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ORIGINAL ARTICLE

Evaluation of the specificity of antibodies raised against cannabinoid receptor type 2 in the mouse retina

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Abstract Cannabinoid receptors (CB1R and CB2R) are among the most abundant G protein-coupled receptors in the central nervous system. The endocannabinoid system is an attractive therapeutic target for immune system modulation and peripheral pain management. While CB1R is distributed in the nervous system, CB2R has traditionally been associated to the immune system. This dogma is currently a subject of debate since the discovery of CB2R expression in neurons using antibody-based methods. The localization of CB2R in the central nervous system (CNS) could have a significant impact on drug development because it would mean that in addition to its effects on the peripheral pain pathway, CB2R could also mediate some central effects of cannabinoids. In an attempt to clarify the debate over CB2R expression in the CNS, we tested several commercially or academically produced CB2R antibodies using Western blot and immunohistochemistry on retinal tissue obtained from wild-type mice and mice lacking CB2R (*cnr2*$^{-/-}$). One of the antibodies tested exhibited a valuable specificity as it marked a single band near the predicted molecular weight in Western blot and produced no staining in *cnr2*$^{-/-}$ mice retina sections. The other antibodies tested detected multiple bands in Western blot and labeled unidentified proteins when used with their immunizing peptide or on *cnr2*$^{-/-}$ retinal sections. We conclude that many commonly used antibodies raised against CB2R are not specific for use in immunohistochemistry, at least in the context of the mouse retina. Moreover, some of them tested presented significant lot-to-lot variability. Hence, caution should be used when interpreting prior and future studies using CB2R antibodies.

Keywords CB2R · Antibodies · CNS · Knockout · Specificity · Retina

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Introduction

Accurate tissue and cellular distributions of a protein can provide insight into its functional role. Because mRNA levels are not always good predictors of protein expression and do not inform on cellular and subcellular distributions of proteins (de Sousa Abreu et al. 2009), localization studies must include protein-targeted techniques, such as immunoassays. Thus, the specificity of antibodies becomes a critical factor governing the reliability of such assays. The most common strategy to generate antibodies against G protein-coupled receptors (GPCRs) consists of the selection of antigens of 10 to 40 amino acids, from peptide sequences in GPCR domains excluding the cellular membrane (N- or C-terminus domains or intracellular loops). This peptide is then synthetized and injected in a host animal, from which serum is collected and purified in order to obtain a GPCR antibody (Hanly et al. 1995). This strategy has proven successful for the study of some GPCRs and their function (Michel et al. 2009).

The conventional method to test the specificity of an antibody is to pre-adsorb it with its synthetic immunizing peptide and examine the remaining immunoreactivity. Although this method demonstrates the specificity of an antibody for its immunogen, it does not rule out the possibility of off-target labeling with "undesired" proteins that share

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sequence homology with the immunogen. Thus, an antibody that binds to its peptide antigen might not be specific to its target protein exclusively. Numerous reports have been published using antibodies that were validated with such an approach. Hence, many of these publications might have reported invalid information since peptide pre-adsorption was the sole confirmation method used to test for antibody specificity. Currently, the best way to test for the specificity of GPCR antibodies with high confidence is through the use of tissues from which the expression of the protein of interest has been silenced, either by RNAi technologies or by genetic mutations.

Of the many signaling systems involving GPCRs, one that would benefit from more cautious antibody testing is the endocannabinoid system. This complex neuromodulatory system consists of cannabinoid receptors; their endogenous ligands, named endocannabinoids (eCBs); and enzymes responsible for their synthesis and degradation (Pertwee et al. 2010). The cannabinoid receptor type 1 (CB1R) has been extensively studied, and its abundant distribution in the central nervous system (CNS) has been extensively described (Herkenham et al. 1991). Since its discovery, the cannabinoid receptor type 2 (CB2R) was identified as the "peripheral cannabinoid receptor" because it had been first localized in many immune structures (Munro et al. 1993; Buckley et al. 2000). However, recent reports suggest that CB2R may also be expressed in neurons (Van Sickle et al. 2005; Ashton et al. 2006; Gong et al. 2006; Suárez et al. 2008), although the extent of neuronal expression of CB2R is controversial since many of these studies lacked appropriate controls (Atwood and Mackie 2010).

