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RESTING-STATE FUNCTIONAL CONNECTIVITY
IN YOUTH WITH GENDER DYSPHORIA

by

FÉLIX L. GARCÍA

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2018

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Resting-State Functional Connectivity in Youth With Gender Dysphoria

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Félix L. García

This manuscript has been read and accepted for the Graduate Faculty in Psychology
in satisfaction of the dissertation requirement for the degree of Doctor of
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ABSTRACT

Resting-State Functional Connectivity in Youth With Gender Dysphoria

by

Félix L. García

Advisor: Deidre Anglin, Ph.D.

Current developmental models of gender identity and gender dysphoria (GD) lack sex-specific profiles of brain function that differentiate between typically-developing and cross-gender identified youth, as postulated by models like the unified theory of the origins of sex differences (Arnold, 2009) and the neurobiological theory of the origins of transsexuality (Swaab & Garcia-Falgueras, 2009). Previously, investigators have used brain imaging modalities such as Resting-State functional Magnetic Resonance Imaging (R-fMRI) to demonstrate differences in resting-state functional connectivity (RSFC) between typically-developing male and female youth, and between typically-developing and GID-diagnosed youth. In the present pilot study, I used R-fMRI to investigate differences in RSFC between typically-developing and cross-gender identified male and female youth subgroups, with the hypothesis that GID-diagnosed subgroups would demonstrate connectivity patterns in between those of typically-developing males and females. Eleven youth diagnosed with gender identity disorder (four males, ages 9 to 20 years; seven females, ages 12 to 20 years) were matched on age and assigned gender with 11 typically-developing youth. All participants completed written informed consent to undergo the IRB-approved research procedures. R-fMRI were collected while the participants were lying down

and resting, with their eyes closed. Primary analyses focused on 14 brain regions selected because they showed sex differences most frequently or reliably in previous studies of R-fMRI in typically-developing youth. Statistical analysis used a 2 x 2 mixed effects analysis (assigned female versus assigned male x typically-developing versus GID-diagnosed), with individual level connectivity maps as the dependent variable. Results showed that significant interaction effects of functional connectivity patterns were associated with 6 of the 14 selected brain regions. GID-diagnosed assigned females exhibited connectivity patterns similar to those of typically-developing males associated with the right medial superior frontal gyrus, right supplementary motor area, left lingual gyrus, right lingual gyrus, left middle frontal gyrus, left medial superior frontal gyrus, left cuneus, right thalamus, left dorsolateral superior frontal gyrus, and left inferior frontal gyrus, triangular part. GID-diagnosed assigned males exhibited functional connectivity patterns similar to those of typically-developing females associated with the right medial superior frontal gyrus and right supplementary motor area; in between those of typically-developing females and males associated with left lingual gyrus, right lingual gyrus, left middle frontal gyrus, left medial superior frontal gyrus, right medial superior frontal gyrus, left dorsolateral superior frontal gyrus, and left inferior frontal gyrus, triangular part; and similar to typically-developing males associated with the right lingual gyrus and left middle frontal gyrus. The right precuneus, hypothesized to show robust findings, did not reveal any effects. In the current study, GID-diagnosed assigned males tended toward demasculinized effects (quantitative interactions showing differences of magnitude), whereas GID-diagnosed assigned females tended toward masculinized effects (qualitative interactions showing differences in direction of correlation). The current findings support the view that brain development associated with gender dysphoria proceeds along separate but overlapping sex-related regions for GID-

diagnosed assigned females and males and provide further evidence of greater cross-gender brain differentiation in assigned females at an earlier age than in assigned males (possibly due to earlier onset of puberty in females). These data suggest that any future use of patterns of brain function for diagnosing gender dysphoria may require separate criteria (e.g., different sets of brain regions) for assigned males and assigned females but will require replication on larger samples.

Keywords: cross-gender identification, transgender, child, adolescent, resting-state functional magnetic resonance imaging (R-fMRI), seed-based connectivity analysis

Dedication

For my partner, Kiersten, and our sons, Félix Christian and Víctor Gray, who mean everything to me.

To any of us whose lives may be improved through our commitment to understanding.

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This work would not have been possible without the mentorship and generosity of Dr. Brad Peterson, who has supported my training in neuroimaging from my days as a lab volunteer to research assistant to doctoral candidate. I also appreciate tremendously Dr. Peterson's research staff at the Pediatric Brain Imaging Lab at the New York State Psychiatric Institute and Columbia University Medical Center, whose expertise and professional standards were invaluable in the collection of the data used in this dissertation. I will forever be indebted to Dr. Heino Meyer-Bahlburg, whose tutelage has instilled in me a confidence that can come only from the praise of a mentor who demands that a student recognize his own potential. This trajectory of research training would not have been available to me without the initiative of Dr. Ann Miller, who in collaboration with Drs. Peterson and Meyer-Bahlburg began the project that led to the existence of these data. I greatly appreciate the contributions of Dr. Lissa Weinstein and Dr. Eric Fertuck, who as readers voiced the intellectual diversity

I have come to identify as an integral part of my training at City College. I cannot thank enough the unflappable Dr. Deidre Anglin, for showing me through apprenticeship and by example how to practice social justice through clinical research, and for always knowing just what to do.

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Introduction

Resting-State Functional Connectivity in Youth With Gender Dysphoria

At the time of birth, most humans possess a fully differentiated reproductive tract that is sex-dimorphic between males and females, assuming fetal development has proceeded without incident. It has traditionally been assumed that these individuals will eventually develop identities consistent with socially-defined expectations associated with their reproductive organs and are on this basis assigned to the male or female gender. Most individuals identify with their assigned gender from childhood (e.g., as boy or girl) to adulthood (e.g., as man or woman): In a longitudinal study including a subsample of N=818 individuals assessed at ages 7.5 and 30.9 years (approximately), Steensma, van der Ende, Verhulst, and Cohen-Kettenis (2013) observed that 98.8% of children who did not express cross-gender identification per parental report also did not endorse cross-gender identification as adults (Zucker, Lawrence, & Kreukels, 2016). In individuals for whom reproductive sex and assigned gender coincide, gender identity is differentiated subsequent to the development of operational thought around ages 5 to 7 years (Berenbaum, Martin, & Ruble, 2008; Martin, Ruble, & Szkrybalo, 2002).

Gendered behavior, or gender expression, also differs between males and females and has been observed in children as young as 6 months of age (Martin et al., 2002; Zucker et al., 2016). However, unlike reproductive sex, gender expression is not always sex dimorphic, meaning that some female-type behavior in males and male-type behavior in females are both naturally-occurring phenomena, and both exhibit some within-gender variability. For instance, scales with separate, multi-item gender dimensions, such as the Career Questionnaire (CareerQ), have demonstrated adequate inter-item reliability for gender role behavior, operationalized as interest in 70 different male-type or female-type occupations selected from census data (Meyer-

Bahlburg, Dolezal, Baker, Ehrhardt, & New, 2006). In the general population, girls exhibit male-type behavior more frequently than boys exhibit female-type behavior, and to a lesser extent, explicitly identify as boys more often than do boys explicitly identify as girls: In a sample of non-clinically-referred (or “nonreferred”) youth ages 4 to 11 years, the parent-report Child Behavior Checklist (CBCL) revealed that 1.0% of boys and 2.3% of girls often behaved like the opposite gender (Item 5), while 0.0% and 1.0% explicitly endorsed the wish to be of the opposite gender (Item 110) (Achenbach & Edelbrock, 1981; in Zucker, Bradley, & Sanikhani, 1997).

Individuals with markedly (statistically) sex-atypical gender expression may come to experience doubts about their assigned genders and dysphoric feelings related to a subjective sense of disconnect between assigned gender and experienced gender (or gender incongruence), and eventually transition to living as their experienced gender. Gender incongruence accompanied by clinically-significant distress or impairment associated with living as one’s assigned gender may be referred to as gender dysphoria (Fisk, 1974; Knudson, De Cuypere, & Bockting, 2010). In clinical settings in the United States, gender dysphoria is formally recognized as the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) diagnosis by the same name (American Psychiatric Association, 2013). For the sake of clarity, in this manuscript, lowercase letters are used to refer to a working conceptualization of the distress associated with incongruence between assigned and experienced gender (i.e., gender dysphoria), and capital letters to denote the more circumscribed DSM-5 definition of the relevant diagnosis (i.e., Gender Dysphoria). More colloquially, transgender is a general term referring to individuals who self-identify as any gender other than the one’s assigned gender (American Psychological Association & National Association of School Psychologists, & American Psychological Association, 2015; American Psychological Association, 2015; Meier, Pardo, Labuski, &

Babcock, 2013). Transsexual refers specifically to individuals who have socially transitioned to living as the experienced gender and may opt to receive hormone treatment or gender confirmation surgery (elsewhere referred to as “sex reassignment surgery”) of the breasts and genitals. Transsexual individuals are often referred to in the literature as either male-to-female (MtF) or female-to-male (MtM).

Given that behavior in general is governed by the brain, investigators have looked to brain anatomy and function to explain differences in behavior between genders. This hypothesis has been substantiated in various animal studies (Guillamón, Junque, & Gómez-Gil, 2016). As one example, the accessory olfactory system, a neural network that is distributed across multiple brain regions and is involved in mating and maternal behaviors, exhibits sex dimorphism in rats (Segovia & Guillamón, 1993) and rabbits (Segovia et al., 2006). Similarly, the human olfactory system also exhibits sex differences, albeit not fully dimorphic as those seen in rodents and lagomorphs (Garcia-Falgueras et al., 2006). While many sex differences have been observed in brain anatomy and function in humans, the connection with gendered behavior is not always as obvious as it is in animal studies.

In terms of the anatomy of the human brain, sex differences are well established: Men have larger brains (Allen, Damasio, Grabowski, Bruss, & Zhang, 2003; X. Chen, Sachdev, Wen, & Anstey, 2007; Cosgrove, Mazure, & Staley, 2007; Nopoulos, Flaum, O’Leary, & Andreasen, 2000; Shin et al., 2005), while women have a greater proportion of gray matter to white matter (Allen et al., 2003; Goldstein et al., 2001; Gur et al., 1999; E. Luders et al., 2005) and cortical thickness (Im et al., 2006; Eileen Luders et al., 2006; Sowell et al., 2007). More specifically, men showed larger volumes than women in the midbrain, left inferior temporal gyrus, right occipital lingual gyrus, right middle temporal gyrus, and bilateral cerebellum, (X. Chen et al., 2007) and

larger primary and associative visual processing areas (Brun et al., 2009). Women showed larger regional volumes (of gray matter) in the anterior, middle, posterior, and ventral cingulate gyri and the right inferior parietal lobule (X. Chen et al., 2007), and greater neuronal density and volume in regions associated with dorsal and ventral attentional networks (Keller & Menon, 2009).

Anatomical connectivity studies using Diffusion Tensor Imaging (DTI) to visualize white matter tracts have revealed sex differences associated with the thalamus, cingulate gyrus, and corpus callosum (Gong, He, & Evans, 2011; cf., Westerhausen et al., 2011 in which corpus callosum differences were observed only for the genu and truncus subregions), and in the fronto-occipital fasciculus, area under the parahippocampal gyrus, bilateral internal capsule, area under the medial frontal gyrus, fusiform gyrus, hippocampus, insula, postcentral gyrus, and various regions throughout the frontal and temporal lobes (Chou, Cheng, Chen, Lin, & Chu, 2011; F. Liu, Vidarsson, Winter, Tran, & Kassner, 2010; Menzler et al., 2011; Westerhausen et al., 2003, 2004). In another study, women compared to men showed greater whole-brain connectivity associated with the left Heschl's gyrus, superior temporal gyrus, superior parietal gyrus, inferior parietal gyrus, and insula and right fusiform gyrus, and an overall effect of greater connectivity among left-hemisphere regions, while men compared to women showed greater connectivity associated with the right Rolandic operculum and right inferior frontal gyrus, triangular part (Gong et al., 2009; Yan et al., 2011).

Regarding task-related brain function, a comprehensive review of differences between males and females in terms of sex-typed "perceptual, cognitive and emotional" processes is given by Sacher and colleagues (2013, pp. 368–371). Briefly, an example of a perceptual sex difference is an olfaction study finding that women showed greater activation than men in the

left orbitofrontal cortex when presented with pleasant or unpleasant stimuli (Royet, Plailly, Delon-Martin, Kareken, & Segebarth, 2003). An example of a perceptual sex difference that also relates to psychosocial functioning is the finding from speech processing research that women recruited the inferior frontal gyri to a greater extent than did men in response to hearing emotional prosody that was incongruous with semantic content (Schirmer, Zysset, Kotz, & Yves von Cramon, 2004). In terms of cognitive sex differences, executive and visuospatial functioning appear to be lateralized in opposite hemispheres for men and women: Based on findings of impaired capacity for activities of daily living associated with lesions of the ventromedial prefrontal cortex and amygdala, Kosciak, Bechara, and Tranel (2010) concluded executive function in healthy individuals to be right-lateralized in men and left-lateralized in women. Visual processing studies have demonstrated men engage the right hemisphere more than do women while participating in visuospatial tasks (Gur et al., 2000). Similarly, while engaged in a mental rotation task, men exhibited greater superior parietal lobule activation, and women greater inferior frontal gyrus activation (Hugdahl, Thomsen, & Ersland, 2006). In all, sex differences in brain function related to emotion perception, fear responses, and visuospatial processing have been reliably observed across a number of brain imaging studies (Sacher et al., 2013).

The most consistent findings on sex dimorphism in human brain function have been in the domain of emotion processing. In an emotion processing meta-analysis, Fusar-Poli and colleagues (2009) concluded that women engage the right subcallosal gyrus, and men the limbic and prefrontal cortices, in response to emotional faces. Furthermore, sex differences specific to various emotions have been observed in response to faces affecting sadness (Tatia M. C. Lee et al., 2002) and contempt, disgust, or neutral affect (Aleman & Swart, 2008; McClure et al., 2004).

In another study, men exhibited greater activation in visual cortices and in the anterior cingulate gyrus compared to women in response to fearful or angry faces of male (but not female) actors (Fischer et al., 2004), which the investigators suggested reflects male-typed heightened sensitivity to potential threat. With regard to effects specific to modality of emotional stimulus, another study demonstrated that men, but not women, consistently engaged the right insula and left thalamus in response to happy and sad stimuli regardless of modality (i.e., faces, scenes, or words) (T. M. C. Lee, Liu, Chan, Fang, & Gao, 2005). Moreover, sexual content consistently evoked sex differences in neurofunction: Erotic images evoked increased activation in the bilateral amygdalae and hippocampi and right cerebellum for men but not women (Hamann, Herman, Nolan, & Wallen, 2004). Similarly, when presented with sentences that elicit jealous feelings, men exhibited greater activation in the amygdalae and hippocampi—possibly in connection with male-typed aggressivity—while women showed greater activation in the posterior superior temporal sulci (Takahashi et al., 2006). In a meta-analysis of sex differences observed specifically with functional Magnetic Resonance Imaging (fMRI), Sacher and colleagues (2013) identified across 13 studies greater activation in men compared to women in the following brain regions: anterior cingulate gyrus, superior frontal gyrus, hippocampus, amygdala, insula, posterior cingulate gyrus, superior temporal gyrus, inferior orbitofrontal cortex, putamen, pallidum, caudate, supramarginal gyrus, parahippocampal gyrus, middle occipital gyrus, thalamus, median cingulate gyrus, vermis, and dorsolateral superior frontal gyrus; and across 14 studies, greater activation in women compared to men in the follow regions: frontal gyrus (nonspecific), thalamus, paracingulate gyrus, temporoparietal junction, superior occipital lobe, amygdala, orbitofrontal cortex, insula, hippocampus, cerebellum, anterior cingulate gyrus, and caudal dorsolateral prefrontal cortex.

Finally, using the imaging modalities Positron Emission Tomography (PET) and resting-state fMRI (abbreviated as R-fMRI or rs-fMRI), investigators have observed baseline sex differences in the resting brains of typically-developing humans, especially in regions implicated in visual, attentional, and language processing (H. Liu, Stufflebeam, Sepulcre, Hedden, & Buckner, 2009). Earlier studies using PET demonstrated that women compared to men exhibit greater global cerebral blood flow (CBF) (Devous, Stokely, Chehabi, & Bonte, 1986; Gur et al., 1982), and faster cerebral glucose utilization (Baxter et al., 1987), especially in the inferior frontal gyrus (Andreason, Zametkin, Guo, Baldwin, & Cohen, 1994). More recently, a multi-site R-fMRI study with a sample of N=1,414 participants revealed stronger resting-state functional connectivity (RSFC) in women compared to men associated with the posterior cingulate gyrus, medial superior frontal gyrus, and inferior parietal lobule, and in men compared to women, associated with the median cingulate gyrus, superior temporal gyrus, supramarginal gyrus and several primary and associative visual cortices in the occipital lobe (Biswal et al., 2010). In a study of functional homotopy (connectivity between the same region in opposite hemispheres) using the same dataset as Biswal et al. (2010), the amygdalae and dorsolateral superior frontal gyri exhibited age-by-sex interactions (Zuo et al., 2010). An R-fMRI study focused on the periaqueductal gray (PAG) revealed that women express greater connectivity between the PAG and the median cingulate gyrus, and men between the PAG and the left ventromedial inferior frontal gyrus, right insula, right operculum, and prefrontal regions (Kong, Tu, Zyloney, & Su, 2010). Several of the regions found to exhibit sex differences in resting-state functional connectivity—the amygdala, dorsolateral superior frontal gyrus, and PAG—are all involved in emotion processing and/or regulation (Kong et al., 2010; Zuo et al., 2010). Beyond detecting regional sex differences, R-fMRI has also been used to observe higher order differences in

properties of neural networks such as greater left hemisphere efficiency in women compared to men, and right hemisphere efficiency in men compared to women (Tian, Wang, Yan, & He, 2011; cf., Weissman-Fogel, Moayedi, Taylor, Pope, & Davis, 2010).

Considering the range and replicability of sex differences in brain anatomy and behavior in typically-developing individuals, it stands to reason that sex-atypical gender role behaviors and identities are associated with atypicalities in neuroanatomy and/or neurofunction. It logically follows that individuals who wish to or do live as the “other” gender¹ express brain anatomy or function comparable to that in typically-developing individuals of the same experienced gender, or at a minimum, trending away from that in typically-developing individuals of the same assigned gender. In other words, we can reasonably expect individuals with gender dysphoria (and more specifically, cross-gender identification) to exhibit gender-atypical if not cross-gender patterns of brain structure and function. To the extent that gender is a fairly stable construct, at least some of these determinant differences are likely to emerge in early life. The current study seeks to use R-fMRI to test whether youth with cross-gender identification exhibit cross-gender, gender-atypical, or same-gender (or cisgender) neurofunction associated with sex-differentiated brain regions.

To date, only one study of neuroconnectivity in gender dysphoria-diagnosed (GD-diagnosed) youth has been published (Nota et al., 2017). In that study, Nota and colleagues (2017) found that adolescent (but not child) GD-diagnosed assigned males had stronger functional connectivity in the right cerebellum compared to GD-diagnosed assigned females, typically-developing males, and typically-developing females; GD-diagnosed assigned males

¹ The Western concept of cross-gender identification is premised on a two-gender system, but outside of this formulation, there are many cultures that recognize more than two genders.

exhibited functional connectivity patterns similar to their experienced gender (i.e., female) associated with the right posterior cingulate gyrus (part of the posterior default mode network) and the right supplementary motor area; and GD-diagnosed assigned females showed a functional connectivity pattern similar to that observed in typically-developing males associated with the right supplementary motor area. Importantly, those findings were obtained only in the adolescent subgroup, suggesting that child and adolescent gender dysphoria may reflect qualitatively distinct neurofunctional profiles, which one may speculate to be related to the presence or absence of anatomic dysphoria. The current study complemented the findings of Nota et al. (2017) by examining an independently identified set of sex-differentiated brain regions, specifically in cross-gender identified youth (rather than those with gender incongruence more generally), using a different approach to measure resting-state functional connectivity.

Gender Dysphoria in Children and Adolescents

Terminology.

For the purposes of this study, I will adhere to the definitions of sex and gender recognized by the American Psychiatric Association in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5, 2013, pp. 451–459). In the DSM-5, sex refers to biological aspects of reproductive function, either male or female, including “sex chromosomes, gonads, sex hormones, and nonambiguous internal and external genitalia,” while gender refers to one’s public expression as “boy or girl, man or woman,” (American Psychiatric Association, 2013, p. 451). The concept of gender arose from the need to disambiguate one’s subjective sense of maleness and femaleness in the context of individuals with ambiguous genitalia resulting from endocrine abnormalities, also termed intersex conditions or disorders of sexual development (DSD). Thus, gender assignment refers to the gender ascribed to an individual, usually at birth,

which is referred to as assigned gender or natal gender (however, the latter expression misleadingly implies that gender development is already completed at birth and does not involve psychosocial factors). Somatic features or behaviors that are (statistically) atypical for a given culturally-defined gender are referred to as gender-atypical, or also gender-nonconforming when referring to behavior in particular. Gender identity is one's subjective sense of identification with the categorical, social constructs of male, female, or alternative genders. Gender dysphoria can refer to either the experience of "affective/cognitive discontent" living as one's assigned gender, or to the DSM-5 diagnosis, which, as previously stated, will here be denoted by capital letters, i.e., Gender Dysphoria (American Psychiatric Association, 2013, pp. 451–459).

Diagnosis.

The DSM-5 defines the primary indicator of 302.6 Gender Dysphoria in Children as a "marked incongruence between one's experienced/expressed gender and assigned gender" including a "strong desire to be of the other gender or an insistence that one is the other gender (or some alternative gender different from one's assigned gender)," and five of seven other indicators relating to gender-atypical behavior or explicit identification with a non-assigned gender, or intense discomfort with one's own primary sex characteristics or the anticipation of secondary sex characteristics (or anatomic dysphoria). For 302.85 Gender Dysphoria in Adolescents and Adults, criteria for "marked incongruence" are met by any two of six indicators relating to any of the following areas: anatomic dysphoria, other- or alternative-gender identification, discomfort with assigned gender role, and an alternative gender-typed perceptual or emotional subjective experience. As with other DSM conditions, the indicators of gender dysphoria must result in "clinically significant distress or impairment" for one to meet criteria for a formal diagnosis of Gender Dysphoria in Children or Gender Dysphoria in Adolescents and

Adults. Formal subcriteria for these diagnoses are given in Table 1A and Table 1B, respectively, below.

