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Review

CNS effects of CB2 cannabinoid receptors: beyond neuro-immuno-cannabinoid activity

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Abstract

There are two well characterized cannabinoid receptors (CBRs), CB1-Rs and CB2-Rs, with other candidates, such as GPR55, PPARs and vanilloid TRPV1 (VR1) receptors, which are either activated by cannabinoids and/or endocannabinoids (eCBs). The neuronal and functional expression of CB2-Rs in the brain has been much less well characterized in comparison with the expression of the ubiquitous CB1-Rs. CB2-Rs were previously thought to be predominantly expressed in immune cells in the periphery and were traditionally referred to as peripheral CB2-Rs. We and others have now demonstrated the expression of CB2-Rs in neuronal, glial and endothelial cells in the brain, and this warrants a re-evaluation of the CNS effects of CB2-Rs. In the present review we summarize our current understanding of *CNR2* genomic structure, its polymorphic nature, subtype specificity, from mice to human subjects, and its variants that confer vulnerabilities to neuropsychiatric disorders beyond neuro-immuno-cannabinoid activity.

Keywords

Brain, CB2 cannabinoid receptors, *CNR2*, neuronal and glial distribution, neuropsychiatry

Introduction

There are two well characterized cannabinoid receptors (CBRs), CB1-R and CB2-R, with other candidates, such as GPR55, PPARs and vanilloid TRPV1 (VR1) receptors, which are either activated by cannabinoids and/or endocannabinoids. Cannabinoids are the constituents in marijuana and endocannabinoids (eCBs) are the endogenous marijuana-like substances found in animals and humans (Onaivi, 2009). The endocannabinoid system (ECS) consists of genes encoding CBRs, their endogenous ligand eCBs (anandamide, partial agonist; 2-arachidonyl glycerol, full agonist and principal eCB), and the enzymes involved in their synthesis (NAPE-PLD, PLA_2, PLC, DAGL, PI-PLC and Lyso-PLC) and degradation (FAAH, MAGL) of these eCBs (Ahn et al., 2008). CBRs are abundantly distributed in the brain and peripheral tissues. However, the neuronal and functional expression of CB2-Rs in the brain has been much less well characterized in comparison with the expression of the ubiquitous CB1-Rs. Although earlier evidence suggested that CB2-Rs are present in the central nervous system (CNS) (Benito et al., 2003, 2005; Golech et al., 2004; Nunez et al., 2004; Sheng et al., 2005), they were referred to as the peripheral CBRs because many investigators were not able to detect neuronal CB2-Rs in healthy brains (Galiegue et al., 1995; Griffin et al., 1999; Ibrahim et al., 2003; Munro et al., 1993).

The functional presence of neuronal CB2-Rs in the CNS was therefore controversial (Ghose, 2009) and CB2-R has been considered as a CBR with an identity crisis (Atwood and Mackie, 2010). Nevertheless, the role of CB2-Rs in the immune system, its therapeutic promise in pain, inflammation and consequently in autoimmune and neurodegenerative disorders is receiving a great deal of attention and the subject of a number of studies and reviews (Ashton and Glass, 2007; Benito et al., 2008; Cabral et al., 2008, 2009; De Filippis et al., 2009; Ellert-Miklaszewska et al., 2007; Fernandez-Ruiz et al., 2006, 2008; Jean-Gilles et al., 2010; Lunn et al., 2008; Marriott and Huffman, 2008; Murikinati et al., 2010; Nagarkatti et al., 2009; Patel et al., 2010; Rivers and Ashton, 2010; Ruiz-Valdepenas et al., 2010; Tanasescu and Constantinescu, 2010). Therefore, as CB2-Rs are associated with immune regulation and function, it is of interest to probe the role of CB2-Rs not only in neurological disorders associated with neuroinflammation but also in neuropsychiatric disturbances. Indeed our studies provided the first evidence for neuronal CNS effects of CB2-Rs and its possible role in drug addiction, eating disorders, psychosis, depression, and autism

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spectrum disorders (Ishiguro et al., 2007, 2010a, 2010b; Onaivi et al., 2008a, 2008b). Previous reviews have focused on covering the evidence for the functional neuronal presence and the emerging role of brain CB2-Rs and its potential involvement in neuropsychiatric disorders (Onaivi et al., 2009; Roche and Finn, 2010). In the present review we summarize our current understanding of *CNR2* genomic structure, its polymorphic nature, subtype specificity, from mice to human subjects, and its variants that confer vulnerabilities to neuropsychiatric disorders beyond neuro-immuno-cannabinoid activity.

CNR2 genomic structure and CB2-receptor sub-type specificity

The *CNR2* cannabinoid gene structure has been poorly defined. However, many features of the cannabinoid *CNR2* gene structure, regulation and variation are beginning to emerge with the discovery and functional identification of CB2-Rs in mammalian CNS (Brusco et al., 2008a, 2008b; Gong et al., 2006; Liu et al., 2009; Onaivi, 2006; Onaivi et al., 2006a; Van Sickle et al., 2005). This prior poor definition could be related to the previously held view that *CNR2* gene and CB2-Rs were not expressed in neurons in brain but mainly in immune cells. It was therefore less investigated for CNS roles except for the association with brain cells of macrophage lineage. Recently, a number of studies from the laboratory of Gardner at the National Institute on Drug Abuse and other laboratories including that of Manzanares from Spain have mapped the cellular distribution of *CNR2* gene expression in mouse brain and those over-expressing CB2 cannabinoid receptors (Garcia-Gutierrez et al., 2010). Over-expression of CB2-R in the hippocampus of transgenic CB2xP mice reduces depressive-related behaviors, such as tail suspension test, novelty-suppressed feeding test and unpredictable chronic mild stress test (Garcia-Gutierrez et al., 2010). The human *CNR2* gene and its mouse and rat orthologs are located on chromosomes 1p36, 4QD3, and 5Q36, respectively. Genome-sequencing projects have also identified *CNR2* genes in chimpanzee, dog, cow, chicken, amphibian, puffer fish, and zebra fish. It appears that the human, rat, mouse, and zebra fish genomes contain two isoforms of CB2-Rs that have differential distribution patterns in the brain and peripheral tissues. Interestingly the puffer fish *Fugu rubripes* has two *CNR1* genes and one *CNR2* gene in contrast to zebra fish *Danio rerio* that has two *CNR2* genes and one *CNR1* gene (McPartland et al., 2006; Rodriguez-Martin et al., 2007; Yamaguchi et al., 1996).