There is increasing indications that the eCB system is implicated in retinal functions, where its activation would likely generate most of the visual effects associated with cannabis consumption. The presence of CB1R was shown in several species from fishes to primates (reviewed in Yazulla (2008)). Its activation affects several retinal processes such as cone photoreceptors' response to light and glutamate synaptic release (Fan and Yazulla 2003; Straiker and Sullivan 2003; Fan and Yazulla 2007), inhibition of calcium and potassium rectifying currents in bipolar cells (Straiker et al. 1999; Yazulla et al. 2000), and modulation of GABA release from amacrine cells (Warrier and Wilson 2007). A few studies also reported the presence of CB2R in the rodent retina. Lu et al. (2000) observed the presence of CB2R mRNA in the ganglion cell layer, the inner nuclear layer, and the inner segments of photoreceptor cells. Lopez et al. (2011), using immunohistochemistry techniques, localized CB2R in the inner segment of photoreceptors, in horizontal, amacrine, displaced amacrine, and ganglion cells of the adult rat retina. Despite these findings, CB2R expression in the CNS is subject to an intense debate (Ashton 2012). Given the physiological importance of the presence of CB2R in neurons, we systematically tested the specificity of a library of antibodies raised against different epitopes of the CB2R for use in immunohistochemistry in the mouse retina.

Methods

Animals and tissue preparation

All procedures were performed in accordance with the guidelines set out by the Canadian Council on Animal Care and were approved by the ethics committee on animal research of the Université de Montréal. CB2R mutant ($cnr2^{-/-}$) and wild-type ($cnr2^{+/+}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). These colonies were maintained in-house and kept under a normal lighting environment (12-h dark/12-h light).

Mice were euthanized by isoflurane overdose. One eye was immediately removed for Western blot analysis. The retina was dissected on ice, promptly frozen, and kept at −80 °C until further processing. Subsequently, a transcardiac perfusion was conducted with phosphate-buffered 0.9 % saline (PBS; 0.1 M, pH 7.4), followed by phosphate-buffered 4 % paraformaldehyde (PFA), until the head was lightly fixed. Two small holes were made in the cornea, prior to a first postfixation in PFA for a period of 30 min. The cornea and lens were then removed, and the eyecups were subsequently postfixed for 30 min in PFA. Several fixation times and protocols were tested, and this method provided the optimal signal-to-noise ratio. The eyecups were washed in PBS, cryoprotected in 30 % sucrose overnight, embedded in Neg 50 tissue embedding medium (Fisher Scientific, Ottawa, Ontario, Canada), flash-frozen, and kept at −80 °C until processing. Sections (14 μm thick) were cut with a cryostat (Leica Microsystems, Exton, PA, USA) and mounted on slides coated with gelatin/chromium (double-frosted microscope slides, Fisher Scientific, Ottawa, Ontario, Canada).

Western blot

Retinas from wild-type mice were homogenized on ice in radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 20 mM Tris (pH 8.0), 1 % NP-40, 0.1 % sodium dodecyl sulfate (SDS), 1 mM EDTA), supplemented with a protease inhibitor mixture (aprotinin, leupeptin, pepstatin (1 μg/ml) and phenylmethylsulfonyl fluoride (0.2 mg/ml); Roche Applied Science, Laval, Quebec, Canada). Thirty micrograms of protein/sample of the homogenate was resolved on a 10 % SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The blocking solution and antibody dilution solution were both 5 % skim milk in Tris-buffered saline containing 0.1 % Tween

20. After blocking, membranes were incubated overnight with various affinity-purified CB2R antibodies at 4 °C (see Table 1 for details). The following day, membranes were exposed to the appropriate HRP-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Detection was carried out using homemade enhanced chemiluminescent (ECL) Western blot detection reagents (final concentrations: 2.5 mM luminol, 0.4 mM *p*-coumaric acid, 0.1 M Tris–HCl (pH 8.5), 0.018 % H_2O_2).

Immunohistochemistry

Frozen sections from wild-type and *cnr2*$^{-/-}$ mice were washed in PBS, postfixed for 10 min in cold acetone, rinsed in PBS with 0.03 % Triton X-100, and blocked in 1 % bovine serum albumin, 0.02 % bovine gelatin, and 0.5 % Triton X-100 diluted in PBS for 1 h. The sections were incubated overnight with antibodies directed against CB2R (see Table 1 for details). The sections were then washed in PBS, blocked for 30 min, and incubated for 1 h with Alexa Fluor donkey anti-rabbit 647 secondary antibody (Molecular Probes, Eugene, OR, USA). After washes, the sections were mounted with a homemade PVA–Dabco mounting medium. The sections were examined with a confocal scanning microscope (TCS SP2, Leica Microsystems), with a ×40 oil immersion objective (NA 1.25). Image stacks (1,024×1,024 pixels×0.5 μm per stack) were captured using the LCS software (version 2.6.1, Leica Microsystems). Offline processing was done with the Fiji software (Schindelin et al. 2012). Gaussian noise from images was partially removed using the PureDenoise plugin for Fiji (Luisier and Blu 2008), and stacks were collapsed by maximal intensity projection.

