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Research Article

Molecular Genetic Study to Reveal the Association Between RYR3 Gene Expression and Gender Dysphoria

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ABSTRACT
Gender dysphoria may have a genetic component, but association study data are conflicting. Brain development diverges in males and females in response to androgen production by the foetal testis. This sexual differentiation of the brain occurs during a sensitive window and induces enduring neuroanatomical and physiological changes that profoundly impact behavior. We hypothesised that gender is an innate part of identity, and we hypothesised that RYR3 would be a part of individuals' transsexuality. By utilising quantitative real-time PCR (RT-PCR) gene expression, we analysed the RYR3 expression level in 50 transgender blood samples. The gene expression level of RYR3 was analysed statistically. A significant association was identified between gender dysphoria and RYR3 expression level. A significant difference in the gene expression level of RYR3 was observed in various age groups. The gene expression ratio was observed in the age groups of 17–20 (0.2826), 20–29 (0.5468), 30-39 (0.2425), 40–49 (0.2006), and control patients (1.000). Our result proposed that there is a low level of expression observed in all the groups when compared to the control group.

KEYWORDS: Gender dysphoria, RYR3, brain development, transsexuality, RT-PCR

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INTRODUCTION

The broad term "transgender" (TG) refers to a person whose gender self-identification differs from the gender assigned at birth. The term "gender dysphoria" (GD) is used to describe this extreme form of TGism when the former becomes overwhelming and has a significant psychological impact (Kuper et al., 2012). People who have gender dysphoria (GD) frequently seek medical attention, either surgically or medically, in an effort to reconcile the gap between their physical appearance and gender identity (Hembree et al., 2017). In recent years, India has made rapid progress in recognising the transgender community and providing legal support for them. Gender-affirming medical interventions are being sought after by more people than ever before. In the Indian subcontinent, many medical and surgical professionals who work with transgender patients face social and clinical obstacles (Srinivasan and Chandrasekaran, 2020). Since ancient times, people have known that there are people whose

gender does not match their biological sex. Transgendered people have been portrayed as heroes and deities in Indian mythology, a country with a long history. The consideration of people with orientation dysphoria is a moderately new peculiarity within the clinical local area (Cook, 2004). Magnus Hirschfeld established the first known centre for the treatment of gender dysphoria patients in 1918 in Berlin. He was a German doctor who had offered care in light of his "variation treatment" at his Foundation for Sexual Science, while his contemporaneous partners were attempting to view it as a "fix" for the condition. He advocated for people to live "according to their nature" rather than according to their gender. In low- and middle-income nations like India, where people are being given a voice through a variety of legal and constitutional changes, this is becoming more and more prominent (Srinivasan and Chandrasekaran, 2020). The medical community is at a crossroads right now, having to deal with this minority group's needs, which may differ from those of other groups.

Gender dysphoria is the pain that an individual feels because of a confound between their orientation personality and their sex doled out upon entering the world. Through the various classification systems for mental health, this term has undergone numerous iterations (Evans, 2017). Gender is a social construct, in contrast to sex, which is biologically determined and, in a sense, immutable. In the society we live in, there are aspects of masculinity and femininity that have "norms." In addition, even among youth who attend college, there is a lack of awareness of sexuality and sexual practices in India (Mikkola, 2008). Transgender people in India face a severe lack of emotional and informational support from family and society. The majority of transgender people learn to hide their orientation when confronted with their own sense of alienation and confusion, as well as the overwhelming negative messages in their home and society (Mikkola, 2008; Evans, 2017). Constant discomfort with one's biological sex is the cause of gender dysphoria (GD), a contentious diagnostic category in the American Psychiatric Association's (APA) Diagnostic Statistical Manual of Mental Disorders (DSM5) (Davy, 2015). Gender identity disorder, transsexuality, and transgender are just a few of the many additional terms that are currently used to describe this condition. It is estimated that less than one male out of 10,000 and one female out of 30,000 adults born experience GD (Zucker et al., 2016). GD is thought to be caused by a combination of biological (hormonal and genetic) and psychological (social) factors. Gender identity is thought to be located anatomically in the brain (Bao, A. M., & Swaab, D. F., 2011; Smith et al., 2015). There are region-specific differences in human brain structure, with some structures larger in males (like the hypothalamus and amygdala) and smaller in females (like the caudate nucleus and hippocampus). However, the significance of these dimorphisms in the emergence of GD remains a mystery. GD is caused by a genetic factor, according to heritability studies (Yang et al., 2017).