Human Cnr2 genomic structure and isoforms

The most striking discovery of CB2 genomic structure is species- and tissue-specific expression patterns and differences between CB2 in human and mouse (Liu et al., 2009). A novel human CB2A isoform was discovered by alignment of EST sequences and sequencing RT-PCR fragments amplified from human brain cDNA (Liu et al., 2009). The CB2A isoform is predominantly expressed in human testis and the promoter of CB2A is located 45 kb upstream of the promoter of the previously identified CB2B isoform that is predominantly expressed in spleen (Munro et al., 1993). The quantitative RT-PCR revealed that CB2A mRNA was expressed in the human brain regions of caudate, amygdala, hippocampus, cerebellum, nucleus accumbens, putamen, and cortex, with similar levels of peripheral tissue expression, such as muscle, spleen, intestine, leukocytes, and kidney, except testis expression, which is more than 100 times that of other tissues. In contrast, CB2B mRNA expression could not be detected in brain regions at a significant level and is predominantly expressed in spleen and to a lesser extent in leukocytes, muscle, intestine, liver, and heart (Liu et al., 2009). The different levels of CB2A mRNA (0–1% of testis) and the absence of CB2B mRNA in brain regions imply specific expression other than homogeneous expression in brain immune-related cells. The human CB2A and CB2B isoforms contain different 5'untranslated region (5'UTR) and their protein coding sequences are the same. The promoter region of CB2A contains CpG islands and several CCAAT boxes with a transcription factor binding site for stress response such as AP-1 (activator protein 1), HSF (heat shock factor), and STRE (stress response element). In contrast, the promoter region of CB2B contains neither CpG islands nor CCAAT boxes, but has transcription factor binding sites of GATA (GATA-binding factor), HSF, Ntx2.5 (homeo domain factor, tinman homolog) and AP-4 (activator protein 4) (TFsearch, http://molsun1.cbr-c.aist.go.jp/research/db/TFSEARCH.html). It is therefore likely that human CB2A might target CB2 to specific tissues and neuron or glia cell components in response to physiological stressors.

We further analyzed human *CNR2* genomic locus (1p36.1) and its neighboring genes. As shown in Figure 1, CB2A is transcribed into more than 90 kb hnRNA that is spliced into mRNA (2.37 kb) that contain exon 1A, 1B, and 3. CB2B is transcribed into 45 kb hnRNA that is spliced into CB2B mRNA (2.29 kb) that contain exon 2 and 3. Upstream of CB2A is located the *PNRC2* (proline-rich nuclear receptor co-activator 2) gene, which is transcribed in the opposite direction of CB2B. With a short 5' flanking sequence (751 bp) between CB2A and *PNRC2*, the promoter might co-regulate these two genes. *PNRC2* plays an essential role in nonsense mediated decay (NMD) of aberrant mRNA (Cho et al., 2009), and the *PNRC2* knockout mice have a higher metabolic rate and are resistant to obesity (Zhou et al., 2008). We also found a transcribed *BTBD6P* (BTB domain containing 6, a zinc finger protein) pseudogene in the intron 2 region between exon 1b and 2. *BTBD6* plays an important role in neuronal differentiation by acting as an ubiquitination adaptor protein that targets the transcription factor *PLZFA* for cytoplasmic degradation (Sobieszczuk et al., 2010). More than 5 kb downstream of the *CNR2* gene is located *FUCA1* (fucosidase, alpha-L-1) and its mutation causes lysosomal fucose storage defect and psychomotor retardation (Kousseff et al., 1976). The functional interaction and consequences of *CNR2* in close proximity with other genes remains to be determined.

The epigenetic regulation of *CNR2* gene locus might play an important role in receptor regulation because CpG islands are found in the promoter regions of the four

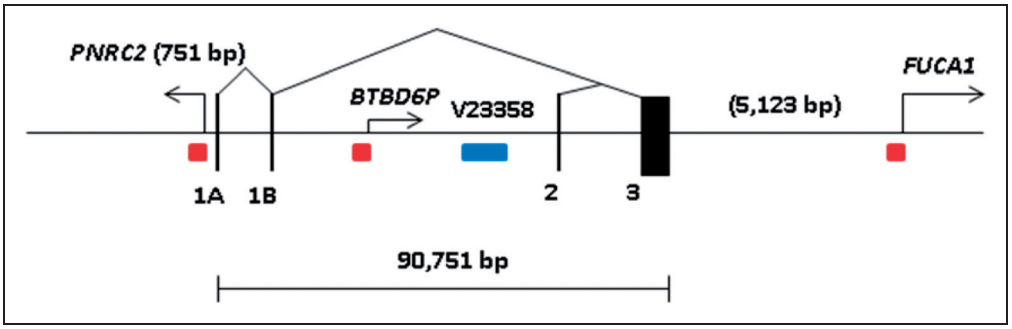

Figure 1. Human CB2 (*CNR2*, 1p36.1) genomic structure and neighboring genes. The gene size is in bp; black vertical bars represent exons; triangles represent splicing patterns, arrows represent neighboring gene transcription directions; red squares represent CpG islands; blue rectangle represents copy number variant.

