Review

Sexual orientation, fraternal birth order, and the maternal immune hypothesis: A review

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ABSTRACT

In 1996, psychologists Ray Blanchard and Anthony Bogaert found evidence that gay men have a greater number of older brothers than do heterosexual men. This "fraternal birth order" (FBO) effect has been replicated numerous times, including in non-Western samples. More recently, strong evidence has been found that the FBO effect is of prenatal origin. Although there is no direct support for the exact prenatal mechanism, the most plausible explanation may be immunological in origin, i.e., a mother develops an immune reaction against a substance important in male fetal development during pregnancy, and that this immune effect becomes increasingly likely with each male gestation. This immune effect is hypothesized to cause an alteration in (some) later born males' prenatal brain development. The target of the immune response may be molecules (i.e., Y-linked proteins) on the surface of male fetal brain cells, including in sites of the anterior hypothalamus, which has been linked to sexual orientation in other research. Antibodies might bind to these molecules and thus alter their role in typical sexual differentiation, leading some later born males to be attracted to men as opposed to women. Here we review evidence in favor of this hypothesis, including recent research showing that mothers of boys develop an immune response to one Y-linked protein (i.e., H-Y antigen; SMCY) important in male fetal development, and that this immune effect becomes increasingly likely with each additional boy to which a mother gives birth. We also discuss other Y-linked proteins that may be relevant if this hypothesis is correct. Finally, we discuss issues in testing the maternal immune hypothesis of FBO.

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1. Introduction

Recent research has provided support for a biological basis to human sexual orientation (for reviews, see [63,67,74,96,101]). This support has been provided in part by studies examining genetic factors. For example, along with supportive heritability studies [5], studies using molecular genetic analyses indicate that the Xq28 region of the X chromosome, along with sites on the autosomes, may be linked to male homosexuality [45,49,68], but see [76]. Such research support also includes studies examining neuroanatomy and brain functioning [62,65,89]. For example, the third interstitial nucleus of the anterior hypothalamus (INAH-3), a sexually dimorphic structure [2] that is similar to a reproductively relevant sitein male mammals (i.e., the sexually dimorphic nucleus of the preoptic area (SDN-POA) [41,75], differs between heterosexual and homosexual men [62]; for a partial replication, see [32]. Research also indicates that sexual partner preferences in rams are

related to structural differences in SDN-POA [77]. Rams are good animal models of human, male heterosexuality/homosexuality, as these animals show clear preferences (i.e., approach and mounting behaviors) for either male or female partners (see [77] for a review). This line of research on neuroanatomy and brain functioning is consistent with prenatal hormonal theories of human sexual orientation, which suggest prenatal hormones (e.g., testosterone) organize site(s) of the fetal brain relevant to sexual orientation during sexual differentiation, e.g., [30,37,43]. Research support for a biological basis to sexual orientation also includes studies on handedness, which develops prenatally [48]. Lalumière and colleagues' meta-analysis of existing literature has indicated a reliable relationship between handedness and sexual orientation in both men and women [59].

Other research in support of a biological basis to men's sexual orientation includes studies on birth order. Early research on sexual orientation suggested that homosexual men have a higher (or later) than expected birth order, that is, they tend to have a greater number of older siblings (i.e., later birth order) than comparable heterosexual men [87]. In 1996, this research advanced when Ray Blanchard and Anthony Bogaert demonstrated that the later birth order of homosexual men could be attributed solely to an ele-

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vated number of older brothers (and not number of older sisters) [14,15]. Their first study [15] examined 302 homosexual men and 302 individually matched heterosexual Canadian men. Logistic regression analysis showed that homosexuality correlated positively with number of older brothers and not with other sibling types (e.g., older sisters). The odds of homosexuality increased 33% with each additional older brother. In the same year, Blanchard and Bogaert found a very similar older brother effect in the original Kinsey data, a very large and historically significant data base [14]. This older brother phenomenon was later deemed the fraternal birth order effect (FBO effect) [12], because older sisters are not associated with men's sexual orientation, and no type of sibling (e.g., older brothers or older sisters) is reliably related to women's sexual orientation, e.g., [22].

The FBO effect is independent of potential confounds such as year of birth, age, socioeconomic status [23], and sibship size, e.g., [92]. There is also little evidence that a later parental age underlies the FBO effect [15,24], although parental age may be independently related to sexual orientation development and/or it may enhance FBO's effect [28,24].

