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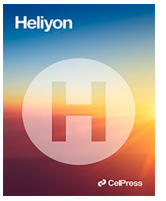

Research article

Rare *CRHR2* and *GRM8* variants identified as candidate factors associated with eating disorders in Japanese patients by whole exome sequencing

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ABSTRACT

Eating disorders (EDs) are a type of psychiatric disorder characterized by pathological eating and related behavior and considered to be highly heritable. The purpose of this study was to explore rare variants expected to display biological functions associated with the etiology of EDs. We performed whole exome sequencing (WES) of affected sib-pairs corresponding to disease subtype through their lifetime and their parents. From those results, rare single nucleotide variants (SNVs) concordant with sib-pairs were extracted and estimated to be most deleterious in the examined families. Two non-synonymous SNVs located on corticotropin-releasing hormone receptor 2 (*CRHR2*) and glutamate metabotropic receptor 8 (*GRM8*) were identified as candidate disease susceptibility factors. The SNV of *CRHR2* was included within the cholesterol binding motif of the transmembrane helix region, while the SNV of *GRM8* was found to contribute to hydrogen bonds for an α-helix structure. CRHR2 plays important roles in the serotoninergic system of dorsal raphe nuclei, which is involved with feeding and stress-coping behavior, whereas GRM8 modulates glutamatergic neurotransmission. Moreover, GRM8 modulates glutamatergic neurotransmission, and is also considered to have effects on dopaminergic and adrenergic neurotransmission. Thus, identification of rare and deleterious variants in this study is expected to increase understanding and treatment of affected individuals. Further investigation regarding the biological function of these variants may provide an opportunity to elucidate the pathogenesis of EDs.

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1. Introduction

Eating behavior can be severely affected by eating disorders (EDs), including anorexia nervosa (AN) and bulimia nervosa (BN). The main manifestations of AN are dietary restriction, fasting, excessive exercise, or other weight control or loss behaviors (e.g., self-induced vomiting, laxative abuse) as resistance to maintenance of normal body weight. On the other hand, BN is characterized by repeated binge eating and inadequate compensatory behaviors (e.g., fasting, extreme exercise, purging) to avoid weight gain. Manifestations shared by these disorders include aspiration for weight loss, fear of weight gain, and self-evaluation unduly influenced by body shape and weight. The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) provides diagnostic criteria for a subgroup of AN defined as restricting type (AN-R), which include severe dietary restriction without regular binge eating and/or purging behaviors, as well as binge-eating/purging type (AN-BP) with regular binge eating and/or purging behaviors.

A study conducted in the United States estimated lifetime prevalence of AN and BN in women to be 0.9% and 1.5%, respectively, and 0.3% and 0.5%, respectively, in men [1]. Among psychiatric disorders, female-biased sex-differences in the onset of EDs are most often seen. The mortality rate for patients with AN is greater than that of any psychiatric disorder [2]. A meta-analysis of 36 studies indicated that weighted annual mortality rates for AN and BN were 5.10 and 1.74 deaths, respectively, per 1000 person-years [3]. Furthermore, it was found that one in five individuals with AN who died had committed suicide [3]. In a study conducted in Sweden, the peak age ranges for AN incidence in females and males were 14–15 and 12–13 years, respectively, while those for other EDs in the same cohort were 16–17 and 18–19 years, respectively [4]. Although these data were mainly derived from Caucasian, the lifetime prevalence and the peak age ranges of EDs in Japanese were similar to them [5].

A study of twins resulted in heritability estimates for both AN and BN of approximately 0.5 [6], while a familial study noted relative risks in female relatives of anorexic and bulimic probands of 11.3 and 12.3, respectively [7]. Moreover, other twin and familial studies demonstrated that genetic predisposition to AN and BN is shared, at least in part [7–9]. On the other hand, environmental factors such as stress, culture and early adverse experiences, may also contribute to a significant proportion of ED cases, and the interpretation of genetic findings should also take those factors into account.