Antigen retrieval

Different tissue processing techniques were tested in order to get the best detection of the antigen in immunohistochemistry. A simple antigen retrieval protocol for cryostat frozen sections was tried for all antibodies, based on the methods described by Brown et al. (1996). Briefly, the sections were immerged in 1 % SDS solution for 5 min at room temperature. Then, they were rinsed and the immunohistochemical staining steps were completed as described previously. We did not observe any enhancement in the immunoreactivity (data not shown).

Blocking peptides

When available, we used the supplier's blocking peptides. For one antibody (PA1-746), a custom peptide sequence was ordered from W.M. Keck Biotechnology Resource Laboratory (Yale University, New Haven, CT, USA). The crude peptide was produced specifically to match the antibody's immunogen (amino acids 1 to 32 of rat CB2), with an N-terminus acetyl cap and a C-terminus $CONH_2$ cap.

Results

We have tested anti-CB2R commercial antibodies from Cayman Chemical (101550), Pierce Biotechnology (PA1-746), Alpha Diagnostic (CB22-A), and Sigma-Aldrich (SAB2500191), as well as two CB2R antibodies kindly provided by Pr. Ken Mackie (KMCB2-CT and KMCB2-NT, Indiana University, Bloomington, IN, USA), for use in immunohistochemistry using *cnr2*$^{-/-}$ mice. All antibodies used in this study were polyclonal, raised against a portion of N-terminus or C-terminus epitopes of CB2R (Table 1).

Western blot

The potential selectivity of the various antibodies was first tested using Western blot assays. The 101550 antibody, directed against the N-terminus epitope of the human CB2R, labeled a single band at around 45 kDa in wild-type mice retina homogenates (Fig. 1a). This labeling was lost when the antibody was pre-incubated with its immunizing peptide. However, a band at around 45 kDa was detected in *cnr2*$^{-/-}$ retina lysate. The PA1-746 antibody, raised against the N-terminus portion of the rat CB2R, also detected two bands at around 30 and 45 kDa (Fig. 1b). No band was visible when this antibody was pre-adsorbed with its blocking peptide. The same two bands were observed in *cnr2*$^{-/-}$ tissues. No immunoreactivity was found when testing the CB22-A antibody, directed against the C-terminus fragment of the rat CB2R (Fig. 1c), and expectedly, no band was observed when the antibody was pre-incubated with its blocking peptide and in *cnr2*$^{-/-}$ tissues. The KMCB2-CT antibody, raised against the C-terminus part of the rat CB2R, labeled at least six bands ranging from 35 to 100 kDa (Fig. 1d) in both *cnr2*$^{+/+}$ and *cnr2*$^{-/-}$ retinas. The KMCB2-NT antibody, directed against the N-terminus epitope of the rat CB2R, detected six bands from 25 to 150 kDa (Fig. 1e) in both *cnr2*$^{+/+}$ and *cnr2*$^{-/-}$ tissues. The SAB2500191 antibody, raised against the C-terminus epitope of the human CB2R, marked six bands from 28 to 45 kDa (Fig. 1f) in both *cnr2*$^{+/+}$ and *cnr2*$^{-/-}$ retina lysates. Thus, of all antibodies tested, only the 101550 antibody resulted in the detection of a single band on wild-type mice retina homogenates. However, all antibodies showed immunoreactivity when tested against *cnr2*$^{-/-}$ retinal tissue extracts.

Table 1 Characteristics of antibodies tested

Antibodies	Supplier	Lot number	Host species	Reactive species	Epitope	Immunogen	Dilution IB	Dilution IHC
101550	Cayman Chemical Ann Arbor, MI	0424681-1	Rabbit	Human	N-terminus	AA 20–33 (NPMKDYMILSGPQK)	1:3,000	1:200
PA1-746	Pierce Biotechnology Rockford, IL	ME154351	Rabbit	Rat	N-terminus	AA 1–32 (MAGCRELELTNGSNG GLEFNPMKEYMILSDAQ)	1:1,000	1:500
CB22-A	Alpha Diagnostic San Antonio, TX	549596A3.2	Rabbit	Rat	C-terminus	AA 328–345 (GKEEAPKSSV TETEAETL)	1:1,000	1:200
KMCB2-CT	Ken Mackie Indiana University, IN	3/16/07	Rabbit	Rat	C-terminus	AA 328–342 (GKEEAPKSSVTETEA)	1:1,000	1:1,000
KMCB2-NT	Ken Mackie	7/29/09	Rabbit	Rat	N-terminus	AA 1–30 (MAGCRELELTNGSN GGLEFNPMKEYMILSD)	1:1,000	1:1,000
SAB2500191	Sigma-Aldrich Oakville, Ontario	6946P1	Goat	Human	C-terminus	AA 337–351 (VTETEADGKITPWPD)	1:1,000	1:1,000
GAPDH	Sigma-Aldrich	080M4806	Mouse	Rabbit	–	The full-length rabbit muscle GAPDH protein	1:20,000	–