[TABLE 1A]

[TABLE 1B]

The relevant diagnoses in the DSM-IV-TR are 302.6 Gender Identity Disorder (GID) in Children and 302.85 Gender Identity Disorder in Adolescents or Adults. Important differences between the DSM-IV-TR diagnosis of GID and DSM-5 diagnosis of Gender Dysphoria (GD) are: (a) the DSM-IV-TR labels identity as “disordered,” while the DSM-5 focuses on affect (feelings of dysphoria) as the principal indicator of clinical significance; (b) the definition of GID assumes a binary gender system, while the definition of GD recognizes more than two gender categories (or alternative genders); (c) the DSM-IV-TR does not have a duration criterion, while the DSM-5 requires indicators be present for at least six months; (d) GID criteria excludes individuals with any form of somatic intersexuality, while GD criteria do not; (e) the DSM-IV-TR requires four indicators be present to establish a diagnosis in children, while the DSM-5 requires six; and most importantly, (f) to meet childhood diagnostic criteria, the DSM-IV-TR does not require explicit acknowledgement of alternative-gender identification, while the DSM-5 does require that individuals explicitly endorse the wish to live (or do live) as a member of a gender other than their assigned gender. Formal subcriteria for the DSM-IV-TR diagnoses are given in Table 1C, below.

[TABLE 1C]

As much of the available data from children and adults with gender dysphoria were collected prior to the publication of the DSM-5, clinical samples identified using DSM-IV-TR criteria are still common in the literature. Such is the case in the current exploratory study, for

which data were collected between 2008 and 2010. Therefore, the current clinical subsamples were denoted as the GID-diagnosed subgroups: GID-diagnosed assigned females and GID-diagnosed assigned males.

Though conceptually the DSM-5 is better able to discern dysphoria related to cross-gender or alternative-gender identification (that is, gender dysphoria) from gender-atypical appearance and behavior,² previous work suggests comparable sensitivity and specificity to gender-related clinically-significant distress or impairment between DSM-IV-TR and DSM-5 criteria (Zucker, 2010, pp. 484–486; Zucker et al., 2013, p. 904). In keeping with current conceptualizations in the field, the results from this study are interpreted in the Discussion chapter first in terms of differences in brain function between typically-developing and cross-gender identified youth, and then contextualized within the broader dialogue of differences in brain structure and function between cisgender individuals and those with gender dysphoria.

Phenomenology.

Gender dysphoria is a condition in which one’s experienced or expressed gender feels persistently incongruent with one’s assigned gender, resulting in clinically-significant distress or discomfort (American Psychiatric Association, 2013, p. 453; Fisk, 1974; Knudson et al., 2010). While the onset of gender dysphoria can occur during childhood, adolescence, or adulthood, gender-atypical appearance and behavior have been observed in children as early as ages 2 to 4 years (American Psychiatric Association, 2013). Early-onset gender dysphoria (prior to puberty) is more common than late-onset (beginning around puberty or later), especially in assigned females. An estimated 2.2% to 30% of assigned males and 12% to 50% of assigned females who

² Interestingly, though the DSM-IV-TR diagnosis is conceptualized in terms of “identity,” identity need not be explicitly known to establish a diagnosis of GID in children.

meet criteria for gender dysphoria in childhood will also meet criteria as adults. Adolescents and adults are more likely than children to endorse strong feelings of discomfort with their bodies, or anatomic dysphoria (Drescher & Byne, 2012a; Green, 1987; Kinsey, Pomeroy, & Martin, 1948; Korte et al., 2008).

Early-onset gender dysphoria is associated with sexual attraction to individuals of the same assigned gender, regardless of whether indicators of gender incongruence persist through adulthood. This association between sexual orientation and gender dysphoria has not been consistently observed in late-onset cases. Many late-onset cases—per parental or retrospective self-report—did not express any gender-atypical appearance or behavior in childhood, suggesting the possibility of at least two distinct developmental trajectories leading to gender dysphoria in adults, of which sexual orientation may be an important marker (American Psychiatric Association, 2013, pp. 455–456). The current study was unable to shed light on the matter of sexual orientation as a predictor of persistence since the sexual orientation of participants was not recorded.

While there are well-established diagnostic measures and protocols for distinguishing gender atypicality from gender dysphoria in children (American Psychological Association, 2015; Coleman et al., 2011), we are not yet able to reliably distinguish between children who will continue to experience gender dysphoria in adulthood (persisters) from those who will not (desisters) (Singh, 2014; Steensma, McGuire, Kreukels, Beekman, & Cohen-Kettenis, 2013). The best behavioral predictor of persistence of gender dysphoria appears to be social transition before puberty (Steensma, McGuire, et al., 2013; Zucker et al., 2016). Efforts at utilizing biological markers (especially genetics) to diagnose gender dysphoria have been unsuccessful (American Psychiatric Association, 2013; Zucker et al., 2016). As it stands, a model of gender

development linking biological factors of sex differentiation with psychosocial factors of identity formation remains elusive (de Vries, Kreukels, Steensma, & McGuire, 2014; Guillamón et al., 2016; Kreukels & Guillamón, 2016; Zucker et al., 2016).

A challenge to distinguishing persisters from desisters is that experienced gender is an evolving process rather than a static trait. Much of social identity development—including gender identity development—occurs during the latency period, during which time there is no sex-hormone activity since the gonads are dormant. Most individuals who at some point met criteria for childhood gender dysphoria do not experience clinically-significant distress associated with gender incongruence as adults (Drummond, Bradley, Peterson-Badali, & Zucker, 2008; Singh, 2014; Wallien & Cohen-Kettenis, 2008), and not all individuals who express sex-atypical gender roles necessarily experience dysphoria (Coleman et al., 2011). Furthermore, many individuals experience gender identity as a fluid construct, at times more masculine and at other times more feminine, and may identify as genderqueer or genderfluid (American Psychological Association, 2015). Other developmental outcomes include (a) identification as neither male nor female; (b) expression of cross-gender behaviors but not identity; (c) most individuals who met criteria as children will be sexually attracted to the same assigned gender but no longer meet GID criteria for gender dysphoria as adults.

Consequently, although there is consensus in the psychiatric community regarding best treatment practices for adults with gender dysphoria (Coleman et al., 2011), there continues to be debate about the use of partially reversible (and irreversible) treatments with adolescents. The main concern among clinicians is that interventions such as cross-sex hormone therapy to induce the desired male or female secondary somatic sex characteristics or use of hormone blockers to delay puberty may alter the course of development irreversibly, which may prove harmful to

individuals for whom gender dysphoria eventually spontaneously desists. Brain imaging research may help to elucidate developmental factors associated with desistance or persistence of gender incongruence.

General theory of sex differentiation of brain and behavior

The normative model of sex differentiation of brain and behavior in humans (Bocklandt & Vilain, 2007; Zucker et al., 2016) is derived from animal studies on the developmental effects of prenatal and pubertal hormone activity in rabbits (Jost, 1947, 1972), guinea pigs (Phoenix, Goy, Gerall, & Young, 1959), and dogs and rhesus monkeys (Goy, McEwen, & Neurosciences Research Program, 1980). Lasting structural effects of sex hormones on neural tissues during particularly sex-hormone-sensitive periods are referred to as organizational effects. Short-term or transient effects of sex hormones on previously organized neural tissues and their functions are labelled activational effects. The normative model of sex differentiation has oriented much of the research on biological factors in the development of gender dysphoria (Wallen, 2009; Zucker et al., 2016). Under the neurobiological theory of the origins of transsexuality (Swaab & Garcia-Falgueras, 2009), gender dysphoria reflects a combination of incongruence between organizational effects on reproductive sex and brain sex, the influence of psychosocial factors in identity development during latency and adolescence, and activational effects during puberty and adulthood (Arnold, 2009; Jürgensen et al., 2013; Steensma, Kreukels, de Vries, & Cohen-Kettenis, 2013; Steensma, McGuire, et al., 2013; Wallen, 2009; Zucker et al., 2016).

A useful paradigm for considering developmental effects among cross-gender identified individuals is given by Becker and colleagues (2005; in Guillamón et al., 2016): Masculinizing and feminizing effects refer to traits and behaviors more similar to typically-developing males and females, respectively, while demasculinizing and defeminizing effects are ones leading to

traits or behaviors less similar to those of typically-developing males and females, respectively. Two additional relevant terms are hypermasculinization and hyperfeminization, which I use here to denote effects that exceed the means of the referent typically-developing subgroups (i.e., assigned males and females, respectively) and occur outside the bipolar range anchored by the typically-developing subgroup means.³ Finally, the terms GID-specific and GD-specific are used to refer to effects that occur outside the range anchored by typically-developing subgroup means when no difference was observed between typically-developing males and females. All of these effects are interpreted with respect to sex differences between typically-developing males and females. A summary of the interpretation of these effects is provided in Table 2A, below.

[TABLE 2A]

Genetic factors.

Researchers have extrapolated hypotheses about genetic markers associated with gender dysphoria from genetics research with rodents and intersex humans (Arnold, 2009; Zucker et al., 2016). In rats and mice, suppression or removal of the *Sry* gene, which is located on the Y chromosome in both rodents and humans, disrupts development of the testes (Arnold, 2009; Dewing et al., 2006). There are also certain genes unrelated to endocrine development that are incidentally located on the X and Y chromosomes and thus indirectly sex-linked (Arnold, 2009).

Two examples of naturally-occurring conditions relevant to sex differentiation of brain and behavior in humans are complete androgen insensitivity syndrome (CAIS) and congenital adrenal hyperplasia (CAH). Chromosomal 46,XY individuals with CAIS express feminized

³ Hypermasculinization has been observed, for instance, in the index-to-ring finger length (2D:4D) ratios of gay men in Britain, suggesting a possible role for androgen insensitivity as a causal factor in the development of atypical sexual orientation in assigned males (Collaer, Reimers, & Manning, 2007; Rahman, 2005; cf., McFadden et al., 2005, in which a follow-up analysis with 2D:4D data showing hypomasculinization and hypermasculinization in samples from the United States and Britain, respectively, was inconclusive).

somatic development and female gender identity in adulthood, while 46,XX individuals with CAH develop ambiguous genitalia resulting from excess fetal androgens in early development. Research on CAIS and CAH has helped identify genes for androgen receptors and androgen synthesis, respectively, which have subsequently been investigated in transsexual adults. Relevant studies with transsexual adults have examined associations between gender-atypical behaviors and androgen receptor genes (Fernández et al., 2014; Hare et al., 2009); estrogen receptor genes (Henningsson et al., 2005); *CYP19*, a gene involved in the aromatization of androgens into estrogens (Fernández et al., 2014; Hare et al., 2009; Henningsson et al., 2005; Ujike et al., 2009; Zucker et al., 2016); and *CYP17*, which is involved in the synthesis of androgens and estrogens (Bentz et al., 2008; Zucker et al., 2016). Notably, Henningsson and colleagues (2005) reported an abnormality in the estrogen receptor gene, *ERβ*, among MtFs (Zucker et al., 2016). To date, however, no compelling evidence has been found for any single gene tied directly to the development of gender dysphoria, and it is thought that any sort of gender dysphoria phenotype(s) is/are likely to arise from the expression of multiple genes, or polygenic genotype (Zucker et al., 2016).

Hormonal factors.

Organizational effects during fetal development are moderated by both the fetus's own testosterone production and maternal androgens during pregnancy, while activational effects during pubertal development and adulthood arise from individuals' circulating hormones (Swaab & Garcia-Falgueras, 2009). Organizational effects of testosterone on prenatal somatic sex differentiation were first demonstrated in gonadectomized 46,XY rabbit embryos that developed into phenotypic females (Jost, 1947, 1972, in Guillamón et al., 2016). The connection between organizational and activational effects was first demonstrated by Phoenix, Goy, Gerall, and

Young (1959) in gonadectomized female guinea pigs treated with testosterone during fetal development, which when treated with testosterone again as adults exhibited masculinized behavior, and with estrogen, defeminized behaviors (Guillamón et al., 2016; Zucker et al., 2016). More recently, researchers have observed sex-differentiated behavior in rhesus monkeys and dogs following from the effects of prenatal hormones alone, demonstrating that organizational effects can influence sex-differentiated behavior (and not just somatic features like reproductive organs) independently of activational effects (Goy et al., 1980; Guillamón et al., 2016).

Psychosocial factors.

Zucker, Lawrence, and Kreukels (2016) draw a distinction between causal psychosocial processes in early life and perpetuating psychosocial processes, such as parental relationships. Early psychosocial hypotheses regarding parental relationships can be summarized as, for MtF adults, Stoller's (1968) maternal overcloseness hypothesis and Green's paternal distance hypothesis (1987); and for FtM adults, maternal undercloseness hypothesis and paternal overcloseness hypothesis (Stoller, 1975). Cohen-Kettenis and Arrindell (1990) tested these hypotheses by collecting questionnaires about parental rejection, emotional warmth and overprotection from a sample of MtF and FtM adults, and found some evidence supporting the paternal distance in the MtF data, and the maternal undercloseness hypothesis in FtM data. Interestingly, FtM respondents experienced fathers as rejecting, contradicting the paternal overcloseness hypothesis (Cohen-Kettenis & Arrindell, 1990; Zucker et al., 2016). However, these retrospective studies were limited in their capacity to demonstrate direction of effects in parent-child dynamics, lacked random sampling, and failed to include clinical controls to test for possible effects of psychosocial influences common in the lives of clinically-referred individuals

(Zucker et al., 2016). None of the above psychosocial developmental models lead to any obvious hypotheses regarding sex differentiation in the brain.

Possible mechanisms underlying atypical sex differentiation in the brain.

The neurobiological theory of the origins of transsexuality⁴ holds that atypical gender development reflects atypicalities in brain anatomy and function (Swaab & Garcia-Falgueras, 2009). The neurobiological theory suggests that those atypicalities are at least partly attributable to changes in concentrations of prenatal hormones between the first and second trimesters, during which reproductive sex and brain sex differentiation occur, respectively (Wallen, 2009; Zucker et al., 2016). However, manipulation of prenatal and pubertal hormone exposure in animal studies has failed to show categorical changes in sex-differentiated behaviors. For example, it has not been possible to induce the full range or intensity of male-type behaviors in gonadectomized 46,XX guinea pigs at any level of dosage of prenatal or pubertal testosterone treatment (Arnold, 2009; Jost, 1972; Phoenix et al., 1959; Wallen, 2009).

In humans, the effects of hormonal and psychosocial factors on the development of gender role behavior and identity differ between assigned genders. An example of such effects in the domain of sexual orientation is that gay men tend to have more older brothers than do straight men, while for lesbian women, no such effect of sibling order on psychosocial development has been observed (Blanchard, 2004; Zucker et al., 2016). Sex-specific effects in the development of gender dysphoria include the greater likelihood of assigned females to experience persistence of early-onset gender incongruence, and of assigned males to experience

⁴ Unlike the relevant DSM-5 definitions provided above, the use of the term “transsexuality” by Swaab and Garcia-Falgueras (2009) should not be interpreted as suggesting that this neurobiological theory applies only to individuals with cross-gender identification, as the general concept also applies in the case of gender incongruence, though the extent of this overlap is currently debated.

late-onset gender incongruence (American Psychiatric Association, 2013; Bockting, Benner, & Coleman, 2009; Chivers & Bailey, 2000; Zucker et al., 2016). In the domain of genetics, several developmental processes have been observed that are unique to gene expression in 46,XX and therefore may influence the development of gender role behavior and identity in a sex-specific way. For example, individuals with more than one X chromosome have a paternal X chromosome; express X-inhibition, or the suppression of certain alleles on the paternal or maternal X-chromosome; and express “mosaic” phenotypes, that is, express half of their X-genes from a maternal chromosome, and half from a paternal chromosome (Arnold, 2009).

Sex differences in gender development may be related to the fact that at the time of birth, sex differentiation in 46,XX individuals has progressed closer to its developmental endpoint than has sex differentiation in 46,XY individuals (Arnold, 2009; Jost, 1972), suggesting gender development may be less susceptible to psychosocial and activational factors in assigned females compared to assigned males. Previously, sex-specific developmental models (or within-sex models) have been proposed to explain sex differences in cellular responses to hormones and onset and duration of sensitivity periods for activational effects (Wallen, 2009; Zucker et al., 2016). Within-sex models are readily accommodated by the unified theory of sex differentiation in mammalian tissue, whereby gender development represents the interaction of “hormonal and direct genetic factors” that promote or inhibit sex differentiation along male-type and female-type trajectories (Arnold, 2009, p. 576). In the context of this study, a within-sex formulation consistent with the unified theory is that there will be a limited degree of overlap between brain anatomy and function associated with cross-gender identification in assigned males and assigned females.

Functional Connectivity in the Brain

Functional connectivity research allows us to make inferences about the relationships among brain regions that may not be obviously connected with one another anatomically, by observing changes in blood flow patterns in the brain over time. More specifically, resting-state functional connectivity allows for inferences about the relatedness of cortical brain regions based on similarity in blood flow patterns in participants who are awake but not involved in an active task. Functional connectivity has been observed through positron emission tomography as regional Cerebral Blood Flow (rCBF), and in fMRI as fluctuations in Blood Oxygen Level Dependency (BOLD) signal.

Resting-state fMRI.

As mentioned above, resting-state fMRI (R-fMRI) allows us to observe networks of brain regions linked through functional connectivity. This brain imaging modality is well-suited for research on gender dysphoria, for which a diagnosis-specific neuropsychological task currently does not exist. Functional connectivity in R-fMRI has been operationalized as the degree of similarity in the frequency, phase, or amplitude of BOLD signal fluctuations across the voxel-by-picture elements (or voxels) in a three-dimensional array (or volume), in which each voxel represents change in blood flow over time at a specific point in the brain. Resting-state fMRI has been used to observe large-scale functional connectivity networks that persist from infancy through adulthood (Power, Fair, Schlaggar, & Petersen, 2010). As such, functional connectivity is an ideal measure for studying complex, subjective psychological constructs such as gender identity, which is likely to be represented across multiple neurofunctional networks. At the time of data collection, this study was the first to obtain such data from GID-diagnosed youth.

Clinical applications of resting-state functional connectivity.

Functional connectivity findings often resemble anatomical connectivity findings, and sometimes are able to show impaired connectivity between regions that may not otherwise be observed using traditional imaging modalities such as anatomical MRI or task-related fMRI. Task-related fMRI has been used in clinical settings to identify brain regions that are functionally impaired in conditions, as with epilepsy and localized brain hemorrhaging. However, task-related fMRI is not sensitive to mediating brain regions with no change in signal between active task and resting-state, but to which other regions assimilate during mental activity (Jessica S. Damoiseaux & Greicius, 2009). Such mediating brain regions can be detected with R-fMRI.

In R-fMRI studies with adults, functional connectivity has been correlated with abnormal behavioral performance and emotional processing in neuropathology such as Alzheimer's disease, depression, and schizophrenia. Resting-state studies using fMRI and other modalities, have been conducted with sedated children to illustrate developmental problems associated with disorders such as ADHD and autism (Freilich & Gaillard, 2010). Functional connectivity profiles with good diagnostic validity have been demonstrated for depression and ADHD, and may offer a promising direction for observing gender development in children and adolescents with gender dysphoria (Posner, Park, & Wang, 2014; Yao, Wang, Lu, Liu, & Teng, 2009a, 2009b).

Measuring resting-state functional connectivity.

A number of R-fMRI analytic approaches and algorithms are available through standard software packages (Cole et al., 2010). Five types of measures that investigators have utilized to detect sex-related differences in research with typically-developing youth are independent components analysis (Agcaoglu, Miller, Mayer, Hugdahl, & Calhoun, 2015); network centrality (Zuo et al., 2012); regional homogeneity (ReHo, Zang, Jiang, Lu, He, & Tian, 2004); homotopic

connectivity (Zuo et al., 2010); and direct region-to-region correlational analysis (Alarcón, Cservenka, Rudolph, Fair, & Nagel, 2015; Solé-Padullés et al., 2016).

Independent components analysis (ICA) is a frequency-domain, data-driven analytic approach similar to confirmatory factor analysis. As ICA is used to identify separate resting-state networks of brain regions, or nodes, it is typically used as a preliminary analysis to orient seed-based analyses. ICA in brain imaging research usually yields around 30 separate resting-state networks, called independent components (Z. Wang & Peterson, 2008).

Network centrality is a type of graph-theory analysis conducted in the frequency domain that measures the interconnectedness or “hubness” of a region (called a node), with respect to functional networks at the region, network, and system levels (Zuo et al., 2012). Degree centrality (or node degree) refers to the total number of regions correlated with a seed region. Node efficiency is an inverse metric of the number of direct and indirect correlations separating a seed region from other regions in the same network. Node betweenness represents the number of pairs of regions that are indirectly correlated with one another via direct correlations with a common seed region (Wu et al., 2013).

Regional homogeneity is a type of seed-based analysis that is conducted in the spatial domain. ReHo measures the extent of clusters of adjacent voxels with similar hemodynamic time series. The resulting individual-level statistic extracted through ReHo is the non-parametric Kendall’s Concordance Coefficient, W (Yao et al., 2009a; Zang et al., 2004; Zuo & Xing, 2014).

Functional homotopy (also called voxel-mirrored homotopic connectivity) is another seed-based analysis conducted in the spatial domain, where seeds are defined by voxels-of-interest (VOI) that are mirrored in the left and right hemispheres. The resulting individual-level statistics extracted through VMHC analysis is Pearson’s product-moment correlation coefficient, r .

