Tips and tricks for cannabinoid receptor 1 detection, interaction and interpretation

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Proteomic approaches are currently used to explore and identify interacting partners of proteins involved in diverse biological processes or with therapeutic potential. The cannabinoid receptor 1, CB_1, is the main mediator of cannabinoids effects in the central nervous system. The role of the endocannabinoid system in development and physiology and as a target of pharmacological and cellular therapies is the subject of intense research. Therefore, the scarcity of proteomic studies on the interactome of CB_1 is, somehow, surprising (Mattheus et al., 2016). We addressed the proteomic study of the CB_1 receptor interactome in rat primary cortical neuron cultures after immunoprecipitation (IP) of the receptor, but were puzzled by the confusing and disparate data regarding the identification of the CB_1 receptor by western blot (WB). Therefore, we established handling conditions for IP and WB detection of CB_1 that resulted in the consistent and specific detection and immunopreciptitation of CB_1 with different antibodies. Based on WB results after deglycosylation and IP of the CB_1 receptor performed with different antibodies, we propose a new interpretation of the molecular identity of CB_1 multiple apparent molecular weights reported in the literature (Esteban et al., 2020). We believe our findings may contribute to clarify the identification of the receptor by WB and IP and make proteomic studies more solid and robust. The reinterpretation of WB and IP results discussed in our report may open new lines of research which will contribute to the understanding of the molecular nature of the CB_1 receptor.

During the first stages of our proteomic approach, we checked for the presence of the receptor after IP by WB following published protocols requiring the boiling of samples before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), but were unable to identify CB_1 by WB both in lysates or immunoprecipitates. To solve this problem, as a previous and necessary step to continue with the project, we decided to find appropriate sample handling conditions for IP and WB detection of CB_1. We used wild-type and CB_1 knockout mouse (Zimmer et al., 1999) cerebral cortex and cerebellar lysates and tested four commercial CB_1 antibodies extensively referenced in the literature: two raised against the C-terminal region of CB_1 (Ct FS from Frontier Institute Co., Ltd., RRID AB_2571593 and Ct Cay from Cayman Chemical, RRID AB_409026) and two raised against the N-terminal region of the receptor (Nt Alo from Alomone Labs, RRID AB_2039795 and Nt Cay from Cayman Chemical, RRID AB_327840; for a complete reference of these antibodies see Esteban et al., 2020).

Our initial results indicate that the use of a negative control and the avoidance of heat denaturation of the samples result in the clear identification of the CB_1 receptor as a protein with an apparent molecular weight of 53 or 64 kDa, depending on the antibody used. In this sense, the Nt Cay antibody detected a 64 kDa protein while the other three antibodies detected the 53 kDa band. Heating samples above 65°C produces a clear reduction in the amount of CB_1 that can be detected by WB and at 95°C the 53–64 kDa CB_1 bands disappear and CB_1 immunoreactivity can be detected as a high molecular weight aggregate in the upper part of the gel and within the stacking gel. The formation of high molecular weight aggregates of membrane proteins such as G-protein coupled receptors after boiling the samples before SDS-PAGE has been reported before (Sagné et al., 1996; Corin et al., 2011; Hislop and von Zastrow, 2011), and our own data confirm that this is the case for CB_1. We also tested different detergents and their effect on CB_1 WB detection and IP. We conclude that the non-ionic detergent n-dodecyl-β-D–maltoside (DDM) was the detergent of choice since it yielded a significant higher amount of CB_1 detected by WB and especially by IP.

The detection of 53 and 64 kDa CB_1 bands has been attributed to the existence of glycosylated forms of the receptor which would be specifically recognized depending on the antibody used (Song and Howlett 1995; Grimsey et al. 2008; Hebert-Chatelain et al. 2014). In this manner, the Nt Cay antibody would specifically recognize a glycosylated form of 64 kDa, while the rest of the antibodies used would detect a non-glycosylated form of around 53 kDa in agreement with the molecular weight of 52.85 kDa calculated with different bioinformatics tools. Standard deglycosylation protocols require sample heat denaturation for a better access of the glycosidase to the N-glycosylation sites, but due to the formation of CB_1 high molecular weight aggregates after heating at 95°C, we used a mutant PNGase F that works on native proteins at room temperature. When brain and cortical neuron culture lysates were treated with PNGase F we obtained unexpected results, since we were able to observe a downshift in the apparent molecular weight of the two CB_1 forms, which would indicate they are both glycosylated. The migration of the CB_1 protein in SDS-PAGE could be dependent on the packing state of the receptor in SDS micelles (Therien et al., 2001; Andersson et al., 2003). The WB results observed after CB_1 deglycosylation could be then explained hypothesizing that a unique fully glycosylated protein is in fact present. Depending on the folding and packing status of the receptor in the presence of SDS, a mixed population of receptor molecules could have different epitopes accessible to the antibodies and show different electrophoretic mobility and, therefore, different apparent molecular weights (**Figure 1**).