Two recent discoveries, also by Blanchard and Bogaert, moved this line of research significantly forward. Using a sample of 944 homosexual and heterosexual participants, Bogaert showed that biological older brothers increase the odds of homosexuality, even if these older brothers were reared in a different household than a younger gay brother [24]. This study also demonstrated that non-biological siblings (e.g., adopted or step older brothers) had no effect on men's sexual orientation [24]. Thus, this pivotal study demonstrated that the FBO effect operates during prenatal life, not during childhood or adolescence. Blanchard, Cantor, Bogaert, Ellis, and Breedlove produced indirect evidence that the FBO effect is biological rather than psychosocial in nature [19]. They found, in a sample of 3146 men, that the FBO effect was contingent on handedness: The effect of older brothers on the likelihood of being gay only occurred in right-handed males; the effect of older brothers did not alter the likelihood of homosexuality in left-handed and ambidextrous men [19]. More recently, Bogaert found the FBO effect may be limited to only moderately right handed men, with extreme right-handers also not showing a FBO effect [25]. This interaction with handedness suggests, at least indirectly, that the FBO effect operates prenatally, because, as mentioned, hand preference is an important marker of prenatal development [48].

Although there have been a few failures to replicate the FBO effect [26], the original finding by Blanchard and Bogaert [15] has been confirmed many times, including replications by independent investigators [42,50]. The FBO effect has also been demonstrated in diverse samples, including very different clinical samples of men with same-sex attractions, including homosexual transsexual men (see review in Blanchard [12]), gay men from different historical eras [15,29], men with same-sex attractions from different cultures [92], in male GID (gender identity disorder) children who will very likely be gay in adulthood [18], and in gay men from convenience [15] and representative, national probability samples [22]. The finding that FBO interacts with handedness has also been confirmed by subsequent research (see review in Blanchard [13]).

In summary, FBO is likely the most reliable epidemiological finding in almost a century of research on sexual orientation. Blanchard and Bogaert have also greatly narrowed down the range of possible explanations for the FBO effect by demonstrating that the effect of older brothers on the sexual orientation of younger brothers operates in prenatal life and not in childhood. Finally, these authors have advanced a detailed theory—the maternal immune hypothesis (MIH)—to explain how the occurrence of prior male fetuses could influence the eventual sexual orientation of subsequent male fetuses [15]; for an early version of MIH, see [64]. This hypothesis, summarized in the next section, has only indirect support to date [12,94]; however, no plausible alternative hypothesis has been advanced to explain the relevant findings since it was introduced 15 years ago.

2. Maternal immune response

According to the MIH of male sexual orientation development, FBO results because some mothers develop an immune response to a substance important in male fetal development. This immune effect would increase in likelihood with each male fetus gestated by the mother; thus, an affected son would exhibit a heightened number of older brothers (hence a "FBO effect"). Presumably, the process would begin when cells (or cell fragments) from a male fetus enter a mother's circulation during pregnancy or childbirth. Given these cells originate from males, they would include on their surfaces (or inside them) male-specific substances (e.g., Y-linked proteins) that are antigenic to the mother; as such, her immune system would recognize these substances as "foreign" given that she herself is female. Despite immunomodulation occurring in pregnancy [73], the mother would develop antibodies against these substances, and antibodies would cross the placental barrier and enter the fetal compartment.[1] These anti-male antibodies would also then cross the blood/brain barrier (BBB) of the immature fetal brain, and ultimately affect brain development. Specifically, these antibodies would alter sex-dimorphic brain structures (e.g., INAH-3 and/or other regions) relevant to sexual orientation, and the affected son would ultimately become attracted to men as opposed to women. The degree to which this immune effect alters brain development would depend on the number of antibodies that reach the relevant brain structure and the binding strength of these antibodies to male-specific substances. As both the number and binding affinity of antibodies generated significantly increase during a memory immune response—i.e., in subsequent male pregnancies when male-specific substances are encountered the second (or third, etc.) time by the maternal immune system—the likelihood of the immune effect on sexual orientation becomes higher in subsequent male pregnancies, and hence the FBO effect. A medical model for a maternal immune response underlying the FBO effect is Hemolytic Disease of the Newborn (HDN). When a mother does not have the Rh factor in her blood (i.e., a mother is Rh negative), after gestating and giving birth to an Rh positive (Rh +) fetus, she may mount an immune response against the Rh factor. This immune response may affect subsequent Rh + fetuses, potentially attacking their red blood cells and causing anemia. The likelihood of an immune response becomes increasingly likely with each Rh + fetus a mother gestates. Thus, like men's sexual orientation, this phenomenon exhibits a birth order effect [1].