Finally, direct correlational analysis of resting-state functional connectivity (RSFC) is computationally like homotopic connectivity, except it is not restricted to correlations between bilateral regions in opposite hemispheres. In this way, direct correlational analysis combines the specificity of hypothesis-driven studies (that is, seed regions must be defined by the investigator), with the flexibility of data-driven studies (the regions with which a seed could be correlated are unrestricted, allowing a full view of the relationship between a given region and the entire brain). RSFC correlational analysis can be further subdivided by the type of seed region used, either from preparamcellated, standard atlases, or manually defined by the investigator as spheres or cubes (usually around 3mm³) using either standardized or subject-specific coordinates.

A methodological article by Biswal et al. (2010) demonstrated that robust sex and age effects with high validity can be reliably detected in resting-state fMRI data. The investigators reported high concordance across the three most common analysis techniques: seed-based functional connectivity, independent component analysis, and frequency-domain analysis (e.g., amplitude of low frequency fluctuation, ALFF). As such, the investigator hypothesized regions of interest in the current study based on previous research using a variety of functional connectivity analysis techniques.

Functional connectivity in typically-developing youth.

Recent studies on functional connectivity in typically-developing youth have identified several key regions that exhibit differences between females and males. The current study examines 14 of those regions most frequently or reliably observed: (1) left dorsolateral superior frontal gyrus (Alarcón et al., 2015; Zuo et al., 2010), (2) left inferior frontal gyrus, triangular part (Agcaoglu et al., 2015; Wu et al., 2013), (3) right medial superior frontal gyrus (Alarcón et al.,

2015; Wu et al., 2013; Zuo et al., 2010), (4) left posterior cingulate gyrus (Zuo et al., 2010), (5) right posterior cingulate gyrus (Zuo et al., 2010), (6) left amygdala (Alarcón et al., 2015; Zuo et al., 2010), (7) right amygdala (Alarcón et al., 2015; Wu et al., 2013; Zuo et al., 2010), (8) left cuneus (Wu et al., 2013), (9) left lingual gyrus (Agcaoglu et al., 2015; Wu et al., 2013), (10) right lingual gyrus (Agcaoglu et al., 2015; Alarcón et al., 2015; Wu et al., 2013), (11) left fusiform gyrus (Wu et al., 2013; Zuo et al., 2010), (12) right fusiform gyrus (Wu et al., 2013), (13) right angular gyrus (Alarcón et al., 2015; Wu et al., 2013), (14) left precuneus (Agcaoglu et al., 2015; Alarcón et al., 2015; Solé-Padullés et al., 2016; Wu et al., 2013). The findings relevant to the selection of each region is presented below.

(1) Left dorsolateral superior frontal gyrus

In a resting-state functional connectivity study with N=122 youth (71 boys, 51 girls, ages 10–16 years), Alarcón et al. (2015) observed an age-by-sex interaction associated with the left dorsolateral superior frontal gyrus, which exhibited an increase in connectivity with the right amygdala for boys, and decrease for girls, with age. Also, in a voxel-mirrored homotopic connectivity study with N=214 participants (96 males, 118 females, ages 7–85 years), Zuo et al. (2010) observed an age-by-sex interaction in which homotopic connectivity between the left and right dorsolateral superior frontal gyrus decreased for females, and increased for males, with age.

(2) Left inferior frontal gyrus, triangular part

The left inferior frontal gyrus, triangular part, has previously been found in typically-developing females compared to males to exhibit a main effect of greater node degree (Wu et al., 2013), as well as to trend toward connectivity with other brain regions limited to one hemisphere or the other (called lateralization) and to constitute a member region in a connectivity network (or node) that also showed a main effect of lateralization (Agcaoglu et al., 2015).

(3) Right medial superior frontal gyrus

In a sample of N=291 typically-developing participants (146 males, 145 females, ages 5.6–18.4 years, Wu et al. (2013) identified a main effect of greater node efficiency in typically-developing females compared to males. Similarly, Zuo et al. (2010) reported a main effect of greater homotopic connectivity between the left and right medial superior frontal gyrus in females compared to males.

(4) Left posterior cingulate gyrus & (5) Right posterior cingulate gyrus

The left posterior cingulate gyrus and right posterior cingulate gyrus were both found by Zuo et al. (2010) to show greater homotopic connectivity in typically-developing females than males. These regions were included here also because the posterior cingulate cortex is an important seed region for observing the posterior Default Mode Network (DMN), which has been found to exhibit sex-related differences in (Moussa, Steen, Laurienti, & Hayasaka, 2012; Raichle, 2011; Rosazza & Minati, 2011; Yeo et al., 2011).

(6) Left amygdala & (7) Right amygdala

In a previous study, homotopic connectivity between the left amygdala and right amygdala decreased in typically-developing males, and increased in females, with age (Zuo et al., 2010). The left and right amygdala were also the hypothesized seeds for an RSFC study demonstrating a variety of sex-related differences in connectivity profiles for four subdivisions of the left and right amygdalae (Alarcón et al., 2015).

For the right amygdala only, Wu et al. (2013) showed greater node degree in typically-developing females than males.

(8) Left cuneus

Wu and colleagues (2013) observed greater node degree and node efficiency in typically-developing females than males, and an age-by-sex interaction in which females showed less node betweenness, and males more (*ns*), with age. The left cuneus was included in the current selection in part because it shows both main and interaction sex-related effects associated with a common region, more specifically, an interaction effect that diminishes over time, showing convergence in connectivity patterns between typically-developing males and females. Of note, the right cuneus was considered as a potential seed as it has previously been observed to show an age-by-sex interaction in which connectivity between the right cuneus and left superficial amygdala became stronger in typically-developing females, and weaker in males, with age (Alarcón et al., 2015). However, that finding was better accounted for by the left amygdala (already included in the list of the hypothesized seed regions) and was not accompanied by any main effects.

(9) Left lingual gyrus & (10) Right lingual gyrus

A previous study found a main effect of lateralization associated with the bilateral lingual gyri in typically-developing females compared to males and a main effect of greater lateralization in typically-developing males compared to females associated with the left lingual gyrus (Agcaoglu et al., 2015). Another study found main effects of greater node degree and node efficiency in typically-developing females compared to males in the left lingual gyrus and right lingual gyrus (i.e., not as a single, bilateral node, but as two separate nodes) (Wu et al., 2013). Finally, an age-by-sex interaction in the connectivity between the right lingual gyrus and the left amygdala has been observed, in which typically-developing males show stronger connectivity, and females weaker connectivity, with age (Alarcón et al., 2015).

(11) Left fusiform gyrus & (12) Right fusiform gyrus

Zuo and colleagues (2010) observed a main effect of greater homotopic connectivity between the left and right fusiform gyri in typically-developing males than in females. Another study revealed main effects of node degree and node efficiency for typically-developing females compared to males at the left fusiform gyrus and right fusiform gyrus (Wu et al., 2013).

(13) Right angular gyrus

Two sex-by-age interaction effects on connectivity have previously been reported at the right angular gyrus, one in which typically-developing males showed greater connectivity with the left basolateral amygdala, and females less, with age (Alarcón et al., 2015); and another in which node betweenness increased for males, and decreased for females (*ns*) for females, with age (Wu et al., 2013). The consistency between previous sex-related interaction effects demonstrated using region-to-region correlations and using measures of network properties was part of the motivation for including the right angular gyrus.

(14) Left precuneus

The left precuneus has been observed in age-by-sex interaction effects on sex-related connectivity in at least three studies: Alarcón and colleagues (2015) found connectivity between the left precuneus and right basolateral amygdala increases for males, and decreases for females, with age. Another study revealed an increase in node betweenness for males, and decrease for females, with age (Wu et al., 2013). The left precuneus also showed, in typically-developing adolescents, stronger intrinsic connectivity with other nodes in the Visuospatial Network among assigned males compared to females, while for children no difference between assigned genders was observed (Solé-Padullés et al., 2016). In addition, another study implicating the left precuneus and bilateral precuneus in two separate resting-state networks showed greater lateralization in typically-developing males compared to females (Agcaoglu et al., 2015).

Although none of the above findings were significant main effects, the left precuneus is included here because of the consistency of reports on this region that have been observed using complementary measures of functional connectivity.

Objective.

The objective of this investigation was to use R-fMRI to observe differences among typically-developing and GID-diagnosed, male and female youth, in terms of patterns of resting-state functional connectivity associated with regions previously reported to express sexual differentiation in typically-developing youths.

Independent variables.

The current study employed a quasi-experimental, mixed-model design that included two subject variables:

- 1) Assigned Gender: female vs. male
- 2) Diagnostic Subcategory: typically-developing vs. GID-diagnosed

The resulting study groups are given in Table 2B below.

[TABLE 2B]

Typically-developing and GID-diagnosed youth were paired according to age and assigned gender.

Dependent variable.

Resting-state functional connectivity was measured as BOLD signal using functional Magnetic Resonance Imaging (fMRI) and operationalized as connectivity correlations associated with a hypothesized set of regions—or seed regions—reported in previous studies of sex differences in the brain. The boundaries of these seed regions were defined by the popular

preparcellated brain anatomical atlas, the Automated Anatomical Labelling (AAL) atlas (Tzourio-Mazoyer et al., 2002).

Group-level analyses.

Mixed-effects analyses of seed-to-voxel RSFC correlation values were used to assess whether, 1) sex-differentiated patterns of brain function differed between typically-developing and GID-diagnosed youth of the same assigned gender, per the neurobiological theory of the origins of transsexuality, and 2) whether differences in brain function between typically-developing and GID-diagnosed youth were modified by assigned gender, per the unified theory of the origins of sex differences and neurobiological theory of the origins of transsexuality. Main effect terms for assigned gender and diagnostic subcategory, and an interaction effect term for assigned gender-by-diagnostic category, were estimated for each region of interest listed in Table 3, below.

[TABLE 3]

Hypotheses.

The current study addresses the research question: Do resting-state functional connectivity patterns associated with regions previously reported to exhibit sex-related differences in typically-developing youth differ among GID-diagnosed assigned females, typically-developing females, GID-diagnosed assigned males, and typically-developing males? The investigator employed a mixed-model design to allow for the observation of two main effects (assigned gender and diagnostic subcategory), and their interaction. The corresponding null and alternative hypotheses are given below:

Main effect of assigned gender.

H_0 = no differences in RSFC exist between assigned males and assigned females

= assigned males (GID-diagnosed + typically-developing) = assigned females (GID-diagnosed + typically-developing)

H_1 = at least one seed region differs in RSFC pattern between assigned males and assigned females

= assigned males (GID-diagnosed + typically-developing) \neq assigned females (GID-diagnosed + typically-developing)

Main effect of diagnostic subcategory.

H_0 = no differences in RSFC exist between GID-diagnosed and typically-developing youth

= GID-diagnosed (assigned female + assigned male) = typically-developing (assigned female + assigned male)

H_1 = at least one seed region differs in RSFC pattern between assigned GID-diagnosed and typically-developing youth

= GID-diagnosed (assigned female + assigned male) \neq typically-developing (assigned female + assigned male)

Interaction effect of assigned gender-by-diagnostic subcategory.

H_0 = no difference in RSFC will exist among the four subgroups formed by combining the factors assigned gender and diagnostic subcategory

= GID-diagnosed assigned female = typically-developing assigned females = GID-diagnosed assigned males = typically-developing assigned males

H_1 = at least one seed region will demonstrate differences in RSFC patterns associated with assigned gender will be modified by diagnostic subcategory
= mean RSFC correlations will not be equal among the four subgroups

Research Plan

Measuring resting-state neurofunction may provide insights into the development of gender identity. In this study, the investigator sought to demonstrate that gender dysphoria in cross-gender identified youth modifies neuroconnectivity associated with male-type and female-type resting-state functional connectivity patterns. R-fMRI was used to observe resting-state functional connectivity correlations in the brains of typically-developing male and female children and adolescents, and GID-diagnosed assigned male and female children and adolescents. RSFC was probed at 14 regions of interest previously associated with sex differences between typically-developing male and female youth. The investigator expected to observe a combination of effects in both GID-diagnosed subgroups, including masculinized, feminized, demasculinized, and defeminized functional connectivity patterns (Kreukels & Guillamón, 2016).

Methods

The proposed study would utilize existing brain scans collected through the Columbia University Medical Center study, “MRI Studies of the Brain in Health and Illness,” (IRB protocols 5321 and 6435), under principal investigators Bradley S. Peterson, MD.

Sample

This study included n=11 GID-diagnosed participants, ages 9.17 to 20.29 years, meeting DSM-5 criteria for gender dysphoria, and n=11 typically-developing youth, ages 8.83 to 19.98 years, matched to the GID-diagnosed participants in assigned gender, age, handedness, and race.

GID-diagnosed youth.

Eligibility.

To be included in the GID-diagnosed subgroups, participants needed to be at least 6 years of age, and meet full DSM-IV-TR criteria for Gender Identity Disorder of Childhood or Adolescence. Data were originally collected from a GID-diagnosed sample of size n=12 with a mean age of 16.02 years (SD=3.85, range: 9.17–20.29). Exclusion criteria were any history of disorders of sexual development (DSD); Autism Spectrum Disorder, psychotic disorders, or substance abuse disorder; premature birth (gestational age ≤ 37 weeks); evidence of brain damage; exposure to exogenous sex hormones; and conditions compromising procedural safety (e.g., ferromagnetic implants, metal braces or retainers, transdermal medicine patches, claustrophobia, or a positive pregnancy test).

Source.

Recruitment was conducted through clinical referral, community outreach, and online advertisement. Most of the enrolled participants came from New England and Mid-Atlantic states.

Sample selection.

Out of 47 families of gender-referred children or adolescents located and invited to participate in the study, 36 expressed interest in enrolling. Of these, 8 could not be reached for prescreening to determine whether any eligibility to participate in a Magnetic Resonance Imaging (MRI) scan, and 2 were screened out due to the presence of metal in the body (e.g., braces), which can become hazardous during a brain scan, or interfere with image acquisition. Five of the remaining 26 enrolled participants were unable to accommodate a laboratory visit due to distance and time constraints. Of the remaining 21 participants, two were unable to attend, and one attended the brain scan but became ill during image acquisition, and subsequently could not be reached to reschedule. One participant twice attempted to sit through the brain scan, but was ultimately unable to do so, and for another, data collection was ended prior to the completion of the resting-state sequences due to time constraints.

Thus, usable resting-state fMRI data were collected from 16 gender-referred participants. Based on clinical interviews conducted on the day of the brain scan, four of these did not meet full DSM-IV-TR criteria for GID. After fMRI data processing, one participant was removed from the GID-assigned males subgroup due to excessive head motion during scan acquisition. The final group of $n=11$ GID-diagnosed participants was comprised of four assigned males and seven assigned females, with a mean age of 16.02 years ($SD=3.85$, range: 9.17–20.29), of whom seven identified as White, two Hispanic, one Asian and one as “other.” One GID-diagnosed assigned male and one female were left handed.

Typically-developing children.

Eligibility.

A sample of 12 typically-developing participants, individually matched with GID-diagnosed participants on age, assigned gender, and handedness, were selected from a larger database of resting-state fMRI data collected from more than $N=85$ similarly-aged youth over the same approximate time period

as the GID-diagnosed participants' data (GID-diagnosed participants were scanned between 2009 and 2010 and typically-developing participants between 2008 and 2012). Exclusion criteria were any history of psychiatric conditions; premature birth (gestational age ≤ 37 weeks); evidence of brain damage; and conditions compromising procedural safety (e.g., ferromagnetic implants, metal braces or retainers, transdermal medicine patches, claustrophobia, or a positive pregnancy test).

Source.

Typically-developing youth were recruited from New York, New Jersey and Connecticut via telemarketing lists.

Sample selection.

Given that the investigator selected typically-developing participants from a larger repository of adolescent brain imaging data, usable scan quality was not inclusion criterion as scans with unusable or insufficient data were previously been flagged in the database and removed from consideration prior to subject matching. After fMRI data processing, one participant was removed from the typically-developing assigned males subgroup due to excessive head motion during scan acquisition.⁵ Thus, usable resting-state fMRI data were selected from 11 typically-developing youth (seven female and four male), with a mean age of 15.78 years (SD=3.79, range: 8.83–19.98), of whom six identified as White, three as Hispanic, and two as African-American. All typically-developing participants were right-handed.

Assessment

Prescreening.

⁵ Incidentally, the only other participant removed from further analyses due to excessive head motion was the GID-diagnosed matched pair of the participant removed from the typically-developing males subgroup. This pair was the youngest in the study: the GID-diagnosed participant was age 8.17 years and the typically-developing participant 7.83 years, which comes as no surprise given younger children have a more difficult time remaining still than do adolescents and adults.

Eligibility and safety screening of GID-diagnosed children was performed through an initial structured interview administered to parents, that includes questions about age, gender-atypical behaviors, medical and psychiatric history, pubertal status and pregnancy, handedness, parental education, and metal implants. Individuals whose responses appeared to endorse gender identity disorder, and to satisfy eligibility for undergoing an MRI scan, were invited to participate in the study. On the day of the study, diagnosis and safety information was re-verified.

Participant characteristics.

Participant profile.

Items on this clinician-administered form ask about demographic information, and medical and psychiatric history, from which can be computed the Four-Factor Hollingshead Index of Social Status, an estimate of socioeconomic status with good validity and reliability (Hollingshead, 1975).

Modified sexual maturity scale.

The Modified Sexual Maturity Scale (SMS) is a validated and noninvasive self-report measure of pubertal development. Children are to indicate the two line drawings which most closely resemble their genitals and breasts, from a choice of five images depicting various stages of development (Morris & Udry, 1980).

Edinburgh handedness inventory.

A standard assessment of lateral dominance for use of hands, feet, and eyes demonstrating adequate reliability (Oldfield, 1971; Ransil & Schachter, 1994).

Psychiatric disorders.

Kiddie-schedule for affective disorders and schizophrenia present and lifetime version.

This semi-structured diagnostic interview is administered to child and caregiver separately and is designed to assess current and past psychopathology in children according to DSM-IV-TR criteria (Ambrosini, Metz, Prabucki, & Lee, 1989; Kaufman et al., 1997).

Child behavior checklist (CBCL).

The CBCL is a structured clinical questionnaire completed by parents, which yields competence scores for daily activities, social behavior, and school performance, as well as problem behavior scores representing eight domains that are compiled into indices of internalizing and externalizing problems (Achenbach, 1995a, 1995b; Achenbach & Edelbrock, 1983). The CBCL includes two items that screen for gender dysphoria. In one study of N=127 adolescents who were gender-referred in childhood, CBCL items #5 and #110 predicted persistence of gender dysphoria in adolescence with very large effect sizes (OR = 4.64 and OR = 37.56, respectively) (H. Chen, Cohen, & Chen, 2010; Steensma, McGuire, et al., 2013).

Consensus diagnosis.

At the time of data collection, multi-axial psychiatric diagnoses based on DSM-IV-TR criteria were assigned following consensus between two doctorate-level mental health clinicians regarding diagnostic impressions of the clinical data collected (American Psychiatric Association, 2000).

Neuropsychological testing.

Wechsler abbreviated scale of intelligence (WASI).

The WASI provides indices of verbal comprehension, perceptual reasoning, and full-scale IQ, based on performance on four subtests: Similarities, Arithmetic, Picture Completion, and Block Design. WASI scores can be used to screen participants for intellectual disability, and as covariates in statistical analyses (Wechsler, 1999).

Gender role behaviors, identity, and dysphoria.

The GID diagnosis of the clinical participants had been established on the basis of a comprehensive clinical work-up including various gender measures. The limited screening performed on the day of brain imaging was primarily intended to confirm eligibility for study participation. Below are the diagnostic measures used in conjunction with the non-structured clinical history interviews and systematic semi-structured gender assessment interviews to establish DSM-IV-TR diagnoses of Gender Identity Disorder of Childhood or Adolescence (see Table 1C for full criteria) in GID-diagnosed assigned male and female subgroups.

Measures for child participants.

Gender Identity Questionnaire for Children (GIQC).

Parents responded using a 5-point Likert-type scale to 16 multiple choice questions about the child's expressed gender ("feels like a boy/girl."). The resulting composite index has demonstrated effect sizes of 3.9-5.4 for children meeting criteria for Gender Identity Disorder (Cohen-Kettenis, 2006).

A factor analysis with a clinical sample of 325 gender-referred children, and control sample of 504 proband sibling, clinic-referred, and nonreferred children, revealed a single latent factor on which 14 of 16 loaded with an overall effect size of 3.70 for children in the range of ages 2.5-12 years. At a 95% Type-II error threshold, the GIQC distinguished scores between proband-controls and gender-referred children with 86.8% sensitivity, between proband-controls and only those who met full GIDC criteria (supracritical cases) with 96.3% sensitivity. It also distinguished between sub- and supracritical gender-referred children with 45.4% sensitivity, which increased to 65.7% when the Type-II threshold was relaxed to the standard rate of 80% (Johnson et al., 2004).

Originally adapted from the Gender Identity Questionnaire (Elizabeth & Green, 1984), the GIQC is one of the most widely used measures of cross-gender identification in children, and has excellent specificity and sensitivity.

Gender Identity Interview for Children (GIIC).

This interviewer-administered, 12-item questionnaire is used with children to collect information about gender identity, and has been cross-nationally validated as a diagnostic tool (Wallien et al., 2009).

Diagnostic validity of the GIIC was tested with a sample of 85 gender-referred, and 98 control children, with mean ages 6.8(2.3) and 8.0(2.5) years, respectively. The combination of affirmative responses on any three items achieved 88.8% specificity with 54.1% sensitivity. With four items, specificity and sensitivity both rose to 93.9% and 65.8%, respectively. The GIIC was originally developed based on DSM-III-R criteria for GIDC. Consequently, a confirmatory factor analysis revealed latent variables relating to two dimensions of gender confusion: affective and cognitive (Zucker et al., 1993).

Child Game Participation Questionnaire (CGPQ).

Parents provide dichotomous responses to 69 gender-normed questions about the child's play behaviors and preferences (e.g., baseball, hop-scotch, etc.). The effect size for sex is 3.2 (Meyer-Bahlburg, Sandberg, Dolezal, & Yager, 1994).

Child Behavior and Attitude Questionnaire (CBAQ).

