A Nonsynonymous Polymorphism in Cannabinoid CB2 Receptor Gene is Associated With Eating Disorders in Humans and Food Intake is Modified in Mice by its Ligands

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Summary by Ellery Schlingmann, 4th year MCDB Major

Cannabis constituents activate cannabinoid receptors to produce multiple behavioral effects involved in addiction, mood, and importantly, appetite. Cannabinoid receptors (CB-Rs) are part of the endocannabinoid system and can be broken down into two subtypes: CB1 receptors, which are expressed in the brain and peripheral nervous system and have targets that confer motor activity, thinking, pain perception, and appetite enhancement, among others; and CB2 receptors, which are dominantly expressed in immune cells of the peripheral nervous system. Previous research has shown that stimulation of CB1 receptors stimulates appetite while the role of CB2 receptors in this behavior remains unclear. The goal of this was to better understand possible roles of CB2 receptors in appetite and related diagnoses, more specifically, how the gene that encodes CB2 receptors, CNR2, contributes to eating behavior and appetite.

Many eating disorders, such as anorexia nervosa and bulimia nervosa, manifest in behaviors associated with food restriction. This human DNA study was conducted among 2080 individuals in the Japanese population to understand associations between CNR2 polymorphisms and eating disorder diagnosis. Eating disorder diagnosis was based upon DSM-IIIR criteria and affected 204 of the study participants. The genotypes of the study participants were determined by restricted fragment length polymorphism (RFLP) following polymerase chain reaction (PCR). The authors found that the R63Q polymorphism in the CNR2 gene was significantly more prevalent in patients with eating disorders [P = 0.04, odd ratio 1.24; 95% CI, (1.01-1.53)] compared to the 1876 controls. This nonsynonymous polymorphism, meaning a change in the amino acid sequence of a protein, displays some correlation with eating disorder diagnosis.

In order to further understand the role of the CB1 and CB2 receptors in appetite and disordered eating, the researchers conducted additional experiments in mice to observe food intake behaviors following the administration of a CB2 receptor agonist, antagonist, or CB1 receptor antagonist. The CB2 receptor agonist and CB1 receptor antagonist suppressed food intake in both time- and strain-dependent manners during a period of unrestricted food access following a 12-hr fast. The only exception was that the CB2 receptor antagonist stimulated food intake in the fasted mice.

The results summarized here point towards connections between the endocannabinoid system. eating disorders, and food consumption. More specifically, that CNR2 variation is associated with eating disorders in a human population. The preclinical (rodent) study also reveals

interesting association between CB2 receptors and food intake. While CB1 receptors have been widely studied and shown to be associated with obesity and eating disorders, CB2 receptors were largely unstudied before this investigation. The discoveries made by this research team are promising given that pharmacological treatments for eating disorders have been largely unsuccessful. This work provides new targets for treating patients. Seeing that the CB2 antagonist stimulated appetite in the mice, these many be used in medications to treat those with eating disorders or who lack an appetite. However, before drastic steps are taken, more studies are required to examine the effects of CB2 receptor ligands on depression and suicide risk, in order to ensure that potential medications do not put patients at higher risk for these conditions.

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A Nonsynonymous Polymorphism in Cannabinoid CB2 Receptor Gene is Associated With Eating Disorders in Humans and Food Intake is Modified in Mice by its Ligands

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ABSTRACT Marijuana use activates cannabinoid receptors (CB-Rs) producing several behavioral effects related to addiction, mood, and appetite. We investigated the association between *CNR2* gene, which encodes cannabinoid CB2 receptor (CB2-R) and eating disorders in 204 subjects with eating disorders and 1876 healthy volunteers in Japanese population. The effect of treatment with CB2-R ligands on mouse food consumption was also determined. The CB2-R ligands used suppressed food intake in a time- and strain-dependent manner when food was available ad libitum and during the 12-h fast except, AM 630—the CB2-R antagonist that stimulated food consumption in food-deprived mice. There is an association between the R63Q polymorphism of the *CNR2* gene and eating disorders ($P = 0.04$; Odds ratio 1.24, 95% CI, (1.01–1.53). These results suggest that cannabinoid CB2-R is involved in the endocannabinoid signaling mechanisms associated with the regulation of food intake and in eating disorders. **Synapse 64:92–96, 2010.**