A number of conditions must exist if the MIH is a viable explanation of FBO (see also [12,94]). First, there must be evidence that fetal material enters the maternal circulation. A review of the relevant research indicates that this condition is well-established. High levels of different fetal cells enter the maternal circulation during abnormal pregnancies; however, there is also evidence that a variety of cells regularly enter maternal circulation throughout normal pregnancies [3,9,60,69,71]. There is also evidence of increased fetomaternal transfer of cells when a pregnancy is terminated, and heightened transfer levels in women who have a history of fetal loss [11]. Moreover, fetal cells have been detected in mothers' circulation for many years (i.e., 27 years) after delivery [10]. Interestingly, the finding that FBO is directly associated with homosexuality for approximately 30% of men who develop a sexual orientation toward males [15,16] may be an underestimate be-

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[1] The subclass of IgG antibodies is small enough to cross the placental barrier [31,84].

cause mothers' miscarriages of male fetuses are not included in these calculations. Thus, pregnancies with a male fetus not coming to full term may still immunize a mother against male-specific antigens. Indeed, given the higher likelihood of fetomaternal transfer of cells when a pregnancy was terminated, it is possible that the terminated pregnancies (of male fetuses) may, under certain conditions, be more likely to immunize a mother than a regular male pregnancy and birth.

A second condition for MIH to be a viable explanation of FBO is that a male-specific substance (or substances) should cause immune responses in (i.e., be antigenic to) females. This condition is also clearly established. A class of molecules derived from male tissues known collectively as H-Y (i.e., histocompatibility-Y) antigens cause immune reactions in females exposed to them [98]. The antigenicity of Y-linked substances was first, clearly demonstrated in non-human female animals [47,58,82,83,88,91]. Recent research has confirmed that human females are immunologically reactive to Y-linked substances. For example, women who have received transplants of male organs are more immunologically reactive to a form of H-Y antigen than women not exposed to these male tissues [90]. In addition, and more relevant to FBO and the MIH, mothers who have given birth to sons are more immunologically reactive to a form of H-Y antigen than those who have not given birth to sons [52,72,93].

The most commonly studied form of H-Y antigen was originally detected via transplants, and is designated by the gene SMCY, or JARID1D (also known as HY, HYA, KIAA0234, or KDM5D). A series of peptides (short sequences of amino acids) derived from the protein encoded by SMCY are typically used in H-Y immunological research. For example, Piper and colleagues used two peptides (FIDSYICQV and SPSVDKARAEL) derived from SMCY, finding that mothers with sons (versus mothers with daughters) are immunologically reactive to these peptides for as long as 8 years after their last male pregnancy [72].

In addition to SMCY, there are at least 26 other Y-linked proteins or protein families, and all may play (or are hypothesized to play) some role in sexual differentiation [86]. Some of these other Y-linked proteins have unknown antigenicity, but they satisfy other conditions necessary for the MIH to underlie FBO (see below); thus, it is reasonable to consider some of them as plausible antigens underlying FBO (see also [12]).

Another important condition for the MIH to underlie FBO and its effect on sexual orientation is that the relevant Y-linked substance(s) should play some role in the sexual differentiation of the brain, including, presumably, lower-brain structures hypothesized to be important in sexual orientation development (e.g., INAH-3). There is evidence that H-Y (i.e., protein coded by SMCY) is expressed in the brain [78], and thus may play a key role in sexual differentiation of it. SMCY is also, evolutionarily speaking, a very old Y-linked gene, emerging in our ancestors when they split approximately 200 million years ago from the lineage of monotremes, or egg-laying mammals [86]. Also, notably, a homologous, male-linked gene has been identified in rodents (SMC, or the lowercase *smcy*) [86]. It seems plausible, then, that this gene could contribute to the sexual differentiation of those reproductively relevant brain structures with, presumably, a long evolutionary history, such as hypothalamic sites (e.g., INAH-3) with mammalian homologs (i.e., SDN-POA).

A complication in the SMCY protein underlying the MIH and FBO is that, along with it being expressed in male brain cells, it is also well represented in gonad cells, including sperm [40,56], and in other parts of the male body [53,55,79]. The question, then, is whether a mother's immune system could target H-Y and affect brain mechanisms underlying sexual orientation but not (or only minimally) affect the development of the body, including the genitals and sperm production. This question is important because

there are likely only minor physical differences between gay and heterosexual men (see review below) [26], and there is no evidence that gay men have lower fertility (e.g., lower viable sperm counts) relative to heterosexual men. In their review of the relevant H-Y literature available at the time, Blanchard and Klassen suggested this type of selective immune action on brain development may be plausible [17]. For example, there is evidence in mice that testes can develop in the absence of H-Y [66]. A maternal immune response against H-Y may also not affect sperm in a fetus, as they are immature, underdeveloped and only reach maturity during adolescence.