Parent rates on an 8-point Likert-type scale the frequency with which their child expresses 71 common childhood attributes ("s/he fights," "s/he plays with dolls"). The effect size for sex is 5.7 (Meyer-Bahlburg, Sandberg, Yager, Dolezal, & Ehrhardt, 1994).

Measures for adolescent participants.

Hobby Preferences Scale (HPS).

The original, self-report Hobby Preferences Scale (HPS) prompted respondents to score their interest in 60 sex-typical activities on a 5-point Likert scale, and yielded a single, scaled factor termed “gender diagnosticity,” (Lippa, 1991, 1995). For the purposes of the current study, participants’ responses were simply summed, following (Meyer-Bahlburg, Dolezal, Baker, et al., 2006), in which study the HPS achieved an adequate inter-item reliability (Cronbach’s $\alpha = .76$) among a sample of 46,XX participants with classical and non-classical congenital adrenal hyperplasia.

Career Questionnaire (CareerQ).

This gender role behavior self-report questionnaire lists 70 occupations classified as male-typical or female-typical (based on census data), and participants respond yes or no regarding their interest in those occupations (Berenbaum, 1999; Meyer-Bahlburg, Dolezal, Baker, et al., 2006). The CareerQ demonstrated adequate inter-item reliability for both Male-typical career dimension (Cronbach’s $\alpha = .73$) and Female-typical career dimension (Cronbach’s $\alpha = .72$) in a study with 46,XX individuals with classical and non-classical hyperplasia (Meyer-Bahlburg, Dolezal, Baker, et al., 2006).

Recalled Child Gender Questionnaire-Revised (RCGQ-R).

This 23-item self-report measure was originally introduced by Mitchell & Zucker (1991), and has subsequently been expanded to measure three dimensions: Gender Role, Physical Activity, and Cross-Gender Desire (Meyer-Bahlburg, Dolezal, Zucker, et al., 2006; Zucker et al., 2006). Of these, Gender Role demonstrated good inter-item reliability and a very large effect size

($d = 1.74$) in a sample of 1,305 adolescents and adults (735 female, 570 male) within an age range of 13 to 74 years ($d = 1.74$, Zucker et al., 2006).

Gender Identity/Dysphoria Questionnaire for Adolescents and Adults (GIDYQ-AA).

The Gender Identity/Dysphoria Questionnaire for Adolescents and Adults (GIDYQ-AA), also called the Gender Identity for Adolescents and Adults (GIQAA), is a 27-item measure of gender identity, comparable to the Gender Identity Interview used with child participants. The four dimensions measured by the GIDYQ-AA are the subjective, social, somatic, and sociolegal (Deogracias et al., 2007, p. 372). Collectively, these dimensions load onto a single factor of gender identity capable of distinguishing adult cases of gender dysphoria from typically-developing university students (including heterosexual and nonheterosexual subgroups) with a sensitivity of 90.4% and specificity of 99.7% (Deogracias et al., 2007), and adolescent cases from clinical case controls with a sensitivity of 93.3% and specificity of 87.3% (Singh et al., 2010).

Brain Image Acquisition

MRI pulse sequence.

Images were acquired on a Signa EXCITE HDx 3.0-Tesla whole body scanner (GE, Milwaukee, WI) with 16-channel mobile graphics display hardware platform, 8-channel quadrature coil, and Array Spatial Sensitivity Encoding Technique (ASSET) software.

3 plane localizer.

Scanning parameters include the following: repetition time (TR) = 23.4ms, echo time (TE) = 1.7 ms, flip angle = 300, bandwidth = 31.3 MHz, field of view (FOV) = 24 x 24 cm², slice thickness = 5.0 mm, spacing = 0.0 mm, 9 slices per volume (3 axials, 3 sagittals and 3 coronals), matrix = 256 x 128.

Anatomical fast spoiled gradient recall (FSPGR).

Acquisition of high resolution, high contrast, T1-weighted head images along the desired direction is achieved in sagittal orientation. 3D FSPGR images were acquired with the following parameters: inversion time (TI) = 500 ms, TR = 4.7 ms, TE = minimum full, flip angle = 11, bandwidth = 41.67 MHz, FOV = 25 x 25 cm², phase FOV = 1.0, slice thickness = 1 mm, spacing = 0.0 mm, 128 slices per volume, 2 series x 1 NEX images, matrix = 256 x 256, scanner acceleration factor = 2.

Resting-state gradient-echo (GRE) echo planar imaging (EPI).

Acquisition of T2*-weighted head images along the desired direction was achieved in the axial orientation, with interleaved volume slices, while the participant was in a state of rest. Resting-state GRE EPI data were acquired with the following parameters: TR = 2200 ms, TR = 30 ms, flip angle = 90, bandwidth = 250 MHz, FOV = 24 x 24 cm², phase FOV = 1.0, slice thickness = 3.5 mm, spacing = 0.0 mm, 34 slices per volume, 6 series x 128 NEX images (+ 6 dummy scans per series), matrix = 64 x 64. At 4:55 minutes per GRE EPI series, total scan time required to collect resting-state fMRI data from subjects was 29:30.

Scan quality control.

To reduce participants' movement during the scan, research assistants and an MRI scan technician applied relaxation scripts and helped accommodate participants inside the scanner with pillows and blankets. Also, a fiducial vitamin E capsule was applied to the left side of participants' heads. Participants with incomplete data collection were removed from the study. A complete data set was considered one with at least two (2) runs of resting-state GRE EPI acquisition containing at least 128 volumes of EPI data, and one (1) complete run of anatomical MRI acquisition. For participants with three or more runs of EPI data, the first two were used for the current analyses.

Procedure

Research setting.

Data were collected at the New York State Psychiatric Institute (NYSPI), on the Columbia University Medical Center campus. Statistical analyses were conducted at the University of Southern California, Children's Hospital of Los Angeles campus.

Protection of human participants.

Modifications to the NYSPI Magnetic Resonance Unit (MRU) protocols #5321R and #6435 for the GIV Study have been approved by the IRB of NYSPI since October 2007.

Informed consent.

The first step for families arriving for their study day were the procedures of informed parental consent and child assent. It was explained to children and parents, separately, that 1) participation was entirely voluntary, and that they could withdraw from the study at any time with no negative repercussions, 2) there were no direct benefits to participation in the study, 3) the study included psychological testing which may cause momentary discomfort, as well as an MRI scanning session in which they would hear loud noises and may become drowsy, 4) they were to be compensated with \$150 in the form of a check or gift certificate upon completion of the MRI session and associated screening procedures and neuropsychological testing, and 5) their data would remain strictly confidential and would be deidentified and archived, and made available for use to only the principal investigator, co-investigators, and research assistants.

Administration of assessment instruments.

Participants in the GID-diagnosed subgroups completed in-depth clinical gender assessments (detailed above) prior to enrollment in this study. The clinical gender assessments were not administered to participants in the typically-developing subgroups.

All study participants completed comparable neuropsychological assessments and confirmatory eligibility screening on or around the day of the scan. The protocol for participant

assessment followed routine MRU procedures and was performed by MRU research assistants trained by personnel with a clinical master's degree or higher, and have demonstrated mastery of structured assessment procedures. Participant characteristics were obtained using self-assessments, structured and semi-structure interviews, neuropsychological testing, and other instruments completed by the participant (and for children, the participant's parent as well). While subjects were completing the MRI component of the protocol, parent-report questionnaires were administered to one or both parents.

Brain imaging.

Participant scanning was performed by a certified MR technologist with the assistance of trained MRU personnel. Several protocols developed at the MRU were used to help prepare participants who were new to MR imaging or had found it difficult in the past.

Compensation.

Participants who completed the majority of study tasks received a \$150 check or gift certificate.

Statistical Analyses

Descriptive statistics.

The open-source statistical analysis software, R, was used to compute descriptive statistics (R Core Team, 2013). Prior to group-level analyses of brain imaging data, the investigator computed descriptive statistics to ensure there were no significant differences among subgroups in terms of subject variables that may lead to confounding effects, namely age and intelligence.

fMRI data pre-processing.

Prior to extraction of resting-state functional connectivity correlations, functional brain images were spatiotemporally preprocessed to maximize signal-to-noise ratio. To do this, brain

images were adjusted for changes in BOLD signal during the time that elapses while one “frame” (or, volume) of functional imaging data is collected (slice-time corrected); realigned to reduce artificial signal due to participant motion in the scanner; transformed to fit within a standard brain image template space (spatial normalization, sometimes called warping); smoothed, or averaged among adjacent voxels to reduce the effect of outliers in image intensity; and bandpass-filtered, to detrend from the BOLD signal data any patterns coming from physiological processes other than neurofunction. Preprocessing of functional and anatomical brain imaging data were conducted within the Matlab2017b platform (The MathWorks, 2017), using the toolbox, Data Processing & Analysis of Brain Imaging (DPABI, Yan, Wang, Zuo, & Zang, 2016).

Extraction of individual-level connectivity correlations.

DPABI was also be used to extract individual-level, 4-dimensional matrices of correlation values—or correlation maps—between hypothesized seed regions and all other voxels in the brain, which in turn were submitted to group-level analyses. Connectivity correlations were Fisher’s r-to-Z transformed to allow correlation values to be treated as individual observations, then standardized to adjust for differences in absolute ranges of signal intensities observed between participants.

Group-level mixed-effects analyses.

Hypothesis testing of subgroup differences in functional connectivity patterns were conducted through a 2-Diagnostic subcategory (typically-developing versus GID-diagnosed) x 2-Assigned gender (female versus male) mixed-effects analysis, to which individual-level connectivity correlation maps were submitted as the dependent variable. Group-level statistical procedures were also performed in DPABI.

Power issues & Inferential scope.

The current study includes a very small sample, a common limitation of research on sex differentiation in the brain (Mueller, De Cuypere, & T'Sjoen, 2017). Indeed, Agcaoglu and colleagues (2015) computed a minimum sample size of $N=172$ is needed to identify a resting-state network in regions relevant to this study using data-driven techniques. Notwithstanding, cross-gender brain morphometry has been observed in histology studies with $n=1$ FtM, at the hypothalamic nucleus INAH3 (Garcia-Falgueras & Swaab, 2008; Smith, Junger, Derntl, & Habel, 2015), and at the stria terminalis (BSTc) (Kruijver et al., 2000; Smith et al., 2015). However, a more recent study with a sample of $n=1$ FtM, failed to obtain either cross-gender (masculinizing) or intermediate (defeminizing) effects using seed-based correlational analysis of resting-state fMRI. To maximize the inferential power in this small sample, the current study employs a seed-based (model-driven) approach to measuring resting-state functional connectivity. In terms of explanatory power, the limited scope of the current investigation qualifies it as a pilot study. However, exploratory findings from this research stand to provide valuable insights for orienting future research in an area about which much remains to unknown.

Results

The raw outcome measure was correlations between the voxelwise blood oxygen level-dependent (BOLD) activity between preparcelled seed brain regions, and each individual voxel in the brain. Fourteen seed regions were selected from the 90 preparcelled cortical regions in the commonly used Automated Anatomical Labeling atlas (see the 14 regions in Table 4). Details about previous findings supporting the selection of seed regions used presently can be found in the Introduction.

[TABLE 4]

Imaging Data Preprocessing

To prepare imaging data for extraction of connectivity correlations, participants' anatomical and functional brain images were first preprocessed using the software package DPABI⁶. The preprocessing steps used to prepare data for analysis were: 1) slice time correction to reduce signal noise due to scan acquisition latency, 2) realignment to reduce noise from participants' movement in the scanner, 3) spatial normalization to transform participant's brain images into a common template space, 4) spatial smoothing to reduce the impact of outliers in voxel BOLD signal intensities, and 5) bandpass filtering to remove BOLD signal trends arising from physiological processes other than neurofunction.

Slice-time correction adjusts for imaging data being collected not instantaneously but rather as a series of slices over a period of time (here, 34 slices and 2.2 seconds, respectively), such that each slice in a single frame (also called a volume) represents a slightly different state of brain activity. Presently, slice-time correction was used to interpolate volume slices to the middle slice within each volume (temporally).

⁶ Data Processing & Analysis for (Resting-State) Brain Imaging (Yan, Wang, Zuo, & Zang, 2016).

Then, for each participant, slice-time corrected functional images were realigned to the 3-dimensional space defined by the first volume collected in the second run, which in turn was coregistered to the participant's anatomical image. To estimate realignment parameters, the investigator used a 24-dimensional model (Friston-24) consisting of three axes of translation and three of rotation (together, referred to as rigid-body image transformations); the temporal derivatives of those axes; and the squares of the rigid-body and derivative parameters (Friston, Williams, Howard, Frackowiak, & Turner, 1996). During realignment, the investigator visually inspected and manually reoriented anatomical and functional brain images approximately about the bicommissural line to reduce the extent of interpolation required to estimate realignment parameters.

Subsequently, realigned images were submitted to the spatial normalization and smoothing algorithm, Diffeomorphic Anatomical Registration Through Exponentiated Lie algebra (DARTEL, Ashburner, 2007). At the cost of increased computational load compared to traditional spatial normalization algorithms, DARTEL first estimates a standard space using averages of participants' brain images, which are then coregistered to a commonly used, large-sample template image space (here, Montreal Neurological Institute conventional space). By coregistering individuals' brain images to one another in addition to the large-sample template, DARTEL offers greater precision than traditional spatial normalization procedures, which coregister individuals' data directly to only a large-sample template. Smoothing—the averaging of all voxels with adjacent voxels to reduce the impact of outliers in voxel intensity values—was applied simultaneously with spatial normalization to help preserve regional integrity among smoothed voxels. In this way, spatial smoothing in DARTEL helps control the increased Type-II error rate resulting from the loss of spatial resolution when rescaling measurement units from 3

mm³ (typical raw data voxel dimensions) to 8 mm³ (the conservative smoothed data voxel dimensions used in this study).

Finally, a bandpass filter was applied to functional images to reduce any signal noise coming from physiological processes (other than neurofunction) known to affect patterns of blood flow in the brain, such as respiration. The bandpass filter removed trends in the functional imaging data occurring at frequencies below 0.01 Hz and above 0.1 Hz.

Preprocessing Quality Control

Prior to extracting individual-level connectivity correlation maps from preprocessed imaging data, anatomical and functional images were visually inspected, and participants' head motion while in the scanner was formally assessed, to ensure suitability for analysis.

Visual inspection.

Raw anatomical images were visually inspected for anatomical abnormalities that could interfere with spatial normalization. After brain extraction—an automated procedure that strips bone and non-brain tissue, leaving grey matter, white matter, and cerebrospinal fluid— anatomical images were inspected for distortions resulting from failed tissue identification. Finally, following spatial normalization, images were again inspected for distortions resulting from failed coregistration to the target anatomical template image.

Raw functional images were visually inspected for signal dropout, or large areas of missing data resulting from interference with radiofrequency signal detection, as can occur, for example, with participants with braces or metal implants. After automated realignment to the structural image from the same participant, functional images were again inspected for distortion resulting from failed coregistration between subjects' anatomical and functional images. Finally,

after spatial normalization and smoothing, functional images were inspected for distortion resulting from failed coregistration to the target anatomical template image.

Visual inspection of the anatomical and functional imaging data in this study did not reveal any obvious image distortions that would interfere with the extraction and standardization of individual-level correlational data, also called postprocessing.

Head motion assessment.

To reduce motion-related confounds from individual participants representing outliers in terms of head motion during brain scan acquisition, the investigator screened participants' translational and rotational movement between functional imaging volumes, as well as a ratio estimate of overall movement (framewise displacement). Of the original sample of N=24 participants, one case-control pair was removed from further analyses due to movement in the scanner. In the first run, both participants reached at least 2.0mm of translation or 2.0° of rotation, and in the second run, 6.0mm of translation and mean framewise displacement >1.2 frames per volume. Mean framewise displacement was calculated using the algorithm described in Jenkinson, Bannister, Brady, and Smith (2002). Following subject exclusion due to excessive head motion, the total number of study participants decreased to N=22.

Imaging Data Postprocessing

Individual-level correlation maps.

Individual-level connectivity correlation maps were extracted from two functional imaging runs for each subject, for each of the 14 hypothesized seed regions selected from the Automated Anatomical Labeling atlas. Correlation values here refer to the relationship between the mean BOLD signal of all voxels in a given seed region, and all other voxels throughout the

brain. Fisher's r-to-Z transformation was applied to correlation maps to facilitate subsequent postprocessing procedures.

Missing data.

Given that everyone has differently shaped brains, MRI data are not evenly collected over the exact same volume of space in all participants, resulting in voxels that appear to contain no BOLD signal even after brain images have been spatially normalized into a standard space. These “empty” voxels, or signal voids, typically occur at the edges of brain images, and their specific locations are assumed to be randomly distributed among participants. However, to ensure signal voids did not skew group-level results, any voxels that did not contain valid data (i.e., BOLD signal) in at least 90% of subjects (i.e., $n=20$) were omitted from the sample by restricting standardization and group-level analyses to voxels within a study-specific template space called a group mask. The group mask was created from participants' individual masks, that is, realigned functional imaging data first binarized using a script based on AFNI's 3dAutomask (Cox, 1996), then spatially normalized to the same participant-specific parameters estimated during preprocessing. The group mask represents an aggregate of the individual binarized masks, in which voxels were included only if they contained valid BOLD signal in at least 20 participants. Voxels that did not contain valid data in at least 20 participants were omitted for all participants from subsequent postprocessing steps and group-level analyses.

As a check of internal validity, results estimated using the group mask were compared to results estimated using the canonical whole-brain and grey-matter masks used as default options in popular software packages like the Statistical Parametric Mapping suite (SPM8) from the Wellcome Trust Centre for Neuroimaging, University College London. Results with the group mask were largely consistent with the two others, albeit with fewer significant seed regions and

smaller significant voxel clusters. Below, the investigator reports results estimated with the study-specific group mask, which provided the most conservative range of voxels (i.e., fewest possible voxels submitted to group-level analyses) and greatest internal validity (i.e., study-specific voxel range tailored to actual participant brains).

Standardization.

To eliminate confounding effects arising from between-subjects differences in global signal (the distribution of BOLD signal intensities observed within a given participant), r-to-Z transformed correlation maps were standardized using mean regression and standard deviation division. This standardization approach has demonstrated good sensitivity to global signal effects in general, and to motion-related global signal effects in particular (Yan, Craddock, Zuo, Zang, & Milham, 2013). For each subject, r-to-Z transformed correlation maps were standardized across all voxels within the space defined by the group mask.

Averaging of functional imaging runs.

As participants' data were collected across two functional imaging runs (that is, two sessions of resting-state fMRI data collected in one visit), the standardized, r-to-Z transformed correlation maps from each participant's first and second runs were averaged to yield a single individual-level correlation map for each participant. These averaged correlation maps were then submitted to group-level statistical analyses.

Group-Level Inferential Statistics

Participants' postprocessed correlation maps were submitted to mixed-model analyses in which assigned gender (assigned female versus assigned male) was treated as a between-subjects variable, and diagnostic subcategory (typically-developing versus GID-diagnosed) as a paired-samples variable. In addition, as an added measure to control for motion-related confounds in

group-level analyses, mixed-effects models included as participants' average framewise displacement as a nuisance variable.⁷

In identifying significant clusters, multiple comparisons correction was achieved using Gaussian Random Field (GRF) theory to adjust minimum cluster sizes (i.e., the minimum number of correlated voxels considered to constitute a valid cluster). In this way, GRF correction was used to identify clusters of voxels that were 1) correlated with seed regions at a significance level of $p < .005$, and 2) adjoined by a minimum number of significant voxels determined on a cluster-by-cluster basis at a thresholded of $p < .0357$ ($\alpha < .01$, Bonferroni-corrected for the number of seeds examined in the study by dividing by 14). In addition, FSL's smoothest script (Jenkinson, Beckmann, Behrens, Woolrich, & Smith, 2012) was utilized to estimate effective image smoothness for use in GRF calculations in response to findings that simply importing the preprocessing smoothing kernel from image header information underrepresents actual image smoothness in postprocessed image files, resulting in an inflated rate Type-I error (Bennett, Wolford, & Miller, 2009). *F*-values obtained from the mixed-model analyses were used as the inferential test statistic.

Presently reported are the brain regions for which the factors, assigned gender and diagnostic subcategory, predicted connectivity differences among the four study subgroups: typically-developing females, GID-diagnosed assigned females, typically-developing males, and GID-diagnosed assigned males. Seed regions represent brain regions hypothesized to show significant differences among the study subgroups. Also reported are the groups of brain regions with which seed regions exhibited connectivity differences, termed clusters. Clusters are

⁷Modeling framewise displacement is a viable alternative to the popular motion correction procedure known as scrubbing, or removing and replacing (usually by interpolation) volumes exceeding a given movement threshold, which was presently contraindicated due to the small sample size (Fair et al., 2013).

identified by the coordinates at which the strongest correlations were observed, also called the peak, and by the name of the brain region in which cluster peaks were observed. Additional brain regions that appeared in observed clusters, but were not themselves peaks, are referred to here as cluster member regions.

Descriptive Statistics

The removal of two participants from the data due to motion changed the sample age distribution slightly ($M = 15.90$ years, $SD=3.73$, range: 8.83–20.29). Seven GID-diagnosed assigned females ($M = 16.66$ years, $SD=3.46$, range 12.36–20.29) and four GID-diagnosed assigned males ($M = 14.88$ years, $SD=4.78$, range 12.12–19.98) were paired with typically-developing participants according to assigned gender (male or female) and by age. In the matched-pair control groups, typically-developing females had a mean age of 16.45 years ($SD=3.29$, range 12.12–19.98), and males 14.62 years ($SD=4.84$, range 8.83–19.57).

Although differences in race/ethnicity were not expected to affect results, case-control pairs were nonetheless matched along this variable as closely as possible. Of the assigned female pairs, three identified as White, one Black/African-American, and two Latino/Hispanic; one GID-diagnosed participant who identified as “Other” was paired with a Latino/Hispanic control. Of the assigned male pairs, three identified as White, and one White GID-diagnosed participant was paired with a Black/African-American control.