This table is based upon information provided by the respective suppliers

AA amino acid, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase

Immunohistochemistry

Although Western blot assays gave some insights into the antibodies' specificity on retinal extracts towards highly denatured proteins, the ultimate goal of this study was to find an adequate antibody to perform immunohistochemistry on retinal tissue sections. Immunohistochemistry performed with the 101550 antibody using wild-type ($cnr2^{+/+}$) mice retina labeled the outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL) (Fig. 2a). When this antibody was pre-adsorbed with its blocking peptide, no immunoreactivity was visible (Fig. 2b). Furthermore, no staining was observed with $cnr2^{-/-}$ tissues (Fig. 2c). The PA1-746 antibody labeled the ONL, OPL, and IPL in wild-type mice (Fig. 2d). However, we observed the same distribution pattern when the antibody was used with its immunizing peptide (Fig. 2e). No signal was detected in the $cnr2^{-/-}$ mice (Fig. 2f). The CB22-A antibody failed to detect CB2R in wild-type mice (Fig. 2g), showed a weak unspecific signal when pre-incubated with its blocking peptide (Fig. 2h), and did not label CB2R in $cnr2^{-/-}$ mice (Fig. 2i). The KMCB2-CT antibody marked the ONL, OPL, and IPL in wild-type mice (Fig. 2j). When applied with its immunizing peptide, no staining was visible (Fig. 2k). However, a strong staining was observed in the $cnr2^{-/-}$ mice (Fig. 2l). The KMCB2-NT antibody labeled the ONL, INL, IPL, and GCL in wild-type mice (Fig. 2m). Immunoreactivity was detected when this antibody was pre-adsorbed with its blocking peptide (Fig. 2n) or tested in $cnr2^{-/-}$ mice (Fig. 2o). The SAB2500191 antibody labeled the external segments of photoreceptor cells and the ONL, OPL, INL, IPL, and GCL

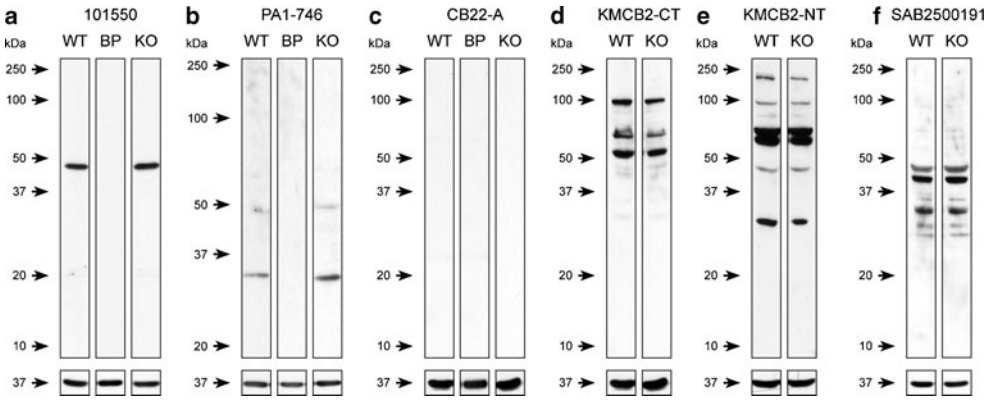

Fig. 1 Western blots using different CB2R antibodies. The 101550 (**a**), PA1-746 (**b**), CB22-A (**c**), KMCB2-CT (**d**), KMCB2-NT (**e**), and SAB2500191 (**f**) antibodies were tested against retina lysate from wild-type (*WT*) mice with and without pre-adsorption with its blocking peptide (*BP*; when available) and from $cnr2^{-/-}$ (*KO*) mice. The *lower lane* represents GAPDH antibody, which was used as a loading control. The *arrows* indicate the position of molecular weight markers

