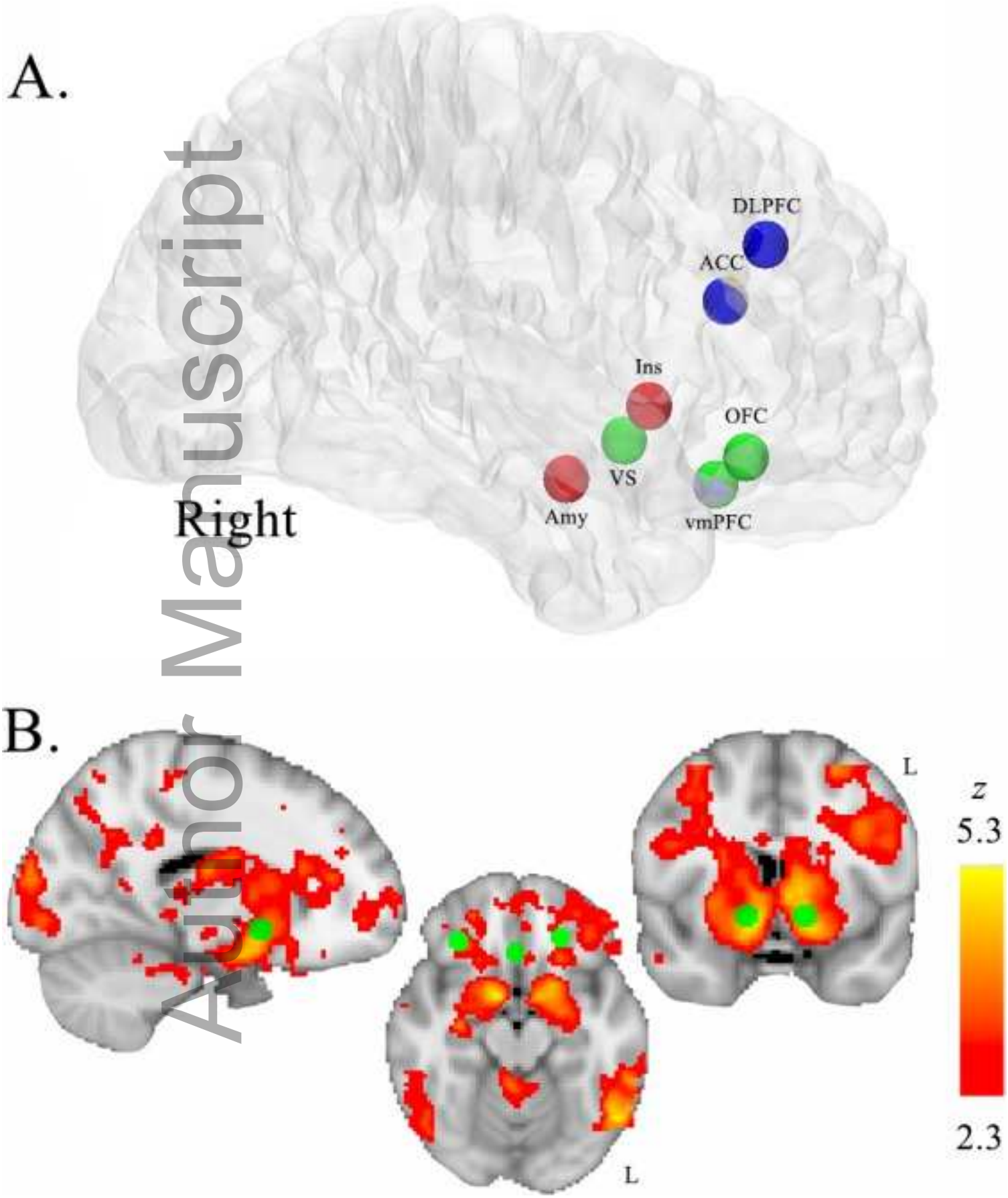
Figure 1. *A priori* regions of interest (ROIs) used in group iterative multiple model estimation (GIMME) connectivity analyses. A.) Right hemisphere and midline ROIs overlaid on a standard template brain. Blue regions are in the regulatory network (contralateral left DLPFC not shown), red regions are in the salience network (contralateral left Amy and Ins not shown), and green regions are in the approach network (contralateral left VS and OFC not shown). See Table 1 for ROI abbreviations. B.) Full-sample ($N = 20$) functional activation during *win* > *loss* trials of the reward processing task (thresholded at $z = 2.3$, $p < .01$), with overlaid approach network ROIs (at x = 17, y = 7, z = -16), showing that the task elicits anticipated activation.