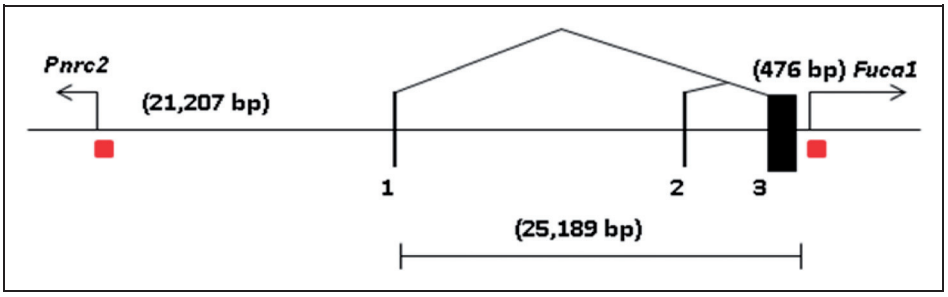

Figure 2. Mouse CB2 (*Cnr2*, 4D3) genomic structure and neighboring genes. The gene size is in bp; black vertical bars represent exons; triangles represent splicing patterns, arrows represent neighboring gene transcription directions; red squares represent CpG islands.

neighboring genes. The close proximity of *PNRC2* and *BTBD6P* genes with *CNR* gene indicates that CB2A gene transcription might be co-regulated. In addition, there is also a copy number variant (CNV) of 2.4 kb (Zhang et al., 2006) located in intron 2 whose genetic significance awaits further investigation.

*Mouse **CNR2** genomic structure and isoforms*

The size of mouse CB2 gene is almost four times smaller than that of human *CNR2* gene, a rare case in the orthologous genes of human and mouse. Although mouse *Cnr2* gene contains two promoters that transcribe mouse CB2A and CB2B isoforms, the activities of the mouse promoters deviate from those of human. Both mouse CB2A and CB2B are expressed predominantly in spleen, which is more than a hundred times the level of mouse brain regions and testis. There is no mouse testis predominant CB2 transcript. Furthermore, mouse CB2A mRNA level is five times that of CB2B mRNA. In brain stem of the C-terminus knockout mice, both CB2A and CB2B are up-regulated, which might indicate compensatory CB2 activation effects and implies brain-specific expression other than homogeneous expression in brain immune-related cells.

We further analyzed mouse *Cnr2* genomic locus (4D3) and its neighboring genes. As shown in Figure 2, mouse CB2A is transcribed into about 25 kb hnRNA that is spliced into mRNA (3.88 kb) that contain exon 1 and 3. CB2B is transcribed into 8.4 kb hnRNA that is spliced into CB2B mRNA (3.91 kb) that contain exon 2 and 3. Similar to human *CNR2* gene, upstream of mouse CB2A is located *Pnrc2* (proline-rich nuclear receptor co-activator 2) and downstream of *Cnr2* is located *Fuca1* (fucosidase, alpha-L-1) gene. The CpG islands are found in the promoter regions of the *Pnrc2* and *Fuca1* genes, not the mouse CB2 gene. The epigenetic regulation of mouse CB2 gene is thus very different from human CB2 gene. The distance between the neighboring genes is also drastically different between mouse and human. *Pnrc2* is upstream of more than 21 kb of CB2A promoter while *Fuca1* is just 476 bp downstream of the coding exon. There is no transcribed *Btbd6p* pseudogene in the mouse CB2 locus. The promoter region of mouse CB2A does not contain CpG island and CCAAT box. However, the CB2A promoter region contains transcription factor binding sites for stress response such as AP-1, HSF, STRE, and homeo-domain factor Nkx2.5. Mouse CB2B promoter region contains not only transcription factor binding sites of AP-1, HSF, Ntx2.5 (homeo domain factor, tinman homolog) but also Nf-kB, Sox-5, and C/EBP (CCAAT enhancer binding protein). Both mouse CB2A and CB2B promoters share similar transcription factor binding sites responsive to stressors.

Table 1. Genomic comparison between mouse and human CB2 genes

Gene symbol	Isoforms	Gene size	Exons	Promoters	Promoter span	CpG islands	Amino acids	mRNA size	Tissue expression
CNR2	hCB2A and hCB2B	90 kb	4	2	45,717 bp	Yes	360	5.0 kb and 2.5 kb	Spleen, leukocytes, testis, muscle, brain
Cnr2	mCB2A and mCB2B	25 kb	3	2	16,290 bp	No	347	4.0 kb	Spleen, leukocytes, brain