This new interpretation of CB_1 WB data, based on the folding and packing status of the receptor in the presence of SDS, could also explain the results obtained after the IP of CB_1 from cortical neuron lysates with antibodies that in WB recognize the 53 or the 64 kDa CB_1 bands specifically. We found that the Nt Alo and the Ct Cay antibodies were able to immunoprecipitate the 53 kDa band they recognize in WB, but surprisingly, they were also able to immunoprecipitate the 64 kDa band that was detected in WB only by the Nt Cay antibody (**Figure 1B** and **C**). The solubilization of lysate proteins as well as the IPs were performed using DDM, a non-ionic detergent that preserves the structural integrity of G-protein coupled receptors in solution (Roy et al., 2013). In these conditions, CB_1 would maintain a native or close to native structure. The different epitopes would be accessible and the three antibodies would be able to immunoprecipitate the receptor (**Figure 1A**). The conditions after IP are quite different and the SDS in the loading and running buffers and in the polyacrylamide gel used for WB would condition CB_1 folding and packing state. Again, a mixed population of CB_1 molecules with variable epitope accessibility and altered electrophoretical mobility could be present, which would be recognized as 53 and 64 kDa bands by the different antibodies (**Figure 1B** and **C**). The only discrepant piece of data found is the inability of the Ct FS and Nt Alo antibodies to detect the 53 kDa band after IP with the Nt Cay antibody. We hypothesize that after IP, the Nt Cay-bound CB_1 should transition to a denatured state in loading buffer at 50°C in the presence of SDS and in the denaturing conditions of the electrophoresis. This could affect the folding and packing of the receptor resulting in the masking of the epitopes recognized by the Ct FS and Nt Alo antibodies and thus, in the absence of the 53 kDa band in the Nt Cay IP.

With all these data in hand, we asked whether these new conditions were appropriate to resume the study of the CB_1 interactome using a proteomic approach

based on the co IP of CB_1 and interacting proteins. As a straightforward approach to answer this, we checked for the presence of G protein subunits already identified as CB_1 associated partners essential for its signaling. We used the Nt Alo and the Ct Cay CB_1 antibodies and were able to coimmunoprecipitate CB_1 and the G_o, G_{i3} and G subunits known to participate in cannabinoid signaling through this receptor.

Overall, the interpretation of CB_1 detection data by WB has been rather difficult due to the disparate molecular weights reported for the receptor, the variety of sample preparation conditions and the frequent lack of use of appropriate negative controls such as CB_1 KO tissue. Additionally, a great amount of research papers on CB_1 presenting pharmacological data do not exhibit WB results or show trimmed WB images, making the interpretation and further validation of the results difficult. Our data demonstrate that the control of sample preparation heating and the use of a proper detergent are crucial parameters for the immunoblot detection and IP of CB_1. The adoption of these basic guidelines together with the use of an appropriate negative control, CB_1 KO tissue or untransfected cells lysates, and the presentation of untrimmed WB images showing a wide range of molecular markers, will contribute to the correct interpretation of CB_1 WB and IP data and will facilitate the replication of experimental results.

We also propose a new interpretation of CB_1 WB and IP data based on the variable folding and packing status of the receptor in the presence of SDS or DDM that could explain the data obtained in our deglycosylation experiments on native CB_1 at room temperature and the IP and WB results observed. The expression in cell lines of CB_1 plasmids carrying different point mutations for the N-glycosylation sites and N-terminal and C-terminal tags could be a valuable strategy to better understand the molecular structure of the receptor, although the efficiency of the expression of the receptor in cell lines is also an issue that would have to be addressed.