In general, common disease susceptibility variants have a very low contribution to a disease per single locus, while they have a high frequency in the general population (common variant) or a high contribution to EDs, and are very rare (rare variant), except for variants located within the human leukocyte antigen (HLA) genomic region, associated with late onset, and undergone positive selection [10]. Common variants have been explored using genome-wide association study (GWAS) with very large populations, whereas rare variants have been detected by sequencing patient families. A recent GWAS for AN in European identified eight significant common variants [11]. However, that study captured only 1.7% of the phenotypic variance, thus additional subjects would be required for future replication studies [11]. On the other hand, a sequencing study with linkage analysis was performed using two large families

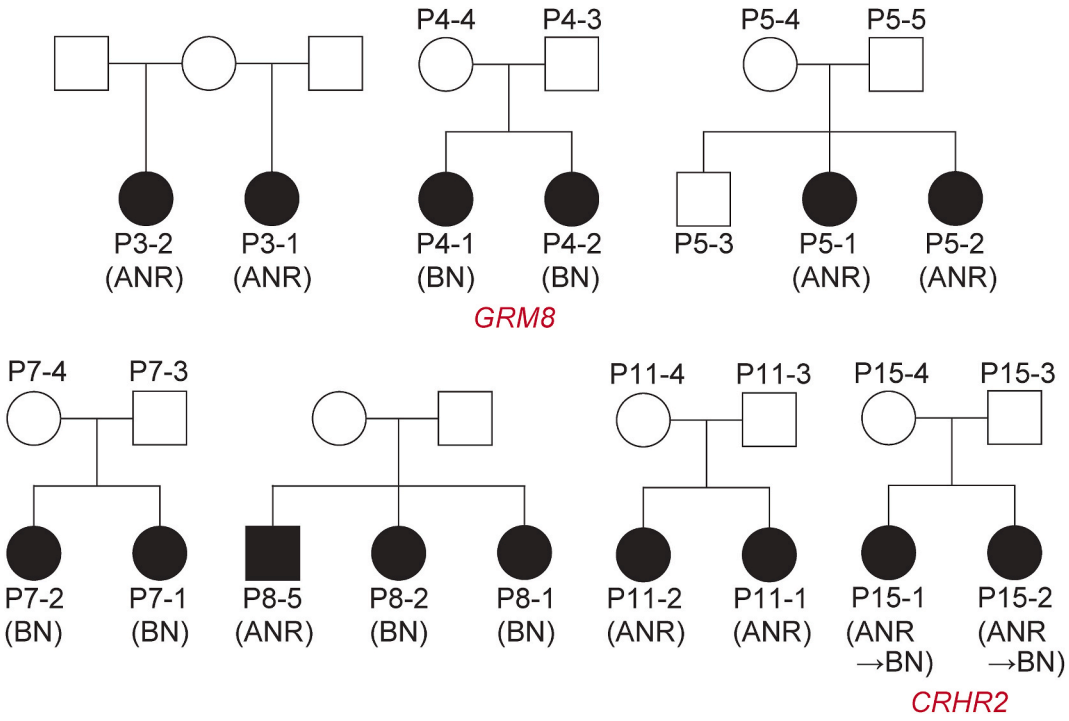

Fig. 1. Sib-pair families affected by EDs were examined using whole exome sequencing. Individuals labeled with an ID number were sequenced. The disease phenotype of affected sib-pairs in the P15 family was transferred from ANR to BN. The rare variants *GRM8* and *CRHR2* were observed in the P4 and P15 families, respectively. Abbreviations: ANR, anorexia nervosa restricting type BN, bulimia nervosa.

with AN and BN in USA, and those findings suggested that each rare missense variant in estrogen-related receptor α (*ESRRA*) and histone deacetylase 4 (*HDAC4*) is associated with EDs [9]. *ESRRA* null mice also display behavioral deficits relevant to EDs such as AN [12]. Moreover, *HDAC4*^{A778T} mice carrying the missense variant demonstrate several ED-related feeding and behavioral deficits in a gender-dependent manner [13,14]. Furthermore, an exome sequencing study using affected sib-pairs in French identified a deleterious variant of *CCKAR* regulating food intake and energy [15], and candidate sequencing studies also identified deleterious variants of *SLC6A4* encoding a serotonin transporter in Mexican patients [16] and *NNAT* regulating food intake in Italian patients [17]. Thus, it is important to explore deleterious variants of patients beyond ethnic group for understanding the pathogenesis of EDs.