All study participants were right-handed except one GID-diagnosed assigned female.

Absent significant demographic differences between case and control groups, these were treated as matched pairs in inferential analyses.

Overview of Findings

For six of the 14 hypothesized seed regions, mixed-effects analyses with matched-pairs factor diagnostic subcategory (GID-diagnosed versus typically-developing) and between-groups factor assigned gender (female versus male) revealed significant interaction effects in multiple brain regions. The four seed regions yielding significant clusters were the right medial superior frontal gyrus, left lingual gyrus, right lingual gyrus, and left cuneus. Two additional seed regions that did not yield significant clusters but were supported indirectly were the left dorsolateral superior frontal gyrus, and the left inferior frontal gyrus, triangular part. No main effects of assigned gender or diagnostic subcategory survived clusterwise Bonferroni correction for multiple comparisons.

Figure 1 below shows patterns of connectivity from atlas-based, parcellated seed regions to voxels in the whole brain. The one-sample T-maps show uncorrected p -values and were spatially masked to include only regions revealed by the corresponding mixed-effects interaction F-maps. Although hypothesis testing was conducted using a cluster-formation voxel threshold of $p < .005$, for display purposes, the mixed-effects F-maps show p -values GRF-corrected at a cluster-formation voxel threshold of $p < .01$. Seed region labels, from top to bottom, identify: SFGmed.R, right medial superior frontal gyrus; LING.L, left lingual gyrus; LING.R, right lingual gyrus; and CUN.L, left cuneus. Interaction column region labels: $z = +54$, SMA, supplementary motor area; $z = +45$, SFGmed, medial superior frontal gyrus, SFGdor, dorsolateral superior frontal gyrus; $z = +36$: MFG, middle frontal gyrus, SFGmed, medial superior frontal gyrus; $z = +3$: CAU, caudate, PUT, putamen (lenticular nucleus), THA, thalamus, PAL, globus pallidus (lenticular nucleus). All region labels are indexed in the AAL atlas (Tzourio-Mazoyer et al., 2002). Accompanying plots of subgroup cluster peak t -values (uncorrected) are given in Figure 2.

[FIGURE 1]

[FIGURE 2]

Hypothesized Seed Regions

Table 6 below gives a summary of cluster peaks, size, and region membership. Formal post hoc hypothesis testing was not possible due to the small sample sizes in the current study. However, connectivity patterns may be informally characterized to facilitate interpretation of findings and comparison with the current literature. Table 7 below shows a breakdown of cluster peaks by participant subgroup.

[TABLE 6]

[TABLE 7]

Left dorsolateral superior frontal gyrus.

The hypothesis that resting-state functional connectivity (RSFC) associated with the left dorsolateral superior frontal gyrus varies was not directly supported in the current findings. However, this region did appear as a member region in a cluster with seed left lingual gyrus and peak left middle frontal gyrus, and another with seed right lingual gyrus and peak right superior medial frontal gyrus, indirectly supporting the hypothesis that connectivity at this region is predicted by the interaction of assigned gender and diagnostic subcategory. In both clusters, RSFC between seed and cluster regions was negatively correlated in (from strongest to weakest) typically-developing males, GID-diagnosed assigned males, and GID-diagnosed assigned females, and positive in typically-developing females.

Left inferior frontal gyrus, triangular part.

The hypothesis that RSFC associated with the left inferior frontal gyrus, triangular part varies as a function of the interaction of assigned gender and diagnostic subcategory was not

directly supported. However, it did constitute a member region of a cluster with seed left lingual gyrus (and peak left middle frontal gyrus), indirectly supporting the hypothesis that connectivity at this region is predicted by the interaction of assigned gender and diagnostic subcategory.

Right medial superior frontal gyrus.

The hypothesis that RSFC associated with the right medial superior frontal gyrus varies as a function of the interaction of assigned gender and diagnostic subcategory was supported in the current findings. This seed region was positively correlated with a single cluster with a peak in the right supplementary motor area in typically-developing males, and to a lesser extent, in GID-diagnosed assigned females. The correlation between seed and cluster was negative in typically-developing females, and more so in GID-diagnosed assigned males. In addition, this region emerged as the peak of a cluster with seed right lingual gyrus, demonstrating the same relative pattern of correlation direction and magnitude among subgroups as in the previously described cluster.

Left lingual gyrus.

The hypothesis that RSFC associated with the left lingual gyrus varies as a function of the interaction of assigned gender and diagnostic subcategory was supported in the current findings. The left lingual gyrus was negatively correlated with two clusters, with peaks in the left middle frontal gyrus and left medial superior frontal gyrus in (from strongest to weakest correlation) typically-developing males, GID-diagnosed assigned males, and GID-diagnosed assigned females; and positively correlated in typically-developing females.

Right lingual gyrus.

The hypothesis that RSFC associated with the right lingual gyrus varies as a function of the interaction of assigned gender and diagnostic subcategory was supported. Like its left-

hemisphere counterpart, the right lingual gyrus was negatively correlated with a cluster with a peak in the left middle frontal gyrus in (from strongest to weakest correlation) GID-diagnosed assigned males, typically-developing males, and GID-diagnosed assigned females; and positively correlated in typically-developing females. The right lingual gyrus also was negatively correlated with a cluster with the right medial superior frontal gyrus in (from strongest to weakest) typically-developing males, GID-diagnosed assigned males, and GID-diagnosed assigned females; and positively correlated in typically-developing females.

Left cuneus.

The hypothesis that RSFC associated with the left cuneus varies as a function of the interaction of assigned gender and diagnostic subcategory was supported. The right lingual gyrus was negatively correlated with a cluster with peak right thalamus in (from strongest to weakest) GID-diagnosed assigned females, typically-developing males, and typically-developing females; and positively correlated in GID-diagnosed assigned males.

Cluster Breakdown

As can be seen in Figure 1 above, significant connectivity was observed in association with seed regions in the frontal lobe (left dorsolateral superior frontal gyrus, right medial superior frontal gyrus, and inferior frontal gyrus, triangular part), and the medial occipital lobe (left lingual gyrus, right lingual gyrus, and left cuneus).

The seed right medial superior frontal gyrus exhibited the strongest peak intensity (here, peak intensity refers to *F*-values associated with the interaction terms of mixed-effects analyses). This cluster had a peak in the right supplementary motor area (SMA.R), and included the right median cingulate gyrus (DCG.R), left median cingulate gyrus (DCG.L), and right dorsolateral

superior frontal gyrus (SFGdor.R), $F(1, 8) = 102.99$, MNI coordinates [15, -3, 54], cluster size $k = 211$.

The left and right lingual gyri (LING.L and LING.R, respectively) were associated with two distinct clusters each. Both seeds exhibited clusters with peaks in the left middle frontal gyrus (MFG.L), and including the left inferior frontal gyrus, opercular part (IFGoperc.L), and left precentral gyrus (PreCG.L). Additionally, the cluster associated with seed LING.L included the left inferior frontal gyrus, triangular part. That cluster included 101 voxels with peak coordinates at [-39, 15, 36], $F(1, 8) = 63.99$. The cluster originating from the seed LING.R included 125 voxels with peak coordinates at [-39, 15, 36], $F(1, 8) = 85.66$.

The left and right lingual gyri also exhibited cluster peaks within their corresponding, ipsilateral medial superior frontal gyri (SFGmed.L and SFGmed.R, respectively). The corresponding clusters of both peaks included the contralateral, medial superior frontal gyrus (SFGmed.R and SFGmed.L, respectively), and bilateral superior frontal gyri (SFG.L and SFG.R). The cluster originating from the seed LING.L included 117 voxels, with peak coordinates at [-3, 42, 45], $F(1, 8) = 59.11$. The cluster originating from the seed LING.R included 94 voxels, with peak coordinates at [6, 54, 36], $F(1, 8) = 59.35$.

Finally, the left cuneus had significant connectivity with a cluster in the basal ganglia that was focused on the right thalamus (THA.R) and included the right pallidum (PAL.R), right putamen (PUT.R), and right caudate (CAU.R). That cluster included 93 voxels, with coordinates at [18, -9, 3], $F(1, 8) = 82.95$.

Discussion

The purpose of this study was to identify differences in resting-state functional connectivity (RSFC) associated with 14 brain regions across assigned gender and diagnostic subcategories of gender dysphoria. To that end, the objective was to examine main effects in connectivity between assigned males and assigned females, and between typically-developing and GID-diagnosed youth, as well as the interaction (e.g., typically-developing females, GID-diagnosed assigned females, typically-developing males, and GID-diagnosed assigned males).

There were no main effects in any of the 14 hypothesized regions between assigned females and assigned males, or between typically-developing youth and GID-diagnosed youth. As no main effects were observed, the investigator fails to reject the null hypotheses that 1) neurofunction associated with sex-differentiated brain regions does not differ between assigned males and assigned females when groups are comprised of both typically-developing and GID-diagnosed youth, and 2) neurofunction associated with sex-differentiated brain regions does not differ between typically-developing and GID-diagnosed youth when groups are comprised of both assigned males and assigned females. Significant interactions revealed connectivity patterns similar to those in typically-developing males for GID-diagnosed females, and patterns in between those of typically-developing males and females for GID-diagnosed males. In addition, GID-diagnosed assigned males showed a more variable pattern of effects, possibly indicating a more heterogeneous sample in this subgroup.

Primary Findings

The primary findings were interaction effects in resting-state functional connectivity associated with six of the 14 hypothesized brain regions. Differences distinguishing among typically-developing females, GID-diagnosed assigned females, typically-developing males, and

GID-diagnosed assigned males were observed in connectivity patterns associated with the (1) left dorsolateral superior frontal gyrus, (2) left inferior frontal gyrus, triangular part, (3) right medial superior frontal gyrus, (4) left inferior frontal gyrus, triangular part, (5) left cuneus, and (6) right cuneus. Moreover, the nature of these interaction effects varied among regions (see Figure 2, above). Connectivity profiles of GID-diagnosed youth suggested by the current findings are summarized in Table 8, below.

[TABLE 8]

Hypothesized seed regions with significant effects.

Below, the results of the current study are contextualized in relation to previous findings of sex-related functional connectivity differences in typically-developing males and females. Previous studies have reported the following types of measures: seed-to-voxel RSFC correlations, as in the current study (Alarcón et al., 2015; Solé-Padullés et al., 2016); lateralization, or degree to which the networks associated with a seed region are concentrated in either the left or right hemisphere (Agcaoglu et al., 2015); network centrality measures: node degree, node efficiency, and node betweenness, the number of pairs of regions that are indirectly correlated with one another via direct correlations with a via direct correlations with a common seed region (Wu et al., 2013); and voxel-mirrored homotopic connectivity, the degree of connectivity between pairs of symmetrical brain regions in the left and right hemispheres (Zuo et al., 2010).

Through the lens of those studies, the current findings are interpreted as revealing in GID-diagnosed subgroups relative to typically-developing subgroups, effects of connectivity that patterned either: 1) after the same assigned gender, 2) after the opposite assigned gender (cross-gender connectivity, either masculinized for assigned females or feminized for assigned males),

3) in between the same and opposite assigned genders (intermediate connectivity, either defeminized for assigned females or demasculinized for assigned males), or 4) after neither assigned gender (GD-specific connectivity). To facilitate discussion, the investigator refers to interactions that involve changes in magnitude of correlation among subgroups as quantitative, and interactions that involve changes in direction of effect as qualitative. The investigator interpreted qualitative interactions as indicating cross-gender connectivity in either one or both GID-diagnosed subgroups (depending on the cluster in question), and quantitative interactions as intermediate connectivity.

All the effects reported here are original findings, unless otherwise noted.

Left dorsolateral superior frontal gyrus.

The seed (1) left dorsolateral superior frontal gyrus was a member region in clusters associated with the seed (4) left lingual gyrus (peak left medial superior frontal gyrus), and (5) right lingual gyrus (peak (3) right medial superior frontal gyrus). In both clusters, a positive seed-to-cluster correlation was observed in TD-developing females, while negative correlations were observed in typically-developing males, typically-developing females, and GID-diagnosed males. These findings suggest that the (1) left dorsolateral superior frontal gyrus expresses cross-gender patterns functional connectivity in GID-diagnosed assigned females, and intermediate connectivity in GID-diagnosed assigned males. The latter effect was more evident in the cluster associated with the (4) left lingual gyrus, which showed greater difference between the two male subgroups (typically-developing and GID-diagnosed) relative to the cluster associated with the (5) right lingual gyrus (see Figure 2, Table 7, and Table 8, above).

Previously, RSFC connectivity has been found to decrease for typically-developing females, and increase for typically-developing males, with age, in correlations between the seed

(1) left dorsolateral superior frontal gyrus and the right basolateral amygdala (part of the basal ganglia) Alarcón et al. (Alarcón et al., 2015), and between the seed (1) left dorsolateral superior frontal gyrus and the right dorsolateral superior frontal gyrus (Zuo et al., 2010). Considering those findings, the current results suggest that connectivity between the (1) left dorsolateral superior frontal gyrus and the right basolateral amygdala and right dorsolateral superior frontal gyrus (which was also observed in both clusters) may be sensitive to gender dysphoria, especially in females.

Left inferior frontal gyrus, triangular part.

The seed (2) left inferior frontal gyrus, triangular part, constituted a member region of a cluster with seed (4) left lingual gyrus (and peak left middle frontal gyrus). In that cluster, both assigned male subgroups showed negative correlations with the seed, while for assigned females, the typically-developing subgroup showed a positive correlation, and the GID-diagnosed subgroup a negative one. These findings suggest that at the (2) left inferior frontal gyrus, triangular part, expresses cross-gender connectivity in GID-diagnosed assigned females, and intermediate connectivity in GID-diagnosed assigned males.

These findings suggest a cross-gender connectivity pattern in GID-diagnosed assigned females, and an intermediate connectivity pattern in GID-diagnosed assigned males, at the (2) left inferior frontal gyrus, triangular part. Previously, the (2) left inferior frontal gyrus, triangular part, has been reported to show a main effect of node degree in typically-developing females compared to males (Wu et al., 2013), and formed part of a network showing greater lateralization in typically-developing females compared to males (Agcaoglu et al., 2015). Considering those findings, the current results may reflect masculinized node degree and lateralization in GID-diagnosed assigned females, and demasculinized node degree and lateralization in GID-

diagnosed assigned males, associated with connectivity at the (2) left inferior frontal gyrus, triangular part.

Right medial superior frontal gyrus.

The seed (3) right medial superior frontal gyrus yielded a single cluster with peak right supplementary motor area, emerged as the peak region of a cluster associated with the seed (5) right lingual gyrus, and appeared as a member region in a cluster associated with seed (4) left lingual gyrus.

For the first cluster (peak right supplementary motor area), RSFC correlations with the seed (3) right medial superior frontal gyrus were positive in GID-diagnosed assigned females and typically-developing males, and negative in typically-developing females and GID-diagnosed assigned males. Of note, the magnitude of the negative correlation observed in GID-diagnosed assigned males was much stronger than that in typically-developing females.

For the second and third clusters (with peaks left medial superior frontal gyrus and (3) right medial superior frontal gyrus, respectively), typically-developing females showed positive correlations between seed and cluster, and GID-diagnosed assigned females negative correlations, while typically-developing and GID-diagnosed assigned males both showed negative correlations. More specifically, the negative correlations observed in the typically-developing and GID-diagnosed assigned male subgroups were considerably stronger than in the GID-diagnosed assigned female subgroup, and while the cluster with seed (5) right lingual gyrus revealed a slightly weaker correlation in GID-diagnosed assigned males than in typically-developing males, this difference was more pronounced in the cluster associated with the seed (4) left lingual gyrus, placing connectivity in GID-diagnosed assigned males in between that of GID-diagnosed assigned females and typically-developing males. These findings suggest that in

GID-diagnosed assigned females, the (3) right medial superior frontal gyrus, (4) left lingual gyrus, and (5) right lingual gyrus may express cross-gender functional connectivity patterns. In GID-diagnosed assigned males, the (3) right medial superior frontal gyrus may be implicated in at least one RSFC network (shared with the right supplementary motor area) that expresses hyperfeminized connectivity, and in two, apparently lateralized, parallel networks that include the (1) left dorsolateral superior frontal gyrus, (3) right medial superior frontal gyrus, and either the (4) lingual gyrus or (5) right lingual gyrus, respectively.

Previously, the (3) right medial superior frontal gyrus has been associated with greater node efficiency in typically-developing females compared to typically-developing assigned males (Wu et al., 2013), and increased connectivity with the right superficial amygdala for typically-developing assigned males and decreased connectivity for typically-developing assigned females, with age (Alarcón et al., 2015). In addition, the left and right medial superior frontal gyri have demonstrated greater homotopic connectivity in assigned females than males (Zuo et al., 2010). It is possible the present findings reflect masculinized node efficiency in GID-diagnosed females, and hyperfeminized node efficiency in GID-diagnosed males (Wu et al., 2013). Given that the seed (3) right medial superior frontal gyrus and its opposite hemisphere counterpart, the left medial superior frontal gyrus, appeared together two clusters (one associated with the (4) left lingual gyrus, and the other with the (5) right lingual gyrus), these findings may reflect masculinized homotopic connectivity in GID-diagnosed assigned females, and defeminized homotopic connectivity in GID-diagnosed assigned males (Zuo et al., 2010). Finally, one can speculate that connectivity between the seed (3) right medial superior frontal gyrus and right superficial amygdala may be sensitive to gender dysphoria, and possibly to age-

related effects that mediate the complex relationship between brain function and experienced gender (Alarcón et al., 2015).

Left lingual gyrus.

The (4) left lingual gyrus was associated with two independent clusters. The first cluster included as a member region the hypothesized (2) seed left inferior frontal gyrus, triangular part. The second cluster included as member regions the hypothesized seeds (1) left dorsolateral superior frontal gyrus, and (3) right medial superior frontal gyrus. As can be seen in Figure 2, both clusters showed differences in magnitude between the negative correlations observed in typically-developing and GID-diagnosed assigned males, while in assigned females, the typically-developing subgroup showed a positive correlation, and the GID-diagnosed subgroup a negative one. These results suggest GID-diagnosed assigned females express cross-gender connectivity patterns, and GID-diagnosed assigned males intermediate connectivity patterns, associated with the hypothesized seeds (1) left dorsolateral superior frontal gyrus, (2) seed left inferior frontal gyrus, triangular part, (3) right medial superior frontal gyrus, and (4) left lingual gyrus.

Previously, the (4) left lingual gyrus has been implicated in sex-related functional connectivity differences under typical development. The (4) left lingual gyrus exhibited greater node degree and node efficiency in typically-developing females than males (Wu et al., 2013). Similarly, Agcaoglu and colleagues (2015) reported greater lateralization associated with the (4) left lingual gyrus in males compared to females. Interestingly, that study also revealed bilateral trends toward greater lateralization in assigned females than males, suggesting that the (4) left lingual gyrus is involved in at least two resting-state networks for which sex-related lateralization effects are inversely correlated. However, no evidence of these putative

complementary connectivity patterns was presently observed. Given the previous findings, the current results may reflect masculinized node degree, node efficiency, and lateralization, in GID-diagnosed assigned females, and demasculinized node degree, node efficiency, and lateralization in GID-diagnosed assigned males, in resting-state connectivity networks that involve the (1) left dorsolateral superior frontal gyrus, (2) seed left inferior frontal gyrus, triangular part, (3) right medial superior frontal gyrus, and (4) left lingual gyrus.

Right lingual gyrus.

The (5) right lingual gyrus was also associated with two independent clusters, one of which had a peak in the (3) right medial superior frontal gyrus and included as a member region the hypothesized seed (1) left dorsolateral superior frontal gyrus. For both clusters, typically-developing assigned females showed positive correlations, and GID-diagnosed assigned females negative correlations. That is, GID-diagnosed assigned females showed cross-gender connectivity patterns associated with the (1) left dorsolateral superior frontal gyrus, (3) right medial superior frontal gyrus, and (5) right lingual gyrus. In the cluster with peak left middle frontal gyrus, GID-diagnosed assigned males showed a stronger negative correlation than did typically-developing assigned males, suggesting a hypermasculinized connectivity pattern. Incidentally, the former cluster also included as a member region the left precentral gyrus, which stands out as one of only two regions involved in sensorimotor brain function observed in the current study (the other being the right supplementary motor area, the peak of a cluster associated with seed (1) right medial superior frontal gyrus). Interestingly, in the cluster with peak (3) right medial superior frontal gyrus, GID-diagnosed assigned males showed a slightly weaker correlation relative to typically-developing males, opposite the direction of effect as the cluster with peak (1) right medial superior frontal gyrus. These findings suggest that in GID-

diagnosed assigned males, the (5) left lingual gyrus may be associated with at least two networks, the first of which exhibits hypermasculinized connectivity and may possibly be connected with sensorimotor functioning (vis-à-vis the inclusion of the left precentral gyrus in the first cluster), while the second exhibits demasculinized connectivity, possibly in connection with executive control networks given that the cluster is essentially comprised of medial and dorsal superior frontal gyri (Raichle, 2011).

Previously, the right lingual gyrus has been reported to show greater node degree and node efficiency in typically-developing assigned females than males (Wu et al., 2013), and an age-by-sex interaction such that connectivity (with the left superficial amygdala) increased in females, and decreased in males, with age (Alarcón et al., 2015). Another study revealed trends toward greater lateralization in females than males associated with the bilateral lingual gyri (Agcaoglu et al., 2015). We may conjecture that the current results reflect masculinized node degree and node efficiency in GID-diagnosed assigned females, and defeminized node degree and efficiency in GID-diagnosed assigned males, relative to typically-developing females and males, respectively. It is possible the hypermasculinized connectivity observed in the (5) right lingual gyrus may in fact reflect a GD-specific connectivity pattern relevant only to assigned males, although potential explanations for such an effect remain unknown. Finally, the possible implication of separate sensorimotor- and executive control-related resting-state networks both connected with the left and right lingual gyri is consistent with recent body-representation theories of the neurobiology of transsexuality, which posit that the incongruence between one's internal body representation as corresponding to one sex and sensory input to the contrary will result in functional connectivity patterns unique to individuals with gender dysphoria relative to both typically-developing and clinical populations (Lin et al., 2014).