INTRODUCTION

Eating disorders including anorexia nervosa, bulimia nervosa, and atypical eating disorders have some features in common associated with restriction of food intake resulting in loss in body weight (Fairburn and Harrison, 2003). On the other hand worldwide obesity epidemic is a chronic complex disease of multifactorial origin, resulting in excessive body fat, in which both genetic and environmental factors are involved (Costa, 2007; Cota, 2008; Di Marzo and Matias, 2005; Lillo, 2007; Monteleone et al., 2005; Robson, 2005; Siegfried et al., 2004). Eating disorders have high rates of comorbidity (Di Marzo and Matias, 2005), particularly with substance use disorders. Although the majority of studies in this area have focused on alcohol use (Costa, 2007; Cota, 2008; Lillo, 2007; Monteleone et al., 2005; Robson, 2005; Siegfried et al., 2004), some have described the degree of use of illicit drugs as well (Herzog et al., 2006). Furthermore, eating disorders and obesity in particular are global health problems that are partly associated with disruption of endocannabinoid signaling mechanisms involved in appetite control (Costa, 2007; Cota, 2008; Di Marzo and Matias, 2005; Monteleone et al., 2005; Siegfried et al., 2004). The endocannabinoid system consists of (1) cannabinoid receptors (CB-Rs), (2) endogenous compounds termed endocannabinoids (eCBs) that activate these receptors, and (3) enzymes that synthesize and degrade the eCBs. Marijuana use, administration of synthetic cannabinoids or

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endocannabinoids activates cannabinoid receptors (CB-Rs) and stimulates appetite (Beal et al., 1995, 1997; Fride et al., 2005; Nauck and Klaschik, 2004; Robson, 2005; Siegfried et al., 2004; Strasser et al., 2006). This is the basis for the use of marijuana or synthetic formulations of the active ingredient, Δ^9-THC (dronabinol) for the treatment of anorexia associated weight loss in patients with AIDS, (Beal et al., 1995, 1997) and cancer-related anorexia-cachexia syndrome (Nauck and Klaschik, 2004; Strasser et al., 2006). Although several classes of drugs including cannabinoids, (Corey, 2005; Rockwell et al., 1984; Volicer et al., 1997) have been tried as therapy for anorexia nervosa, to date no medication, alone or in combination with other therapies, has been demonstrated to be effective in the treatment of the primary disorder (Israel, 2005). However, anorexia, marked by decreased food intake has been reported as one of the withdrawal effects in over 50% of individuals who smoke marijuana repeatedly through out the day, 6–7 days per week in laboratory and clinical studies (Haney et al., 2008; Horcajadas, 2007). In one study, Haney et al., (2008) reported that a combination of THC and lofexidine, a centrally acting α_2-agonist, produced the most robust improvements in marijuana withdrawal effects relative to either medication alone(Haney et al., 2008; Horcajadas, 2007). While a number of eating disorders including anorexia nervosa and bulimia nervosa occur most commonly in young women often beginning around the age of puberty, and much less frequent in men (Fairburn and Harrison, 2003), anorexia of aging in long term care has also been reported and examined (Gelegen et al., 2007; Wilson et al., 2007). Subjects with anorexia of aging gained weight with dronabinol; a synthethetic cannabinoid agonist during a 12-week treatment period. Oral cannabinoid-containing medications was reported to be effective for the management of interferon and ribavirin-induced anorexia, nausea, and weight loss in patients treated for chronic hepatitis C virus (Costiniuk et al., 2008). Cannabinoids, endocannabinoids and marijuana use activate two well characterized cannabinoid receptors; CB1-Rs and CB2-Rs. The expression of CB1-Rs in the brain and periphery has also been well studied, but CB2-Rs have received much less attention than CB1-Rs. CB2-Rs were previously thought to be predominantly expressed in immune cells in the periphery and were traditionally referred to as peripheral CB-Rs. We and others have now demonstrated the presence of CB2-Rs in neuronal, glial, and endothelial cells in the brain (Brusco et al., 2008; Gong et al., 2006; Onaivi et al., 2008; Van Sickle et al., 2005), and this warrants a reevaluation of the CNS effects of CB2-Rs. However, many features of CB2-R gene structure, variants, and regulation remain poorly characterized compared to the CB1-R. In view of our finding of the expression and regulation of *CNR2* gene in the mammalian brain, we tested the hypothesis that genetic variants of *CNR2* gene might have significant effect in eating disorders. Therefore, we examined a possible role of CB2-R ligands on mouse food consumption and an association between a non-synonymous polymorphism, Q63R in the *CNR2* gene and eating disorders in Japanese population.