Swedish twin study for EDs demonstrated that concordance rates in monozygotic twins were higher than dizygotic twins within the same disease subtype, on the other hand, the rates were similar in the different subtype [8]. Therefore, in the current study, we selected seven affected sib-pairs corresponding to disease subtype for whole exome sequencing (WES) to investigate rare variants functionally related to the pathogenesis of EDs. However, the statistical power was quite insufficient to perform genetic association and linkage analysis. Therefore, we explored the most deleterious coding variants shared with sib-pairs in each family.

2. Materials and methods

2.1. Patients and families

Upon approval of the experimental procedures from the relevant ethical committees at the National Institute of Mental Health, National Center of Neurology and Psychiatry (number: A2013-054) and Tokai University (number: 14I-60), we obtained written informed consent in accordance with the Declaration of Helsinki from 30 sib pairs and their family members prior to collection of DNA samples. Among all of the subjects, 7 sib pairs showed correspondence to disease subtype throughout their lifetime, thus findings from those 7 sib pairs were used for sequencing to reduce sample heterogeneity and elevate the likelihood of identification of susceptibility variants [18], though family P8 included a brother who displayed a different subtype (Fig. 1 and Supplementary Table 1). There were 15 individuals affected with EDs and 11 unaffected individuals, all of Japanese origin. They were also three ANR and three BN families, and one family displayed a diagnostic crossover from ANR to BN. Each of these cases was diagnosed according to the DSM-IV criteria used in Japan. DSV-IV eating disorder diagnosis was determined by an expert psychosomatic doctor or psychiatrist with expertise regarding eating disorders. All had been diagnosed before the DSM-V diagnostic criteria were established, thus the DSM-IV criteria were applied for present study. DNA was extracted using a QIAamp DNA blood kit (QIAGEN, Hilden, Germany).

2.2. Genomic library construction and sequencing

For exon fragment capture and sequencing, an Agilent SureSelect Target Enrichment, v. 5 (50 Mbp), was used. Sequence analysis was performed using an Illumina Genome Analyzer IIx, HiSeq2000, or HiSeq2500 platform.

2.3. Sequencing data analysis

Reads that passed quality control were mapped to the reference genome (UCSC Genome Browser assembly GRCh37/hg19, http://genome.ucsc.edu/) with a Burrows-Wheeler Aligner, v. 0.5.9. Potential PCR duplicates were flagged with Picard MarkDuplicates, v. 1.88 (http://picard.sourceforge.net/). Genome Analysis Toolkit v. 2.2–8, was used to perform map quality score recalibration and variant detection. Single nucleotide variants (SNVs) and indels were then annotated for functional consequences at the gene using the ANNOVAR. Predictions of variants with risk were also performed using ANNOVAR, with non-synonymous variants (LJB, v. 3.0) employed for the effects on protein function (SIFT, PolyPhen, MutationTaster, Muration Assesor, LRT, FATHMM) and evolutional conservation (GERP++, phyloP, Siphy). We used all nine methods reported by Liu et al. [19] and mainly applied the criteria described by Fu et al. [20]. Criteria used for deleterious variants in each prediction are shown following:

SIFT (sift): D: deleterious, PolyPhen 2 HDIV: D: Probably damaging, LRT: D: Deleterious, MutationTaster: A (disease_causing_automatic) or D (disease_causing), MutationAssessor: H: high.

FATHMM: D: deleterious, GERP++ >5, phyloP >2.0, SiPhy >15.

2.4. Confirmation of SNVs

For PCR and sequencing (Greiner Bio-one) of the SNV of *CRHR2*, forward (CCTGGCAGGGGGAGAAGAGC) and reverse (CCCCAAGCTGCCTCCTGACA) primers, and for the SNV of *GRM8*, forward (GCAACTCCAAGTCATCCATTTTCTTCA) and reverse (TGGAGCGAATTGCTCGGGATT) primers, were used. Sequencing reactions were carried out using a BigDye Terminator Cycle Sequencing kit, v. 3.1 (Thermo Fisher Scientific). Automated electrophoresis was performed with an ABI PRISM 3730 Genetic Analyzer (Thermo Fisher Scientific).