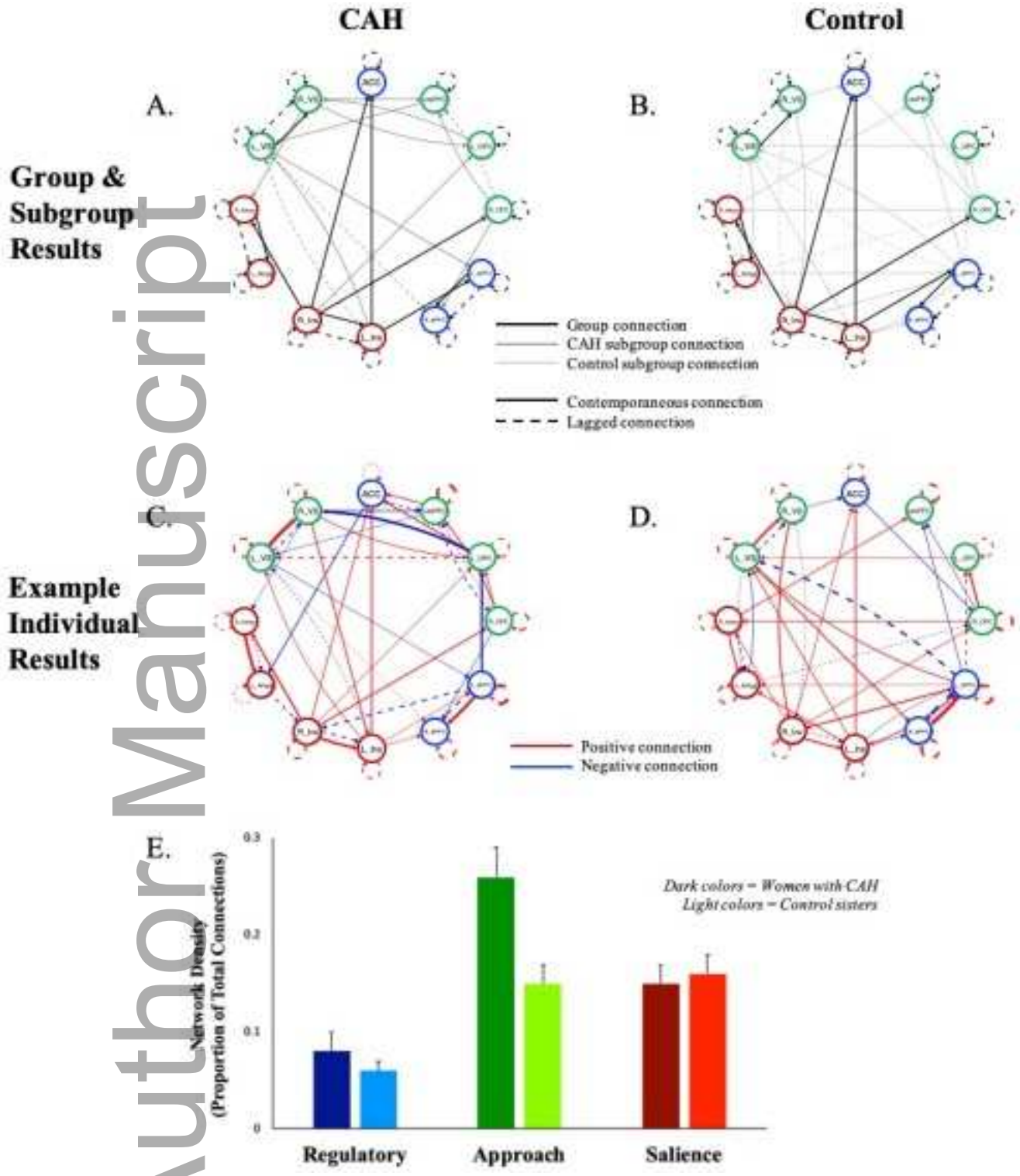
Figure 2. Results of group iterative multiple model estimation (GIMME) connectivity analyses. A.) Subgroup networks for women with CAH, showing full sample connections (black) and CAH-specific connections (dark gray) among regulatory (blue), approach (green), and salience (red) regions of interest. B.) Subgroup networks for control women (i.e., unaffected sisters), showing full sample connections and control-specific connections (light gray). C.) Final map for a women with CAH (unaffected sister shown in D.) that fits her data well: $\chi^2(160) = 876.50$, $p < .001$, RMSEA = .098, SRMR = .046, CFI = .950, NNFI = .914, with positive red connections, negative blue connections, and connection magnitude reflected by width. D.) Final map for a control woman (sister with CAH shown in C.) that fits her data well: $\chi^2(161) = 753.78$, $p < .001$, RMSEA = .089, SRMR = .042, CFI = .953, NNFI = .919. E.) Density of regulatory (blue),

approach (green), and salience (red) networks in women with CAH (dark colors) compared to their unaffected control sisters (light colors).

Table 1. A Priori Regions of Interest (ROIs) in Illustrative Analyses on Reward Processing

Network	ROI	Abbreviation	MNI Central Coordinate (x, y, z)
Regulatory	Left Dorsolateral Prefrontal Cortex	L_DLPFC	-42, 34, 28
	Right Dorsolateral Prefrontal Cortex	R_DLPFC	43, 37, 29
	Anterior Cingulate Cortex	ACC	3, 29, 21
Approach	Left Ventral Striatum	L_VS	-12, 8, -8
	Right Ventral Striatum	R_VS	15, 8, -9
	Left Orbitofrontal Cortex	L_OFC	-22, 34, -14
	Right Orbitofrontal Cortex	R_OFC	32, 33, -14
	Ventromedial Prefrontal Cortex	vmPFC	3, 27, -17
Salience	Left Amygdala	L_Amy	-22, -5, -19
	Right Amygdala	R_Amy	26, -4, -18
	Left Insula	L_Ins	-38, 12, -9





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