Numerous studies have suggested that transgender identity is genetically influenced (Fernández et al., 2015; Polderman et al., 2018; Lippa and Hershberger, 1999; Bentz et al., 2007; Smith et al., 2015; Fisher et al., 2018; Foreman et al., 2019). Heritability has been found to range from 38 to 47 percent in twin studies for female adolescent mothers and 25 to 43 percent in male adolescent mothers; however, this range drops to 11 to 44 percent and 27 to 47 percent in adults (Theisen et al., 2019; Polderman et al., 2018; Fisher et al., 2018). These studies yielded a number of genes, many of which had already been identified in studies of sexual differentiation in animal models. COMT, PIK3CA, RYR3, SRD5A2, STS, and SULT2A1 are among these, in addition to variants of genes that code for aromatase, androgen receptor (AR), estrogen receptor (ER), and 17-hydroxylase (Smith et al., 2015; Theisen et al., 2019; Fernández et al., 2015, 2018; Fisher et al., 2018; Yang et al., 2017; Foreman et al., 2019).

In light of the fact that heritable factors play a role in gender identity variation, a number of studies over the past ten years have focused on individual candidate genes. The majority of studies on candidate genes have focused on genes that are involved in sex hormone pathways or sex hormone receptors like androgen and oestrogen receptors (Heilmann-Heimbach et al., 2020; Trabert et al., 2011). An overview of candidate-gene studies on gender identity has not yet yielded any conclusive connections (Fisher et al., 2017). The inconclusive findings thus far are most likely due to the focus on individual genes and small sample sizes, as the genetic architecture of the majority of complex human traits is characterized by very small effect sizes of multiple common genes. A sufficiently powered Genome Wide Association Study (GWAS), which has been demonstrated to be successful for many other complex human traits, would be an important next step in comprehending the genetic background of gender identity (Visscher et al., 2017). In order to carry out a GWAS with sufficient power in the near future, we strongly urge gathering all existing genetic and phenotypic data on gender identity and gender dysphoria. In recent times, Yang et al. (2017) used a genetic sequencing design to investigate the effects of rare genetic variants on 14 self-identified transgender Han Chinese individuals (Frolov et al., 2020). They found an effect caused by RYR3, a gene that controls intracellular calcium homeostasis and is highly expressed in the brain. This gene, on the other hand, ought to be regarded as merely one of many potentially contributing genetic factors rather than a major causal genetic mechanism because of the likely polygenic architecture of gender identity and the extremely small sample size of this study (Frolov et al., 2020). In addition, this finding must be replicated before a firm connection between RYR3 and gender dysphoria can be established. To support this study, we performed a gene expression study of RYR3 in transgender people using real-time PCR.

EXPERIMENTAL WORK

Blood Samples Collection

50 transgender people were chosen for our experiment. 3 to 6 ml of whole blood was collected in sodium heparin tubes (green tubes) and acts as an anti-coagulant by preventing the formation of thrombin. The collected blood samples were immediately subjected to total RNA isolation.

Total RNA Isolation

The experimental procedure was similar to the one that was previously described (Zheng YM et al., 2005). Total RNAs were obtained from freshly collected whole blood in sodium heparin tubes. QIAamp RNA Blood Mini Kits were utilised to isolate total RNA from 1.5 ml of freshly collected human whole blood.

Gene Expression Analysis by Real-Time Quantitative (RT-PCR)

The gene expression level of RYR3 was quantified by Real Time PCR (RT-PCR) method. Using a HiMedia Insta Q96 Plus Real Time PCR Machine and a specific target gene (RYR3) forward and reverse primers with SYBR Green Supermix, cDNAs were synthesized and amplified. The primary cDNA synthesis reaction mixture contained one µl, which was used for RT-PCR PCR amplification. Depending on the linear range of PCR amplification for RYR3 gene, RT-PCR runs were typically carried out for 20 to 35 cycles. Each PCR cycle included denaturation at 94°C for 30 s, anneling at 55°C for 1 min, and extension at 72°C for 5 min. After the previous cycle, a 10 minute cycle at 72°C was run to trim polymerizations that were not completely completed. The forward and reverse primers of RYR3 gene was: 5′-AGGATATGGAGCTGGATGCC-3′, and 5′-GCACACGACAGGAGAAAGTC-3′. Endogenous control was provided by the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each biological duplicate was used for RNA extraction and then qRT-PCR in triplicate for each of the qRT-PCR reactions. The six biological duplicates, each with a triplicate, formed the mean of the six values for the final threshold cycle (Ct). The relative amounts of each amplified product in the samples were evaluated using the comparative Ct calculation. The HiMedia Insta Q96 Plus Real Time PCR System used default parameters to calculate the $^{\Delta\Delta}$Ct for each reaction. A negative control containing water rather than cDNA was included in each qRT-PCR reaction set. The mean values of the triplicate measurements were used in subsequent calculations after they were averaged to determine the level of RYR3 and GAPDH gene expression in transgender patients' whole blood for the purpose of quantifying RYR3 gene expression.