Left cuneus.

The (6) left cuneus was associated with one cluster in the basal ganglia, with peak right thalamus. As Figure 2 shows, correlations in both typically-developing and GID-diagnosed assigned female subgroups were negative, while correlations in assigned males were positive in the GID-diagnosed subgroup, and negative in the typically-developing subgroup. The GID-diagnosed assigned female subgroup showed the strongest negative correlation, followed by the typically-developing assigned male subgroup, and finally, the typically-developing assigned female subgroup. These findings suggest connectivity between the (6) left cuneus and right basal ganglia regions exhibits a hypermasculinized pattern in GID-diagnosed assigned females, and given that the GID-diagnosed assigned male subgroup was the only one to show a positive correlation, possibly a connectivity pattern that may be unique to GID-diagnosed assigned males.

Previously, Wu and colleagues (2013), reported greater node degree and node efficiency in typically-developing females compared to males, and an age-by-sex interaction such that node efficiency decreased more rapidly with age for typically-developing females than for males.⁸ Since the GID-diagnosed assigned males were the only subgroup showing a positive correlation associated with the left cuneus, this finding cannot be readily related to either typically-developing assigned female or male subgroups. It is possible the current results reflect hypermasculinized node degree and node efficiency associated with the (4) left cuneus in GID-diagnosed assigned females, and subgroup-specific node degree and node efficiency in GID-diagnosed assigned males.

⁸ Interestingly, another study revealed an age-by-sex interaction with opposite effects in the connectivity between the right cuneus and left superficial amygdala, suggesting a divergence of connectivity associated with the left and right cuneus between typically-developing males and females with age (Alarcón, Cservenka, Rudolph, Fair, & Nagel, 2015), however, this pattern was not observed in the current study.

Hypothesized seed regions with no significant effects.

Seed regions that did not yield significant findings were those in association cortices in the temporal lobes (left amygdala and right amygdala), occipital lobe (left fusiform gyrus and right fusiform gyrus), and parietal lobe (right angular gyrus and left precuneus); and limbic cortices in the parietal lobe (left posterior cingulate gyrus and right posterior cingulate gyrus). Of these, the left precuneus was the most surprising, as it was the most frequently reported region in the literature on sex differences in functional connectivity in typically-developing youth (Agcaoglu et al., 2015; Alarcón et al., 2015; Solé-Padullés et al., 2016; Wu et al., 2013).

Secondary Findings

Almost all cluster peak and member regions observed in the current study have previously been implicated in functional connectivity studies of sex effects in either typically-developing humans (Agcaoglu et al., 2015; Alarcón et al., 2015; Solé-Padullés et al., 2016; Wu et al., 2013; Zuo et al., 2010), typically-developing adults (Kilpatrick, Zald, Pardo, & Cahill, 2006; Scheinost et al., 2015; L. Wang, Shen, Tang, Zang, & Hu, 2012), or transsexual adults (Lin et al., 2014; Santarnecchi, Vatti, Déttore, & Rossi, 2012).

Non-hypothesized cluster peak regions.

To the limited extent that cluster peak regions represent focal points of neurofunctional activity, the present findings suggest these non-hypothesized regions may also be implicated in gender development. Non-hypothesized regions that emerged as cluster peaks in the present study were the: (7) right supplementary motor area (Wu et al., 2013), (8) left middle frontal gyrus (Agcaoglu et al., 2015; Zuo et al., 2010); (9) left medial superior frontal gyrus (Zuo et al., 2010); and (10) right thalamus (Kilpatrick et al., 2006; Scheinost et al., 2015). Therefore, although not specifically hypothesized to show group differences in connectivity, the cluster

peak regions observed presently coincide with previous findings implicating the same regions in sex differences in functional connectivity between typically-developing male and female youth.

Right supplementary motor area.

The (7) right supplementary motor area emerged as the peak of a cluster with seed (3) right medial superior frontal gyrus. For this cluster, RSFC correlations between the (3) right medial superior frontal gyrus and (7) right supplementary motor area in assigned females was positive for the GID-diagnosed subgroup, and positive for the typically-developing subgroup, while for males, this pattern was mirrored such that the GID-diagnosed male subgroup showed a (strongly) negative correlation, and typically-developing male subgroup a positive correlation. These findings suggest there is at least one resting-state functional connectivity network that includes the (7) right supplementary motor area and (3) right medial superior frontal gyrus, associated with hyperfeminized functional connectivity in GID-diagnosed assigned males, and with masculinized connectivity in GID-diagnosed assigned females.

Previously, Wu and colleagues (2013) observed greater node degree associated with the right supplemental motor area in typically-developing assigned females compared to males. Therefore, it is possible the current findings represent hyperfeminized network centrality in GID-diagnosed assigned males, and masculinized network centrality in GID-diagnosed assigned females, associated with the (7) right supplementary motor area.

More recently, in a study with adolescent samples of n=19 GID-diagnosed assigned males, n=21 GID-diagnosed assigned females, n=20 typically-developing assigned males, and n=21 typically-developing assigned males, Nota and colleagues (2017) observed patterns of functional connectivity associated with the (7) right supplementary motor area that in adolescents with gender dysphoria were more similar to patterns observed in typically-developing youth of

the opposite rather than same sex. Using ICA, the investigators were able to identify the resting-state network (RSN) within which the (7) right supplementary motor area exhibited differences to be the sensorimotor network II (SMN-II) in the sensorimotor system (Nota et al., 2017; Raichle, 2011). Therefore, one may speculate that the current findings on the (7) right supplementary motor area, and by extension, the (3) right medial superior frontal gyrus, reflect cross-gender functional connectivity in both GID-diagnosed assigned males and females associated with the SMN-II network.

Left middle frontal gyrus.

The (8) left middle frontal gyrus was the peak of two clusters: one associated with seed left lingual gyrus, and one with seed right lingual gyrus. Both clusters showed differences in the direction of correlations observed in assigned females, which was positive in the typically-developing subgroup, and negative in the GID-diagnosed subgroup. For assigned males, the cluster associated with the left lingual gyrus showed a strong negative correlation in the typically-developing subgroup, and a weak correlation in the GID-diagnosed subgroup. Meanwhile, the cluster associated with the right lingual gyrus showed strong negative correlations of similar magnitude in both the typically-developing and GID-diagnosed assigned male subgroups. This pattern of findings suggests that the (7) left middle frontal gyrus shares networks with the (1) left and (5) right lingual gyri that express cross-gender connectivity patterns in GID-diagnosed assigned females, and in assigned males, shares a network with the (4) left lingual gyrus that expresses intermediate connectivity.

Presently, the (8) left middle frontal gyrus revealed cross-gender connectivity patterns in GID-diagnosed assigned females, and an intermediate connectivity pattern in GID-diagnosed assigned males. Previously, the middle frontal gyrus has been reported to show greater

homotopic connectivity for females than males, with an increase in homotopic connectivity for males, and decrease for females, with age (Zuo et al., 2010). In addition, the left middle frontal gyrus has shown trends toward greater lateralization for females than males in both voxelwise and cofactor regression analyses (Agcaoglu et al., 2015). It is possible the present findings reflect masculinized homotopy and lateralization in GID-diagnosed assigned females, and demasculinized homotopy and lateralization in GID-diagnosed assigned males.

Left medial superior frontal gyrus.

The (9) left medial superior frontal gyrus emerged as the peak region of a cluster associated with the (4) left lingual gyrus, and as a member region in a cluster associated with the (5) right lingual gyrus. In both clusters, the GID-diagnosed assigned male subgroup showed weaker correlations relative to typically-developing males. This difference was much more noticeable in the cluster with seed (4) left lingual gyrus, in which the seed-to-cluster correlation in GID-diagnosed assigned males was more similar to that in GID-diagnosed assigned females than to that in typically-developing assigned males. Also in both clusters, the typically-developing assigned female subgroup showed positive correlation, while the GID-diagnosed assigned female subgroup showed negative correlations. These findings suggest that the (9) left medial superior frontal gyrus may express cross-gender (masculinized) functional connectivity in GID-diagnosed assigned females, and intermediate demasculinized connectivity in GID-diagnosed assigned males.

This is the first study to observe cross-gender functional connectivity in GID-diagnosed assigned females, and developmentally-atypical connectivity (i.e., dissimilar from both typically-developing assigned females and typically-developing assigned males) in GID-diagnosed assigned males associated specifically with the left medial superior frontal gyrus. However,

homotopic connectivity between the left and right medial superior frontal gyri has previously demonstrated a stronger correlation in typically-developing assigned females than in typically-developing assigned males (Zuo et al., 2010), consistent with the present findings of negative correlations in GID-diagnosed assigned females, and positive correlations in typically-developing assigned females, between the left medial superior frontal gyrus and both the (4) left lingual gyrus and (5) right lingual gyrus. It is possible the present findings reflect masculinized homotopic connectivity among GID-diagnosed assigned females, and demasculinized homotopy among GID-diagnosed assigned males, associated with the (9) left medial superior frontal gyrus. More speculatively, the left medial superior frontal gyrus may help to mediate homotopic connectivity between the left lingual gyrus and right lingual gyrus under typical development.

Right thalamus.

The (10) right thalamus emerged as the peak of a cluster associated with seed (6) left cuneus. For this cluster, the typically-developing and GID-diagnosed assigned female subgroups both showed negative correlations of similar magnitude, while for assigned males, the GID-diagnosed subgroup showed a strong positive correlation and the typically-developing subgroup a weak negative correlation. Of note, the magnitude of the negative correlation corresponding in the typically-developing male subgroup was stronger than that of the typically-developing assigned female subgroup, but weaker than that of GID-diagnosed assigned female subgroup. These findings suggest the (10) right thalamus expresses hypermasculinized connectivity in GID-diagnosed assigned females, and in GID-diagnosed assigned males, a connectivity pattern that may possibly be unique to this subgroup.

To the best of the investigator's knowledge, to date no studies on sex differences in connectivity associated with the right thalamus have included typically-developing youth.

However, functional connectivity at the left thalamus has been found to increase with age for women but not men (Scheinost et al., 2015). Also, Kilpatrick and colleagues (2006) found significantly greater connectivity between the bilateral thalamus and right amygdala for men compared to women, and greater connectivity between the left thalamus and left amygdala for women compared to men. By contrast, one study focused specifically on thalamic connectivity in adults did not reveal any differences between men and women (Weissman-Fogel et al., 2010), consistent with the findings on the typically-developing subgroups in this study. Interestingly, while the typically-developing assigned male and female subgroups showed little difference in magnitude of correlations, both GID-diagnosed subgroups showed correlations notably different from their typically-developing counterparts.

Given the findings in typically-developing adults summarized above, there may be a key role for the left amygdala and/or right amygdala in connection with one or more networks including the (6) left cuneus and (10) right thalamus. It is tempting to conclude that there exists at least one resting-state network connecting the left cuneus with the right thalamus (and other right basal ganglia structures: pallidum, putamen, and caudate nucleus), which may eventually serve to help differentiate typical development and development associated with gender dysphoria in assigned male and female youth, however this finding will require replication.

Non-hypothesized cluster member regions.

The significance of the non-hypothesized cluster member regions reported presently, while interesting to compare with previous findings, should be interpreted with caution given that they are less representative of core findings than either hypothesized seed regions or observed cluster peak regions. Regarding non-hypothesized cluster member regions reported here, the inspired reader is referred to the following sources: for the left precentral gyrus, left

median cingulate gyrus, right median cingulate gyrus, and right caudate, (Agcaoglu et al., 2015); right pallidum, (Santarnecchi et al., 2012); right putamen, (L. Wang et al., 2012); left inferior frontal gyrus, opercular part, (Wu et al., 2013); for the right dorsolateral superior frontal gyrus, (Zuo et al., 2010); and for the left anterior cingulate gyrus, recent research with transsexual adults (Feusner et al., 2017; Manzouri, Kosidou, & Savic, 2017).

Relation to Previous Functional Connectivity Findings in Youth with Gender Dysphoria

To date, only one other study exists against which to compare the present findings. Nota and colleagues (2017) examined resting-state functional connectivity in GD-diagnosed youth and observed greater connectivity associated with the bilateral supplemental motor area in cisgirls versus cisboys or transboys, and transgirls versus cisboys in adolescents (but not children). Similarly, in the present study, the (7) right supplementary motor area (peak of a cluster associated with the seed (1) right medial superior frontal gyrus) exhibited a cross-over interaction in which seed-to-cluster correlations were positive for typically-developing assigned males and GID-diagnosed assigned females, and negative for typically-developing assigned females and GID-diagnosed assigned males. Moreover, the negative correlation in GID-diagnosed assigned males was considerably stronger than that in typically-developing assigned males. Interestingly, the direction of effects in the current study run counter to those reported by Nota and colleagues (2017), though the pattern of effects coincide insofar as GID-diagnosed assigned males and females both showed correlations more similar to the opposite rather than same assigned sex. The similarity between the current findings and those reported by Nota et al. (2017) calls for further inquiry into the role of the right supplementary motor area in the development of gender dysphoria.

Furthermore, the investigators of that study conducted an independent components analysis (ICA) to guide the seed-to-seed correlational connectivity analyses, and were thus able to specify the resting-state network within which the (7) right supplementary motor area was exhibiting group differences to be the sensorimotor network-II (SMN-II), part of the sensorimotor system of functional connectivity networks (Nota et al., 2017). Although the present study did not include ICA, the cluster with peak (7) right supplementary motor area included member regions left anterior cingulate gyrus, left median cingulate gyrus, and right middle, which form part of a resting-state network implicated in passive detection of situationally-relevant perceptual stimuli, called the salience network (SN) (Raichle, 2011). The salience network has been observed to mediate the relationship between the so-called default mode network (DMN), associated with internal processes such as rumination, and the executive control network (ECN), associated with metacognitive functions like decision making and deliberate action (Goulden et al., 2014).

Previously, Lin and colleagues (2014) applied a graph-theory analysis to R-fMRI data collected from 23 pre-treatment transsexual and 23 age-matched cissexuals adults, and found that the transsexual group compared to the cissexual group showed greater degree centrality in the bilateral superior parietal lobule, and in the primary somatosensory cortex, two key regions of the body-representation network. The transsexual group also exhibited a negative correlation between self-report ratings of cross-gender identification and strength of connectivity between the right insula (involved in affective processes) and bilateral primary somatosensory cortices. Taken together, these findings suggest that adults with cross-gender identification engage body-representation neurofunctional networks more often than do cisgender adults (perhaps owing to heightened awareness of primary and secondary sex characteristics and therefore their bodily

selves), which must then be counteracted by dampening communication between neurofunction related to bodily self-awareness (e.g., body-representation networks) and passive attention to environmental cues (i.e., salience network) in order to reduce psychological discomfort and distress.

Despite the limited extent to which regional connectivity correlations (as in the current study) may be compared with network measures proper (as in Nota et al., 2017), the observation of common regions in similar samples using distinct measures of connectivity suggests important roles for the (7) right supplementary motor area (sensorimotor network) and left anterior cingulate gyrus, left median cingulate gyrus, and right median cingulate gyrus (salience network), in neurofunction related to gender dysphoria. In the current study, the seed (1) right medial superior frontal gyrus, previously identified as an important region within the default mode network (M. H. Lee et al., 2012; Moussa et al., 2012; Raichle, 2011), was associated with the bilateral anterior cingulate gyri and median cingulate gyri, which are limbic cortices previously implicated in the default mode network (Moussa et al., 2012) and salience and sensorimotor networks (Raichle, 2011); with the right dorsolateral superior frontal gyrus, a key member region of a local network focused on the supplementary motor area (Kim et al., 2010); and with peak (7) right supplementary motor area, previously implicated in the sensorimotor system (Kim et al., 2010; Raichle, 2011). The pattern of mean correlations in this cluster is consistent with the body-representation hypothesis for the GID-diagnosed assigned male subgroup, which showed a strong negative correlation between seed and cluster. In contrast, in the GID-diagnosed assigned female subgroup, the neurobiological theory of origins of transsexuality was supported to the extent that connectivity between seed and cluster was positive in both GID-diagnosed assigned females and typically-developing assigned males, and

negative in typically-developing females, indicating a categorical effect of GID-diagnosed females exhibiting resting-state neurofunctional patterns similar to those in typically-developing males, and dissimilar from those in typically-developing females.

Implications for Models of Sex Differentiation of Brain & Behavior

Differences between the types of connectivity measures used in the present study versus previous studies limit direct comparison of magnitude and direction of effects. However, given that the purpose of this exploratory analysis was to help locate regions that may eventually be included in neurofunctional models of gender dysphoria (and more specifically, resting-state functional connectivity profiles associated with cross-gender identification), any overlap between the regions observed presently with those implicated in previous studies represent important regions to consider moving forward.

Table 8, above, provides a summary of functional connectivity profiles suggested by the current findings, along with implications for neurobiological models of gender dysphoria. As can be seen in Table 8, the most consistent effect was that of masculinized (and hypermasculinized) connectivity among GID-diagnosed assigned females. By contrast, GID-diagnosed assigned males showed a more variable pattern of connectivity profiles, most commonly intermediate between typically-developing assigned females and typically-developing assigned males (defeminized), but also including one hypermasculinized cluster (with seed right lingual gyrus and peak left middle frontal gyrus), one hyperfeminized (with seed right medial superior frontal gyrus and peak right supplementary motor area), and in one cluster, a profile specific to GID-diagnosed assigned males (that is, dissimilar to both typically-developing assigned males and typically-developing assigned females).

Neurobiological theories of gender dysphoria.

In the context of the current exploratory study, the neurobiological theory of the origins of transsexuality predicts that among GID-diagnosed youth, resting-state functional connectivity patterns associated with sex-differentiated brain regions will be consistent with patterns in typically-developing youth of the same experienced gender, or at least, in between patterns associated with typically-developing males and females (Swaab & Garcia-Falgueras, 2009). In the current study, this theory held for six of the 14 hypothesized seed regions of interest (left dorsolateral superior frontal gyrus, right medial superior frontal gyrus, left lingual gyrus, right lingual gyrus, left cuneus, and left inferior frontal gyrus, triangular part) and for four non-hypothesized cluster peak regions (right supplementary motor area, left middle frontal gyrus, left medial superior frontal gyrus, and right thalamus). It remains unclear why such differences did not obtain in the remaining hypothesized regions (left posterior cingulate gyrus, right posterior cingulate gyrus, left amygdala, right amygdala, left fusiform gyrus, right fusiform gyrus, right angular gyrus, and left precuneus).

Normative models of sex differentiation in mammalian tissue posit that there are distinct genetic and hormonal factors associated with male and female brain differentiation (Arnold, 2009; Jost, 1972; Phoenix et al., 1959), suggesting that resting-state functional connectivity networks associated with gender dysphoria may differ between GID-diagnosed assigned males and assigned females. This view was generally supported in the current study: While GID-diagnosed assigned females showed a consistent pattern of masculinized connectivity, GID-diagnosed assigned males showed a range of effects including hyperfeminized, demasculinized, hypermasculinized, and subgroup-specific effects.

Body representation incongruence hypothesis.

The body representation incongruence hypothesis predicts that a potential neurofunctional marker for gender dysphoria is a dissociation in connectivity between somatosensory regions involved in internal body representation, and frontal and limbic/basal ganglia regions associative cortices involved in complex social and emotion processing (Ku et al., 2013). That hypothesis was proposed to explain findings of a main effect of decreased connectivity between the ventral tegmental area (VTA, involved in sex-dimorphic internal representation of the genitals) and the pregenual and dorsal anterior cingulate gyrus (social processing, autobiographical thought processes, error detection, and punishment-based learning) in transsexual adults (21 female-to-male (FtM) and 20 male-to-female (MtF) transsexual) compared with typically-developing adults (19 cisgender females and 19 cisgender males) (Ku et al., 2013). A follow-up study with a smaller, entirely pre-treatment sample of 12 FtM and 11 MtF adults extended those findings by demonstrating that transsexual adults' "selfness" ratings—which measured degree of identification with male and female actors viewed in erotic versus neutral film clips—were inversely correlated with connectivity between the right insula (involved in emotion processing) and the bilateral primary somatosensory cortex (Lin et al., 2014). Through the lens of the body representation incongruence hypothesis, these findings may represent the existence of a coping mechanism unique to individuals with gender dysphoria, whereby distress specifically associated with body dysphoria is alleviated by a compensatory disconnect between the neurofunction in networks involved in one's internal bodily representation and social (including self-referential) processing.

Support for the body representation incongruence hypothesis was recently observed in an adolescent study with 19 typically-developing assigned males, 21 typically-developing assigned females, 20 GD-diagnosed assigned males, and 21 GD-diagnosed assigned females (Nota et al.,

2017), which revealed connectivity patterns consistent with experienced gender in both GD-diagnosed assigned males and females associated with the right supplementary motor area. That finding resonates with the connectivity patterns observed in the GD-diagnosed assigned male and GD-diagnosed assigned female subgroups, which exhibited patterns consistent with typically-developing assigned females and typically-developing assigned males, respectively, in the cluster with seed right medial superior frontal gyrus and peak right supplementary motor area.

In addition, GD-diagnosed assigned males showed a unique connectivity pattern associated with the visual network-I (more specifically, in the right cerebellum) (Nota et al., 2017), consistent with the present findings in the cluster with seed left cuneus and peak right thalamus, in which GD-diagnosed assigned males showed a strong positive correlation, while typically-developing assigned males, typically-developing assigned females, and GD-diagnosed assigned females showed negative correlations. It is possible this effect reflects hypervigilance in GD-diagnosed males associated, perhaps associated with heightened awareness of undesired gender-marking aspects of one's own body or social interactions, or more generically, stigma-related traumatic stress (Balleur-van Rijn, Steensma, Kreukels, & Cohen-Kettenis, 2013).