MATERIALS AND METHODS

Human DNA subjects and genotyping

About 204 patients with eating disorders (composed of 94 anorexia nervosa and 111 bulimia nervosa), age 25.0 ± 7.0, including 7 males and 197 females were research volunteers from mid-north main island area with diagnosis based on DSM-IIIR criteria without other psychiatric diagnoses. We used large Japanese population set of 1876 healthy controls for association analysis, to ascertain statistical power of detection. Mean age and ratio of male in the control population were higher in this study. The unscreened but gender-matched control group were unrelated healthy Japanese (age 46 + 12.9 years) and had no known history of psychiatric illness. We obtained informed consent for the genetic study from all volunteers. The genotype was determined by restricted fragment length polymorphism (RFLP) method after polymerase chain reaction (PCR), as described in our previous report (Ishiguro et al., 2007). The study was approved by the ethics committee of the University of Tsukuba.

Chemicals used

The following cannabinoid ligands were used. *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM 251), 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl) methanone (AM 630) and Palmitoylethanolamide (PEA) all obtained from Tocris Biosciences Missouri, USA.

Subjects in food consumption tests

Three strains of mice including, C57BL/6J, Balb/C and DBA/2 male or female mice were housed in individual cages with access to mouse chow and water ad libitum, in a 12 by 12 h light:dark cycle. These mice were used in the control and treatment groups for the food consumption experiments at the beginning of the night cycle and following 12 h of food deprivation. The animal care and experimental protocol were approved by IACUC committee of William Paterson Universities, in accordance with NIH guidelines for the Care and Use of Laboratory Animals and the principles presented in the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience. Using $n = 10$ mice of each strain, ani-

TABLE I. Distribution of R63Q polymorphism in the CNR2 gene

Population		Genotype count (frequency) RR/RR	RR/QQ	QQ/QQ	*P*	RR	QQ	*P*
Affected	$n = 204$	79 (0.39)	89 (0.44)	36 (0.18)		247 (0.61)	161 (0.39)	
Controls	$n = 1876$	591 (0.32)	890 (0.47)	395 (0.21)	0.10	2072 (0.55)	1680 (0.45)	0.04
Affected	$n = 94$	42 (0.45)	30 (0.32)	22 (0.23)		114 (0.61)	74 (0.39)	
Controls	$n = 1876$	591 (0.32)	890 (0.47)	395 (0.21)	0.01	2072 (0.55)	1680 (0.45)	0.14
Affected	$n = 111$	37 (0.33)	59 (0.53)	15 (0.14)		133 (0.60)	89 (0.40)	
Controls	$n = 1876$	591 (0.32)	890 (0.47)	395 (0.21)	0.16	2072 (0.55)	1680 (0.45)	0.17

R allele is significantly more abundant in eating disorders than in controls ($P = 0.04$; 95% CI: 1.24(1.01–1.53)). There was no difference in allele frequency between the patients with anorexia nervosa and those with bulimia nervosa. For the RR/RR, the ratio in parentheses (frequency/total) for the affected is higher in the affected individuals than in controls.

mals were acclimatized to the food consumption tests with and without 12-h food deprivation. Five mice of each strain were then injected with AM 251 (3 mg kg^{-1}), a CB1-R antagonist, AM 630 (10 mg kg^{-1}), a CB2-R antagonist, or PEA (10 mg kg^{-1}), a CB2-R putative agonist, with the remaining five mice from each group injected with the vehicle (1:1:18) emulphur: ethanol: saline) mixture. Following the intraperitoneal administration of vehicle or the cannabinoid ligands, mouse food consumption was measured at 0.5, 1, 2, 4, 12, and 24 h after the start of the night cycle. For the 12-h food deprivation tests, a similar protocol as described above was used except that the separate groups of mice were deprived of food for 12 h before drug and vehicle treatment and food consumption was measured at same time intervals after the start of the light cycle.