Also relevant is that alternate transcripts of SMCY occur, coding for three different forms of the protein (i.e., different isoforms). Thus, it may seem plausible that one form of the protein, if predominantly expressed at one tissue site (e.g., brain), may be the target of an immune reaction, whereas the other, different forms expressed in other tissues (i.e., the body) may not be the target of an immune reaction. However, we do not know any evidence that a SMCY isoform is indeed specific to the brain. An additional complication is that the SMCY protein is expressed *within* and not on the surface of cells; thus, in the protein's whole form and its functioning, it is intracellular. As a result, in its whole protein form, it is inaccessible to antibodies. However, SMCY is broken down into peptides after the protein performs its intracellular function and degrades, and these peptides are expressed on the surface of cells (including brain cells) by the Major Histocompatibility Complex (MHC) class I protein. These SMCY peptides on the surface of cells do not have a biological function, except for allowing the body to recognize them as "self" through MHC processes. Thus, antibodies (and other parts of the immune system) could target these cell-surface peptides, although they likely would not interfere with (or inactivate) the direct function of this protein within the cell; rather, the immune effect, if inflicted, would likely be more drastic, killing the entire cell. It is also notable that some SMCY-derived peptides, including those studied by Piper and colleagues mentioned above [72], are ubiquitously expressed throughout the body, whereas others seem to be more restricted in their expression [36]. Perhaps some of these restricted peptides are more limited to the brain (although we have no evidence for this).

Aside from H-Y (e.g., SMCY), other Y-linked proteins likely affect male fetal brain development [86]. Three are of particular relevance here, as they are well-expressed in the brain. The first is protocadherin 11-linked, PCDH11Y (also known as PCDH22 and PCDHY) [20]. PCDH11Y codes for a protein important in cell adhesion and is predominantly expressed on the surface of brain cells [20,54,86]. It is involved with synapse formation and neural pathway development. Given that PCDH11Y is, predominantly expressed in the brain, this makes it an appealing candidate to underlie FBO, as a maternal immune effect would likely be limited to brain/behavior differences and not to bodily differences, which, as mentioned, are likely minimal between gay and heterosexual men (see below). In addition, there is evidence that alternative transcripts of PCDH11X/Y (different isoforms) are expressed in different parts of the body and brain [54]. Thus, this gene ultimately codes for different protein isoforms at different tissue sites, only one of which may be relevant to sexual orientation development (and thus the target of a maternal immune response underlying FBO). Indeed, there is evidence of a PCDH11Y isoform (isoform-a) that seems to be unique to the brain [54].

PCDH11Y is not expressed in our closest living relatives, chimpanzees and gorillas [97], and it evolved near the time of the human divergence from the chimpanzee lineage (i.e., 6 million years ago) [95]. Given that PCDH11Y is specific to hominids, evolved very recently, and is predominantly expressed in the brain, it may play an important role in gender differences in behavior and

cognitive processing unique to humans. Indeed, Crow has speculated that PCDH11Y underlies human sex differences in handedness, language, and cerebral asymmetry [35]. However, to date, we are not aware of any study showing direct evidence that PCDH11Y is associated with sex-linked cognitive functioning. Interestingly, a minority of men lack the PCDH11Y gene; unfortunately, there is no data on their cognitive functioning or cerebral asymmetry profile [53].

If PCDH11Y evolved recently and its effects are specific to (sex-linked) cognitive processing in humans, the question arises whether this gene contributes directly to the sexual differentiation of those structures with, presumably, a long evolutionary history, such as the hypothalamus. This question is relevant because, as mentioned, hypothalamic (e.g., INAH-3) sites with mammalian homologs (i.e., SDN-POA) have been implicated in human sexual orientation development. There is evidence that PCDH11Y is expressed in some areas of the lower brain (e.g., pons; thalamus; olfactory bulb) [54], but we know of no evidence that it is expressed in the hypothalamus.

Even if PCDH11Y does not directly affect the hypothalamus, however, it may still underlie higher-order brain structures/processes relevant to sex-linked traits/behaviors, and ultimately affect sexual attraction patterns. For example, gay and heterosexual men differ, on average, in a number of sex-linked traits/behaviors, including personality, occupational and other interests, handedness, language processing, and cerebral asymmetry (for a review, see [63,67,74,96,101]). Indeed, PCDH11X/Y is expressed in regions of the brain relevant to this type of processing (e.g., cerebral cortex; corpus callosum) [54]. Thus, an immune response against the PCDH11Y protein may alter one or more of these sex-linked traits/behaviors and ultimately lead to atypical sexual attraction patterns in men. For example, Bemhas speculated that sex-linked temperament/personality, in conjunction with the interaction of peers in childhood, may underlie sexual orientation development in humans [8], but see [21].