Statistical Analysis

Means ± SE were used to represent each cycle threshold data set. The independent t test was used to determine whether differences between observations were statistically significant. As a level of statistical significance, $p<0.05$ was accepted.

RESULT

Ryanodine receptor 3 (calcium release channel), expressed in cardiac and neonatal skeletal muscle in the aorta,sophagus,, lung, has alternative spliced isoforms. RYR3 is coexpressed with ITPR3 in neurons and in the hippocampal CA1 pyramidal cell layer, in the caudale/putamen, olfactory bulb, and olfactory tubercle, smooth muscle cells, and is involved in a special form of hippocampal synaptic plasticity (for adaptation of acquired memory flexibility to environmental changes). During the physiological processes of sexual brain development, intracellular calcium homeostasis may be largely regulated by RYR3. Mutations of RYR3 may cause an imbalance of intracellular calcium homeostasis, leading to impairment of neuronal function. Males and females differ in (primordial stage) genes, as is factual from genomic examinations. Notwithstanding, their sex- and gender-specific explicit way of behaving and physiology is in any case significantly unique, regardless of whether they experienced childhood in a comparative way. By expanding the "Calcigender-idea" to show that the sex-steroid hormone-dependent intracellular calcium concentration is an important third factor, In our study, a total of 50 patients (informed consents) were enrolled. All the patients have undergone gender dysphoria surgery and are known to be transgender. From the selected patients, blood samples were obtained using sodium-heparinized tubes. Patients with a history of transgender behaviour and gender dysphoria surgery were only utilised in this study (Table 3). Table 1 summarises the number of patients included in our study age-wise (17 years to 49 years). Table 2 represents the cycle threshold (Ct) value of the RYR3 and GAPDH genes obtained in the RT-PCR study.

Table No.1: No. of Patients who experienced transsexuality behaviour were Selected for the Study with Age (in years)

No. of Patients with Age (in years)	**Transgender men**	
	n = 50	**% of n**
18-20	3	6 %
20-29	19	38 %
30-39	13	26 %
40-49	15	30 %
Standard Deviation (SD)	5.89	11.78

Table 2 : No. of Patients who experienced transsexuality behaviour were Selected for the Study with Age (in years).

S. No.	Patients Name	Age (in years)	Average Ct Values	
			GAPDH	RYR3
1.	Patient 1	18	26.04	24.25
2.	Patient 2	18	25.09	23.03
3.	Patient 3	19	21.03	19.38
4.	Patient 4	20	26.98	25.07
5.	Patient 5	21	26.09	24.76
6.	Patient 6	23	24.56	22.45
7.	Patient 7	23	25.08	24.23
8.	Patient 8	24	26.27	24.13
9.	Patient 9	25	27.07	24.27
10.	Patient 10	26	25.09	23.84
11.	Patient 11	27	25.56	24.29
12.	Patient 12	27	26.45	24.21
13.	Patient 13	28	27.51	25.54
14.	Patient 14	23	26.06	24.83
15.	Patient 15	25	25.81	24.82
16.	Patient 16	21	24.44	23.12
17.	Patient 17	26	25.94	24.57
18.	Patient 18	23	24.34	23.17
19.	Patient 19	28	25.78	24.04
20.	Patient 20	29	25.19	23.97
21.	Patient 21	24	26.45	24.79
22.	Patient 22	25	24.87	22.33
23.	Patient 23	30	26.09	24.08
24.	Patient 24	34	26.13	23.95
25.	Patient 25	31	23.08	21.97
26.	Patient 26	37	25.56	24.01
27.	Patient 27	33	23.66	22.54
28.	Patient 28	32	23.18	22.65
29.	Patient 29	30	26.67	25.13
30.	Patient 30	38	26.32	24.65
31.	Patient 31	32	25.07	23.96
32.	Patient 32	39	25.33	23.65
33.	Patient 33	30	25.88	23.43
34.	Patient 34	31	26.08	24.03
35.	Patient 35	34	24.33	22.34
36.	Patient 36	40	23.54	21.99
37.	Patient 37	43	25.61	23.33
38.	Patient 38	45	26.17	24.02