*Comparison of human **CNR2** and mouse **Cnr2** gene structure and expression*

Table 1 shows the genomic comparison of human *CNR2* and mouse *Cnr2* genes. Although these gene orthologs share molecular and functional similarities, the genomic structure and gene expression patterns are strikingly different and require special attention in developing CB2-specific agonists and antagonists in treatment of human disorders. The evolutional pace of *CNR2* gene was accelerated in human with added regulatory elements such as creation of new exons, new promoter with CpG island that could be shared by *PNRC2* gene, insertion of *BTBD6P* expressed pseudogene containing promoter of CpG island, and drastic expansion of intron sequences (Li et al., 2010). Human *CNR2* gene expression could be affected by the proximity of *PNRC2* and *BTBD6P* genes that reside only 751 bp upstream of the first exon and within intron 2 of human *CNR2* gene respectively (Piontkivska et al., 2009). The expression and regulation of human *CNR2* gene deviate from its ancestral counterpart, such as mouse *Cnr2* gene does not have CpG island containing promoter. The distance of the mouse promoter of CB2A and CB2B is about 16 kb indicating that the two promoters are still within the same open chromatin conformation domain that confers predominant spleen expression of both isoforms. In contrast, the distance of the human promoters of CB2A and CB2B is about 46 kb, which might be located in different chromatin conformation domains that confer different tissue specific expressions (Boyle et al., 2008). Therefore, human CB2A is predominantly expressed in testis and to a much lesser extent in several brain regions. The possible DNA methylation of CpG islands and stress responsive transcription binding sites might enable human CB2A promoter to be inhibited and activated in brain regions in spatial and temporal modes and the unique 5'UTR sequence coded by human specific exon 1a and 1b might target human CB2 to specific neuronal regions such as pre- or post-synaptic structures. To investigate the brain specific and regulated expression of human CB2-R requires a highly specific CB2-R antibody that is not currently available, to our knowledge (Atwood and Mackie, 2010; Patel et al., 2010). The finding of novel human CB2-R isoforms (Liu et al., 2009) with possible brain expression and regulation has opened up a new frontier to study CB2 function in brain under normal and abnormal brain functions. Our data shows that there are two forms of the CB2-Rs in human, rat, and mouse (Liu et al., 2009) with differential subtype distribution specificities in the brain and peripheral organ tissues. The promoter-specific CB2-R isoform distribution may in part explain why CB2-Rs were previously undetectable in both human and rodent brains.

There are reports of endogenous and exogenous retroviral integrations found in rodent CB2 3'UTR. A frequent provirus insertion site (*Evi11*), about 400 bp, was found after Ca-Br-M murine leukemia virus inoculation of NIH/Swiss mice that developed malignancies (Joosten et al., 2000; Valk et al., 1999). In rat *Cnr2* gene, there is a rat B2 retroposon insertion (flanked by direct repeat) into the 3'UTR region of the major coding exon in the reported rat rCB2 cDNA clone (Brown et al., 2002). This B2 insertion was also found in the NCBI rat sequence and not in the Celera rat genome sequence. We demonstrated that the Sprague-Dawley and Long Evans rats that we used in some of our studies did not contain B2 retroposon insertion (Liu et al., 2009). Since insertion of Ca-Br-M murine leukemia virus (MuLV) into mouse CB2 3'UTR sequence induced primary tumor, the B2 retroviral insertion into rat CB2 3'UTR might also contribute additional CB2-R activity. Both human *FUCA1* and *CNR2* genes map to 1p36 and near the common virus integration site *Evi11*, making *CNR2* a candidate gene for viral interference (Valk et al., 1997).

Brain cannabinoid receptor genetic variation in neuropsychiatry

Cannabinoids in marijuana and eCBs acting on CBRs are important regulators of various aspects of neurophysiological, psycho-behavioral, immunological, and metabolic functions. There is an emerging understanding that brain CB2-Rs appear to be associated with vulnerability to several neuropsychiatric disorders including alcoholism, eating disorders, depression, schizophrenia, autism spectrum disorders (ASDs), and anxiety-related disorders (Ishiguro et al., 2007, 2010a, 2010b; Onaivi et al., 2008a). High comorbidity in these disorders and CB2-R function may explain certain common phenotypes in some of these neuropsychiatric disturbances. For example, common genetic contributions were suggested across alcohol and cannabis misuse (Sartor et al., 2010). Marijuana withdrawal discomfort and alcohol craving

positively correlated with alcohol drinks per day in patients with marijuana abstinence (Peters and Hughes, 2010). Substance use problems, including cannabis, in adolescents with eating disorders are frequently found (Castro-Fornieles et al., 2010). Associations between marijuana use and schizophrenia have been also suggested in some studies. A dose–response relationship has been found between the amount of cannabis used in adolescence and the subsequent risk of developing schizophrenia (Andreasson et al., 1987; Zammit et al., 2002). More psychotic symptoms are experienced by schizophrenic patients who use cannabis (Leweke, 2007). Schizophrenia-like symptoms can occur in non-schizophrenic people after cannabis use (Morgan and Curran, 2008). Furthermore, there is a decrease in gray matter density in the right posterior cingulate cortex in first-episode schizophrenics who use cannabis compared with those who do not use cannabis (Bangalore et al., 2008). As a biomarker probably related to eCBs, significantly higher amounts of anandamide in the blood occur more frequently in patients with acute schizophrenia than in healthy volunteers (De Marchi et al., 2003) and significantly higher levels of anandamide are detected in the cerebrospinal fluid (CSF) of first-episode schizophrenic patients than in that of healthy volunteers (Koethe et al., 2007; Leweke et al., 1999). Another important area of growing interest is brain CBR pharmacogenomics and potential therapeutic applications targeting the endocannabinoid system, as reviewed previously (Onaivi, 2009, 2010).

*Genetic polymorphisms of **CNR2** gene in neuropsychiatry*

Human *CNR2* gene is located on chromosome 1p36, and there was no linkage with the psychiatric disorders found by previous linkage studies in the locus. The previous studies found weak linkages to alcoholism centered on chromosome 1p31 (Agrawal et al., 2008; Lappalainen et al., 2004), and a strong linkage was reported in the locus on chromosome 1p31.1 in a homogenous population from India (Holliday et al., 2009); these were not very likely to cover *CNR2* locus. Furthermore, genome wide association studies (GWASs) for schizophrenia (Cichon et al., 2009; Duan et al., 2010; Holmans et al., 2009) and alcoholism (Edenberg et al., 2010; Lind et al., 2010; Treutlein et al., 2009) have been conducted worldwide. GWAS technologies can detect disease-vulnerability genes with nucleotide polymorphisms or copy number variants (deletions and duplications) in the genome. These analyses provided genetic markers for statistical evaluation for prediction of association between SNPs and disease. However, the association between *CNR2* polymorphisms and the disease were unknown because GWAS datasets with Affymetrix 500 K or Illumina HumanHap550 platforms did not include the SNPs of *CNR2* (including rs12744386 and/or rs2501432). We have found and reported *CNR2* polymorphisms to be associated with a number of disorders in Japanese subjects from our studies described below. The Japanese population is good for genetic analysis because of few stratification problems, as indicated in the study (Yamaguchi-Kabata et al., 2008). However, we have not yet confirmed a possibility for generalization of the associations to other ethnic populations.