Finally, as mentioned above, given the amount of effort put into the study of the endocannabinoid system, the scarcity of proteomic studies on the interactome of the CB_1 receptor is somehow, puzzling, and could reflect the use of inadequate sample handling conditions. Our results show that the study of the interactome of the CB_1 receptor and other G-protein coupled receptors could benefit from the use of the most advantageous conditions for sample preparation, the coimmunoprecipitation of the CB_1 receptor and the ulterior analysis of the results by WB.

This work was funded by the Ministry of Science, Innovation and Universities of Spain, Grant ID SAF2015-69927 (cofunded by FEDER-European Union) (to EMH).

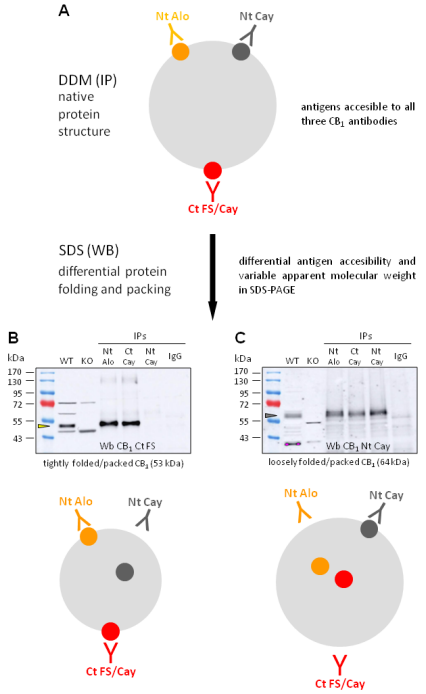

Figure 1 | **CB_1 apparent molecular weight in WB could be dependent on the folding and packing status in the presence of SDS.**
With the WB results obtained after the deglycosylation and IP of the CB_1 receptor, we propose a new interpretation on the nature of the variable apparent molecular weigth of CB_1 obseved in WB. We argue that a unique fully glycosylated protein may be present in brain lysates and that the 53 and 64 kDa forms found in WB with different antibodies could be the result of a mixed poulation of differentially folded and packed receptor molecules in the presence of SDS used for electrophoresis. (A) When solubilizied in non-ionic detergents such as DDM, the receptor would conserve a native structure and present different antigens accesible to the antibodies used in our study. Therefore, the Nt Alo and the Ct Cay antibodies would immunoprecipitate the native receptor that in the presence of SDS and due to a more tightly packed status would be recognized by the Ct FS and Nt Alo antibodies as a 53 kDa protein whose folding would mask the Nt Cay antigen (B; yellow arrowhead). The absence of the 53 kDa CB_1 form in the Nt Cay IPs could be due to the inability of the Nt Cay-bound receptor to adopt this tightly packed form recognized by the Nt Alo, Ct FS and Ct Cay antibodies when denatured in the presence of SDS (B). The more relaxed packed form would be recognized as a 64 kDa protein (C, grey arrowhead) by the Nt Cay antibody and would have the antigenes for the Nt Alo, Ct Cay and Ct FS antibodies masked. Rat brain lysates were immunoprecipitated with the Nt Alo, Ct Cay and Nt Cay antibodies or purified control rabbit IgG. The IPs were resolved by SDS-PAGE and the presence of CB_1 was revealed with the Ct FS antibody (B) and the Nt Alo antibody (data not shown). The membrane was stripped and reprobed with the Nt Cay antibody (C). The two western blots are reprinted from Esteban et al. (2020). DDM: n-Dodecyl-β-D–maltoside; IP: immunoprecipitation; SDS: sodium dodecyl sulfate; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WB: western blot.

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Date of submission: May 17, 2020
Date of decision: July 9, 2020
Date of acceptance: November 21, 2020
Date of web publication: January 7, 2021

https://doi.org/10.4103/1673-5374.300984
How to cite this article: *Esteban PF, Molina-Holgado E (2021) Tips and tricks for cannabinoid receptor 1 detection, interaction and interpretation. Neural Regen Res 16(8):1535-1536.*
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Peer review: *Externally peer reviewed.*

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C-Editors: Zhao M, Qiu Y; T-Editor: Jia Y