39.	Patient 39	41	26.12	24.91
40.	Patient 40	42	23.06	21.45
41.	Patient 41	49	24.88	23.33
42.	Patient 42	42	24.32	23.89
43.	Patient 43	40	26.72	24.91
44.	Patient 44	42	25.94	24.02
45.	Patient 45	41	24.76	23.34
46.	Patient 46	40	25.07	23.78
47.	Patient 47	45	25.32	23.19
48.	Patient 48	47	26.03	25.05
49.	Patient 49	49	25.09	23.13
50.	Patient 50	48	24.97	23.09
51.	Control Sample	27	18.21	19.21

Table 3 : Ct Value of RYR3 (Target Gene) and GAPDH (Housekeeping gene)

S. No.	**Patients Name**	**Age (in years)**	**RYR3**	
			Average $^{\Delta\Delta}$Ct Value	**Expression Ratio**
1.	Patient 1	18	-1.79	3.458
2.	Patient 2	18	-2.06	4.170
3.	Patient 3	19	-1.65	3.138
4.	Patient 4	20	-1.91	3.758
5.	Patient 5	21	-1.33	2.514
6.	Patient 6	23	-2.11	4.317
7.	Patient 7	23	-0.85	1.803
8.	Patient 8	24	-2.14	4.408
9.	Patient 9	25	-2.80	6.964
10.	Patient 10	26	-1.25	2.378
11.	Patient 11	27	-1.27	2.412
12.	Patient 12	27	-2.24	4.724
13.	Patient 13	28	-1.97	3.918
14.	Patient 14	23	-1.23	2.346
15.	Patient 15	25	-0.99	1.986
16.	Patient 16	21	-1.32	2.497
17.	Patient 17	26	-1.37	2.585
18.	Patient 18	23	-1.17	2.250
19.	Patient 19	28	-1.74	3.340
20.	Patient 20	29	-1.22	2.329
21.	Patient 21	24	-1.66	3.160
22.	Patient 22	25	-2.54	5.816
23.	Patient 23	30	-2.01	4.028
24.	Patient 24	34	-2.18	4.532
25.	Patient 25	31	-1.11	2.158
26.	Patient 26	37	-1.55	2.928

27.	Patient 27	33	-1.12	2.173
28.	Patient 28	32	3.54	1.444
29.	Patient 29	30	-1.54	2.908
30.	Patient 30	38	-1.67	3.182
31.	Patient 31	32	-1.11	2.158
32.	Patient 32	39	-1.68	3.204
33.	Patient 33	30	-2.45	5.464
34.	Patient 34	31	-2.05	4.141
35.	Patient 35	34	-1.99	3.972
36.	Patient 36	40	-1.55	2.928
37.	Patient 37	43	-2.28	4.857
38.	Patient 38	45	-2.15	4.438
39.	Patient 39	41	-1.21	2.313
40.	Patient 40	42	-1.61	3.053
41.	Patient 41	49	-1.55	2.928
42.	Patient 42	42	-0.43	1.347
43.	Patient 43	40	-1.81	3.506
44.	Patient 44	42	-1.92	3.784
45.	Patient 45	41	-1.42	2.676
46.	Patient 46	40	-1.29	2.445
47.	Patient 47	45	-2.13	4.377
48.	Patient 48	47	-0.98	1.972
49.	Patient 49	49	-1.96	3.891
50.	Patient 50	48	-1.88	3.681
51.	Control Sample	29	1.00	0.500

The statistical calculation was determined to find out the standard deviation observed in the expression level of the RYR3 gene (Table 4).