A second Y-linked gene of relevance is neuroligin 4 Y-linked, NLGN4Y (also known as KIAA0951) [51]. Like PCDH11Y, it also codes for a protein involved in cell adhesion and is well-expressed in the brain, although it is also expressed in other regions, including the genitals and prostate [51,86]. This protein is primarily expressed at the postsynaptic side of the synapse and is thought to play an important role in synaptic functioning [51]. It is also expressed on the surface of cells and thus makes it accessible to antibodies. Even though NLGN4Y is well-expressed on the surface of brain cells, a maternal immune effect against the protein – similar to the maternal immune effect against the SMCY protein – may also affect the body due to NLGN4Y's expression in the body. However, like PCDH11Y, there is evidence that alternative transcripts are expressed in different parts of the body and brain [51]. Thus, this gene may also ultimately code for different protein isoforms at different tissue sites, only one of which may be relevant to sexual orientation development (and thus the target of a maternal immune response underlying FBO). However, we do not know of any evidence that an NLGN4Y isoform is unique to the brain. NLGN4Y's evolutionary history is much older than PCDH11Y, estimated as emerging in our ancestors when they split from the lineage that contains new world monkeys, i.e., approximately 40–50 million years ago [86]. Thus, given its evolutionary age, it may be more likely, at least relative to PCDH11Y, to contribute directly to the sexual differentiation of reproductively relevant sites of the lower brain (e.g., INAH-3) with mammalian homologs (i.e., SDN-POA). However, it is unknown to us whether it is expressed in the hypothalamus. Also, unlike PCDH11Y, this gene has been, directly linked to sex-linked brain functioning. In particular, mutations of this gene are linked to autism, a sex-linked disorder [51,61,81,99]. Interestingly, autism has been recently suggested to be affected

by a maternal immune response to (as yet unidentified) fetal brain proteins [100]. However, we know of no consistent evidence of a birth order effect related to autism (e.g., [100]).

A final Y-linked protein of relevance is TBL1Y. This protein too is well-expressed in the brain, although it is expressed within cells and not on their surface; thus, in its whole form, it is not accessible to antibodies. However, like SMCY and other intracellular proteins, it is broken down into peptides after it performs its function and degrades, and these peptides are expressed on the surface of cells by the MHC class I protein. Unfortunately, unlike SMCY, these peptides are not well studied. TBL1Y is also expressed throughout the body. Thus, like NLGN4Y and SMCY, a maternal immune effect against this protein might also affect the body and not just brain structures relevant to sexual orientation development. Also, like SMCY, the degree to which TBL1Y peptides are differentially expressed in various parts of the body/brain is unknown; thus, it is unclear whether a TBL1Y peptide could be relatively exclusively expressed in the brain. There is also no evidence of alternative transcripts (i.e., no isoforms) of TBL1Y. However, there is evidence that this protein could be inactivated without obvious phenotypic consequences to the body because men of certain lineages lack TBL1Y [53]. TBL1Y has an evolutionary history that is much older than PCDH11Y or NLGN4Y, estimated as emerging in our ancestors when they split from the lineage that contains placental mammals approximately 100 million years ago [86]. Thus, given its evolutionary age, it may be more likely, at least relative to PCDH11Y or even NLGN4Y, to contribute directly to the sexual differentiation of sites of the lower brain (e.g., INAH-3) with mammalian homologs (i.e., SDN-POA).

TBL1Y has been indirectly linked to cognitive functioning. The homolog gene on the X-chromosome, TBL1X, has been linked to deafness [7]. Moreover, there is some evidence that gay and heterosexual men differ in the structure and function of the auditory system. For example, there is evidence that gay and heterosexual men differ in auditory functioning (i.e., auditory evoked potentials), although this evidence is contrary to a standard prediction from prenatal hormonal theories (e.g., [37,30]), as gay men seem to have a hypermasculinized pattern relative to heterosexual men [65], see also [27,43]. What relevance TBL1Y plays in these putative hearing differences in gay versus heterosexual men is unknown.

In summary, four Y-linked proteins are plausible candidate antigens because they satisfy a third condition necessary for the MIH to plausibly underlie FBO: i.e., these proteins are well-expressed in the brain.

A fourth condition for the MIH to be a plausible explanation of FBO is that there should be evidence that a maternal immune response to Y-linked proteins affects fetal development, including, potentially, sexual differentiation of the brain. There is a variety of indirect evidence for this, including the finding that male fetuses are more prone to HDN than female fetuses [44], suggesting that exposure to Y-linked substances may enhance an Rh negative mother's immunological reactivity in conjunction with exposure to Rh positive fetuses. In addition, there is evidence that male (versus female) pregnancies increase the likelihood of secondary recurrent miscarriage [33]. The authors suggest that exposure to H-Y (i.e., SMCY peptides) in the first pregnancy increases the likelihood that mothers will have an immune reaction against H-Y antigen expressed in, and ultimately induce a miscarriage to, a subsequent male fetus. As yet, there is no evidence that a mother's exposure to SMCY peptides in an initial pregnancy alter reproductive tendencies in sons produced from subsequent pregnancies. However, there is evidence in rodents that those females immunized to H-Y (i.e., SMC, via exposure to male spleen cells) sire pups not able to reproduce, despite little evidence of gross physical abnormalities in the genitalia [85].