Data analysis

Deviations of the observed allele and genotype distributions from Hardy-Weinberg equilibrium were calculated by HWE computer program, and differences in allele frequencies between groups were tested for significance with Fisher's exact test on 2×2 contingency tables. Differences in food intake in the mouse strains were analyzed by analysis of variance for multiple comparisons followed by Turkey's test. We considered $P < 0.05$ as significant in these tests.

RESULTS

Association between R63Q polymorphism in CNR2 gene and eating disorders

The R allele was significantly more abundant in patients with eating disorders than in controls [$P = 0.04$, odd ratio 1.24; 95% confidence interval (1.01–1.53)]. When we discriminate the patients with anorexia nervosa and with those with bulimia nervosa, there was no difference in allele frequency, while more robust association between the genotype distribution and anorexia nervosa was observed in comparison with or without the RR genotype (Table I).

CB2-R ligands modifies food intake across the strains

Food intake was compared in mice following administration of the putative CB2-R agonist, palmitoylethanolamide (PEA, 10 mg kg^{-1}), and antagonist, (AM 630, 10 mg kg^{-1}) to that of a known CB1-R antagonist (AM 251, 3 mg kg^{-1}). The doses were selected from dose response curves and those of previous studies. Baseline food consumption varied according to mouse strains used and whether food was available or during deprivation. When food was available ad libitum, the suppressant effect of CB2-R ligands used were strain- and time-dependent. This effect was similar to that of CB1-R receptor antagonist AM 251. However, during a 12-h food deprivation, AM 630 and PEA increased and suppressed food consumption

TABLE II. Modification of food intake by CB2-R ligands in mice

Drug/intake	C57Bl/6J Vehicle	C57Bl/6J Treated	DBA/2 Vehicle	DBA/2 Treated	BALB/C Vehicle	BALB/C Treated
PEA						
Normal	3.2 ± 0.4	2.5 ± 0.6*	3.1 ± 0.4	2.6 ± 0.3*	2.9 ± 0.9	2.5 ± 1.1
Fasted	10.7 ± 0.4	8.2 ± 1.4*	10.4 ± 2.1	8.9 ± 0.7*	12.2 ± 1.1	10.5 ± 2.0*
AM 630						
Normal	4.4 ± 0.3	3.7 ± 0.5*	5.5 ± 0.6	4.5 ± 0.4*	6.2 ± 0.7	5.2 ± 0.6*
Fasted	7.9 ± 1.5	8.3 ± 1.0*	8.7 ± 1.7	7.9 ± 1.2	6.3 ± 0.7	7.4 ± 0.9
AM 251						
Normal	8.6 ± 0.9	7.1 ± 0.7*	7.9 ± 0.9	6.3 ± 2.5*	8.9 ± 1.2	8.9 ± 1.3*
Fasted	5.6 ± 0.3	5.1 ± 1.7*	7.7 ± 0.6	6.1 ± 1.9*	7.6 ± 0.9	6.3 ± 0.9*

The effects of CB2-R ligands and AM 251 on food intake in three mouse strains. Food consumption was evaluated in control mice with vehicle treatment and after administration of PEA (10 mg kg^{-1}) or AM 630 (10 mg kg^{-1}) in comparison with that of AM 251 (3 mg kg^{-1}). The data shows mean ± SEM of food consumption during normal food intake and after a 12-h food deprivation. The data shown is the food consumption over a 24-hr period of measurement of food intake. Significant differences in food intake are indicated ($P < 0.05$). The summary of the food consumption monitored at 0.5, 1, 2, 4, 12, and 24 h after the start of the night cycle is summarized in Table III.
*Denotes significant difference in food intake compared to control animals.

TABLE III. CB2-Rs: Plays a role in food intake

PEA	C57B1/6	DBA/2	Balb/c
Normal	↓	↓	—
Fasted	↓	↓	↓
AM630			
Normal	↓	↓	—
Fasted	↑	—	—
AM251			
Normal	↓	↓	↓
Fasted	↓	↓	↓

Food consumption was compared in mice following administration of the putative CB2-R agonist, palmitoylethanolamide (PEA, 10 mg kg^{-1}) and antagonist, (AM 630, 10 mg kg^{-1}) to that of a known CB1-R antagonist (AM 251, 3 mg kg^{-1}). ↓: Decrease in food intake. ↑: Increase in food intake. —: No effect in food intake.

respectively in a strain- and time-dependent manner, whereas AM 251 suppressed food intake in all strains used in a time-dependent fashion (summarized in Tables II and III). At the doses used however no overt behavioral changes were recorded. The threshold for food consumption was compared between the normal food consumption and when animals had been fasted.