The existence of genetic polymorphisms has been associated with CB2 function in diverse human populations, with two currently known functional polymorphisms in the *CNR2* gene. One of the SNPs, rs35761398, makes the substitution of arginine at amino acid position 63 by glutamine (R63Q: two base pairs replacement polymorphism). The receptor transcribed from the gene with R63 allele appeared to have reduced responses to three CBR ligands, 2-arachidonylglyerol (2-AG), AM630, a CB2 receptor inverse agonist and JWH-015, a CB2 receptor agonist, observed in cAMP activity in cultured cells (Ishiguro et al., 2010a). Other sets of SNPs show correlation between their alleles and altered *CNR2* gene expression in *cis*-acting fashion (for example, rs12744386C allele, effect $= -0.490$, $H^2 = 11.64$, LOD $= 8.819$) in lymphoblast cells. Further, in our study rs12744386 genotypes also show difference of *CNR2* gene and protein expression in postmortem prefrontal cortex between CC and CT genotypes of rs12744386 and with TT genotype (Ishiguro et al., 2010a). Taken together, the low function alleles of these polymorphisms have high linkage disequilibrium (Ishiguro et al., 2010a). Thus in human populations, there could be groups with high or low *CNR2* function, and other minor ones with intermediate function of the receptor. The allelic distributions in different ethnicities are similar in European and Asian but there are differences between African and others, according to the NCBI database and our study (Ishiguro et al., 2010a). We also investigated associations between psychiatric disorders and *CNR2* polymorphism in Japanese populations. Interestingly, vulnerabilities to alcoholism, depression, schizophrenia, and anorexia nervosa appear to be associated with the R63Q polymorphism in the gene (Ishiguro et al., 2007, 2010a, 2010b; Onaivi et al., 2008a). The power to detect the association, if the odds ratio (OR) equals 1.2, seems to be in the 15–60 percentage range in our sample size (http://pngu.mgh.harvard.edu/~purcell/cgi-bin/cc2k.cgi). Strong association between the low function haplotype described above and schizophrenia was observed (Ishiguro et al., 2010a).

Low function of the receptor seemed to have impact on several other physical disorders, such as autoimmune disorders as well as neuropsychiatric disorders. R63 was reported to be associated with autoimmune disease (Karsak et al., 2005; Sipe et al., 2005) and with human osteoporosis (OR: 1.43 (1.07–1.92)). Sipe et al. (2005) reported its functional change from the polymorphism in the immune system in vitro. Schizophrenic patients have lower bone mass than the community population since they are young, while aging and menopausal transition effect on bone mass in the general female population cannot be seen in the schizophrenic patient group (Renn et al., 2009). Abnormalities in peripheral immune cells have been indicated in schizophrenia and numerous epidemiological studies have associated schizophrenia with autoimmunity and allergies (Muller and Schwarz, 2006; Patterson, 2009; Strous and Shoenfeld, 2006). The impact of CB2-Rs in inflammation and their role in autoimmune disorders is an area of current interest. Although the biologic and genetic mechanisms common between osteoporosis and alcoholism are not known, heavy

alcohol intake and alcoholism disrupt calcium and bone homeostasis, which reduces bone mineral density and increases the incidence of fractures. Alcohol abuse has been suggested as a lifestyle factor for secondary osteoporosis (Berg et al., 2008; Malik et al., 2009; Sampson, 2002). Little has been reported about association between autoimmune disorders and alcoholism (Sammarco, 2007); however, alcohol abuse altered immune regulation leading to immunodeficiency and autoimmunity (Achur et al., 2010; Plackett and Kovacs, 2008). Such action may be mediated by the glutamatergic system (Ward et al., 2009).

Brain CB2 receptor distribution and sub-cellular localization

Some studies could not detect expression of CB2-Rs in the brain (Brown et al., 2002; Griffin et al., 1999; Munro et al., 1993) because the PCR primers may not have been specific to detect CB2-R isoforms. In addition, the specificity of the available antibodies for both CB1-Rs and CB2-Rs has also been controversial as some could not detect the native and in some cases the transfected CBR antigen, although they recognized proteins in Western blot and in immunohistochemical analysis (Grimsey et al., 2008). There are also problems with the antibodies because of the species differences between human and rodent CB2 gene (Liu et al., 2009). We have resolved some of these issues by using CB2 isoform specific TaqMan probes that could differentiate the isoform-specific expression patterns and are more sensitive and specific than the CB2 probes and primers previously used (Liu et al., 2009). The controversial CB2-R brain expression could also be due to the low expression levels of CB2A isoform in brain regions and the less specific CB2 commercial antibodies in immunohistochemical studies, especially those studies using antibodies against human hCB2 epitopes for rodent brain immunostaining. There are also problems with the use of the CB2 knockout (ko) mice (Buckley et al., 2000) in Western blots and in behavioral analysis. When we analyzed the CB2 ko mice using the three TaqMan probes against two promoters of mouse CB2 gene and the deleted part of CB2 gene, we found that the promoters of CB2-R ko mice were still active and that a CB2 truncated version was expressed, indicating that the CB2 ko mice with ablation of the C-terminal peptides of 131 amino acids was an incomplete CB2-R knockout (Liu et al., 2009). Another mouse CB2-R ko mice that has now been generated with ablation of N-terminal peptide 156 amino acid (Deltagen, Inc., San Mateo, CA) may clarify the specificity of the antibodies that were used against the N-terminal epitopes.