3. Results

3.1. WES

We obtained rare 4032 SNVs, 113 splice site variants and 76 insertion/deletion variants (allele frequency <0.001) from a total of

633,030 variants (Fig. 2). In general, thresholds of 0.01 and 0.05 are commonly applied for common complex traits, though even lower allele frequency (e.g., 0.001 or 0.0005) has been used for investigation of rare Mendelian diseases [21]. In the present study, a threshold of 0.001 was applied because rarer mutations are likely to be more deleterious, though EDs are not rare. Initially, we attempted to identify recessive and compound variants concordant with the affected sib-pairs, however none were found among these rare variants. Next, heterozygote variants concordant with affected sib-pairs were extracted as dominant candidates. Finally, we estimated the most deleterious SNV in each family by use of six functional prediction (SIFT, PolyPhen, MutationTaster, Muration Assesor, LRT, FATHMM) and three conservation-based (GERP++, phyloP, Siphy) methods [19].

A previous large exome sequencing study indicated a strong inverse correlation between average SNV age and number of methods utilized in our current study for the prediction of deleterious variants. Based on results of multiple methods, it is considered that SNVs predicted to be deleterious are younger age and very rare in general populations [20], and suggested as candidates for disease pathogenesis. Moreover, most of the very-rare variants have arisen within the past 5–10,000 years and are population-specific [22]. Therefore, 381 SNVs and 4 *de novo* SNVs were evaluated (Fig. 2, Supplementary Table 2) based on the number of methods that predicted a variant to be deleterious and a feature of the gene. Among those, only two were predicted to be deleterious by all nine methods, an SNV in corticotropin-releasing hormone receptor 2 (*CRHR2*) noted in family P15 and an SNV in glutamate metabotropic receptor 8 (*GRM8*) noted in family P4 (Table 1 and Figs. 1–2). These were considered likely to be specific for a Japanese population, though a single allele for *CRHR2* was previously observed in Europeans (Table 1). In addition, SNVs supported by eight of the methods were observed in the P7, P8, and P11 families, while those supported by seven of the methods were observed in the P3 and P5 families. Among the 10 genes, *CRHR2, GRM8*, and glutamate ionotropic receptor α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) type subunit 2 (*GRIA2*) were indicated to have higher levels of expression in the brain and related tissues (Table 1).

Four *de novo*, five splice site SNVs, and five insertion and deletion variants concordant with affected sib-pairs were identified as heterozygotes (Supplementary Tables 3–5). However, in consideration of their features, these fourteen genes are unlikely to be causative factors of EDs.

3.2. Evaluation of function of CRHR2, GRM8 and GRIA2 as causative factors for EDs

CRHR2 encodes a neuropeptide receptor for corticotropin-releasing factor (CRF), a critical coordinator of the hypothalamic-pituitary-adrenal axis [23] and for three urocortins (UCN1–UCN3) [24]. CRHR2 is also a specific receptor for UCN2 and 3, which are associated with regulation of stress, anxiety, and food intake [25–28]. *GRM8* (family P4) encodes a G-protein coupled metabotropic glutamate receptor that influences inhibition of the cyclic AMP cascade and regulates presynaptic glutamate release [29], and also has been found to have genetic associations with psychiatric phenotypes, including depression [30,31] and eating behavior [29]. The two variants were also confirmed by Sanger sequencing (Supplementary Fig. 1). Thus, it is considered that *CRHR2* and *GRM8* are potent candidate genes due to the pathogenesis of EDs in individuals included in this study. *GRIA2* has a significant role in excitatory neurotransmission [32], and has been identified as a causative gene of intellectual disability and developmental epileptic encephalopathy. Therefore, we considered that *GRIA2* was a weaker candidate gene for EDs in this study, as the phenotypes generated by mutations may not be concordant with EDs. Furthermore, among the 381 SNVs considered to be candidates for dominant, all other SNVs were thought to be unlikely as causative factors of EDs after considering the descriptions and locations of expression (**Table 1**).