The current findings provide further support for the view that body representation incongruence in youth with gender dysphoria may be reflected as decreased functional connectivity between brain regions involved in self-awareness and sensorimotor processing. Table 9 (below) gives a breakdown of cluster seed, peak and member regions according to the resting-state networks with which those regions have been previously implicated. The information presented in Table 9 was obtained from the following sources: default mode network (J.S. Damoiseaux et al., 2008; M. H. Lee et al., 2012; Moussa et al., 2012; Raichle, 2011; Rosazza & Minati, 2011; Yeo et al., 2011); dorsal attention network (Moussa et al., 2012;

Raichle, 2011); ventral attention network (Kiviniemi et al., 2009; M. H. Lee et al., 2012; Moussa et al., 2012); salience network (Raichle, 2011); visual system (M. H. Lee et al., 2012; Raichle, 2011; Rosazza & Minati, 2011); executive control network (Raichle, 2011; Rosazza & Minati, 2011); fronto-parietal control (M. H. Lee et al., 2012; Rosazza & Minati, 2011); temporo-parietal control (Rosazza & Minati, 2011); sensorimotor system (Kim et al., 2010; M. H. Lee et al., 2012; Raichle, 2011); basal ganglia/limbic system (Moussa et al., 2012; Roy et al., 2009).

[TABLE 9]

Limitations

The statistical power of the present findings was limited by the small sample size and the quantity of seed regions tested. Other challenges to internal validity included a restricted age range and participants' head motion while in the scanner. The investigator addressed these limitations by implementing a hypothesis-driven approach, matched-subjects design, conservative multiple comparisons corrections in inferential statistics, and computational rigor in image processing.

The statistical approach in the present study diverged from the convention of employing a data-driven, Independent Components Analysis prior to conducting hypothesis testing on region-to-region functional connectivity. For example, Wang and Peterson (2008) used Partner-Matching Independent Components Analysis (PM-ICA) to reliably identify 50 resting-state networks in as few as 13 healthy control subjects, and then in a single adult male meeting criteria for Tourette Syndrome. However, because those fMRI scans were acquired during active tasks and from adult participants, the data were less susceptible to movement- and age-related confounds than in the current study. In lieu of hypothesis tests guided by data-driven detection of study-specific seeds—which may impart confounds related to study-specific connectivity

profiles—the current study instead tested functional connectivity directly using a limited set of seed regions identified from the existing literature on sex-related neuroconnectivity differences in typically-developing youth.

Reliable findings with small samples like in the current study were previously obtained in a connectivity study with a sample of one pre-transition young adult (Santaracchi et al., 2012), and in a single photon emission computed tomography (SPECT) study with a sample of 11 pre-transition female-to-male young adults (Nawata et al., 2010).

Other limitations associated with an underpowered sample were insufficient data for regression analysis of age, puberty, and symptom severity effects, and unequal sample sizes. A restricted developmental range in the current study may have obscured group differences. Only in age-by-sex interactions had connectivity differences been previously reported in the left precuneus (Alarcón et al., 2015; Solé-Padullés et al., 2016; Wu et al., 2013) and temporal pole: superior temporal gyrus (Wu et al., 2013). In addition, incomplete data collection in the present study prevented comparison of subgroups using Tanner pubertal development scales. However, the age distribution in the current sample was clearly skewed toward late adolescence. To minimize the impact of age- and development-related confounds, subjects were individually matched in age (among other factors).

Multiple comparisons correction for the quantity of seed regions examined may have resulted in a substantially elevated rate of Type-II error relative to other studies. Fourteen seeds were selected to account for the inconsistency of age ranges, connectivity measures, and findings in the literature. However, inclusion of a representative set of seed regions came at the expense of stringent corrected p -values during inferential tests. Therefore, group-level analyses included

a conservative multiple comparisons correction approach (GRF-based cluster size adjustment), applied with a stringent, Bonferroni-corrected cluster-level error threshold.

Two other issues that could not be readily controlled in the current study were a restricted age range and a lack of predictive or longitudinal information on developmental outcomes in the clinical subsamples. More specifically, the current analysis lacked information on psychosocial and pubertal development (both correlated with age) and the following developmental factors associated with gender dysphoria subtypes: early- versus late-onset (i.e., in the adolescent sample), persistence versus desistence of gender dysphoria or cross-gender identification, and sexual orientation. Without further information about the degree of sample heterogeneity, the extent of the generalizability of the current findings is difficult to establish.

Conclusions

This study adds to the growing body of research on neurobiological factors in the development of gender dysphoria. An assigned gender-by-diagnostic subcategory interaction moderated resting-state functional connectivity associated with six of 14 hypothesized regions of interest: four as cluster seeds (right medial superior frontal gyrus, left lingual gyrus, right lingual gyrus, and left cuneus) and two as cluster member regions (left dorsolateral superior frontal gyrus and right inferior frontal gyrus, triangular part). GID-diagnosed assigned females showed a general pattern of masculinized functional connectivity, while GID-diagnosed assigned males demonstrated a range of effects across clusters, including hyperfeminized, demasculinized, hypermasculinized, and subgroup-specific connectivity patterns. In other words, neurofunction in GID-diagnosed assigned females consistently tended toward a cross-gender profile, and in GID-diagnosed assigned males, less reliably toward an intermediate profile. Finally, a functional connectivity pattern specific to GID-diagnosed assigned males was also observed.

In the current study, the absence of any main effects of assigned gender suggests resting-state neurofunction in the brains of GID-diagnosed individuals varied sufficiently from typically-developing individuals to eliminate otherwise robust effects of assigned gender in typically-developing subgroups. At the same time, the lack of robust cross-over interactions that would arise from complete dissociation of somatic sex and brain sex, as predicted by the neurobiological theory of the origins of transsexuality (Swaab & Garcia-Falgueras, 2009), suggests the existence of both discrete and overlapping functional connectivity networks associated with gender dysphoria in assigned males and females. That is, the present findings are consistent with the views that 1) neurofunction in youth with gender dysphoria differs from that of typically-developing youth of the same assigned gender as well as the same experienced gender, and 2) some connectivity differences between typically-developing and GID-diagnosed youth are specific to either assigned females or assigned males.

More speculatively, the present findings support the notion that neuroconnectivity patterns associated with gender dysphoria change within individuals over the course of development. Previously, age-by-sex interaction effects on functional connectivity have been associated with the three seeds, the right medial superior frontal gyrus and right lingual gyrus (Alarcón et al., 2015), and the left cuneus (Wu et al., 2013), and one region that emerged as the peak of two clusters, the middle frontal gyrus (Zuo et al., 2010). Furthermore, connectivity patterns associated with the left and right lingual gyri have demonstrated quadratic relationships with age, while patterns associated with the seed left dorsolateral superior frontal gyrus and cluster member region right putamen have shown cubic relationships with age (Zuo et al., 2010). Therefore, the left lingual gyrus, right lingual gyrus, left dorsolateral superior frontal gyrus, and

right putamen may represent key regions to examine in longitudinal designs with individuals with gender dysphoria.

The left and right lingual gyri in particular may be some of the most important regions to focus on moving forward for several reasons. In some assigned males with early-onset gender dysphoria, indicators of the condition remit temporarily, during which period individuals tend to identify as gay (American Psychiatric Association, 2013, p. 455). This phenomenon suggests the presence of one or more quadratic effects of age on the resting-state functional connectivity associated with gender dysphoria, at least in assigned males. Based on the current findings, a strong match for such regions would be the lingual gyri because their resting-state connectivity patterns have previously been reported to show quadratic relationships with age (Zuo et al., 2010), and in a separate study, were associated with an age-by-sex interaction effect (Alarcón et al., 2015). Presently, the left and right lingual gyri served as the seeds for four of the six significant clusters observed.

Given the urgency with which we must advance our understanding of gender dysphoria to meet the needs of cross-gender identified youth (Drescher & Byne, 2012b, 2012a), and the current momentum toward the development of a functional connectivity profile for gender dysphoria (Castellanos, Di Martino, Craddock, Mehta, & Milham, 2013; Clemens et al., 2017; Feusner et al., 2017; Manzouri et al., 2017; Smith et al., 2015) the present findings are offered to help advance current clinical and research initiatives toward improved developmental models of gender dysphoria.

Future Directions

There are several considerations for future studies that will advance this line of work: collecting data in a larger sample that includes a childhood-to-adulthood developmental range;

measuring gender identity, gender role behavior, and pubertal development for all groups; and use of ICA followed by seed-to-seed correlational analyses.

Future functional connectivity studies with youth with gender dysphoria may benefit from inclusion of chronological age and pubertal development as separate covariates, despite their high collinearity, to help distinguish any potential cohort effects from neurobiological ones. Other factors of psychosexual development that should be included in future studies are onset subtype (early, late, or rapid) and whenever possible, sexual orientation. In addition, the use of genetic testing would be ideal in order to account for potentially confounding biological factors associated with gender atypicality, such as a concurrent diagnosis of non-classical congenital adrenal hyperplasia in 46,XX assigned females. Longitudinal study designs may help to overcome challenges to this line of research such as a lack of brain imaging data on heterosexual desisters and of behavioral data on sexual orientation in youth samples.

Finally, future studies will benefit from sample sizes that support an independent components analysis (ICA). By identifying sample-specific networks that can be reliably observed across all study subjects, ICA can help guide investigators toward the region-to-region correlations most relevant to their hypotheses, thereby maximizing power while reducing the likelihood of Type-II error. Complex patterns of region-to-region correlations can then be tested directly, such as in path analyses that can be used to confirm and elaborate connectivity models. In order to extract valid independent components using a partner-matching ICA algorithm, Z. Wang recommended a sample size of at least $N=20$ would be needed to reliably identify 18 to 20 sample-specific functional connectivity networks (personal communication, January 29, 2018). However, as part of a neuroconnectivity study on gender effects in typically-developing children, Agcaoglu and colleagues (2015) calculated a sample size of $N=172$ would be required to identify

a network focused on the left lingual gyrus, and $N=294$ for one focused on the inferior frontal gyrus. Future sample size calculations should account for the propensity for interaction and higher-order effects of neurofunction associated with relevant regions, such as the lingual gyri.

Table 1A

DSM-5 Diagnostic Criteria for 302.6 (F64.2) Gender Dysphoria in Children

- A. A marked incongruence between one's experienced/expressed gender and assigned gender, of at least 6 months' duration, as manifested by at least six of the following (one of which must be Criterion A1):
1. A strong desire to be of the other gender or an insistence that one is the other gender (or some alternative gender different from one's assigned gender).
 2. In boys (assigned gender), a strong preference for cross-dressing or simulating female attire; or in girls (assigned gender), a strong preference for wearing only typical masculine clothing and a strong resistance to the wearing of typical feminine clothing.
 3. A strong preference for cross-gender roles in make-believe play or fantasy play.
 4. A strong preference for the toys, games, or activities stereotypically used or engaged in by the other gender.
 5. A strong preference for playmates of the other gender.
 6. In boys (assigned gender), a strong rejection of typically masculine toys, games, and activities and a strong avoidance of rough-and-tumble play; or in girls (assigned gender), a strong rejection of typically feminine toys, games, and activities.
 7. A strong dislike of one's sexual anatomy.
 8. A strong desire for the primary and/or secondary sex characteristics that match one's experienced gender.
- B. The condition is associated with clinically significant distress or impairment in social, school, or other important areas of functioning.

Table 1B

DSM-5 Diagnostic Criteria for 302.85 (F64.1) Gender Dysphoria in Adolescents & Adults

- A. A marked incongruence between one's experienced/expressed gender and assigned gender, of at least 6 months' duration, as manifested by at least two of the following:
1. A marked incongruence between one's experienced/expressed gender and primary and/or secondary sex characteristics (or in young adolescents, the anticipated secondary sex characteristics).
 2. A strong desire to be rid of one's primary and/or secondary sex characteristics because of a marked incongruence with one's experienced/expressed gender (or in young adolescents, a desire to prevent the development of the anticipated secondary sex characteristics).
 3. A strong desire for the primary and/or secondary sex characteristics of the other gender.
 4. A strong desire to be of the other gender (or some alternative gender different from one's assigned gender).
 5. A strong desire to be treated as the other gender (or some alternative gender different from one's assigned gender).

6. A strong conviction that one has the typical feelings and reactions of the other gender (or some alternative gender different from one's assigned gender).
- B. The condition is associated with clinically significant distress or impairment in social, occupational, or other important areas of functioning.

Table 1C

DSM-IV-TR Diagnostic Criteria for Gender Identity Disorder in Children and Adolescents

- A. A strong and persistent cross-gender identification (not merely a desire for any perceived cultural advantages of being the other sex).
- In children, the disturbance is manifested by four (or more) of the following:
- (1) repeatedly stated desire to be, or insistence that he or she is, the other sex
 - (2) in boys, preference for cross-dressing or simulating female attire; in girls, insistence on wearing only stereotypical masculine clothing
 - (3) strong and persistent preferences for cross-sex roles in make-believe play or persistent fantasies of being the other sex
 - (4) intense desire to participate in the stereotypical games and pastimes of the other sex
 - (5) strong preference for playmates of the other sex
- In adolescents and adults, the disturbance is manifested by symptoms such as a stated desire to be the other sex, frequent passing as the other sex, desire to live or be treated as the other sex, or the conviction that he or she has the typical feelings and reactions of the other sex.
- B. Persistent discomfort with his or her sex or sense of inappropriateness in the gender role of that sex.
- In children, the disturbance is manifested by any of the following: in boys, assertion that his penis or testes are disgusting or will disappear or assertion that it would be better not to have a penis, or aversion toward rough-and-tumble play and rejection of male stereotypical toys, games, and activities; in girls, rejection of urinating in a sitting position, assertion that she has or will grow a penis, or assertion that she does not want to grow breasts or menstruate, or marked aversion toward normative feminine clothing.
- In adolescents and adults, the disturbance is manifested by symptoms such as preoccupation with getting rid of primary and secondary sex characteristics (e.g., request for hormones, surgery, or other procedures to physically alter sexual characteristics to simulate the other sex) or belief that he or she was born the wrong sex.
- C. The disturbance is not concurrent with a physical intersex condition.
- D. The disturbance causes clinically significant distress or impairment in social, occupational, or other important areas of functioning.

Table 2A

Interpretation of Effects

<u>Observed Effect</u>	<u>Typically-Developing Direction of Effect</u>	
	<u>TD-F > TD-M</u>	<u>TD-F < TD-M</u>
TD-F > GD-AF	defeminized masculinized	hyperfeminized
TD-F < GD-AF	hyperfeminized	defeminized masculinized
TD-M > GD-AM	hypermasculinized	demasculinized feminized
TD-M < GD-AM	demasculinized feminized	hypermasculinized

Note. Interpretation of effects between typically-developing and GID-diagnosed youth of the same assigned gender relative to the direction of effect between typically-developing females and males. Observed Effect refers to differences observed between diagnostic subgroups of the same assigned gender. The TD-F > TD-M and TD-F < TD-M columns under Typically-Developing Direction of Effect give the categories of effects observed in GID-diagnosed youth in the context of effects between typically-developing males and females (per column label). The parenthetical terms quantitative and qualitative denote the types of interactions that would indicate the referent category.

Table 2B

Study Groups

<u>DSM-IV-TR Diagnostic Subcategory</u>	<u>Assigned Gender</u>	
	<u>Female</u>	<u>Male</u>
Typically-developing	TD-M	TD-F
GID-diagnosed	GID-AM	GID-AF

Table 3

Hypothesized Seed Regions & Direction of Effects

<u>Region of Interest</u>	<u>Previous Findings</u>	<u>Hypothesized Direction of Effect</u>
Left Superior frontal gyrus, dorsolateral (SFGdor.L)	Alarcón et al. (2015): age*sex interaction (RSFC) decr. for TD-F, incr. for TD-M, with age) <i>sample</i> : N=122 (71 boys), ages 10–16 years <i>measure</i> : voxel-wise RSFC correlations <i>seed region</i> : AMYG.R (basolateral)	TD-F < GID-AF < GID-AM < TD-M
	Zuo et al. (2010): age*sex interaction (VMHC) decr. for TD-F, incr. for TD-M, with age) <i>sample</i> : N=214 (96 males), ages 7–85 years <i>measure</i> : homotopic connectivity	
Left inferior frontal gyrus, triangular part (IFGtriang.L)	Wu et al. (2013): TD-F > TD-M <i>sample</i> : N=291 (146 males), ages 5.6–18.4 years <i>measure</i> : node degree	TD-F > GID-AF > GID-AM > TD-M
	Agcaoglu et al. (2015): TD-F > TD-M <i>sample</i> : N=603 (298 males), ages 12–71 years <i>measures</i> : lateralization (voxel-wise RSFC, ICA, small-cluster RSFC; NB: one trend, one indirect finding as network node)	
Right medial superior frontal gyrus (SFGmed.R)	Wu et al. (2013): TD-F > TD-M <i>sample</i> : N=291 (146 males), ages 5.6–18.4 years <i>measure</i> : node efficiency	TD-F > GID-AF > GID-AM > TD-M
	Zuo et al. (2010): TD-F > TD-M <i>sample</i> : N=214 (96 males), ages 7–85 years <i>measure</i> : homotopic connectivity	
	Alarcón et al. (2015): age*sex interaction (RSFC) incr. for TD-F, decr. for TD-M, with age) <i>sample</i> : N=122 (71 boys), ages 10–16 years <i>measure</i> : voxel-wise RSFC correlations <i>seed region</i> : AMY.R (superficial)	TD-F < GID-AF < GID-AM < TD-M
Left Posterior Cingulum (PCG.L)	Zuo et al. (2010): TD-F > TD-M <i>sample</i> : N=214 (96 males), ages 7–85 years <i>measure</i> : homotopic connectivity	TD-F > GID-AF > GID-AM > TD-M

Right Posterior Cingulum (PCG.R)	Zuo et al. (2010): TD-F > TD-M <i>sample</i> : N=214 (96 males), ages 7–85 years <i>measure</i> : homotopic connectivity	TD-F > GID-AF > GID-AM > TD-M
Left amygdala (AMY.L)	Alarcón et al. (2015): age*sex interaction (RSFC) incr. for TD-F, decr. for TD-M, with age) <i>sample</i> : N=122 (71 boys), ages 10–16 years <i>measure</i> : voxel-wise RSFC correlations <i>peak regions</i> : CUN.R, LING.R	TD-F > GID-AF > GID-AM > TD-M
	Zuo et al. (2010): age*sex interaction (VMHC) incr. for TD-F, decr. for TD-M, with age) <i>sample</i> : N=214 (96 males), ages 7–85 years <i>measure</i> : homotopic connectivity	
	Alarcón et al. (2015): age*sex interaction (RSFC) decr. for TD-F, incr. for TD-M, with age) <i>sample</i> : N=122 (71 boys), ages 10–16 years <i>measure</i> : voxel-wise RSFC correlations <i>peak regions</i> : ANG.R, ORBmed.L, IPL.L	TD-F < GID-AF < GID-AM < TD-M
Right amygdala (AMY.R)	Alarcón et al. (2015): age*sex interaction (RSFC) incr. for TD-F, decr. for TD-M, with age) <i>sample</i> : N=122 (71 boys), ages 10–16 years <i>measure</i> : voxel-wise RSFC correlations <i>peak regions</i> : SFGmed.R, IPL.L	TD-F > GID-AF > GID-AM > TD-M
	Wu et al. (2013): TD-F > TD-M <i>sample</i> : N=291 (146 males), ages 5.6–18.4 years <i>measure</i> : node degree	
	Zuo et al. (2010): age*sex interaction (VMHC) incr. for TD-F, decr. for TD-M, with age) <i>sample</i> : N=214 (96 males), ages 7–85 years <i>measure</i> : homotopic connectivity	
	Alarcón et al. (2015): age*sex interaction (RSFC) decr. for TD-F, incr. for TD-M, with age) <i>sample</i> : N=122 (71 boys), ages 10–16 years <i>measure</i> : voxel-wise RSFC correlations <i>peak regions</i> : MOG.L, IPL.L, PoCG.L, PCUN.L	TD-F < GID-AF < GID-AM < TD-M
Left cuneus (CUN.L)	Wu et al. (2013): TD-F > TD-M <i>sample</i> : N=291 (146 males), ages 5.6–18.4 years <i>measure</i> : node degree, node efficiency	TD-F > GID-AF > GID-AM > TD-M

	<p>Wu et al. (2013): age*sex interaction (network centrality decr. for TD-F, incr. for TD-M (<i>ns</i>), with age) <i>sample</i>: N=291 (146 males), ages 5.6–18.4 years <i>measure</i>: node betweenness</p>	<p>TD-F < GID-AF < GID-AM < TD-M</p>
<p>Left lingual gyrus (LING.L)</p>	<p>Wu et al. (2013): TD-F > TD-M <i>sample</i>: N=291 (146 males), ages 5.6–18.4 years <i>measures</i>: node degree, node efficiency</p> <p>Agcaoglu et al. (2015): TD-F > TD-M <i>sample</i>: N=603 (298 males), ages 12–71 years <i>measures</i>: lateralization (voxel-wise RSFC, ICA, small-cluster RSFC, lateralization cofactor analysis; NB: non-significant trends in the Bilateral lingual gyrus)</p>	<p>TD-F > GID-AF > GID-AM > TD-M</p>
	<p>Agcaoglu et al. (2015): TD-F < TD-M <i>sample</i>: N=603 (298 males), ages 12–71 years <i>measure</i>: lateralization (voxel-wise RSFC, ICA)</p>	<p>TD-F < GID-AF < GID-AM < TD-M</p>
<p>Right lingual gyrus (LING.R)</p>	<p>Wu et al. (2013): TD-F > TD-M <i>sample</i>: N=291 (146 males), ages 5.6–18.4 years <i>measures</i>: node degree, node efficiency</p> <p>Alarcón et al. (2015): age*sex interaction (RSFC incr. for TD-F, decr. for TD-M, with age) <i>sample</i>: N=122 (71 boys), ages 10–16 years <i>measure</i>: voxel-wise RSFC correlations <i>seed region</i>: AMYG.L</p> <p>Agcaoglu et al. (2015): TD-F > TD-M <i>sample</i>: N=603 (298 males), ages 12–71 years <i>measures</i>: lateralization (voxel-wise RSFC, ICA, small-cluster RSFC, lateralization cofactor analysis; NB: non-significant trends in the Bilateral lingual gyrus)</p>	<p>TD-F > GID-AF > GID-AM > TD-M</p>
<p>Left fusiform gyrus (FFG.L)</p>	<p>Wu et al. (2013): TD-F > TD-M <i>sample</i>: N=291 (146 males), ages 5.6–18.4 years <i>measures</i>: node degree, node efficiency</p> <p>Zuo et al. (2010): TD-F < TD-M <i>sample</i>: N=214 (96 males), ages 7–85 years <i>measure</i>: homotopic connectivity</p>	<p>TD-F > GID-AF > GID-AM > TD-M</p> <p>TD-F < GID-AF < GID-AM < TD-M</p>