Table 2 shows studies documenting the expression of CB2-R mRNA and CB2-R protein in the mammalian nervous system. In demonstrating neuronal presence of CB2-Rs in vitro, Gong et al. (2006) used sequential double labeling. The hippocampal slide cultured tissue preparation was first labeled with the CB2-R antibody followed by the neuronal marker neuron-specific enolase (NSE). Immunopositive expression was detected in perikarya and in neuronal processes as well as glial cells (Gong et al., 2006). Our studies have demonstrated the expression of the CB2-R mRNA and CB2-R protein in the mammalian nervous system (Brusco et al., 2008a, 2008b; Gong et al., 2006; Liu et al., 2009; Onaivi et al., 2006a, 2006b, 2008a). Many other studies have identified brain CB2-Rs in brain stem neurons (Van Sickle et al., 2005), on neural progenitor cells of the subgranular zone of the dentate gyrus in the hippocampus (Palazuelos et al., 2006), on rat neocortical neurons (Hill et al., 2007), in the rat cerebellum and hippocampus (Suarez et al., 2008, 2009), in the thalamus (Jhaveri et al., 2008), at CNS synapses in the entorhinal cortex (Morgan et al., 2009), in primate cerebral cortex, within layers III and V on pyramidal neurons (Lanciego et al., 2010).

Using mouse and rat brains, the subcellular distribution of CB2-Rs in neuronal, endothelial and glial cells in the cortex, hippocampus and substantia nigra were shown using immunohistochemical electron microscopy (Brusco et al., 2008a, 2008b; Onaivi, 2006). *Cnr2* gene and protein expression in different brain regions of Swiss ICR mice under normal conditions were demonstrated and over-expression of CB2-R in the hippocampus of transgenic CB2xP mice reduces depressive-related behaviors, such as tail suspension test, novelty-suppressed feeding test and unpredictable chronic mild stress test (Garcia-Gutierrez et al., 2010). In the rat brain study and in each region immunoperoxidase labeling for CB2-Rs was detected in neurons as well as in glial and endothelial cells. In neuronal cells, iCB2 was observed in somata and large and medium-sized dendrites. In the soma, iCB2 labeling was mainly associated with the rough endoplasmic reticulum and Golgi apparatus, suggesting its endogenous synthesis. In the dendrites, iCB2 labeling was observed in the cytoplasm and was associated with the plasma membrane near the area of synaptic contact with axon terminals, indicating a post-synaptic distribution of CB2-Rs. In iCB2 glial and endothelial cells, the labeling was also found to be associated with the plasma membrane. In the substantia nigra, some unmyelinated axons were immunoreactive for CB2-Rs, and CB2-R-labeled axon terminals were rarely found. In mice, electron micrographs from different cortical areas show dendrites with immunostaining for CB2-Rs. In some areas, axon terminals were not immunoreactive for CB2-Rs. The pattern of staining in most mouse cortical areas appeared to be mainly post-synaptic localization of CB2-Rs (Brusco et al., 2008a). Our data therefore provided the first ultrastructural evidence that CB2-Rs are mainly post-synaptic in the rat hippocampus and substantia nigra and in some cortical areas in the mouse and rat brain (Brusco et al., 2008a, 2008b; Onaivi, 2006). We cannot exclude that some of the CB2-Rs may be presynaptic (Suarez et al., 2008), just as CB1-Rs are not exclusively presynaptic, with some post-synaptic distribution reported (Ong and Mackie, 1999) in the brain.

A number of studies from mouse to human subjects, using a variety of techniques including those used in pain models, electrophysiological, brain stimulation reward paradigm, histological, immunohistochemical, electron microscopy, molecular biological, behavioral and pharmacological, pharmacological MRI, cerebral occlusion and hemicerebellectomy, transgenic and cell culture studies, show the functional presence of CB2-Rs in neural progenitor cells, neurons, glial and endothelial cells (Brusco et al., 2008a, 2008b; Chin et al., 2008; Garcia-Gutierrez et al., 2010;

Table 2. Expression of the CB2-R mRNA and CB2-R protein in the mammalian nervous system

Area of the nervous system	Cellular type	Subcellular localization	References
Olfactory bulb and tubercle	Neurons		Gong et al., 2006
Cerebral cortex: Orbital, visual, motor and auditory cortex	Pyramidal neurons layers III and V		Gong et al., 2006
Cerebral cortex (*CB2-R mRNA*)	Pyramidal-like neurons layers III and V		Lanciego et al., 2010
Hippocampus:			
CA2 and CA3 areas	Pyramidal neurons and interneurons	Neuronal cytoplasm and dendrites	Gong et al, 2006; Brusco et al., 2008a
CA1 and CA3 areas (*CB2-R mRNA*)	Interneuron-like cells.		Lanciego et al., 2010
Strata oriens and radiatum	Neuropil		Suarez et al., 2008, 2009
Corpus callosum	Glial cells		Gong et al., 2006
Globus pallidus (*CB2-R mRNA*)	PV-IR and NeuN-IR neurons		Lanciego et al., 2010
Midbrain areas:			Gong et al., 2006; Brusco et al., 2008b
Periaqueductal gray matter, paralemniscal, paratrochlear and red nuclei.	Neurons		
Substantia nigra (pars reticulata)	Neurons	Dendrites, axons and axon terminals	
Cerebellum			Suarez et al., 2008, 2009; Baek et al., 2008
Molecular layer	Parallel varicose fibers and neuropil		Gong et al., 2006; Baek et al., 2008; Ashton et al., 2006
Purkinje cells layer	Neurons		
Granular layer	Mossy fibers and neuropil		Suarez et al., 2008, 2009; Baek et al., 2008
Brainstem nuclei			
Vestibular and cochlear nuclei			Gong et al., 2006; Suarez et al., 2008
Parvocellular reticular nucleus, spinal trigeminal tract nucleus			Gong et al., 2006
Dorsal motor nucleus of the vagus, nucleus ambiguous and spinal trigeminal nucleus	Neurons		Van Sickle et al., 2005
Pineal gland	Pinealocytes and intrapineal nerve fibers		Koch et al., 2008
Retina (*CB2-R mRNA*)	Inner photoreceptor segments, inner nuclear layer, ganglion cell layer.		Lu et al., 2000
Retina (*CB2-R protein*)	Inner photoreceptor segments, inner nuclear layer, inner plexiform layer, ganglion cell layer		Lopez et al., 2010