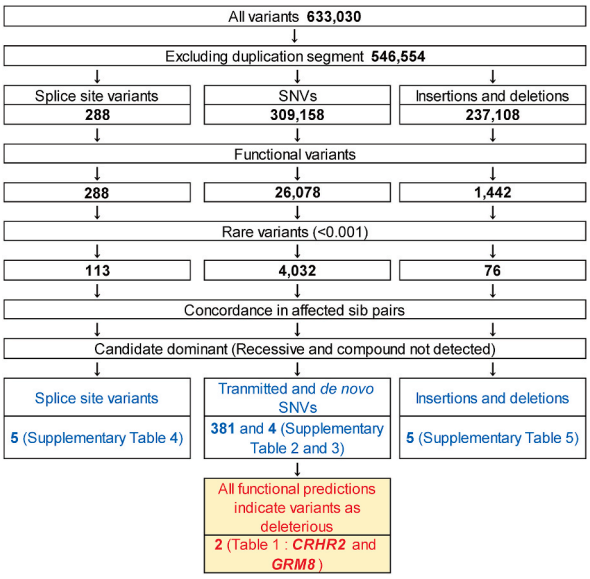

Fig. 2. Scheme for screening variants with whole exome sequencing.

Table 1
Most deleterious variants in each family estimated by functional prediction.

Gene symbol	Family	No. of methods[a]	Chr	Position[b]	Alt[c]	Substitution	SNV	Frequency		Gene description	Gene expression[f]
						Amino acid		Japanese[d]	European[e](non-Finnish)		
CRHR2	P15	9	7	30702316	T	p.G231R	rs569607645	0.000414	0.000009	Corticotropin-releasing hormone receptor 2	Pituitary, brain
GRM8	P4	9	7	126409942	C	p.Y445C	NA	0.000000	0.000000	Glutamate metabotropic receptor 8	Testis, brain
LGR4	P7	8	11	27390603	C	p.N556S	rs754383451	0.000000	0.000016	Leucine-rich repeat-containing G protein-coupled receptor 4	Ovary, pancreas
MYH7B		8	20	33583163	A	p.E951K	rs370896124	0.000535	0.000008	Myosin heavy chain 7B	Heart, skeletal muscle
POC1B		8	12	89866042	T	p.D155 N	NA	0.000826	0.000000	POC1 centriolar protein homolog B	Heart, testis
ACADS	P8	8	12	121176662	T	p.R325W	rs121908006	0.000000	0.000026	Acyl-CoA dehydrogenase, C-2 to C-3 short chain	Liver, skeletal muscle
ALAS1	P11	8	3	52236598	T	p.P92L	rs370171958	0.000826	0.000015	Aminolevulinate, delta-, synthase 1	Adrenal gland, brain
C3orf70	P3	7	3	184800841	A	p.S236L	rs201783027	0.000413	0.000031	Chromosome 3 open reading frame 70	Colon, artery
NRIP1			21	16338385	T	p.R710H	rs139218138	0.000000	0.000008	Nuclear receptor interacting protein 1	Cervix, adipose
GRIA2	P5	7	4	158253975	T	p.T296I	rs200845438	0.000400	0.000015	Glutamate receptor, ionotropic, AMPA 2	Brain, pituitary

Abbreviations: NA, not applicable.
[a] Maximum number of methods for predicting variant as deleterious in each family.
[b] Physical position in UCSC Genome Browser in Human Feb. 2009 Human GRCh37/hg19 Assembly.
[c] Allele observed in this study.
[d] Allele frequency in Japanese in Human Genetic Variation Database (https://www.hgvd.genome.med.kyoto-u.ac.jp/).
[e] Allele frequency in European gnomAD (https://gnomad.broadinstitute.org/).
[f] Top two tissues showing highest expression level in GTEx (release V8, 54 non-diseased tissue sites across nearly 1000 individuals).

3.3. Evaluation of impact of SNVs on CRHR2 and GRM8

CRHR2 is classified as a secretin subfamily among class B G protein-coupled receptors (GPCRs). A GPCR features seven transmembrane helices (TM), three extracellular loops, and three intracellular loops. The rare SNV in family P15 (rs569607645) was found located within the TM4 region. Gly 231, with a codon corresponding with the SNV conserved across species and among the class B GPCRs (Fig. 3), and located within a highly conserved sequence motif, GWGxP, in class B GPCRs [33]. Moreover, structural analysis by Ma et al. revealed that the motif indicated its indispensable role in cholesterol binding to TM4, which helps to stabilize conformational changes in the activated receptors [34]. Thus, the substitution converts hydrophobic (glycine) into hydrophilic (arginine) amino acid, implying that this SNV influences conformation and function of CRHR2. In fact, the previous report also demonstrated that mutations in the cholesterol-binding motifs reduced UCN affinity on CRHR2 [34].

