Genes, Brain and Behavior
Official publication of the International Behavioural and Neural Genetics Society

Genes, Brain and Behavior (2009) ***8:*** *519–530*

Species differences in cannabinoid receptor 2 (*CNR2* gene): identification of novel human and rodent CB2 isoforms, differential tissue expression and regulation by cannabinoid receptor ligands

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Cannabinoids, endocannabinoids and marijuana activate two well-characterized cannabinoid receptors (CB-Rs), CB1-Rs and CB2-Rs. The expression of CB1-Rs in the brain and periphery has been well studied, but neuronal CB2-Rs have received much less attention than CB1-Rs. Many studies have now identified and characterized functional glial and neuronal CB2-Rs in the central nervous system. However, many features of CB2-R gene structure, regulation and variation remain poorly characterized in comparison with the CB1-R. In this study, we report on the discovery of a novel human CB2 gene promoter transcribing testis (CB2A) isoform with starting exon located ca 45 kb upstream from the previously identified promoter transcribing the spleen isoform (CB2B). The 5′ exons of both CB2 isoforms are untranslated 5′UTRs and alternatively spliced to the major protein coding exon of the CB2 gene. CB2A is expressed higher in testis and brain than CB2B that is expressed higher in other peripheral tissues than CB2A. Species comparison found that the CB2 gene of human, rat and mouse genomes deviated in their gene structures and isoform expression patterns. mCB2A expression was increased significantly in the cerebellum of mice treated with the CB-R mixed agonist, WIN55212-2. These results provide much improved information about CB2 gene structure and its human and rodent variants that should be considered in developing CB2-R-based therapeutic agents.

Keywords: Brain, CB2 cannabinoid receptors, CB2A, CB2B, spleen, testis

Received 3 February 2009, revised 28 March 2009, accepted for publication 15 April 2009

Marijuana remains one of the most widely used drugs, and much progress in cannabinoid research has showed the existence of an elaborate endocannabinoid physiological control system (EPCS) in animals and humans (Mackie 2008; Onaivi *et al.* 2008), with increasing new knowledge on the scientific and therapeutic bases for the use of cannabinoids (CBs) as medicine. The EPCS 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. The study of the distribution and function of various components of the EPCS in the brain has provided the basis for the established effects of marijuana and eCBs. Thus, the use of cannabis or administration of phytocannabinoids or synthetic CBs interacts with the EPCS to modulate synaptic transmission. This modulatory action on synaptic transmission has significant functional implications on the interactions between exogenous cannabinoids such as smoking marijuana or administration of Δ^9-THC and eCBs-mediated synaptic plasticity.

The expression of CB1 cannabinoid receptors (CB1-Rs) in the brain and periphery has been well studied, but CB2-Rs have received much less attention than have CB1-Rs (Mackie 2008; Onaivi *et al.* 2006). Both CB1-Rs and CB2-Rs are widely distributed in peripheral tissues with CB2-Rs particularly enriched in immune tissues and cells such as spleen, thymus and leucocytes (Sugiura & Waku 2002). The functional neuronal expression of CB2-Rs in the brain had therefore been ambiguous and controversial (Onaivi *et al.* 2006; Van Sickle *et al.* 2005). The lack of CB2 information on brain expression is because CB2-Rs were thought previously to be expressed primarily in some peripheral tissues such as the spleen and in other immune cells and has therefore been traditionally referred to as the peripheral CB-Rs (Gerard *et al.* 1990; Griffin *et al.* 1999; Munro *et al.* 1993). Several studies have now identified and characterized glial and neuronal CB2-Rs in the central nervous system (CNS). However, many features of CB2-R gene structure, regulation and variation remain poorly characterized (Benito *et al.* 2008; Gong *et al.* 2006; Nunez *et al.* 2008; Onaivi *et al.* 2006; Van Sickle *et al.* 2005). This poor characterization

doi: 10.1111/j.1601-183X.2009.00498.x

of CB2-R gene structure and polymorphisms hampers progress in the determination of the functional role of CB2-Rs, particularly in neurodegenerative and neuropsychiatric disorders. Furthermore, the neurobiological basis for CB2-R physiological activity, and its putative interaction with or without CB1-Rs remains to be determined.

Our recent studies indicate that genetic variants of CB2 gene expression, with a high incidence of Q63R but not the H316Y polymorphism, is involved in eating disorders, substance abuse and depression in a human population (Onaivi *et al.* 2008). Other studies in human tissue samples from participants with Alzheimer's disease showed that CB2-Rs are selectively over expressed in the microglial cells that are associated with Aβ-enriched neuritic plaques (Pazos *et al.* 2004). Data obtained *in vitro* and from animal models showed the inducible nature of CB2-Rs under neuroinflammatory conditions and suggest that the upregulation of CB2-Rs is a common pattern of response against different types of chronic human brain neuropathology (Benito *et al.* 2008). Accruing evidence shows that CB2-Rs and its gene variants may play possible roles in neuroinflammation occurring in multiple sclerosis, traumatic brain injury, HIV-induced encephalitis, Alzheimer's, Parkinson's and Huntington's diseases (Benito *et al.* 2008). It would be important to understand the role of CB2-Rs and its gene variants in disturbances of the brain involving neuroinflammation. Therefore, improved information about the CB2 gene and its human variants might add to our understanding of the role of CB2-Rs during neuroinflammatory conditions in the CNS and beyond immunocannabinoid activity.

To date, the complete gene structure, 5′- and 3′-UTR, and transcription initiation sites of human CB2 receptor have not been fully characterized (Abood 2005; Onaivi *et al.* 2006). Because the identification of mouse CB2 expression in brain regions was reported (Gong *et al.* 2006; Van Sickle *et al.* 2005), the specific expression of human or mouse CB2 isoforms in brain regions was not known. However, the published evidence showed significant species differences of CB2-Rs in human, mouse and rat in terms of peptides, mRNA sizes, gene structure and pharmacology (Brown *et al.* 2002; Munro *et al.* 1993; Shire *et al.* 1996). We thereafter name human, mouse and rat CB2 genes as hCB2, mCB2 and rCB2, respectively. For instance, hCB2 mRNA was detected in the HL60 cell line (human acute promyelocytic leukemia cells) by Northern blot as 2.5-kb and 5.0-kb mRNA bands that were elevated by TPA- (12-O-tetradecanoylphorbol−13-acetate) and DMF-(*N*, *N*-dimethylformamide) induced differentiation to myeloid and granulocyte, respectively (Munro *et al.* 1993). The mRNA 5.0-kb and 2.5-kb bands of hCB2 were also observed in the Jurkat E6−1 cell line (human T-cell leukemia cells); however, only the 2.5-kb band was observed in human peripheral blood acute leukemia cell line (HPB-ALL) cells (Schatz *et al.* 1997). In contrast, mCB2 mRNA were shown to be predominantly expressed as a single band of 4.0 kb in spleen (Schatz *et al.* 1997). Different rCB2 mRNA species were observed as a 2.4-kb band (Schatz *et al.* 1997) and as a 3.9-kb band in rat spleen, thymus, testis and leucocytes (Brown *et al.* 2002). The discrepancies on CB2 mRNA sizes in the literature indicated incomplete gene structure of CB2 gene in different species or polymorphism in the same species. We now report on the discovery of a novel CB2 gene promoter that differentially encodes tissue-specific CB2 gene expression in human tissues. Intriguingly, the testis-specific isoform hCB2A expression level was much higher in brain regions than the spleen-dominant hCB2B isoform that is expressed at higher levels in spleen and other peripheral tissues except testis. Our results showed tissue-specific isoforms of *CNR2* gene that may provide tissue-specific targeting of cannabinoids in inflammatory and neuropathic pain disorders associated with CB2-Rs in the brain and peripheral tissues.