Table No.4: RYR3 $^{\Delta\Delta}$Ct Value and Gene Expression Ratio

Patients with Age (in years) was grouped	**RYR3**	
	Mean $^{\Delta\Delta}$Ct Value	**Expression Ratio**
18-20	-1.8525	3.631
21-29	-1.6222	3.3193
30-39	-1.3016	3.2532
40-49	-1.6113	3.2137
Control	**1.00**	**0.500**

DISCUSSION

The behaviour, interests, appearance, expression, or identity of individuals who do not conform to culturally defined norms expected of their natal gender is referred to as gender variance (Coleman et al., 2012). According to the American Psychiatric Association (APA), 2000, transsexualism can be diagnosed in individuals who have a strong and persistent cross-gender identification as well as persistent discomfort with their biologic sex or the gender role of that sex (Lewis, G. 1996). Persistent discomfort with one's gender and a desire to acquire sexual characteristics of the opposite gender are the distinctive characteristics that make GD unique (Hatami, M., & Ayvazi, S., 2013). Gender identity probably reflects a complicated interaction of biological, cultural, and environmental factors (Rosenthal, S. M., 2014; Hidalgo, M. A. et al., 2013). The term "transgender" refers to people who

have declared a gender identity that is distinct from their actual sex identity. People who identify as transgender have gender identities, behaviours, or expressions that cross or transcend culturally defined gender categories. FTM refers to assigned female individuals who self-identify as males. MTF stands for male assignees who identify as female (Coleman, E. et al., 2012). The profile and prevalence of gender variant behaviour and GD remain largely unknown due to a lack of data from the Indian population. GD is interesting, with an expected overall lifetime commonness of 1:12,900–1:35,000 for MTFs and from 1:33,000–1:00,000 for FTMs. A few international studies that looked at gender differences in behaviour found that 2 to 4 percent of boys and 5 to 10 percent of girls occasionally acted differently. As a result, some previous studies suggest that FTMs outnumber MTFs in both the adult and child populations. However, our study subjects with MTF produced contradictory outcomes: a 3.05:1 ratio of FTM. According to Karla, India is home to between 5 and 6 million eunuchs. Eunuchs are merely MTF transsexuals (Kalra, S. 2012). In India, the rising number of MTF transsexuals reflects the perception of a male-dominated society in which female counterparts (FTM) are unable to effectively assert themselves or present themselves for professional guidance and management (Sanyal, D., & Majumder, A. 2016). A significant portion (16%) of our GD subjects had previously undergone castration or mastectomy, even by nonmedically qualified individuals, due to the delay in endocrine consultation. 16.67% of our MTF subjects had undergone prior castration, with nonmedical individuals performing two-thirds of the castrations (Kalra, S. 2012).

According to the author, Indian eunuchs frequently visit an endocrinologist after undergoing crude surgery, such as an orchidectomy or a partial or complete penectomy (St. Peter et al., 2012; McGovern, S. J., 1995). The major obstacles are hidden social, psychological, and psychiatric conditions, the out-of-pocket cost of an elective orchiectomy, a lack of access to a surgeon willing to perform such operations, long waiting times for sex reassignment surgery, and other factors. Because of these logical ramifications, self-castration may be justified if nonmedical castration is preferred in a developing nation like India (St. Peter et al., 2012; McGovern, S. J., 1995). The average age of presentation for GD patients in our study was 25.66 ± 6.5 years. This was a super postponed endocrine conference considering the middle time of the beginning of GD was 9 to 10 years, going from 9 to 11 years. From a cultural point of view, India is still very conservative, which means that people there deny their sexual orientation and also express it. Because of widespread prejudice, it can be challenging for Indian transgender people to express their sexual identity to family and society, resulting in delayed disclosure (Elischberger et al., 2018). Gender identity, gender non-conforming, gender creative, transgender, and other related terms A strong and persistent identification with the opposite sex and discomfort with one's own sex are hallmarks of GID or GD (Sanyal and Majumder, 2016).

CONCLUSION

The biological basis for transgender identity is unknown, but there does appear to be a genetic component, especially in the brain during development. The RYR3 gene has been observed to be decreased in transgender blood samples. Alteration in RYR3 expression may be one of the genetic components and reasons for the biological basis for transsexuality during brain development, with reference to calcium release. Our results and observations suggest that an alteration in RYR3 expression is a reliable signal for transsexual activity in transgender people. To our knowledge, this is the first study to date of gender dysphoria conducted; the RYR3 gene was examined by RT-PCR.

Ethical Approval

The study was approved by the Mahatma Gandhi Medical College and Research Institute (decision number: MGMCRI/RAC/2021/02/QHEC/60). The consent forms are collected from informed consents for the use of human biological samples, such as blood.

Consent to participate

Our manuscript does not contain any data from any individual person. Hence, this is not applicable.

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Competing interests

The author have no conflicts of interest to declare and publishing the article.

Availability of data and materials

The data and supplement materials will be shared based upon reasonable request.

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