DISCUSSION

A major breakthrough in marijuana-cannabinoid research has been the discovery of the endocannabinoid system in humans with two CB1 and CB2 receptors encoded by *CNR1* and *CNR2* genes located in chromosome 6 and 1, respectively. A number of variations in *CNR* genes have been associated with human disorders including osteoporosis, ADHD, PTSD, drug dependency, obesity, and depression (Ishiguro et al., 2007; Karsak et al., 2005; Sipe et al., 2005; Zhang et al., 2004). The data obtained from this study provide additional evidence that *CNR2* gene variation is associated with eating disorders in a human population and in the mouse food consumption paradigm. The CB2-R ligands used suppressed food intake in a time- and strain-dependent manner when food was available ad libitum and during the 12-h fast except, AM 630—the CB2-R antagonist, stimulated food consumption in food deprived mice. Previous report indicates that the polymorphism at the *CNR2* gene results in the conversion of AA to GG results in the substitution of glutamine by arginine at position 63 (R63Q). There is an association between the R63Q polymorphism of the *CNR2* gene and eating disorders. These results suggest that cannabinoid CB2-R is involved in the endocannabinoid signaling mechanisms associated with the regulation of food intake and in eating disorders. Previous research have documented that the endocannabinoid system plays a crucial role in regulating food intake and energy metabolism (Horcajadas, 2007). While the involvement of CB1-Rs and other components of the endocannabinoid system in eating disorders, rodent feeding paradigms, human obesity, and metabolic regulation have been widely reported (Costa, 2007; Cota, 2008; Di Marzo and Matias, 2005; Frieling et al., 2008; Lillo, 2007; Matias et al., 2008; Monteleone et al., 2005; Norrod and Puffenbarger, 2007; Robson, 2005; Siegfried et al., 2004; Stoving et al., 2008), we now report the modification of food intake in mice by CB2-R ligands and the association of *CNR2* gene variant with eating disorders in a human population. The paucity of data on the involvement of CB2 cannabinoid receptors in feeding and eating disturbances may be related to the previous notion that CB2-Rs were predominantly expressed in immune cells in the periphery and were thought to be absent from neurons in the brain and had traditionally been referred to as peripheral cannabinoid CB2-Rs. Our data therefore adds to the accumulating evidence that functional CB2-Rs may provide novel targets in the nervous system beyond immunocannabinoid activity than previously appreciated (Brusco et al., 2008; Gong et al., 2006; Van Sickle et al., 2005). CB2-Rs have also now been demonstrated to be present in areas of the brain, including different hypothalamic nuclei that are involved in feeding behavior (Gong et al., 2006). Alternatively, the involvement of CB2-Rs in eating disorders and food consumption may indicate that disruption of food intake and eating disorders may be due in part to a dysregulation in the immune system. The involvement of the CB1-R agonists and antagonist in food intake and energy metabolism has been widely reported as discussed above. Our studies provide additional evidence, that CB2-Rs ligands are involved in food intake in mice. Studies in humans are needed to evaluate the role of CB2-R ligands in food intake in humans. This may be a new avenue for future research.

In summary numerous studies have reported on the crucial role of CB1-Rs in eating disorders and obesity and the role of CB2-Rs was unknown. We have investigated the effects of CB2-R agonist and antagonist and compared it with those of known CB1-R antagonist in mouse food consumption test. Our results show that CB2-Rs may play a role in the regulation of food intake. This is especially important as pharmacological treatment of eating disorders have been ineffective. Thus the association of *CNR2* gene and eating disorders in a human population suggest that cannabinoid CB2-R may be involved in the endocannabinoid signaling mechanisms associated with the regulation of food intake and in eating disorders. However more studies are required to determine if the CB2 receptor ligands have the risk of depression or suicide that has led to the withdrawal of rimonabant, the CB1-R antagonist that had been approved for use in Europe as an appetite suppressant in obesity.

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