Right fusiform gyrus (FFG.R)	<p>Wu et al. (2013): TD-F > TD-M <i>sample</i>: N=291 (146 males), ages 5.6–18.4 years <i>measures</i>: node degree, node efficiency</p>	<p>TD-F > GID-AF > GID-AM > TD-M</p>
	<p>Zuo et al. (2010): TD-F < TD-M <i>sample</i>: N=214 (96 males), ages 7–85 years <i>measure</i>: homotopic connectivity</p>	<p>TD-F < GID-AF < GID-AM < TD-M</p>
Right angular gyrus (ANG.R)	<p>Alarcón et al. (2015): age*sex interaction (RSFC incr. for TD-F, decr. for TD-M, with age) <i>sample</i>: N=122 (71 boys), ages 10–16 years <i>measure</i>: voxel-wise RSFC correlations <i>seed region</i>: AMYG.L</p>	<p>TD-F < GID-AF < GID-AM < TD-M</p>
	<p>Wu et al. (2013): age*sex interaction (network centrality decr. for TD-F (<i>ns</i>), incr. for TD-M, with age) <i>sample</i>: N=291 (146 males), ages 5.6–18.4 years <i>measure</i>: node betweenness</p>	
Left precuneus (PCUN.L)	<p>Agcaoglu et al. (2015): TD-F < TD-M <i>sample</i>: N=603 (298 males), ages 12–71 years <i>measures</i>: lateralization (voxel-wise RSFC, ICA, small-cluster RSFC; NB: finding replicated in Bilateral precuneus)</p>	<p>TD-F < GID-AF < GID-AM < TD-M</p>
	<p>Alarcón et al. (2015): age*sex interaction (RSFC decr. for TD-F, incr. for TD-M, with age) <i>sample</i>: N=122 (71 boys), ages 10–16 years <i>measure</i>: voxel-wise RSFC correlations <i>seed region</i>: AMYG.R (basolateral)</p>	
	<p>Wu et al. (2013): age*sex interaction (network centrality decr. for TD-F (<i>ns</i>), incr. for TD-M (<i>ns</i>), with age) <i>sample</i>: N=291 (146 males), ages 5.6–18.4 years <i>measure</i>: node betweenness</p>	
	<p>Solé-Padullés et al. (2016): age*sex interaction (intrinsic connectivity decr. for females, incr. for males, for adolescents but not children) <i>sample</i>: N=113 (55 males), ages 7–18 years <i>measure</i>: RSFC (ICA-identified) <i>network</i>: Visuospatial (VSN)</p>	

Note. The column Regions of Interest gives the seed regions identified from the literature for testing group differences in this study; region abbreviations, in parentheses, follow the convention of the Automated Anatomical Labeling atlas (Tzourio-Mazoyer et al., 2002). The column Previous Findings lists the functional connectivity effects reported in the relevant literature. The column Hypothesized Direction of Effect gives the anticipated pattern of correlations among study subgroups (see section Possible mechanisms underlying atypical sex differentiation of the brain, above); group labels denote: GID-diagnosed assigned female (GID-AF), typically-developing female (TD-F), GID-diagnosed assigned male (GID-AM), and typically-developing male (TD-M).

Table 4	
<i>List of Hypothesized Seed Regions</i>	
<u>AAL Region Label</u>	<u>AAL Region Label Abbr.</u>
Left amygdala	AMYG.L
Right amygdala	AMYG.R
Left angular gyrus	ANG.L
Left cuneus	CUN.L
Left fusiform gyrus	FFG.L
Right fusiform gyrus	FFG.R
Left inferior frontal gyrus, triangular part	IFGtriang.L
Left lingual gyrus	LING.L
Right lingual gyrus	LING.R
Left posterior cingulate gyrus	PCG.L
Right posterior cingulate gyrus	PCG.R
Left precuneus	PCUN.L
Left dorsolateral superior frontal gyrus	SFGdor.L
Right medial superior frontal gyrus	SFGmed.R
<i>Note.</i> The column AAL Region Label refers to the names of brain regions as identified in the Automated Anatomical Label atlas (Tzourio-Mazoyer et al., 2002). AAL Region Label Abbr. refers to the commonly used abbreviations for designated brain regions.	

Table 5	
<i>Non-Hypothesized Peak Regions Observed</i>	
<u>AAL Region Label</u>	<u>AAL Region Label Abbr.</u>
Left middle frontal gyrus	MFG.L
Right medial superior frontal gyrus	SFGmed.R
Right supplementary motor area	SMA.R
Right thalamus	THA.R
<i>Note.</i> (See Table 4 note.)	

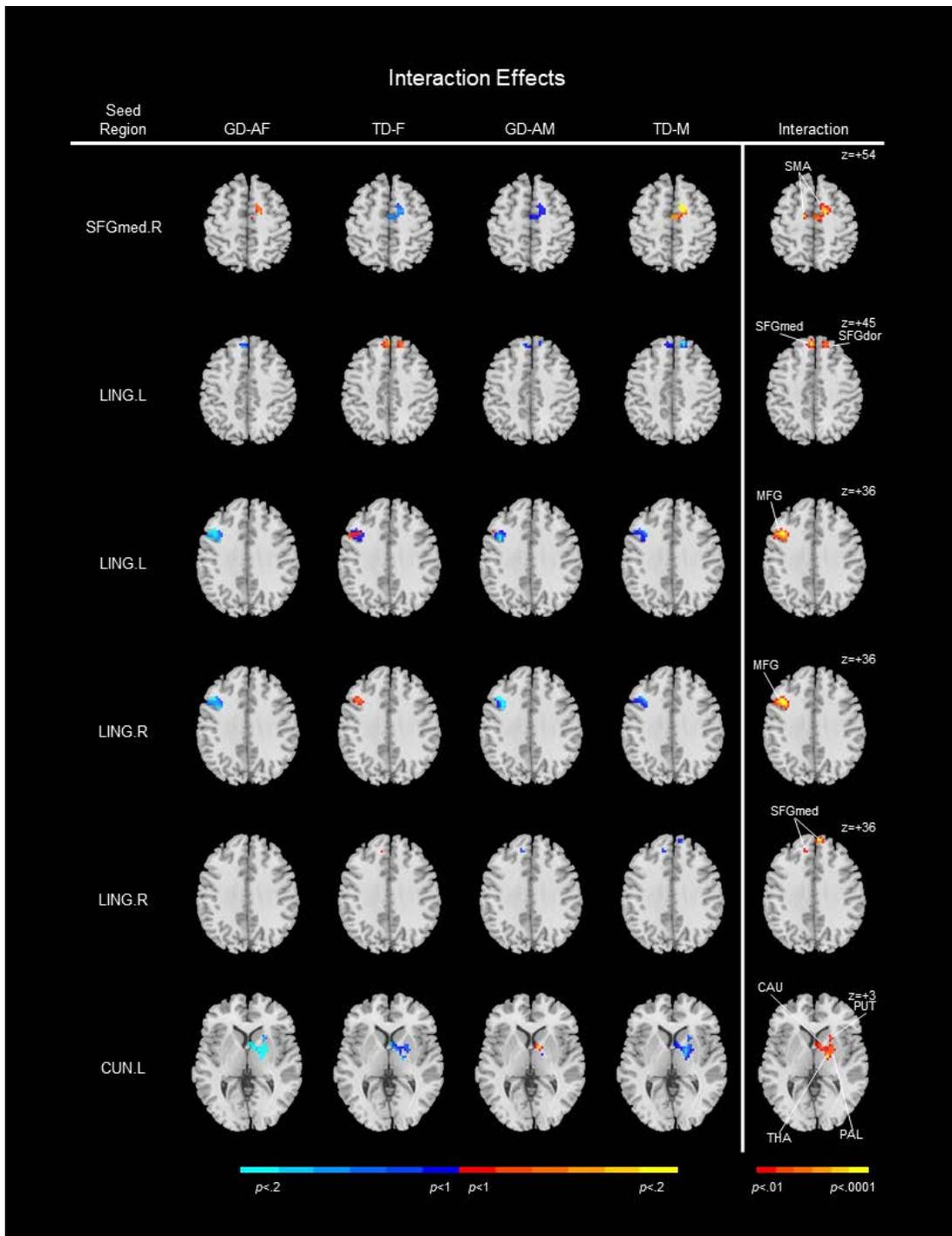


Figure 1. Axial slices in columns 2-5 represent resting-state functional connectivity T-maps generated by GD-diagnosed assigned females (GD-AF), typically-developing females (TD-F), GD-diagnosed assigned males (GD-AM), and typically-developing males (TD-M). Column 6 shows the F-map generated by the interaction between factors assigned gender and diagnostic subcategory. Positively correlated regions (red-to-yellow color bar) represent increased connectivity with seed regions (leftmost column); negatively correlated regions (blue-to-aqua color bar) represent decreased connectivity.

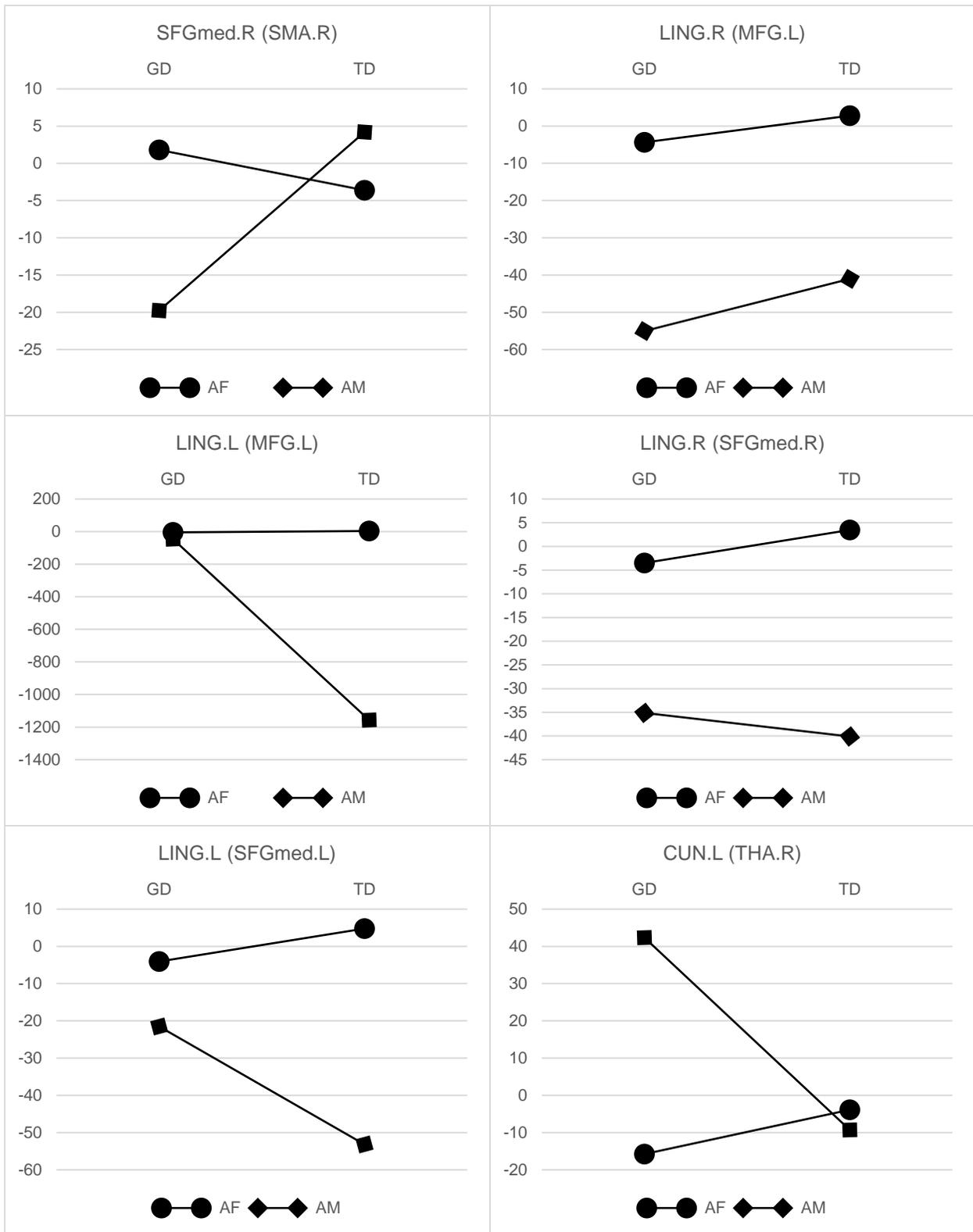


Figure 2. Line graphs depicting group means for significant interactions. Solid line represents assigned females; dashed line represents assigned males. Left hand points represent typically-developing groups; right hand points represent gender-dysphoric groups. Clusters are identified by their seeds, with cluster peaks in parentheses.

Table 6

Summary of Significant Clusters

<u>Seed Region</u>	<u>Cluster Size</u>	<u>Peak Value</u>	<u>Peak MNI</u>	<u>Peak Region</u>	<u>Cluster Member Regions</u>
Right superior frontal gyrus, medial	211	102.99	15, -3, 54	Right supplementary motor area	Right median cingulate gyrus Left median cingulate gyrus Right superior frontal gyrus, dorsolateral Left anterior cingulate gyrus
Left lingual gyrus	101	63.99	-39, 15, 36	Left middle frontal gyrus	Left precentral gyrus Left inferior frontal gyrus, opercular part Left inferior frontal gyrus, triangular part
Left lingual gyrus	117	59.11	-3, 42, 45	Left superior frontal gyrus, medial	Right superior frontal gyrus, medial Right superior frontal gyrus, dorsolateral Left superior frontal gyrus, dorsolateral
Right lingual gyrus	125	85.77	-39, 15, 36	Left middle frontal gyrus	Left precentral gyrus Left inferior frontal gyrus, opercular part
Right lingual gyrus	94	59.35	6, 54, 36	Right superior frontal gyrus, medial	Left superior frontal gyrus, medial Right superior frontal gyrus, dorsolateral Left superior frontal gyrus, dorsolateral
Left cuneus	93	82.95	18, -9, 3	Right thalamus	Right pallidum Right putamen Right caudate

Note. Region labels refer to the Automated Anatomical Labeling atlas (Tzourio-Mazoyer et al., 2002). Cluster sizes are reported for GRF-corrected F-maps with voxel-level threshold $p < .005$, and cluster-level threshold $p < .00357$. Peak Value represents F-values associated with the interaction effect of assigned gender (male vs. female) and diagnostic subcategory (typically developing vs. GID-diagnosed). Peak MNI represents peak coordinates reported in MNI space.

Table information was obtained from cluster reports generated using the xjView toolbox (<http://www.alivelearn.net/xjview>) as implemented in DPABI (Yan et al., 2016).

Table 7

Interaction Cluster Peak Intensity Breakdown

<u>Seed Region</u>	<u>Peak Region</u>	<u>Peak MNI</u>	<u>GD-AF</u>	<u>Peak Values</u>		
				<u>TD-F</u>	<u>GD-AM</u>	<u>TD-M</u>
SFGmed.R	SMA.R	15, -3, 54	1.81	-3.61	-19.76	4.20
LING.L	MFG.L	-39, 15, 36	-4.79	3.82	-45.81	-1156.51
LING.L	SFGmed.L	-3, 42, 45	-4.05	4.78	-21.50	-53.17
LING.R	MFG.L	-39, 15, 36	-4.35	2.80	-55.02	-41.02
LING.R	SFGmed.R	6, 54, 36	-3.49	3.47	-35.12	-40.10
CUN.L	THA.R	18, -9, 3	-15.76	-3.86	42.36	-9.28

Note. A breakdown of cluster peak intensity by study subgroup. Seed and peak region abbreviations are given in Tables 1A and 1B. Peak regions and coordinates refer to clusters reported in Table 6. Peak MNI refers to coordinates oriented in Montreal Neurological Institute (MNI) conventional space. Peak Values represents t-values for each subgroup obtained by estimating one-sample t-tests restricted to voxels within masks created from the clusters observed in mixed-effects analyses. Peak t-values are presented for subgroups GID-diagnosed assigned female (GD-AF), typically-developing female (TD-F), GID-diagnosed assigned male (GD-AM), and typically-developing male (TD-M). As reported t-values were estimated on spatially-masked volumes and uncorrected for multiple comparisons, they are presented solely to aid interpretation of interactions.

Table 8

Connectivity Profiles for GID-Diagnosed Youth

<u>Seed (Peak)</u>	<u>Member Regions</u>	<u>Connectivity Profile</u>				
		<u>Hyperfeminized</u>	<u>Demasculinized/ Defeminized</u>	<u>Feminized/ Masculinized</u>	<u>Hypermasculinized</u>	<u>GID-specific</u>
SFGmed.R (SMA.R)	DCG.R DCG.L SFGdor.R SMA.L ACG.L	AM		AF		
LING.L (MFG.L)	PreCG.L IFGoperc.L IFGtriang.L*		AM	AF		
LING.L (SFGmed.L)	SFGmed.R* SFGdor.R SFGdor.L*		AM	AF		
LING.R (MFG.L)	PreCG.L IFGoperc.L			AF	AM	
LING.R (SFGmed.R*)	SFGmed.L SFGdor.R SFGdor.L*		AM	AF		
CUN.L (THA.R)	PAL.R PUT.R CAU.R				AF	AM

Note. The Seed (Peak) and Member Regions columns refers to observed clusters using AAL abbreviations (see Appendix A, Table A1). Here, AF refers to the GID-diagnosed assigned female subgroup, and AM to the GID-diagnosed assigned male subgroup; assigned gender denotes legal gender entered in the birth certificate. Connectivity Profile columns indicate direction of sex differentiation effect. The column GID-specific denotes effects for which, because no difference between typically-developing males and females was currently observed in the specified cluster, it was not possible to categorize within the paradigm of masculinization and feminization.

Table 9

Resting-State Networks Associated With Significant Clusters

<u>Cluster Regions</u>			<u>Resting-State Network(s)</u>										
<u>Seed Regions</u>	<u>Peak Regions</u>	<u>Member Regions</u>	<u>Default Mode</u>	<u>Dorsal Attention</u>	<u>Ventral Attention</u>	<u>Salience</u>	<u>Visual System</u>	<u>Executive Control</u>	<u>Fronto-Parietal Control</u>	<u>Temporo-Parietal Control</u>	<u>Sensorimotor</u>	<u>Basal Ganglia/Limbic</u>	
SFGmed.R	SMA.R		x					x					
											x		
			DCG.R				x					x	
			DCG.L				x					x	
			SFGdor.R			x			x	x		x	
			SMA.L									x	
			ACG.L	x						x			x
LING.L (cluster 1)	MFG.L		x		x	x							
			PreCG.L		x							x	
			IFGoperc.L							x	x		
			IFGtriang.L						x	x	x		
			SFGmed.L	x					x				
			SFGmed.R	x					x				
			SFGmed.L	x					x				
(cluster 2)	SFGmed.L	SFGmed.R	x					x					
		SFGdor.L			x			x	x		x		
								x					
LING.R (cluster 1)	MFG.L		x		x	x							
			PreCG.L	x								x	
			IFGoperc.L							x	x		
			SFGmed.R	x					x				
(cluster 2)	SFGmed.R	SFGmed.L	x					x					
		SFGdor.L			x			x	x		x		
		SFGdor.R			x			x	x		x		
CUN.L	THA.R						x						
				x		x					x	x	
			PAL.R										x

Appendix A

Table A1			
<i>Automated Anatomical Labelling Atlas Region Names & Abbreviations</i>			
<u>Region</u>	<u>Abbreviation</u>	<u>Region</u>	<u>Abbreviation</u>
Amygdala	AMYG	Middle occipital gyrus	MOG
Angular gyrus	ANG	Middle temporal gyrus	MTG
Anterior cingulate and paracingulate gyri	ACG	Olfactory cortex	OLF
Calcarine fissure and surrounding cortex	CAL	Orbitofrontal cortex, medial	ORBmed
Caudate nucleus	CAU	Paracentral lobule	PCL
Cuneus	CUN	Parahippocampal gyrus	PHG
Fusiform gyrus	FFG	Posterior cingulate gyrus	PCG
Gyrus rectus	REC	Postcentral gyrus	PoCG
Heschl gyrus	HES	Precentral gyrus	PreCG
Hippocampus	HIP	Precuneus	PCUN
Inferior frontal gyrus, opercular part	IFGoperc	Rolandic operculum	ROL
Inferior frontal gyrus, orbital part	ORBinf	Superior frontal gyrus, dorsolateral	SFGdor
Inferior frontal gyrus, triangular part	IFGtriang	Superior frontal gyrus, medial	SFGmed
Inferior occipital gyrus	IOG	Superior frontal gyrus, orbital part	ORBsup
Inferior parietal, but supramarginal and angular gyri	IPL	Superior occipital gyrus	SOG
Inferior temporal gyrus	ITG	Superior parietal gyrus	SPG
Insula	INS	Superior temporal gyrus	STG
Lenticular nucleus, pallidum	PAL	Supplementary motor area SMA	

Lenticular nucleus, putamen	PUT	Supramarginal gyrus	SMG
Lingual gyrus	LING	Temporal pole: middle temporal gyrus	TPOmid
Median cingulate and paracingulate gyri	DCG	Temporal pole: superior temporal gyrus	TPOsup
Middle frontal gyrus	MFG	Thalamus	THA
Middle frontal gyrus, orbital part	ORBmid		

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