Metabotropic glutamate (mGlu) receptors are members of the class C family of GPCRs that modulate cellular responses to the excitatory neurotransmitter L-glutamic acid (L-Glu) in many synapses in the CNS [35]. The amino-terminal domain (ATD) of mGlu receptors contains an orthosteric glutamate recognition site, which is highly conserved across the eight mGlu receptor subtypes [36]. The rare SNV in family P4 was located within an α-helix structure of the ATD region. Tyrosine 441, with a codon corresponding with the SNV, was found to be conserved across species and hydrogen bonds to Asp 422 in an adjacent α-helix structure (Supplementary Fig. 2) [35]. Therefore, the substitution of tyrosine to cysteine is likely to cancel the hydrogen bond. We also evaluated the effect of the SNV on protein stability using X-ray crystal structure data (PDB ID 6BSZ) and ENCoM, which are used to predict the effect of mutations on thermostability and dynamics, as well as to generate geometrically realistic conformational ensembles [37]. Those results indicated that the conformation of GRM8 was expected to be destabilized (−0.822 kcal/mol). Thus, the substitution may have an impact on the structure and functions of GRM8.

4. Discussion

4.1. Limitation of this study

The approach of current study narrowed down to two non-synonymous SNVs in *CRHR2 and GRM8* as candidate genes for EDs. However, this study only observed the deleterious SNVs in one family for each gene and was not able to indicate that the SNVs explained how much of the genetic variance of EDs. Furthermore, sample size in this study was too small. Therefore, these genes must be confirmed by genetic analysis for additional families and sporadic cases and by functional analysis using animals engineered to carry the variants in the future investigations.

4.2. CRHR2

Common SNVs in *CRHR2* were not detected in a large genome-wide association study for anorexia nervosa and other psychiatric disorders [11]. On the other hand, WES with bipolar disorder families identified a rare and functionally relevant nonsense variant

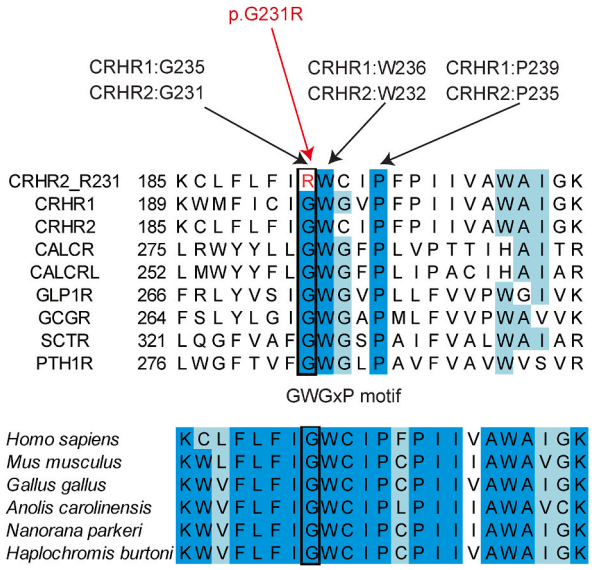

Fig. 3. Multiple amino acid sequence alignments of TM4 helix of human class B GPCRs and CRHR2 in various species. Upper alignment indicates TM4 of a selected set of human class B GPCRs included with the GWG x P motif involved in cholesterol binding. Lower alignment indicates evolutionarily conserved amino acids. The level of blue shading is shown according to sequence conservation. Black square indicates amino acid position corresponding to human G231.

within the intracellular C-terminal region of *CRHR2* [38]. Thus, it is suggested that a rare SNV in *CRHR2* contributes to the pathogenesis of a psychiatric disorder.