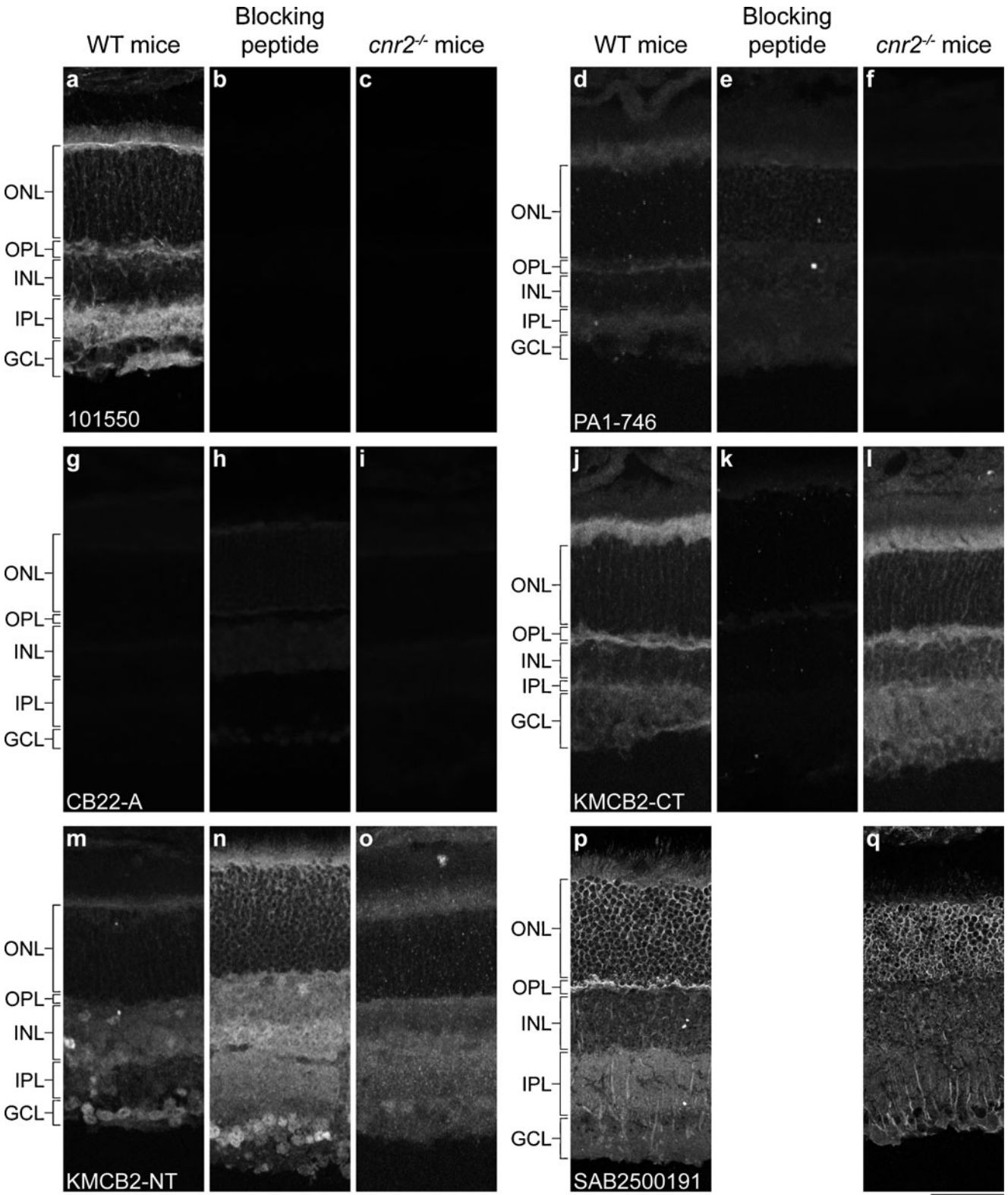

Fig. 2 Immunohistochemical labeling obtained from CB2R antibodies on mouse retinal sections. The 101550 (**a**–**c**), PA1-746 (**d**–**f**), CB22-A (**g**–**i**), KMCB2-CT (**j**–**l**), KMCB2-NT (**m**–**o**), and SAB2500191 (**p**, **q**) antibodies were tested against retinal sections from wild-type (*WT*) mice, pre-incubated with their immunizing peptide, and tested against sections from *cnr2*$^{-/-}$ mice. *ONL* outer nuclear layer, *OPL* outer plexiform layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *GCL* ganglion cell layer. *Scale bar* = 50 μm

in wild-type mice (Fig. 2p). The same immunoreactivity was detected when this antibody was tested in *cnr2*$^{-/-}$ mice (Fig. 2q). Note that the blocking peptide is not commercially available for this antibody. Thus, of all antibodies, only the 101550 did not detect CB2R when incubated with its immunizing peptide and when tested against *cnr2*$^{-/-}$ mice retinal sections.