Materials and methods

RNA isolation, complementary DNA synthesis and real-time polymerase chain reaction quantification

Human total RNA from brain and peripheral tissues were obtained from Clontech (Mountain View, CA, USA) and human brain tissues were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland. Mouse total RNAs were extracted from C57BL/6J, C57BL/10J and the BTBR T+tF/J mouse peripheral tissues and brain regions using RNAzolB protocol (Tel-Test, Inc., Friendwood, TX, USA). Single strand complementary DNA (cDNA) was synthesized from total RNA using SuperScript™ III One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA). Novel hCB2A exons (exons 1a and 1b) were identified by aligning human genomic sequences with expressed sequence tag clone AL527378 isolated 3′-from normalized neuroblastoma cDNA library. For quantitative real-time polymerase chain reaction (RT-PCR) assays, the exon-specific primers and fluorescent FAM-labeled and minor grove binder (MGB) conjugated probes of hCB2, mCB2 and rCB2 genes were designed (Table 1) using Primer Express (Applied Biosystems, Foster City, CA, USA). For hCB2 expression study, an hCB2A-specific probe was designed to span exons 1a and 1b, and an hCB2B-specific probe was designed to span exons 2 and 3. For the mCB2 expression study, a mCB2A-specific probe was designed to span exons 1 and 3 and a mCB2B-specific probe was designed to span exons 2 and 3. For the rCB2 expression study, specific probes (Table 1) were designed to span exons 1 and 3 and exons 2 and 3 to detect rCB2A and rCB2B expression, respectively. The rCB2 primers were also designed (Table 1) to detect retroposon B2 insertion into 3′UTR of rCB2.

Endogenous controls were provided by predeveloped human *GAPDH* or mouse and β-actin with fluorescent FAM-labeling (Applied Biosystems). ABI 7900HT Sequence Detection System default program was used for RT-PCR with 40 cycles as well as rCB2 B2 insertion detection.

The novel isoforms were confirmed by agarose gel analysis of PCR fragments that were in the expected sizes and by dideoxynucleotide sequencing. To confirm hCB2A testis-specific expression, TaqMan assays were carried out independently by different individuals. Mouse mCB2 gene expression and regulation by treatment with cannabinoids were determined in mouse brain regions and other peripheral tissues after subacute treatment with CB-R ligands in comparison with vehicle-treated control mice. Sprague−Dawley and Long Evans rats were decapitated, and the brain and peripheral tissues were dissected for RNA isolation and cDNA synthesis. The different brain regions included striatum, brain stem, cerebellum, hypothalamus and frontal cortex, and peripheral tissues included spleen and testis.

Table 1: TaqMan probes and flanking forward and reverse primer sequences for human, mouse, and rat CB2 genes and PCR primers to amplify rat B2 insertion site

Isoforms	Exons	MGB TaqMan probes	Forward primer	Reverse primer
hCB2A	Exons 1a, 1b	CCAGATGCAGCCGC	GGAAGAAAGAGAATATTGTTCAGTTGATT	GCTGGCCTTGGAGAGTGACA
hCB2B	Exons 2, 3	ACAACACAACCCAAAGC	TGGGAGAGGACAGAAAACAACTG	TGGGCCCTTCAGATTCCA
rCB2A	Exons 1, 3	CTGACAAATGACACCCAGTC	CAGGACAAGGCTCCACAAGAC	GATGGGCTTTGGCTTCTTCTAC
rCB2B	Exons 1, 3	TGGGCCCAGTCTT	GCCACCCAGCAAACATCTCT	GATGGGCTTTGGCTTCTTCTAC
rB2 insert	Exon 3		CCAGGCTGCTCCAATTGCT	TCTGACTCGGACTGCTTCCA
mCB2A	Exons 1, 3	CTGACAAATGACTCCCAGTC	CAGGACAAGGCTTCACAAGAC	GACAGGCTTTGGCTGCTTCTAC
mCB2B	Exons 2, 3	TGGGCCCAGTCCT	GCCACCCAGCAAACATCTAT	GACAGGCTTTGGCTGCTTCTAC
mCB2-KO	Exon 3	ATGCTGGTTCCCTGCAC	AGCTCGGATGCGGCTAGAC	AGGCTGTGGCCCATGAGA

Animal subjects and cannabinoid receptor ligand treatments

Animals (males) from the selected mouse strain C57BL/6Js were treated daily for 7 days with the mixed cannabinoid agonist, WIN55212−2 (2 mg/kg). On test days, animals were treated with the test drug for 30 min before assessment of the performance of the mice in forced swim test (FST) for 5 min. In this study, the effect of WIN55212−2 acutely and on day 6 in the FST was determined and compared with control mice following 7 days of daily administration. Six mice per group were killed, and non-injected BTBR mice tissue parts included for mCB2 gene expression analysis were as follows: vehicle = (V1–V6), WIN55212−2 (2 mg/kg) = (W1–W6) and untreated BTBR = (B1–B6). We also analyzed mCB2 gene expression in chronically treated C57BL/10Js treated with CB1-R antagonist (AM 251, 3 mg/kg) or CB2-R antagonist (AM 630, 10 mg/kg) in comparison with vehicle-treated control mice.

The mCB2 knockout mice were bred at NIDA-IRP from two $CB2^{+/-}$ breeding pairs, generously donated by Dr George Kunos at the National Institute on Alcohol Abuse and Alcoholism (NIAAA). Genotyping was performed by the Charles River National Laboratories. We analyzed mCB2 expression in brain regions, testis and spleen using the three TaqMan probes against two promoters of mouse mCB2 gene and the deleted part of mCB2 gene (Table 1).

Statistical analysis

Prism-3 program , version 3.02 (GraphPad Software, Inc., San Diego, CA, USA) was used for graphical and statistical analyses including *t*-tests and one-way analyses of variance. The accepted level of significance is $P < 0.05$.

Results

Human CB2 genomic structure (1p36.1) and discovery of a novel hCB2A promoter

With the published evidence showing clear species differences of CB2-Rs in human, mouse and rat in terms of mRNA sizes in tissues and cell lines (Brown *et al.* 2002; Munro *et al.* 1993; Shire *et al.* 1996), we set out to identify unidentified human hCB2 isoforms by first aligning the EST sequences with hCB2 genomic sequences and then by PCR amplification. We found that an EST clone (AL527378) derived from a normalized neuroblastoma cDNA library aligned with two upstream human hCB2 exons (exons 1a and 1b) and downstream major coding exon (exon 3) with two conserved exon–intron (AG-GT) junctions (Fig. 1a). We then sequenced the PCR fragments that were amplified using primers designed according to the novel exons, and we deposited hCB2A cDNA sequence in GenBank (EU517121). The novel exons 1a and 1b of hCB2 are located at 85 478 bp and 75 971 bp, respectively, upstream from the major coding exon 3. Both of the novel exons 1a and 1b are 5′UTR sequences because they do not contain open reading frame in frame with the initiation methionine of hCB2 encoded by the exon 3. In contrast, the previously identified upstream 5′UTR exon 2 is located 39 769 bp upstream from the major coding exon 3 (Munro *et al.* 1993). Therefore, the size of the newly identified hCB2A isoform spans a genomic region of 90.8 kb, about twice as large as the previously identified human hCB2B gene of 45.7 kb (Fig. 1a).