The CRHR2 variant was found to be rare in both European (1/10,000 individuals) and Japanese (1/1200) populations, and is expected to be highly deleterious, indicating that it arose within the most recent 10,000-years period [20]. It is thus considered that this variant was derived from different ancestors and independently arose in independent populations. On the other hand, it is also likely that this variant contributes to EDs in European and other populations due to its deleterious effects.

A study that used *Crhr2* knockout mice demonstrated that CRHR2 is essential for sustained feeding suppression induced by UCNs, members of the CRF family of peptides [39]. In another investigation, *Crhr2*-mutant mice showed decreased food intake following food deprivation [23]. Therefore, UCNs are potent for suppression of food intake by CRHR2-specific mediation [40]. Furthermore, *Crhr2*-mutant mice were also reported to have an anxiogenic phenotype and impaired stress recovery [24]. These observations are related to serotoninergic neurons. Serotonin (5-HT), which is a neurotransmitter in the brain, regulates dopamine and noradrenaline to stabilize mental state. Serotonin is also produced by serotoninergic neurons of the dorsal raphe nucleus (DRN) in the brain stem and regulates feeding behavior [41]. On the other hand, CRHR2 is specifically expressed in the DRN serotoninergic neuron [42] and plays important roles in controlling serotonergic neuronal activity [43]. Hammack et al. demonstrated that CRHR2 within the rat DRN mediates behavioral consequences of uncontrollable stress by experiments within the DRN using UCN2, an agonist for the highly selective CRHR2 [44]. Additionally, anxiety-related stimuli by UCN2 were shown to lead to increased activation of serotonergic neurons within the DRN [45]. The DRN projects to the bed nucleus of the stria terminalis, which plays essential roles in threat processing, and is responsible for such emotional states as fear and anxiety [46,47]. The tone of the DRN–5-HT system is regulated in a dynamic manner through CRHR2 activation, and either decreased or increased depending on the level of endogenous or exogenous ligands [48]. Moreover, *Crhr2* mRNA expression in the ventrolateral part of the DRN in subadult female rats was found to be10-fold greater than in males, which may underlie sex-related differences in response to stress-related situations beginning in adolescence [49].Indeed, in malnourished individuals suffering from AN the cerebrospinal fluid has reduced amounts of 5-hydroxyindoleacetic acid which is the major brain metabolite of 5-HT and is thought to reflect extracellular 5-HT concentrations, indicating that abnormal activity of 5-HT system is related to EDs pathogenesis [50]. Thus, CRHR2 mediates not only food intake, but also passive coping behavior and depression-like responses triggered by uncontrollable stress [24] via the serotoninergic neuron in the DRN. Giel et al. proposed that dysfunctional eating behaviors of ED patients represent an attempt to regulate emotions and stress [51]. Therefore, abnormalities in serotonergic neuronal systems caused by a variant of CRHR2 may affect the stress-coping behavior. Further, investigations of functions of *CRHR2* variants may provide a paradigm for understanding ED pathogenesis factors and novel biomarkers as well as potential therapeutic agents.

Both of the variants *CRHR2* and *GRM8* in the affected sib-pairs examined in the present study were transmitted from their healthy fathers (Fig. 1, Supplementary Fig. 1). The DRN expressing *Crhr2* in the brainstem has been found to display a female-biased sexual dimorphism in rats [49], implying that CRHR2 of DRN in humans also has female-specific functions. Therefore, such sexual dimorphism may explain incomplete penetrance for the variant of *CRHR2* in the autosomal dominant inheritance findings noted in the present study, though the function of GRM8 remains unclear.

In rodents, female-biased sex differences in the nucleus are produced by estrogen during adolescence. The previously reported linkage analysis identified a rare missense variant of the *ESRRA* in an EDs pedigree [9], suggesting that this variant may also contribute to the function of CRHR2 via the serotonin neuron in the pathogenesis of EDs.

Structural analysis demonstrated that the motif included with rs569607645 is a cholesterol-binding site, and that artificial mutations in that site reduce the activation potency of UCN1 and CRF to CRHR2, suggesting that the site plays a role in stabilizing peptide binding [34]. Moreover, bound cholesterols in the structure of CRHR2 contribute to GPCR signaling, indicating that the GPCR signalosome carries out its function in the cholesterol-rich lipid raft [34]. Thus, the substitution of G231R provided by the rare SNV rs569607645 impairs the functions of CRHR2, which may decrease the coping mechanism in response to stress [24] and/or feed suppression [40] in patients with EDs.