Lot-to-lot variability

An important aspect of antibody validation is reproducibility. Under our protocols, we noticed that some commercially available antibodies showed inconsistent results in Western blot. For example, a lot of 101550 antibody detected two bands at around 50 and 150 kDa (Fig. 3a) instead of a single band at around 45 kDa, as shown in Fig. 1a. We noted only one inconsistent lot of 101550 antibody out of ten different lots ordered since 2006. Moreover, a lot of PA1-746 antibody labeled six bands ranging from 30 to 90 kDa approximately (Fig. 3b) rather than two bands at around 30 and 45 kDa, previously shown in Fig. 1b. These results highlight the importance of testing the specificity of every new antibody lot.

Discussion

We have tested the specificity of several CB2R antibodies that are currently available from commercial or academic sources using their specific immunizing peptides and *cnr2*$^{-/-}$ mice as negative controls. We have chosen to test these antibodies in the context of the rodent retina because the eCB system is known to exert its effects on this structure and because the presence of CB2R in CNS neurons is controversial (Ashton 2012). We report that many frequently used antibodies raised against CB2R are not specific for use in immunohistochemistry, at least in the context of the mouse retina.

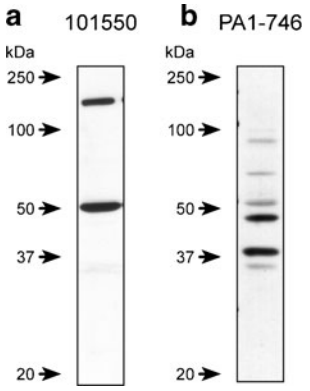

Fig. 3 Lot-to-lot variability with CB2R antibodies. The immunoreactivity of 101550 (**a**) and PA1-746 (**b**) antibodies is different from the one presented in Fig. 1a, b. The *arrows* indicate the position of molecular weight markers

The main objective of this study was to compare different CB2R antibodies in the context of the mouse retina. It has to be noted that none of the antibodies tested was generated against mouse CB2R protein as they were either generated against rat or human CB2R. To our knowledge, there is no antibody generated against mouse CB2R protein. However, rat and mouse CB2R share more than 83 % homology while human and mouse share 82 % (Shire et al. 1996). Furthermore, rat and mouse CB2R share 86 % amino acid identity and 92 % similarity in their N-terminus, while they share 81 % identity and 88 % similarity in their C-terminus (Brown et al. 2002). Moreover, the overlap between the target region in mouse and the immunogen for each antibody is shown in Table 2. Most of the antibodies share more than 85 % in homology with the mouse CB2R protein. For these reasons, we believe that the antibodies used in this study had the potential to label adequately CB2R protein in the mouse retina.

Western blot

Western blot is typically the first validation step to establish an antibody's specificity for immunochemistry purposes. The first sign that an antibody is specific for a particular target would be the observation of a single band at the protein's known molecular weight. The 101550 antibody generated promising results in Western blot as it detected a single band at around 45 kDa near the predicted molecular weight of CB2R (40 kDa). A few reports raise the presence of a glycosylated form of CB2R at around 46 kDa and a non-glycosylated form of CB2R at around 41 kDa (Filppula et al. 2004). Thus, the 45-kDa band observed with the 101550 antibody under our conditions could represent a glycosylated form of CB2R. The other antibodies either failed to detect CB2R (CB22-A) or labeled several bands (PA1-746, KMCB2-CT, KMCB2-NT, and SAB2500191). The presence of multiple bands or a band at an incorrect molecular weight could represent totally different proteins, degradation products, or the desired target at different posttranslational modification steps (Bordeaux et al. 2010). However, to our knowledge, no posttranslational modification has been reported for CB2R that could give rise to the multi-band Western blot profiles observed.

Additionally, a consistent molecular weight for CB2R has been reported across many tissues and species: the rat brain and spinal cord (Gong et al. 2006; Cox et al. 2007; Ramirez et al. 2012; Walczak et al. 2005, 2006; Merriam et al. 2008), the human or mice cultured podocytes (Barutta et al. 2011), and the vervet monkey retina (Bouskila et al. 2013).