There are four probable poly A signals and poly A sites in the extended 3′UTR sequence of hCB2 cDNA. The first poly A site is the previously identified cDNA clone (HCX5.36) with imperfect polyadenylation signal sequence of GATAAA (Munro *et al.* 1993). There are three additional poly A sites with perfect polyadenylation signal sequence AATAAA; the second poly A site is located at 2799 bp of hCB2A (EU517121) with poly A containing 3′EST alignments (AW515513, AI864752 and AI475798). The third is located at 4632 bp with poly A containing 3′EST alignments (AW967543, AW966929, AV715015 and AA250845), and the fourth is located at 5349 bp with polyA containing 3′EST alignments (AW405600, AW294847, AA909480, AI218261 and AI424994). All these 3′EST clones were derived from cDNA libraries of B cells or lymphocytes. The additional evidence of hCB2 mRNA that contains extended 3′UTR sequence is another hCB2 cDNA clone (HCX5.1) with extended sequence beyond the first polyA site (Munro *et al.* 1993). Therefore, human *CNR2* gene contains two separate promoters and several alternative polyA sites, generating multiple mRNA sizes as previously observed in Northern blot of human HL-60 and Jurkat E6-1 cell lines (Munro *et al.* 1993; Schatz *et al.* 1997). The first promoter initiates from 5′UTR exon 1a and is spliced to exon 1b and then to the major coding exon 3 (Fig. 1). The second promoter initiates from exon 2 that is also spliced to the major coding exon 3 (Fig. 1). We named the novel hCB2 isoform that is transcribed from the first exon as hCB2A and the second promoter as hCB2B.

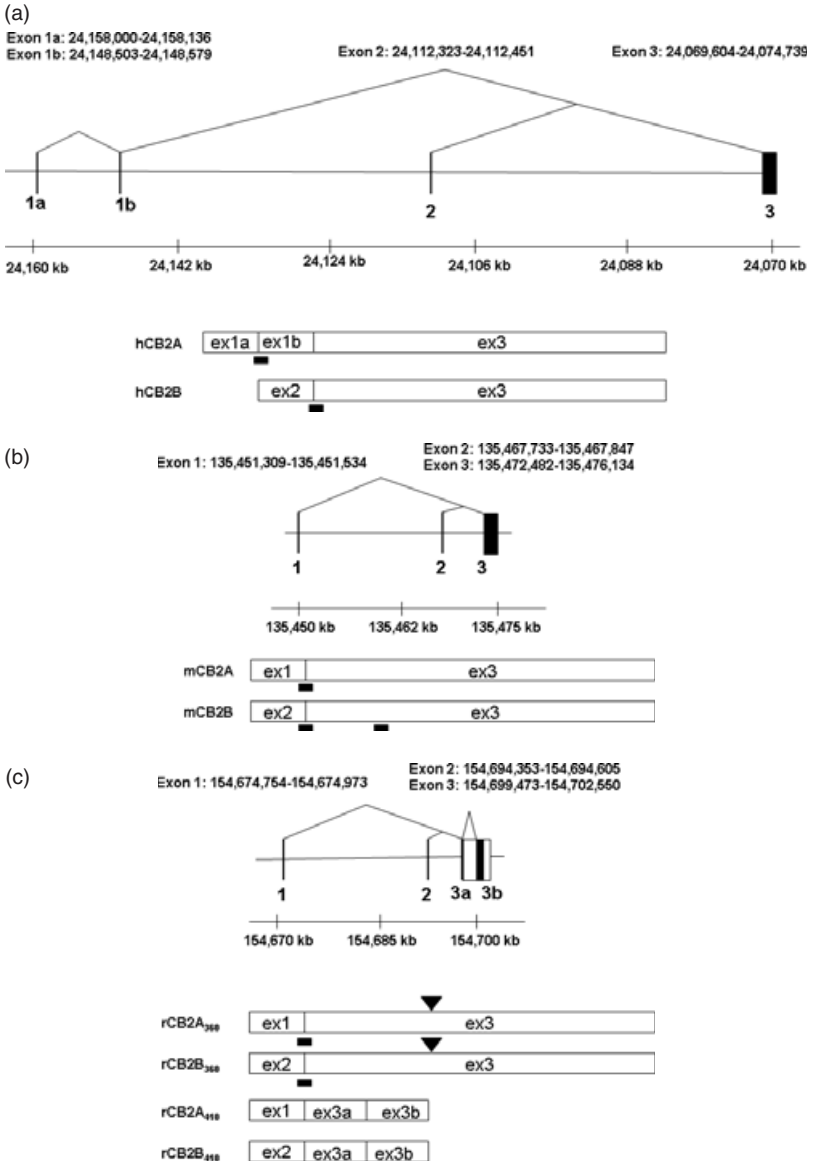

Figure 1: (a) Human hCB2 genomic structure and transcripts. (b) Mouse mCB2 genomic structure and transcripts. (c) Rat rCB2 genomic structure and transcripts. Horizontal line represents NCBI MapViewer's *Homo sapiens* chromosome1p36.1 position Build 36.3, *mus musculus* chromosome 4D3 position Build 37.1, and *Rattus norvegicus* chromosome 5q36 position Build RGSC 34.1. Vertical bars represent exons. Triangular lines represent splicing events. Open boxes represent spliced exons in mRNA transcripts. Black bars represent the location of TaqMan probes. Black boxes represent intraexonal splicing of rat rCB2 major coding exon and black triangles represent rat B2 retroposon insertions sites.

Human hCB2A and hCB2B isoform tissue expression patterns

We next tested the tissue expression patterns of the newly identified hCB2A isoform in comparison with the previously identified hCB2B isoform in various human peripheral tissues and brain regions. The tissue cDNA templates were synthesized from human RNA preparations that consist of intact RNA with no genomic DNA contamination (Clontech). The TaqMan probes were designed across exon 1a/1b and exon 2/3, respectively, for detecting specific hCB2A and hCB2B isoform expression and avoiding the detection of genomic contamination (Table 1 and Fig. 1a). Strikingly, hCB2A expression was observed predominantly in testis, i.e. more than 100-fold than that of spleen and leucocytes. The hCB2A expression was also observed in various brain regions at levels, i.e. zero to twofold using intestine hCB2A as a reference (Fig. 2a). As expected, hCB2B expression was observed predominantly in spleen and leucocytes. Spleen and leucocytes hCB2B mRNA levels were 120- and 30-fold higher than that of intestine reference of hCB2B (Fig. 2b), respectively. However, there was little expression of hCB2B in brain regions. In comparison with intestine hCB2B reference, hCB2A expression levels were significantly lower in peripheral tissues except in testis; however, hCB2A expression in brain regions was observed in amygdala, caudate, putaman, nucleus accumbens, cortex, hippocampus and cerebellum at low levels. The caudate hCB2A mRNA level was about 0.07% of the spleen hCB2B expression level (Fig. 2c). This is the first observation of the isoforms-specific expression of human hCB2A at significant, albeit low levels (<1% of hCB2A