A fifth condition for the MIH to be a plausible explanation of FBO is that the maternal immune response to Y-linked proteins should show an incremental response to previous male fetuses similar to the incremental pattern that the FBO effect has on male sexual orientation for each additional older brother. This has been established for H-Y (i.e., SMCY peptides): Piper and colleagues showed that 37% of women with a male pregnancy had a detectable immune reaction (i.e., H-Y-specific CD8 T cells) to H-Y peptides; and this amount increased to 50% with two or more male pregnancies [72]. These authors also demonstrated that H-Y-specific CD8 T cells in mothers with previous male pregnancies were functional (i.e., able to be primed to produce cytokines) for at least 8 years after their last male pregnancy, suggesting that a possible immune response may affect subsequent fetuses even after large pregnancy intervals. Moreover, this result suggests that the detection of an immune response may be possible many years (e.g., perhaps as many as 20 years or more) after a mother's last pregnancy, although these researchers did not test mothers who had a male pregnancy beyond 8 years ago [72]. Other research indicates, however, that women do retain immunity to H-Y antigen for as long as 20 years or more [93].

3. Summary, and the role of SRY and prenatal hormones

In summary, four male-specific proteins (encoded by SMCY, PCDH11Y, NLGN4Y, TBL1Y genes) are possible candidates to consider as a relevant antigenic substance underlying FBO because they satisfy (or plausibly satisfy) a number of conditions necessary for the MIH to underlie FBO. SMCY is the most studied of the four, and seems to satisfy most of, the conditions necessary (e.g., expressed in the fetal brain, shows an incremental immune response to previous male fetuses, similar to the pattern of FBO). However, SMCY (at least in its functional form) is intracellular in nature and thus is not directly accessible to antibodies. In addition, there is no evidence yet of a maternal immune reaction against three of the proteins (PCDH11Y, NLGN4Y, TBL1Y) and only one (PCDH11Y, isoform-a) is almost exclusively expressed in the fetal brain.

Another Y-linked gene not mentioned above but of note is SRY (sex determining region of the Y chromosome), known to produce a main protein necessary for the development of the testes. SRY has a very old evolutionary history and is thought to have emerged in our ancestors when they split from the lineage that contains birds (approximately 300 million years ago) [86]. This protein is also expressed in the brain, although its main role in sexual differentiation of the brain (and the rest of the body) occurs indirectly by the prenatal masculinizing hormones (e.g., testosterone and dihydrotestosterone) produced by the testes (see [57] for a review). The SRY protein is also not known to be antigenic to females. Moreover, it is expressed intracellularly, and as such, in its whole form is not accessible to antibodies. Thus, although it should not be completely discounted (e.g., perhaps there is a relatively brain-specific isoform of SRY), we expect this protein is not the most plausible Y-linked antigen to underlie a maternal immune response affecting sexual orientation and FBO.

The important role that the SRY gene plays in sexual differentiation—largely via prenatal hormones produced by the testes—is consistent with the traditional biological explanation of sexual orientation, as being driven by prenatal hormones (e.g., [37]). Our hypothesis of a maternal immune response to a Y-linked protein underlying FBO does not oppose this view. Instead, it is consistent with the view that human male brain development depends on two systems: one driven by prenatal androgens (e.g., testosterone indirectly influenced by SRY), and the other driven directly by sex-linked genes (e.g., Y-linked genes/proteins). This view is also consistent with recent perspectives on sexual differentiation of the brain [4,57,70].

4. Physical moderators

Given the FBO effect has also been reliably linked to handedness, a maternal immune response explanation needs to account for the joint effect of FBO and handedness in predicting men's sexual orientation. Certain genes are linked to both immune system functioning and handedness [39,34], and variations in these genes may affect the development of sexual orientation. Thus, a gene may, for example, predispose a mother and her children (e.g., to atypical handedness (e.g., non right or extreme right) but also vary this family's ability to resist a maternal immune response against a Y-linked protein underlying the FBO effect. Major Histocompatibility Complex (MHC) alleles may be relevant here. This gene complex is responsible for widespread immunological functions in vertebrate species, but it also associated with nonright handedness in humans [39]. Genes of the Rh system (e.g., RHD; RHCE) have also been linked to both handedness and immune system functioning (see [46], for a review). As suggested earlier, Rh is a factor in blood associated with HDN, and this phenomenon provides an important medical model of an immune response that may underlie FBO. That Rh genes are also linked to handedness supports the idea that these genes may also play a role in sexual orientation development. It is also interesting that, as mentioned, although the Rh phenomenon is initiated by both male and female fetuses, the effect is more common in male fetuses [44].