Surprisingly, all the antibodies tested showed immunoreactivity with *cnr2*$^{-/-}$ retinal extracts. This implies that none of these antibodies is specific for CB2R in Western blot. These results support the idea that

the specificity of an antibody highly depends on the experimental context in which it is being used (Cernecka et al. 2012). For example, an antibody could be suitable for immunohistochemistry while it may completely lack specificity for Western blot. This could be explained by differences in the conformation of the epitopes under various experimental conditions, resulting in different antibody specificities.

Immunohistochemistry

While the Western blot is a crucial first step in antibody characterization, it does not establish antibody specificity for immunohistochemistry. Indeed, detection of the denatured, linearized protein in Western blot does not guarantee the same reaction with the protein in its native configuration.

In immunohistochemistry, only the 101550 antibody yielded a strong staining in wild-type mice, as well as no unspecific signal when used with its blocking peptide or on *cnr2*$^{-/-}$ mouse retinas. Therefore, we would only recommend the use of this antibody for immunochemistry purposes. This recommendation is supported by the fact that the retinal cellular distribution of CB2R we observed using this antibody is in agreement with CB2R mRNA expression in the GCL, the INL, and the inner segments of photoreceptors (Lu et al. 2000). It is also strengthened by the fact that this antibody has also been validated on renal tissues from *cnr2*$^{-/-}$ mice (Barutta et al. 2011).

Furthermore, a recent report testing different CB2R antibodies, including the 101550, in the brain using the knockout control test concluded that none of the antibodies tested are specific (Baek et al. 2013). An important factor can explain the differences between this study and ours: Baek et al. (2013) tested the CB2R antibodies in the context of the brain. The brain is a complex and heterogeneous cellular composition relative to other tissues, such as the retina. Thus, non-specific interactions and background issues are more important in the brain. These results suggest that the 101550 antibody is only specific to the mouse retina.

It is evident that relying only on the loss of signals with immunizing peptide or the presence of a single band in Western blot is not the best method to prove the specificity of an antibody (Michel et al. 2009). Blocking peptides do not demonstrate exclusive specificity of an antibody since off-target binding activity of the antibody to an irrelevant epitope that is structurally similar to the desired epitope will also be inhibited by pre-adsorption with the immunizing peptide. Thus, blocking peptides can show that an antibody is unspecific, when staining is seen in their presence, but they cannot prove that an antibody is specific (see for review Bordeaux et al. (2010)). Knockout models, in which the coding sequence of the protein of interest has been genetically deleted, thus provide very good negative controls (Lorincz and Nusser 2008), although attention needs to be given to the portion of the gene that has been deleted.

The *cnr2*$^{-/-}$ mouse used in this study was developed by Deltagen Inc. (San Mateo, CA, USA) and distributed by The Jackson Laboratory. It was generated by the insertion of a neomycin coding sequence in the *cnr2* gene, leading to the deletion of sequences encoding the first three transmembrane domains (amino acids 26 to 140 of the mouse *cnr2* cDNA). While the coding sequence for amino acids 1 to 25 remains, it is not known if this sequence is actually translated (Monory and Lutz 2009). Interestingly, the immunogens used to generate PA1-746 and KMCB2-NT antibodies corresponded to amino acids 1 to 32 and 1 to 30, respectively. Consequently, these antibodies could still bind to amino acids 1 to 25 of the native protein sequence in the *cnr2*$^{-/-}$. The 101550 antibody's immunogen is from amino acids 20 to 33. When used with *cnr2*$^{-/-}$ sections, this antibody could bind with amino acids 20 to 25. However, studies have revealed that about 15 to 22 amino acids on the surface on the antigen make contact with a similar number of residues on the antibody's binding site (Alberts et al. 2002; Frank 2002; Goldsby et al. 2002). Therefore, there is virtually no chance that the 101550 antibody is able to react with only six amino acids in native confirmation of the CB2R. This is exactly what was observed, as no immunoreactivity was detected in *cnr2*$^{-/-}$ sections with the 101550 antibody. After the PGK-Neo cassette insertion, the rest of the CB2R coding region was still present in the genome. We cannot eliminate the possibility that there could be a splicing over the PGK-Neo cassette, even if it is reported to be unlikely (Monory and Lutz 2009).

A second *cnr2* knockout mice line is also available, characterized by the ablation of C-terminus amino acid positions 217 to 347 of CB2R (Buckley et al. 2000). Because the first 216 amino acids coding for the first five transmembrane domains of the CB2R protein were unaffected, this mouse line could only be useful for testing antibodies raised against C-terminus epitopes of CB2R. This mouse strain was analyzed using quantitative RT-PCR, and it was discovered that the promoters of *cnr2* knockout mice were still active and that a truncated version of CB2R mRNA was expressed, indicating that this mouse was an incomplete *cnr2* knockout (Liu et al. 2009).