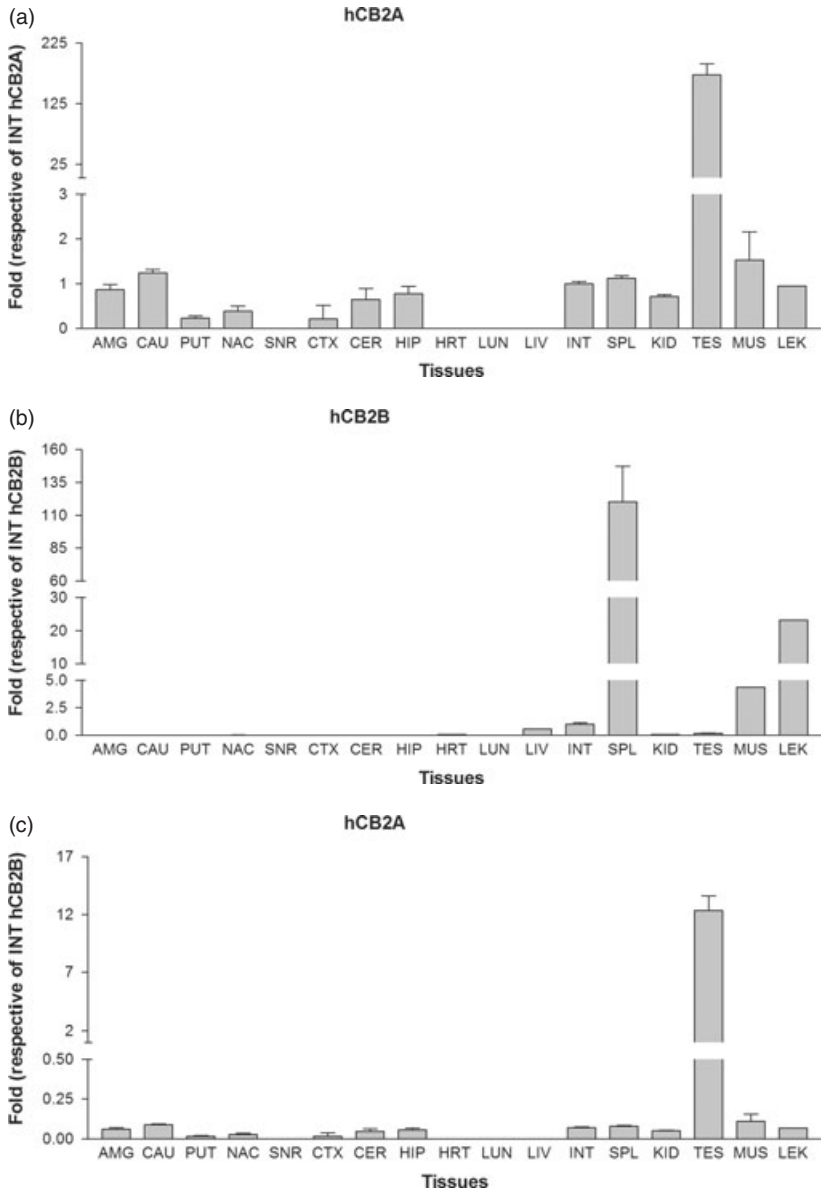

Figure 2: Tissue expression patterns of human hCB2A and hCB2B isoforms. (a) Human hCB2A isoform expression in different brain regions and peripheral tissues using human intestine hCB2A as a reference. (b) Human hCB2B isoform expression in different brain regions and peripheral tissues using human intestine hCB2B as a reference. (c) Human hCB2A isoform expression patterns using human intestine hCB2B as a reference. Error bars were derived from standard deviation (SD) of triplicate TaqMan assay. The abbreviations for brain regions and peripheral tissues are AMG, amygdala; CAU, caudate; PUT, putaman; NAC, nucleus acumbens; SNR, substantia nigra; CTX, cortex; CER, cerebellum; HIP, hippocampus; HRT, heart; LUN, lung; LIV, liver; INT, intestine; SPL, spleen; KID, kidney; TES, testis; MUS, muscle; LEU, leucocytes.

testis expression and <0.1% of CB2B spleen expression) in human brain regions (Fig. 2c).

Mouse mCB2 genomic structure (4D3) and tissue expression patterns

Mouse mCB2 gene contains three exons with two separate promoters (Fig. 1b) (Onaivi *et al.* 2006). Transcription of the first cloned mouse mCB2A cDNA (X86405) was initiated from the first exon (Shire *et al.* 1996) and that of the mouse mCB2B cDNA clones from FANTOM Consortium and Mammalian Gene Collection (AK036658 and BC024052, respectively) was initiated from exon 2 (Fig. 1b) (Okazaki *et al.* 2002; Strausberg *et al.* 2002). However, the tissue expression patterns of the specific mCB2A and mCB2B isoforms were not known. We designed the isoform-specific TaqMan probes across exon 1/3 and exon 2/3 (Table 1 and Fig. 1b) to determine mCB2A and mCB2B tissue expression patterns, respectively. Mouse mCB2A and mCB2B were expressed predominantly in spleen, although the mCB2A and mCB2B isoform transcripts were also detected in brain regions such as frontal cortex, striatum and brain stem at about 1% of the spleen expression (Fig. 3a,b). Although mCB2A and mCB2B tissue expression patterns were similar, mCB2A mRNA levels were generally about fivefold higher than mCB2B using brain stem CB2A as a reference (Fig. 3c); therefore, mouse mCB2A isoform is expressed at higher levels than mCB2 in brain regions and other peripheral tissues.