Although somewhat less consistently linked to FBO than handedness, aspects of physical development, including birth weight and adult stature, have also been linked to FBO (e.g., [23]). This research shows that later born gay men tend to have a lower birth weight and be shorter as adults than earlier born gay men and heterosexual men. One explanation for these interactions of physical development with FBO is that a maternal immune response against a Y-linked antigen de-masculinizes (or feminizes) later born fetuses, thus affecting mechanisms related to both sexual orientation development and physical development. One consequence of a physical development alteration linked to FBO in gay men is that a male-specific protein targeted by a putative maternal immune response need not be (exclusively) expressed in the fetal brain. Thus, a variety of Y-linked proteins that are expressed in both the brain and the body may be relevant if a maternal immune response underlies FBO. However, it must be stressed that there are likely only very minor differences in physical development between homosexual and heterosexual men. In contrast, there are, of course, very large, average differences in physical development (i.e., sex dimorphism) occurring between men and women, particularly after puberty. Thus, if a Y-linked protein is being affected by a maternal immune response underlying FBO and sexual orientation development, then it is likely that it will have only relatively minor effects on the body. It is also important to note that brain mechanisms ultimately partially regulate growth and development; thus, Y-linked proteins preferentially or exclusively expressed in the fetal brain (PCDH11Y, NLGN4Y, TBL1Y) may still have an impact on putative physical differences between gay and heterosexual men via changes in the brain (e.g., hypothalamus).

5. Considerations and challenges in testing the MIH

Although the original formulation of the maternal immune response theory targeted the humoral immune system (e.g., specifically, the production of anti-male antibodies) as the source of the antigenic response, recent work, including the study [72] reviewed above, suggests that a cell-mediated immune response may also be relevant to the FBO effect. Cell-based immunity is not traditionally associated with an antibody response but rather with the activation of macrophages, T-cells, and cytokine release (e.g., Inter-

feron-gamma; IFN-γ). In theory, mothers of gay men who have produced an antibody response to male antigens (i.e., a humoral response) may also have had a cellular immune response, including the production of antigen-specific effector T-cells and the release of cytokines (e.g., IFN-γ, after exposure to one or more of these Y-linked antigens). And, as in Piper and colleagues [72], re-stimulation of antigen-specific effector T-cells in the mothers of gay men, even years later, may show evidence of this exposure, resulting in the release of cytokines (e.g., IFN-γ). Thus, evidence of cytokine release after re-stimulation of antigen specific T-cells could be used as a marker of a cellular immune response that may have occurred in mothers many years earlier. The ability to detect a cellular immune response many years later is important in light of the fact that circulating levels of anti-male antibodies in the mother's blood (via her humoral system) may be undetectable years after they were stimulated by a male fetus. Also, importantly, unlike antibodies, cytokines (e.g., IFN-γ) can cross a normally developed blood–brain barrier, BBB [6]. Consequently, a maternal immune response involving cytokines would not necessarily require the fetus to have a blood–brain barrier (BBB) that is immature and susceptible to antibodies. In sum, cytokines via the cellular system may not only play a direct role in the putative immune response underlying FBO, it may also be more detectable (e.g., by a priming/recall response of a mother's T-cells) than antibodies via a humoral response.

Another consideration in testing MIH concerns the reproductive and general medical history of the mother. Thus, aside from (or in conjunction with) the number of previous male gestations, different factors in the mother may increase the likelihood that she develops an immune response to a Y-linked antigen that ultimately affects a male fetus. One of these additional factors may include whether or not a mother has had heightened exposure to Y-linked antigens through abnormal pregnancies. As mentioned, fetal cells enter into the maternal circulation in elevated quantities during abnormal pregnancies [60,71]. Fetomaternal transfer also increases if the pregnancy was terminated, and heightened levels of fetal cells have been found in women with a history of fetal loss [11]. Thus, prior abnormal pregnancies with male fetuses may be more likely to expose a mother to Y-linked antigens, and thus increase her likelihood of mounting an immune response to a subsequent male fetus. To our knowledge, however, no study has found evidence that gay sons are indeed more likely to occur in mothers with a history of (prior) pregnancy/birth complications (e.g., miscarriages). One study showed that mothers of gay males had 15% of pregnancies lasting less than 6 months, but this was not statistically different than the mothers of heterosexual males (13%) [38]. However, only a small proportion of the lost pregnancies were of a known sex: 9.6%. It also was not clear what percentage of the terminated pregnancies came before or after the gay or heterosexual son. If miscarriages/abortions increase the likelihood of the exchange of blood between the fetus and the mother, and lost male pregnancies increase the likelihood of immunizing her against a Y-linked protein, then the sex of the fetus and the timing of this pregnancy—before or after the proband—are important considerations here.