Lot-to-lot variability

Finally, we would also recommend testing CB2R antibodies from lot to lot. Our experience is that one lot of an antibody may work fine; the next may not. This was the case for 101550 and PA1-746 antibodies, which displayed inconsistent results from one batch to another. Lot-to-lot inconsistency could also explain the absence of immunoreactivity of the CB22-A antibody, an antibody that was otherwise validated in C-terminus epitope *cnr2*$^{-/-}$ mice on brainstem neurons (Van

Table 2 Homology between the mouse CB2R protein and the various epitope sequences of the antibodies tested

Mouse CB2R N-terminus sequence (AA 1–33)	M	E	G	C	R	E	T	E	V	T	N	G	S	N	G	G	L	E	F	N	P	M	K	E	Y	M	I	L	S	S	G	Q	Q
101550 Identity 71 % Similarity 79 %																				N	P	M	K	*D*	Y	M	I	L	S	**G**	**P**	Q	**K**
PA1-746 Identity 84 % Similarity 91 %	M	**A**	G	C	R	E	**L**	E	*L*	T	N	G	S	N	G	G	L	E	F	N	P	M	K	E	Y	M	I	L	S	**D**	*A*	Q	
KMCB2-NT Identity 87 % Similarity 90 %	M	**A**	G	C	R	E	**L**	E	*L*	T	N	G	S	N	G	G	L	E	F	N	P	M	K	E	Y	M	I	L	S	**D**			
Mouse CB2R C-terminus sequence (AA 328–345)	G	K	E	E	G	P	R	S	S	V	T	E	T	E	A	D	V	K	T	T													
CB22-A Identity 72 % Similarity 89 %	G	K	E	E	*A*	P	*K*	S	S	V	T	E	T	E	A	*E*	**T**	**L**															
KMCB2-CT Identity 87 % Similarity 100 %	G	K	E	E	*A*	P	*K*	S	S	V	T	E	T	E	A																		
SAB2500191 Identity 60 % Similarity 60 %										V	T	E	T	E	A	D	**G**	K	I	T	**P**	**W**	**P**	**D**								

Identical amino acids in normal style, similar residues in italics, and dissimilar residues in bold

Sickle et al. 2005). This potential discrepancy between cerebral and retinal tissues highlights the importance of thoroughly testing antibodies in the cellular context in which they will be used.

Commercial GPCR antibodies' specificity

Recently, an increasing number of studies raised concerns regarding the specificity of GPCR antibodies (Grimsey et al. 2008; Bodei et al. 2009; Hamdani and van der Velden 2009; Jensen et al. 2009; Beermann et al. 2012; Cernecka et al. 2012; Baek et al. 2013; Seifert et al. 2013). Since, four criteria have been proposed to demonstrate receptor antibody specificity, of which at least one must be met to consider an antibody to be specific (Michel et al. 2009). Firstly, the reactivity of a specific antibody must be lost upon analysis of tissues obtained from animals genetically deficient in expression of the receptor of interest. Secondly, the reactivity of a specific antibody must clearly decrease after genetic knockdown of the expression of the receptor of interest. Thirdly, the reactivity of a specific antibody must be present when analyzing cells recombinantly expressing the receptor of interest, but must be absent when analyzing closely related receptor subtypes. Finally, the reactivity of a specific antibody must be comparable to that of other antibodies recognizing different epitopes of the same receptor. The 101550 antibody meets correctly the first of these criteria, as its staining disappears in immunohistochemical studies of tissues from animals genetically engineered to lack CB2 receptor. We fully agree with some reports stating that it would be helpful to have "certified" commercial antibodies that fulfill at least one of the criteria to demonstrate sufficient specificity (Pradidarcheep et al. 2008; Michel et al. 2009; Beermann et al. 2012).

Conclusion

Given that many studies using CB2R antibodies did not test their antibodies against KO tissues (Benito et al. 2005; Ashton et al. 2006; Gong et al. 2006; Brusco et al. 2008; Suárez et al. 2008; Lopez et al. 2011; den Boon et al. 2012; Schmidt et al. 2012), their interpretation becomes somewhat debatable given the data presented in this paper. We conclude that at present, there is no perfectly reliable antibody-based method for CB2R detection in adult mice retina for immunohistochemistry, and a great deal of caution, together with appropriate concurrent controls, must be employed in any study using CB2R antibodies. In this study, the 101550 antibody shows the most valuable specificity despite some lot-to-lot variability.

Consequently, we suggest that this antibody can be used, with concurrent knockout controls, for immunohistochemistry expression studies.

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