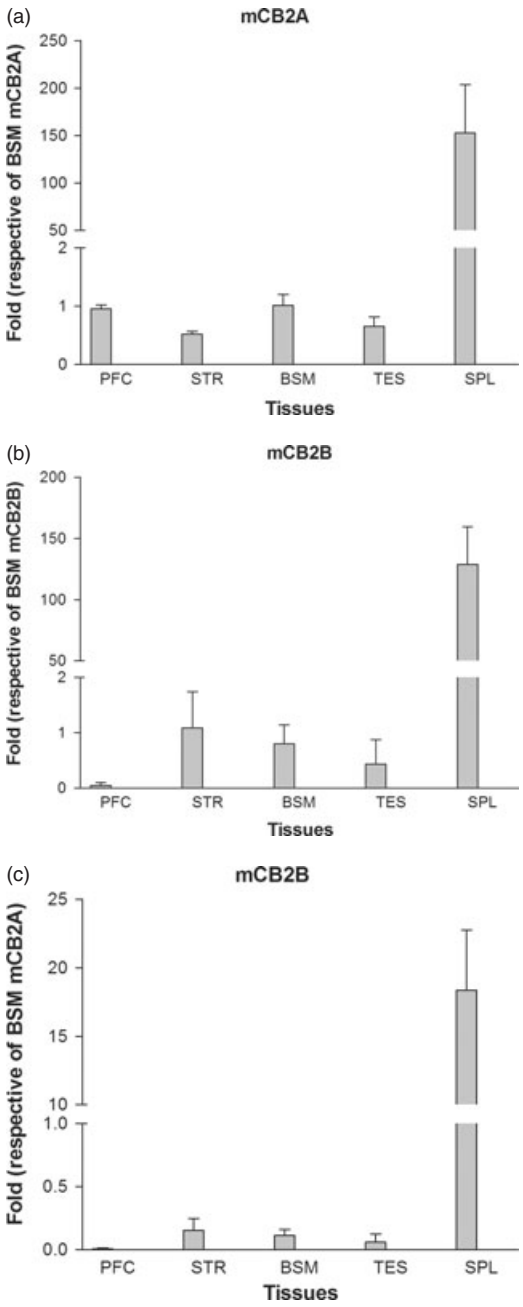

Figure 3: Tissue expression patterns of mouse mCB2A and mCB2B isoforms. Error bars were derived from standard error of means (SEM) from six mice. (a) Mouse mCB2A isoform expression in different brain regions and peripheral tissues using mouse brain stem mCB2A as a reference. (b) Mouse mCB2B isoform expression in different brain regions and peripheral tissues using mouse brain stem mCB2B as a reference. (c) mCB2A isoform expression patterns using mouse brain stem mCB2A as a reference. The abbreviations for brain regions and peripheral tissues are PFC, prefrontal cortex; STR, striatum; BSM, brain stem; TES, testis; SPL, spleen.

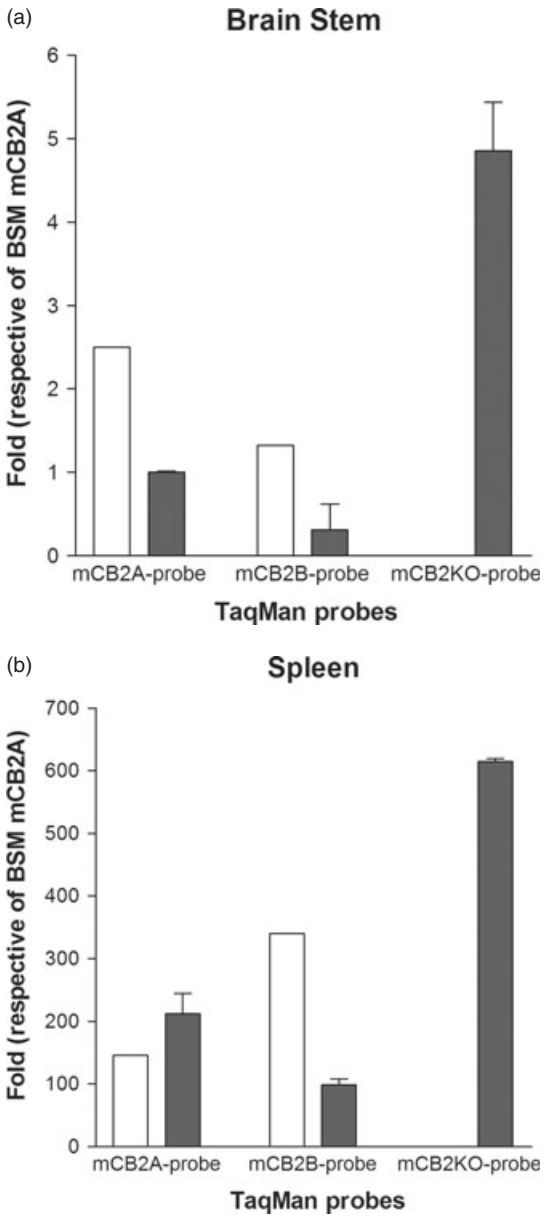

Figure 4: Detection of mCB2 tissue expression in the C-terminus deletion of mCB2 knockout mouse (open bars) and wild type (filled bars) by mCB2A, mCB2B and mCB2-KO TaqMan probes. Figures (a) and (b) represent brain stem (BSM) and spleen (SPL), respectively.

Upregulation of undeleted portion of mCB2 in the C-terminal knockout mouse tissues

We further investigated the promoter activities of mCB2A and mCB2B in the knockout mouse brain and peripheral tissues, because the knockout strain deleted the C-terminal 131 amino acid including last two transmembrane and intracellular domains (Buckley 2008; Buckley *et al.* 2000). We found that there was a compensatory effect of mCB2A

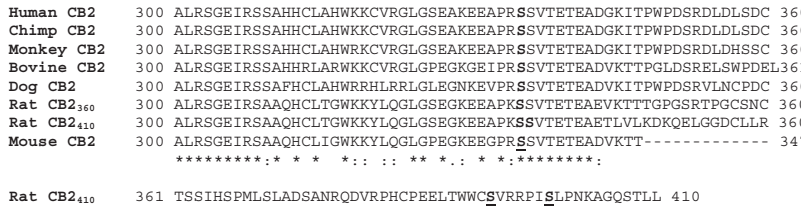

Figure 5: Alignment of the C-terminal intracellular peptide sequences of CB2 receptors from human, mouse, rat $rCB2_{360}$ and $rCB2_{410}$ isoforms. The numbers represent the amino acid numbers in each of the CB2 receptors from different species and isoforms. Asterisks represent identical amino acids; colons and dots represent similar amino acids. Bold and underlined S represents predicted PKA phosphorylation and PKC phosphorylation sites (Score >0.65, NetPhosK 1.0: http://www.cbs.dtu.dk/services/NetPhosK/).

and mCB2B promoter activities that upregulate mCB2A and mCB2B isoforms in the brain stem of mCB2 knockout mouse (Fig. 4a) using wild-type brain stem mCB2A as a reference. The expression level of mCB2B was also upregulated, although mCB2A expression was reduced in the spleen tissue of mCB2 knockout mice (Fig. 4b). We further designed the TaqMan probe mCB2-KO (Table 1) that hybridized to the deleted C-terminus of mCB2 knockout mouse (Buckley *et al.* 2000) to validate the mCB2 expression in the mCB2 knockout mouse. We found that the knockout mouse did not express the deleted portion of mCB2 in the brain stem and spleen; however, the mCB2-KO TaqMan probe could readily detect the C-terminal expression in the brain stem and spleen tissue of the wild-type mouse (Fig. 4a,b).

Rat rCB2 genomic structure (5q36) and novel isoforms without retroposon B2 insertion at 3′ UTR

Rat rCB2 gene was reported previously to contain three coding exons that encode two different intracellular C-terminal peptides with one homologous (Griffin *et al.* 2000) and the other non-homologous with hCB2 and mCB2 C-terminal peptides (Brown *et al.* 2002). The upstream promoters of rCB2 gene were not reported in the literature. In order to identify rCB2 isoforms by PCR cloning, we used homologous region of mouse exons 1 and 2 for forward primers and used downstream exon 3 sequence for the reverse primers (Table 1 and Fig. 1c). We found that rCB2 gene also contains isoform rCB2A and rCB2B that were transcribed from upstream promoters of exons 1 and 2, respectively, similar to mCB2 gene (Fig. 1c). The rCB2 genomic region of 29 kb is also significantly shorter than that of the hCB2 gene of 90 kb. Surprisingly, we found only one coding exon instead of three coding exons (Brown *et al.* 2002) in Sprague–Dawley and Long Evans rat cDNA templates synthesized from spleen and brain regions (Fig. 1c). The uninterrupted coding exon encodes rCB2 of 360 amino acids with shorter intracellular C-terminal peptide (Fig. 5) as Griffin *et al.* reported (Griffin *et al.* 2000) than that of previously cloned rCB2 of 410 amino acids (Brown *et al.* 2002). We named the rat C-terminal isoform as $rCB2_{360}$ and $rCB2_{410}$ for rCB2 isoforms containing 360 and 410 amino acids, respectively. Interestingly, $rCB2_{360}$ isoform contains the same size of hCB2 intracellular C-terminal peptide with 67% identity and is 13 amino acids longer than that of mCB2 intracellular C-terminal region with 72% identity (Fig. 5). We could not detect $rCB2_{410}$ in the rat strains of Sprague–Dawley and Long Evans that were used for the experiment; therefore, we could not compare the expression levels of $rCB2_{360}$ and $rCB2_{410}$ isoforms. The tissue expression patterns of rCB2A and rCB2B were very similar with mCB2A and mCB2B (data not shown; primer sequences are listed in Table 1).