Palazuelos et al., 2006; Viscomi et al., 2009). Potential interactions of CB2-Rs for example, where chronic blockade of CB2-Rs or over-expression of CB2-Rs is associated with the modulation of anxiety response in the mouse model (Garcia-Gutierrez and Manzanares, 2010) have been demonstrated. Furthermore, over-expression of CB2-Rs results in neuroprotection against behavioral and neurochemical alterations induced by intracaudate administration of 6-hydroxydopamine (Ternianov et al., 2010).

Therefore the pharmacological actions at brain CB2-Rs may be more complex than previously appreciated with species and subtype differences and distribution patterns. However, the role of CB2-Rs in CNS disturbances involving neuroinflammation and neuropathic pain has been extensively reported. While the CNS presence of CB2-Rs may no longer be a debate, the neurobiological basis for CB2-R physiological activity and its interaction with or without CB1-Rs remains to be determined. However, functional interactions

between forebrain CB2-R and mu-opioid receptor (MOR) was demonstrated (Paldyova et al., 2008) and CB2-R antagonist SR144528 was reported to decrease MOR expression and activation in mouse brain stem (Paldy et al., 2008). CB2-Rs in the pineal gland along with other components of the ECS may be involved in the control of pineal physiology (Koch et al., 2008). Gender-dependent changes on the expression of hippocampal CB1 and CB2-Rs were demonstrated in the early maternal deprivation model in neonatal rats (Suarez et al., 2008). While CB1-Rs remain one of the most ubiquitous G-protein coupled receptors in the mammalian brain, we have described the multifocal distribution of CB2-Rs, albeit at lower levels than the CB1-Rs in neuronal and glial processes in a number of brain areas (Gong et al., 2006). This multifocal distribution and the presence of CNS brain CB2-Rs suggest a need to re-evaluate the role of these receptors in neurotransmission.

Emerging evidence suggests that CB1-Rs and CB2-Rs modulate some of their physiological effects by acting in opposite directions. For example, it was demonstrated that CB2-Rs participate in the regulation of glucose homeostasis in rat by opposing the actions exerted through CB1-Rs (Bermudez-Silva et al., 2007). The investigators suggested that the opposing roles of CB1-Rs and CB2-Rs in glucose homeostasis were involved in the regulation of glycemia. Using CB1-R and CB2-R agonists and antagonists and their interaction, it was demonstrated and confirmed in the rat model that glucose levels remain high after stimulation of CB1-Rs (Bermudez-Silva et al., 2006) and the levels return to normal after stimulation of CB2-Rs (Bermudez-Silva et al., 2006, 2007). Using the brain stimulation reward paradigm in the rat model, such opposing effects of CB1-Rs and CB2-Rs were shown to modulate brain stimulation in opposite directions. In the study presented at the Society for Neuroscience meeting and at the International Cannabinoid Research Society meeting (Xi et al., 2009), it was reported that the brain stimulation reward (BSR) enhancing effect produced by cannabinoid is mediated by activation of brain CB1-Rs (using THC and WIN55212-2), while the brain stimulation inhibiting effect produced is mediated by activation of brain CB2-Rs.

It is important to understand the role of CB2-Rs and their gene variants in the CNS and their possible involvement in drug addiction and neuropsychiatric disorders. However, research on the involvement of CB2-Rs in neuroinflammatory conditions and in neuropathic pain has advanced more than other areas in neuropsychiatry and drug addiction. Therefore, improved information about *CNR2* gene and its human variants might add to our understanding not only of the role of CB2-Rs during neuroinflammatory conditions in the CNS but also beyond neuro-immuno-cannabinoid activity.

Pharmacology of CNS effects of CB2 cannabinoid receptors

The CNS effects of CB2-Rs had been ambiguous and controversial and their role in depression and substance abuse was unknown. We therefore have conducted studies from mouse to human subjects to examine the following. 1) We investigated the involvement of CB2-Rs in alcohol preference in mice and alcoholism in a human population (Ishiguro et al., 2007); 2) we analyzed the behavioral effects of CB2 cannabinoid receptor activation and its influence on food and alcohol consumption in mice (Onaivi et al., 2008b); 3) we have described the involvement of brain neuronal CB2-Rs in the effects of drugs of abuse and in depression (Onaivi et al., 2008a). Indeed, alcoholism in humans may be caused by both genetics and environmental factors. There is a high incidence (comorbidity) of alcoholism and depression in the human population (Hasin et al., 2005). Several lines of experimental evidence support roles for the ECS in alcoholism and neuropsychiatric disorders (Basavarajappa and Hungund, 2005; Onaivi et al., 2008b; Vinod and Hungund, 2005). We therefore tested whether CB2-Rs in the CNS play a role in alcohol abuse/dependence in an animal model and then examined the association between *CNR2* gene polymorphism and alcoholism in a human population. We found that mice preferring alcohol had reduced *Cnr2* gene expression in the ventral midbrain whereas *Cnr2* gene expression was unaltered in the ventral midbrain region of mice with little or no preference for alcohol. Treatment of mice with the CB2-R agonist JWH 015 enhanced alcohol consumption in mice subjected to chronic mild stress (CMS) and treatment with the CB2-R antagonist AM630 reduced the stress-induced increase in alcohol consumption. This CB2-R agonist or antagonist effect was absent in normal mice that were not subjected to CMS.