ED patients have higher rates of anxiety disorders as compared with normal individuals, suggesting that both disorders might share common etiological factors, which can increase the susceptibility of an individual to either [52]. Stress is prominently involved in the pathogenesis and development of EDs and anxiety disorders. Moreover, coping mechanisms such as a positive attitude, planning, and social support seem to be impaired in ED patients who often do not cope well with emotional distress, implicating that such mechanisms may be disrupted [53]. Therefore, a decay of CRHR2 functions may play a part in the pathogenesis of EDs.

It has been shown that children and adolescents who have experienced adverse childhood experiences (ACEs), particularly household challenges, have greater possibility of developing EDs [54]. Therefore, heritability predicted by epidemiological data should be lower than true heritability. On the other hand, a previous study indicated that social instability stress during adolescence has impact on the function of the paraventricular nucleus (PVN) of the hypothalamus involved in eating behavior and stress response in female rats [55]. Giel et al. proposed that ACEs in early life might modulate the stress response system, and that dysfunctional eating behavior seen in patients with EDs might represent an attempt to regulate emotions and stress [51]. Furthermore, Lukkes et al. suggested that CRHR2 may underlie female-biased sex-differences in the emergence of stress-related psychiatric disorders [49]. Therefore, we consider that a predisposing factor for EDs is the female-biased sexually dimorphic nucleus including the serotonin neuron with CRHR2, and that any stress such as ACEs have structural influence on the nucleus, leading to dysfunctional eating behavior. As a result, the impact on serotonin neurons may be primarily derived from environment factors and only slightly from genetic factors. However, the significance of the present study is the link of EDs to CRHR2, a candidate major factor in the pathogenesis.

4.3. GRM8

As mentioned above, associations between psychiatric disorders and *GRM8* has been indicated with strong evidence in previous reports. On the other hand, a sequencing study for bipolar disorder identified enrichment of damaging mutations in GPCRs including *CRHR2* and the *GRM* gene family, with increased numbers of deleterious variants [38].

The mGlu receptors modulate glutamatergic neurotransmission and are considered to also have effects on dopaminergic and adrenergic neurotransmission, implicating their involvement in a number of neurological disorders [56]. An expression study using animals indicated that GRM8 may play a role in feeding behavior and metabolism via the hypothalamic pathway [29,57].

5. Conclusions

We found two rare SNVs as candidates for predisposition to EDs in affected Japanese sib-pair families. One SNV was found in the coding sequence of the *CRHR2* gene, which plays important roles in the serotoninergic system in the DRN and is involved with feeding and stress-coping behavior. The second SNV is located in the *GRM8* gene, which modulates cellular responses to the excitatory neurotransmitter in the CNS, and may play a role in feeding behavior and metabolism via the hypothalamic pathway. Moreover, the present findings indicate potential for both non-synonymous SNVs to have impact on conformation and functions of molecules. Notably, CRHR2 function as a receptor for UCNs expressed in the DRN which indicates female-biased sexual dimorphism during adolescent development, suggesting a functional relationship of CRHR2 with EDs. Moreover, the rarer variant of CRHR2 observed in European subjects may also be an ED risk factor because of possible deleterious effects. However, this study only observed the deleterious SNVs in one family for each gene and was not able to explain most of the genetic variance of EDs. Furthermore, it is impossible to rule out all candidate variants. Therefore, these genes must be confirmed by genetic analysis for additional families and sporadic cases. Further investigations of the molecules identified in this study should lead to greater elucidation of the pathogenesis of EDs as well as other psychiatric disorders.

Data accessibility statement

Data obtained in this study are available from the authors upon reasonable request.

CRediT authorship contribution statement

Akira Oka: Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Data curation, Conceptualization. **Shinji Hadano:** Writing – review & editing, Writing – original draft. **Mahoko Takahashi Ueda:** Formal analysis, Data curation. **So Nakagawa:** Software, Formal analysis, Data curation. **Gen Komaki:** Resources, Conceptualization. **Tetsuya Ando:** Writing – original draft, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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