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) as well as the NCBI rat genomic sequence. Using primers that flank the B2 retroposon insertion site (Table 1), we showed that the Sprague–Dawley and Long Evans rats that we used did not contain B2 retroposon insertion (data not shown). Indeed, Celera rat genome sequence does not contain the B2 retroposon insertion of the major coding exon of rCB2 gene.

Species differences of CB2 gene among human, mouse and rat

Including the newly identified exons of hCB2A, the human *CNR2* gene spans the 90-kb genomic region, which is about four times larger than the 23-kb mouse mCB2 gene and 29-kb rat rCB2 gene (Fig. 1a–c), respectively. Using the default setting of NCBI Blast 2 program to align human and rodent exon sequences, the only region that aligns significantly is in the protein coding region and no significant alignments could be found in the 5′UTR and 3′UTR sequences between human and rodent rCB2 and mCB2 cDNA sequences. The full length of human hCB2 3′UTR sequence is 2500 and 3000 bp longer than that of mouse and rat 3′UTR sequences, respectively. In contrast, mouse and rat 5′UTR and 3′UTR were well aligned with more than 85% identity. However, mCB2 contains longer 3′UTR (779 bp longer) than that of rCB2. Mouse mCB2 contains a stop codon that precedes the C-terminal 13 amino acids of human hCB2 and rat rCB2 isoforms, although the remaining 12 amino acids of mCB2 after stop codon are completely identical to rCB_{360} isoform. It is possible that a nonsense mutation occurred during mouse

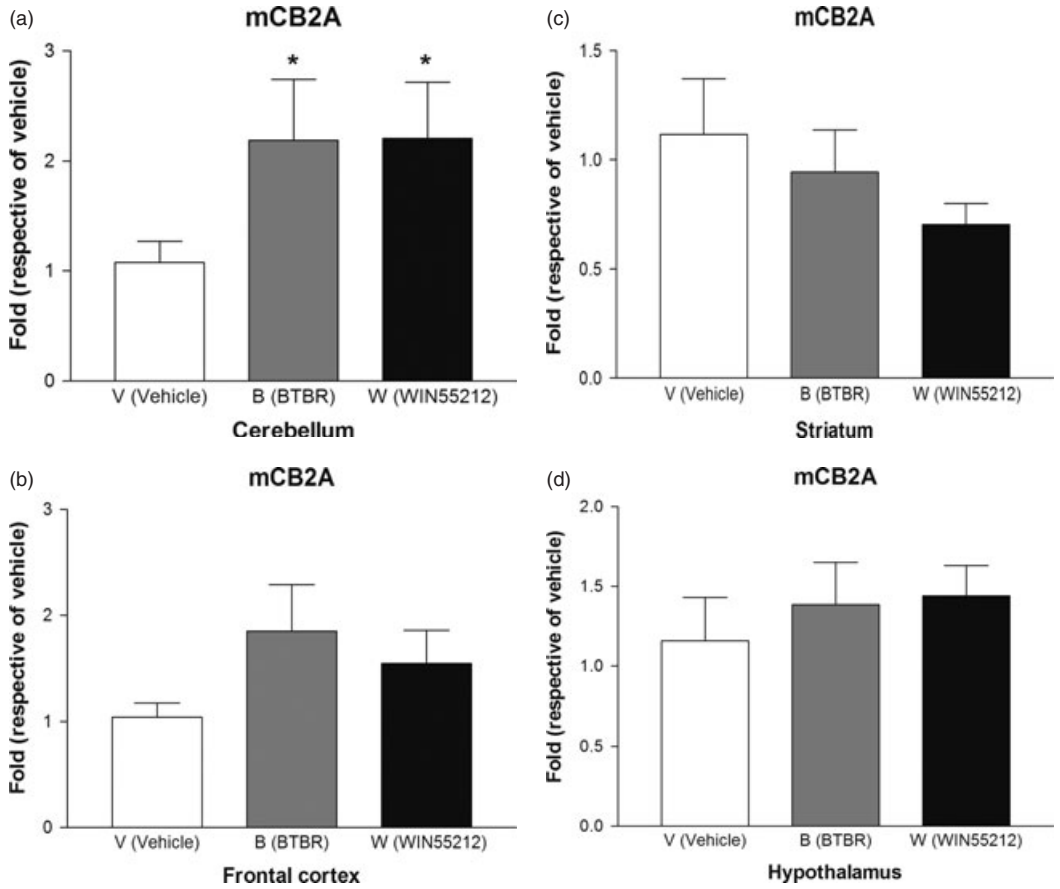

Figure 6: Regulation of mCB2A after 7 days of subacute treatment with the mixed CB-R agonist WIN55212-2 in different brain regions of mice from the inbred strain C57BL/6J and in untreated animals from the BTBR T+tF/J mouse autism model. Error bars were derived from standard error of means (SEM) from six mice. (a) Cerebellum, (b) frontal cortex, (c) striatum and (d) hypothalamus. B (BTBR) mice were untreated whereas W (C57BL/6J) treated animals with WIN55212) had similar upregulation of CB2A gene.

evolution because all other mammalian species such as rat, bovine, sheep, dog, rhesus monkey and chimpanzee do not contain the premature stop codon as does mouse mCB2 (Fig. 5).

Although human and mouse CB2 genes both contain two promoters, the 5′ flanking sequences of the transcription initiation exons share no significant homology and the promoter activities of hCB2 and mCB2 genes are quite different. Human hCB2A is expressed predominantly in testis and hCB2B predominantly in spleen, whereas both rodent CB2A and CB2B are expressed predominantly in spleen. Human hCB2A promoter transcription is stronger in testis and brain regions, whereas hCB2B promoter transcription is stronger in spleen, leucocytes and other peripheral tissues (Fig. 2a–c). In contrast, mouse mCB2A promoter activity is about five times stronger in the expressed tissues than that of mCB2B (Fig. 3a–c). The common features of human and rodent CB2 tissue expression patterns are the isoform-specific predominant tissue expression such as human hCB2A in testis, human hCB2B in spleen, and rodent mCB2A and mCB2B in spleen (Figs 2 and 3). The detectable brain expression of hCB2A was at very low levels at 1% of that of hCB2A in testis and 0.07% of that of hCB2B in spleen; however, the detectable brain expression of mCB2A was measurable at 1% of that of mCB2A in spleen. Therefore, CB2A isoform expression is higher in mouse brain than in human brain. In general, human hCB2B expression levels were higher than hCB2A except in testis and brain regions. Mouse mCB2A expression levels were generally higher in both brain and peripheral tissues. The gene regulation mechanisms might differ in human and mouse CB2 genes.