To further understand the physiological relevance of the expression of CB2-Rs and their gene transcripts, we examined the expression of *Cnr2* gene transcripts in rodents treated with opioids, cocaine, and alcohol in comparison with control animals. Animals treated with cocaine or heroin showed increased *Cnr2* gene transcripts in comparison with controls, indicating the presence of *Cnr2* gene transcripts in the brain is influenced by abused substances (Ishiguro et al., 2007; Onaivi, 2006; Onaivi et al., 2008b). We utilized behavioral and molecular methods to study and determine whether there was a link between depression that may be a factor in drug/alcohol addiction and CNS CB2-Rs. First we established the use of the mouse CMS model of depression, which has been validated and is a widely used model for screening antidepressants. Briefly, the mouse CMS model measures one of the core symptoms of depression, anhedonia, a lack of pleasure. Mice were subjected daily for four weeks to CMS, and anhedonia was measured by the consumption of sucrose solution. Behavioral and rewarding effects of abused substances were determined in the CMS and control animals. The expression of CB2-Rs and their gene transcripts was compared in the brains of CMS and control animals by Western blotting and RT-PCR. CMS induced gender-specific aversions in the test of anxiety, which were blocked by WIN55212-2 and CB2-R agonist. In other studies we demonstrated that direct CB2-R antisense oligonucleotide microinjection into the mouse brain induced anxiolysis, indicating that CB2-Rs are functionally present in the brain and may influence behavior (Ishiguro et al., 2007; Onaivi, 2006; Onaivi et al., 2006a, 2008b; Uhl et al., 2006). Overall it appears that endocannabinoid activity in the nervous system may play a significant

role in a number of neuropsychiatric conditions following neuroinflammation.

The genetic findings of reduced functioning of *CNR2* gene associated with schizophrenia may also be supported by the findings of the pharmacological experiments using the animal model and AM630, the CB2-R antagonist in the study (Ishiguro et al., 2010a). Prepulse inhibition (PPI) is frequently used in pharmaco-behavioral studies of animal models. PPI refers to the reduction in amplitude of the startle reflex that occurs when a brief, sub-threshold stimulus immediately precedes a startle stimulus (Hoffman and Ison, 1980). Deficits in PPI are observed in several psychiatric disorders, especially in schizophrenia (Swerdlow et al., 2006), and it has been postulated that this impairment of sensori-motor gating reflects at least some portion of the cognitive dysfunction observed in patients with schizophrenia (Braff et al., 2001, 2005). The association with the cannabinoid system had been investigated and cannabidiol reverses MK-801-induced disruption of PPI in mice (Long et al., 2006). In our study, we evaluated the effect of pretreatment with AM630 on PPI, combined with MK-801 or methamphetamine treatment separately, in mice. AM630 alone did not affect PPI in mice. AM630 exacerbated MK-801 or methamphetamine induced disturbance of PPI and hyperactivity in C57BL/6JJmsSlc mice (Ishiguro et al., 2010a). When administered to mice in home cages both MK-801 and methamphetamine produced significant hyperlocomotion. Although AM630 alone did not produce significant hyperlocomotion, AM630 pretreatments significantly increased methamphetamine-induced and MK-801-induced locomotion compared with saline pretreatments. Therefore, reduced CB2-R function itself is not likely to cause schizophrenia, but it is hypothesized that, when combined with other risk factors, it could be a risk factor for schizophrenia-susceptible individuals.

There are, however, some limitations with the use of such animal models because the possible effect of AM630 on CB1 and interaction between CB1 and CB2 receptors could not be excluded. A further study using *Cnr1* ko mice would be needed to explore this pharmacological possibility to clarify the functions of CB2 receptors in brain. In a recent abstract presented at the Society for Neuroscience Ortega-Alvaro et al. (2010) reported that deletion of CB2-R induces schizophrenia-related behaviors in mice, which is in agreement with the findings of Ishiguro et al. (2010a).

Comments and future directions

The clinical and functional implication of neuronal CB2-Rs in the brain will gradually become clearer because more research will certainly unravel the contribution and interaction of CB1 and CB2-Rs in neuropsychiatry beyond neuro-immuno-cannabinoid activity. The new knowledge from our data and those of other recent studies that CB2-Rs are present in the brain raises many questions about the possible roles that CB2-Rs may play in the nervous system. These results therefore extend the previous evidence that CB2-Rs are playing an important role in immune function to other putative neuronal function by their apparent presence in neuronal processes. Our studies implicate neuronal and glial CB2-Rs in the chronic mild stress model of depression, and substance abuse. With neuroinflammation known to be associated with a number of autoimmune and neurological disorders, the close association of the immune system with CB2-Rs and their functional expression in neurons warrants a re-evaluation of CB2-Rs in mental disturbances. Both CB1 and CB2 receptors seem likely to work both independently or in opposite directions and/or cooperatively in differing neuronal and/or glial cell populations to regulate important physiological activities in the central nervous system. Thus, many more studies are required to determine the exact role of CB2-Rs and the nature of their interactions with other receptors and CB1-Rs in the brain and therefore determine the therapeutic utility of CB2-R ligands in the clinic.

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Conflict of interest

None declared.

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