It is also important to note that the issue of immune sensitization of the mother to Y-linked proteins may be a broader one than what occurs through her pregnancies with male fetuses. A mother may, in fact, be immunized against Y-linked proteins by exposure to any blood or other tissue from males, as long as this tissue in some form—e.g., cell fragments—enters her circulation [90]. Thus, a mother's history of, say, blood transfusions, grafts, and organ transplantations may be additional important considerations here, if this history indicates exposure to male tissue in some form (e.g., a blood transfusion from a male donor). As such, a mother's broader medical history (beyond her pregnancies) may be an additional

moderator of the maternal immune response directed toward male fetuses and important to consider in testing MIH. That having been said, however, it is important to note that not all male tissues would necessarily contain the relevant Y-linked protein putatively underlying FBO and male sexual orientation development. As mentioned, only certain Y-linked proteins are potentially good candidates underlying this effect, as they are preferentially expressed in the brain. Thus, even though exposure to any male tissue may sensitize her immune system against a variety of Y-linked antigens, only exposure to the Y-linked antigen underlying sexual orientation development is relevant. Thus, male miscarriages and abortions may be particularly relevant as such events may expose a mother to a variety of male tissues, including fetal brain tissues that may contain the relevant Y-linked protein (or one of its isoforms) underlying male sexual orientation.

Although there is evidence that fetal cells enter the maternal circulation in much greater quantities during abnormal pregnancies [9,60,71], it is unknown to us whether a mother's antibodies or cytokines have an elevated likelihood of crossing the placental barrier and ultimately affecting the fetus during abnormal pregnancies. Thus, a mother may have a higher likelihood of being immunized against a Y-linked protein during a first male pregnancy if that pregnancy was abnormal in some way, but it is unknown to us if the product of a mother's immune response (anti-male antibodies; cytokines) may be equally likely to cross the placental barrier and affect a subsequent fetus, regardless of the typicality of subsequent pregnancies. As such, a maternal immune response may be particularly likely to occur in mothers who had a previous male pregnancy that was abnormal in some way, but the subsequent pregnancy, in which an immune response ultimately affects the fetus' future sexual orientation, may not be abnormal. However, it does seem plausible that a subsequent abnormal male pregnancy would increase the likelihood of an immune response, particularly if the placenta is affected (e.g., increased likelihood of subclasses of antibodies other than IgG to cross the placental barrier). Similarly, it seems plausible that an abnormal pregnancy may delay or complicate the development of the fetal blood–brain barrier (BBB), making it not only incapable of preventing the crossing of maternal cytokines but also (maternal) antibodies, which, as mentioned, normally cannot cross this barrier [80].

One additional unknown that arises in testing the immune response hypothesis concerns finding the exact antigen underlying FBO and sexual orientation development. It is possible that a mother was sensitized against a number of the Y-linked proteins during pregnancy with male fetuses and has a detectable immune response to more than one of these antigens years later, but only one of these antigens is relevant to male sexual orientation development. Thus, it may be difficult to discriminate which of the (possible) Y-linked antigens showing immunological reactivity in mothers of gay men underlies sexual orientation and FBO, especially given the limited knowledge of how these proteins operate in brain development. It is also the case that all of the (possibly) reactive antigens that may be tested in mothers of gay men play no specific role in male sexual orientation, and rather only serve as a marker for another, untested Y-linked protein. This is because, as mentioned, there are numerous Y-linked proteins currently identified (at least 27), and not all of these would likely be examined in any given immune assay developed to test FBO. As mentioned, currently we believe the four previously reviewed are the most plausible (PCDH11Y, NLGN4Y, TBL1Y, SMCY), and although these would be the ones most likely to be examined in immunological work testing FBO, one or more of the other 23 Y-linked proteins not tested maybe the relevant antigen underlying FBO. It is also the case that there may be other Y-linked proteins that are not yet identified, and one or more of these may be the relevant

antigen. Thus, confirmation of an immune response to one or more Y-linked antigens in mothers of gay men would be "smoking gun" evidence that, indeed, an immune mechanism to a Y-linked antigen underlies FBO and is one developmental route to homosexual development in men, although there is a possibility that it may not reveal the exact Y-linked antigen underlying it. However, it is time that the maternal immune hypothesis is put to the test, despite the possibility that it will not locate the exact Y-linked antigen.

Acknowledgments

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