Regulation of mCB2 isoform expression by CB-R ligand treatment in mouse brain regions of C57BL/6J mice, similar to untreated autistic mouse model of BTBR T+F/J strain

Are mice mCB2A and mCB2B receptor isoforms regulated differentially by treatment with the mixed cannabinoid agonist and CB2-R ligands? We therefore determined

mCB2 gene expression in mice brain regions after subacute treatment with CB2-R ligands using CB1-R selective antagonist AM251 (3 mg/kg) and CB2-R selective antagonist AM 630 (10 mg/kg) and the mixed cannabinoid receptor agonist WIN55212-2 (2 mg/kg) after 4 weeks of treatment (Fig. 6). We did not observe any significant changes of mCB2A and mCB2B in peripheral tissues such as spleen and testis (data not shown). We did not observe any significant expression changes in AM251 and AM630 treatment for CB1- and CB2-specific antagonist treatments, respectively, in brain regions (data not shown). However, there were mouse strain and brain regional-specific mCB2 gene expression profiles following subacute administration with the mixed cannabinoid receptor agonist WIN55212-2 (2 mg/kg). In the cerebellum, for example, mCB2A was significantly (*t*-test: $t = 2.39$, $df = 10$, $P = 0.0438$) upregulated by treatment of C57BL/6J mice with WIN55212-2 in comparison with vehicle controls. Interestingly, mCB2A was also increased significantly in the cerebellum of the BTBR T+tF/J strain of mouse compared with C57BL/6J strain (Fig. 6a). mCB2A isoform expression was increased about onefold in cerebellum in the mixed agonist WIN55212-2 treatment, although no significant changes were observed in other brain regions such as frontal cortex, striatum and hypothalamus (Fig. 6b–d). mCB2B regulation in mouse brain regions were not shown because mCB2B expression is several times lower than that of mCB2A, and the mRNA levels could not be measured reliably by TaqMan assay.

Discussion

New data obtained from these studies shed some light on the ambiguity and sometimes confusing structure and expression patterns of CB2/*CNR2* gene in different species (Brown *et al.* 2002; Munro *et al.* 1993; Shire *et al.* 1996). Using isoform-specific TaqMan probes, we have identified a novel human hCB2A isoform that is expressed predominantly in testis and at lower levels in brain regions of reward system in contrast to hCB2B that is expressed predominantly in spleen with very low expression in brain. We showed that the different sizes (2.5 and 5.0 kb) of hCB2 mRNA observed in Northern blot (Munro *et al.* 1993; Schatz *et al.* 1997) were because of alternative polyadenylation of the extended 3′UTR of hCB2 mRNA. Mouse mCB2A and mCB2B are both expressed predominantly in spleen with mCB2A expression about five times higher in the expressed tissues. mCB2 knockout strain with the C-terminal deletion (Buckley *et al.* 2000) showed enhanced mCB2A and mCB2B promoter activities that might reflect increased expression of the truncated mCB2 receptor. The N-terminal-specific antibody against mCB2 shall be used in the future experiment to determine whether the truncated mCB2 protein is indeed upregulated. The truncated mCB2 receptor might introduce gain of function and influence interpretation of pharmacological and behavioral experiment results on mCB2 knockout mice.

We also identified a rat $rCB2_{360}$ short isoform (Griffin *et al.* 2000) that contains a shorter intracellular C-terminus peptide than previously identified rat $rCB2_{410}$ long isoform (Brown *et al.* 2002). The $rCB2_{360}$ C-terminus intracellular region is more homologous to human and mouse mCB2 C-terminal peptide than that of $rCB2_{410}$ isoform. It is possible that the B2 retroposon insertion into the 3′UTR of rCB2 alters the splicing pattern and created two cryptic alternative splicing sites that caused a frame shift and generated different intracellular C-terminal sequence of rCB2 protein, which also was shown by different sizes of rCB2 mRNA on Northern blot (Brown *et al.* 2002). Subsequently, Brown *et al.* (2002) noted a G-to-T transversion following codon 343 of rCB2 might introduce a putative GT splice donor site that initiates an intron. However, Ashton *et al.* found that the 54 nucleotides following the G-to-T transversion share high identity with homologous hCB2 cDNA sequence including the stop codon (Ashton *et al.* 2008). However, another cDNA sequence (AF286722) from the same laboratory (Brown *et al.* 2002) contains upstream exon 1 spliced to the uninterrupted exon 3 of the rCB2 with B2 retroposon insertion sequence. Translation of this uninterrupted rCB2A sequence produced the same C-terminal intracellular peptide sequence as did $rCB2_{360}$. It is very likely that the major rCB2 isoform is $rCB2_{360}$. We could not find G-to-T transversion or the B2 insertion in the rat strains that we used for the experiment. The expression levels of $rCB2_{360}$ and $rCB2_{410}$ isoforms, and the 3′UTR with or without B2 insertion, therefore, could not be measured. It would be interesting to use proteomic approach to identify $rCB2_{360}$ and $rCB2_{410}$ isoforms once the G-to-T transversion and B2 insertion are identified in rat strains. The C-terminal intracellular domain of CB2 interacts with Gi/o protein that is negatively coupled with adenylyl cyclase (Bayewitch *et al.* 1995) and activates the MAP kinase signal transduction pathway (Wartmann *et al.* 1995). The differences in rat $rCB2_{360}$ and $rCB2_{410}$ isoform might influence the coupling of Gi/o with adenylyl cyclase and downstream signal transduction because rat $rCB2_{410}$ contains additional protein kinase A (PKA) and protein kinase C (PKC) consensus sites (S393 and S399, respectively) besides the common PKA consensus site (S336) that is conserved in the CB2 isoforms of other mammalian species (Fig. 5). It would be interesting to study the pharmacological and behavioral differences of rat strains that contain different C-terminal intracellular peptide sequences.

Retroposon B2 integration into 3′UTR of rCB2 might also affect rCB2 expression. Retroviral integration was also observed in mCB2 gene, and mCB2 3′UTR contains a frequent provirus insertion site (*Evi 11*) after Ca-Br-M murine leukemia virus (MuLV) inoculation of NIH/Swiss mice that developed malignancies (Joosten *et al.* 2000; Valk *et al.* 1997). The special 3′UTR sequences of rodent CB2 and the chromatin structure in this region might form an open DNA conformation to attract retropositions as observed in other genomic regions (Liu & Chan 1990). The B2 retroposon insertion into similar 3′UTR of rat rCB2 gene might also

change the expression of rCB2 and influence hematopoietic differentiation.

The specific human hCB2A isoform that is expressed predominantly in testis might indicate that human hCB2 has a potential function related to spermatogenesis and fertilization. Chronic administration of (-) -Δ^9-tetrahydrocannabinol (THC) induces impotence, reduction of testosterone and decrease of sperm viability (Hall & Solowij 1998; Murphy *et al.* 1994; Park *et al.* 2004). Mouse immature Sertoli cells of testis express mCB2 receptor on the plasma membrane with a CB2-specific agonist-binding profile (Maccarrone *et al.* 2003). Higher expression of hCB2A isoform in human testis might render humans more vulnerable to male gametoxicity by cannabinoids. The detailed localization of hCB2A in human testis needs to be studied further by *in situ* hybridization and immunohistochemistry using human testis biopsy samples with specific nucleotide and antibody probes.

The species differences between the human and rodent CB2 gene are striking. A meta-analysis of previous studies showed significant differences in ligand-binding affinities between hCB2 and rCB2 (McPartland *et al.* 2007a). Human hCB2 gene is four times larger than that of rodents. If the transcription rates are similar between human and rodents, hCB2A isoform would take much longer time to be transcribed in testis and brain. This is unusual because other gene orthologs between humans and mice are usually within onefold differences in genomic sizes. The CB2A promoter distances to the spliced coding exon and the species-specific splicing mechanisms might reflect tissue-specific expression differences between humans and mice. CB2A isoform could be detected in human and mouse brain regions at such low levels of $<0.1\%$ and $<1\%$ of the highest expressed CB2 isoform of human and mouse spleen expression, respectively. However, a selective postsynaptic localization pattern has been observed in rat hippocampus (Brusco *et al.* 2008). CB2A expression level was higher in mouse brain than in human brain, but such immunohistochemical signals of CB2 has yet to be found in human brain regions.

Such promoter-specific CB2-R isoform distribution may in part explain why CB2-Rs were previously undetectable in both human and rodent brains using PCR primers designed in the region of CB2 exon 3 and Northern blot analysis (Brown *et al.* 2002; Griffin *et al.* 1999; Munro *et al.* 1993). Our study of CB2 isoform expression was more specific because the TaqMan probes were designed to hybridize the promoter exons and the major coding exon. TaqMan assay is more sensitive than *in situ* hybridization that was unable to detect significant mCB2 signals in mouse brain (Griffin *et al.* 1999; Munro *et al.* 1993). The weak mRNA signals of CB2 in brain would possibly be overcome by *in situ* PCR on brain slides using CB2A-specific primers to determine cellular types that express CB2A. Therefore, recent detection of mCB2 brain expression in mice by immunohistochemistry (Baek *et al.* 2008; Brusco *et al.* 2008; Gong *et al.* 2006; Van Sickle *et al.* 2005) might not represent human CB2 brain expression patterns because of different promoter activities in terms of quantity and quality for tissue distributions between human and rodents. The CB2 isoform-specific TaqMan probes could differentiate the isoform-specific expression and are more sensitive and specific than CB2 antibodies that are currently available (N-terminal epitopes; Cayman Chemical Company, Ann Arbor, MI, USA, and Sigma-Aldrich, St. Louis, MO, USA., or C-terminal epitope; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA.) that could not distinguish CB2 isoforms. The controversial CB2 brain expression in the literature could be of low expression of CB2A isoform in brain regions and less-specific CB2 commercial antibodies in immunohistochemistry studies, especially those studies using antibodies against human hCB2 epitopes for rodent brain immunostaining. The specificities of CB1 receptor antibodies were also controversial because they could not detect the native and transfected CB1 antigen, although they recognized unspecific proteins in Western blot and immunohistochemistry (Grimsey *et al.* 2008). The mCB2 knockout strain with ablation of N-terminal peptide of 156 amino acids (Deltagen, Inc., San Mateo, CA, USA) could clarify the specificity of the antibodies that were used against the N-terminal epitopes (human CB2 1–33 amino acids from Sigma and human10–33 amino acids from Cayman Chemical Company). Another CB2 knockout strain with ablation of C-terminal peptide of 131 amino acids (Buckley *et al.* 2000) could clarify the specificity of the antibodies that were used against C-terminal epitopes (human CB2 301–360 amino acids from Santa Cruz and rat 328–342 amino acids from Ken Mackie's lab) (Suarez *et al.* 2008).

CB2-specific agonists are under development for therapeutic treatment of inflammatory and neuropathic pain (Guindon & Hohmann 2008) with minimum psychoactive effects (Malan *et al.* 2003). Although cannabinoids significantly reduce chronic pain in animal models, they have less analgesic effects in human participants (Malan *et al.* 2003). The identification of novel human hCB2A isoform that is expressed at low levels in different brain regions might provide targets for any psychoactive side-effects for high doses of CB2 agonist-mediated anti-nociception treatment in humans that are not observed in mouse and rat models (Campbell *et al.* 2001). Another possible side-effect that should be considered is associated with the reproductive system because hCB2A is expressed at a high level in human testis. The differential polyadenylation at 3′UTR that generates 2.5 and 5.0 kb human hCB2 mRNA species might target hCB2 to different cell types and even different subcellular localizations (An *et al.* 2008; Liu *et al.* 2005) that might also differ from human and rodent.

The differential promoter activities and non-homologous 5′UTR and 3′UTR sequences in human and rodents might explain differential anti-nociceptive effects of CB2 agonists in human and rodent models. CB2 gene is conserved from fish to human and only an ancestral CB-R sequence was identified in some invertebrates (McPartland *et al.* 2006). Human *CNR2* gene structure and transcription mechanisms are quite different from those of rodent *Cnr2* gene. It would be interesting to compare the human *CNR2* gene with other primate *Cnr2* genes; however, the *Cnr2* genomic

sequences are still unavailable from other primate species such as chimpanzee and rhesus monkey. The CB2-R gene might evolve more rapidly in recent evolution time than CB1-R (McPartland *et al.* 2007b) to generate such species differences of CB2 gene structure, tissue expression and regulation that should be taken into account for developing analgesic drugs for different species. hCB2A gene is possibly still evolving because many new transcripts first appear in testes that possess the facilitated transcription mechanism. Some functional genes could then migrate out of testes to other tissues (Vinckenbosch *et al.* 2006).

One interesting result from our study is that the BTBR T+tF/J strain that has been reported to exhibit autism-like behavioral phenotypes showed a higher basal wheel running activity and was significantly sensitive to a low dose of BML-190 that induced wheel running activity in the BTBR mice in comparison with vehicle-treated controls (data not shown). Although recent data indicate increasing rates of autism in children, future studies should investigate the role of the cannabinoid system in the etiology of autism spectrum disorders. Some recent events have linked the anti-obesity drug Rimonabant, a CB1-R antagonist, an appetite suppressant with a higher risk of depression and suicide. However, our results showing an association of *CNR2* gene and depression in a human population (Onaivi *et al.* 2008) and an animal model suggest that cannabinoid CB2-R may be involved in the endocannabinoid signaling mechanisms associated with the regulation of emotionality. Thus, more studies are required to determine whether CB2-R ligands have the risk of depression or suicide that has led to the withdrawal of Rimonabant from use as an appetite suppressant in control of obesity. The studies here provide some of the first opportunities to elucidate candidate promoter regions for the hCB2 gene of continuing interest.

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Acknowledgments

This work was supported in part by NIDA/IRP and ESO acknowledges the technical assistance and financial support from William Paterson University Center for Research, Biology department, the Dean's (Dr De Young) student worker fund, and the Provost office for assigned release time. The CB2 knockout and their wild-type control mice were developed by Buckley *et al.* (2000), and obtained from NIAAA through Dr Kunos. Part of human brain tissue was obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, Maryland. The authors would also like to acknowledge the technical assistance of Dr Xia Li from Chemical Biology Research Branch and English editing by Dr Mary Pfeiffer, Editor and Writer NIDA, IRP. ALB and MPV acknowledges Red de Trastornos Adictivos (RD06/0001/1013). We thank Dr John McPartland for his excellent and in-depth review of the manuscript and for his suggestions.