Faculty Opinions

Proteolytic regulation of calcium channels – avoiding controversy

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EVALUATION OF

Landmark

Proteolytic processing of the L-type Ca^{2+} channel alpha$_1$ 1.2 subunit in neurons [version 2; referees: 3 approved].

Buonarati OR *et al.*
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The publication of papers containing data obtained with suboptimal rigor in the experimental design and choice of key reagents, such as antibodies, can result in a lack of reproducibility and generate controversy that can both needlessly divert resources and, in some cases, damage public perception of the scientific enterprise. This exemplary paper by Buonarati *et al.* (2018)[1] shows how a previously published, potentially important paper on calcium channel regulation falls short of the necessary mark, and aims to resolve the resulting controversy.

This article contains an editorial note.

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Background

Dynamic changes in the intracellular concentrations of calcium ions (Ca^{2+}), mediated by numerous Ca^{2+} channels, pumps and exchangers, are a critical regulator of almost all cellular functions. Among a large family of closely related channels, the plasma membrane L-type voltage-gated Ca^{2+} channel (LTCC) is a major source of Ca^{2+} influx following membrane depolarization in many excitable cells, including neurons, cardiomyocytes, smooth muscle cells, and endocrine cells. The major poreforming LTCC α1 subunit consists of four homologous domains (I–IV), each containing six transmembrane α-helices; distinct genes encode four LTCC α1 subunit variants, known as $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, and $Ca_v1.4$. Most neurons express a mixture of $Ca_v1.2$ and $Ca_v1.3$ LTCCs, and they can regulate excitability, synaptic transmission and gene expression. Mutations in the $Ca_v1.2$ and $Ca_v1.3$ LTCC α1 subunits have been linked to neuropsychiatric and neurodevelopmental disorders, as well as cardiac dysfunction. Thus, it is critical to understand how LTCCs are regulated.

Multiple $Ca_v1.2$ and $Ca_v1.3$ variants are differentially expressed in various cells/tissues, which have been attributed to alternative mRNA splicing and in some cases regulated proteolysis. These variants exhibit different biophysical properties and can differentially regulate downstream signaling. However, there is considerable controversy surrounding the reported sizes and cellular/subcellular distributions of LTCC variants at the protein level.

The Buonarati *et al.* (2018) article directly addresses this issue by rigorously identifying the major $Ca_v1.2$-derived protein species in rodent brain[1]. Their results call into question potentially exciting previous reports that $Ca_v1.2$ is regulated by proteolytic cleavage[2,3]. Buonarati *et al.* attribute those findings to the prior use of inadequately characterized antibodies for immunoblotting and immunohistochemical analyses. This paper provides an exemplary template for the antibody characterizations that are required to avoid creating similar controversies in the future.

Main contributions and importance

The approach to the issues

The 250 kDa full length $Ca_v1.2$ α1 subunit, known as α1c, can be partially proteolyzed in cells to generate multiple fragments with distinct functional properties, including a 210 kDa fragment that remains associated with an ~40 kDa fragment containing the distal cytosolic C-terminal domain[4–6]. Also reported are a C-terminal fragment of ~75 kDa[3] and ~90 and ~150 kDa "hemi-channel" fragments generated by mid-channel proteolysis[2]. However, not all of these fragments have been confirmed. To address this issue, Buonarati *et al.* rigorously validated the specificity of several polyclonal antibodies that recognize different epitopes in the $Ca_v1.2$ α1 subunit and then used them to define the major $Ca_v1.2$ proteins expressed in rodent brain. Major strengths of their approach include:

1. The careful and complete description of the methods used and the data presented, including detailed descriptions of the sources and epitopes recognized by each antibody tested.

2. The use of tissue from $Ca_v1.2$ knockout mice as a negative control to conclusively identify $Ca_v1.2$-specific bands recognized by the antibodies in tissue lysates (see Fig. 2 of Buonarati *et al.*[1]). The identity of these bands is further validated by immunoprecipitation followed by immunoblot. The data clearly demonstrate that antibodies separately raised to identical epitopes can recognize a different set of specific and non-specific bands on immunoblots. This is a significant concern

when investigators use commercially-available antibodies because polyclonal antibodies sold under the same product/catalog number may in fact have been generated in different animals (e.g. different lots), but few companies make this apparent.

3. The extremely careful analysis of the sizes of the proteins recognized by these antibodies, including an exhaustive comparison of apparent sizes of these bands using two different commercially-sourced molecular weight markers, while varying the acrylamide concentrations used in the gel. The data (see Fig. 3) provide a master-class in the dangers of using pre-stained molecular weight markers and the impact of other variables on estimating the sizes of proteins in polyacrylamide gels.

4. The authors confirm that an ~210 kDa putative proteolytic fragment of $Ca_v1.2$ lacking a large piece of the C-terminal domain (including the epitopes for some of the antibodies used) in native tissues co-migrates with a recombinant fragment expressed in heterologous cells (see Fig. 4), providing strong support for the biological source of this band.

The results

The data clearly demonstrate that the full-length form of $Ca_v1.2$ in brain migrates with an apparent molecular weight of ~250 kDa, consistent with the predicted size and with the electrophoretic mobility of recombinant full-length protein. The ~250 kDa protein is detected with all of the fully validated antibodies: three that recognize the II-III loop in the middle of the protein, as well as three that recognize the cytosolic C-terminal domain.

A shorter, ~210 kDa form was also identified by antibodies recognizing epitopes in the II-III loop or the membrane proximal region of the C-terminal domain (pS1700), but not by antibodies recognizing more distal epitopes in the C-terminal domain (pS1928 and CNC2). The ~250 kDa and ~210 kDa forms of $Ca_v1.2$ appear to be present in similar abundance in both mouse and rat brain. These findings are consistent with prior studies showing partial cleavage of ~350 amino acids from the distal C-terminus of the channel in tissues[4–6].

Given these findings, one might also expect to detect a 350 amino-acid cleavage product of $Ca_v1.2$, and it is notable that the CNC2 antibody also recognizes an ~30 kDa protein that is absent from $Ca_v1.2$ knockout brain tissue (see Fig. 9). Although this protein appears somewhat smaller than the predicted ~350 amino acid fragment, this discrepancy was not addressed. Since an ~40 kDa C-terminal domain fragment was also previously reported[6], it is possible that the distal C-terminal domain can be proteolyzed in more than one place in a tissue-specific manner, yielding appreciable levels of these C-terminal fragments with potentially distinct functions.

Another focus of the current studies was to investigate possible mid-chain proteolysis of $Ca_v1.2$ within the II-III linker domain, which was reported to generate N- and C-terminal hemi-channel fragments of ~90 kDa and ~150 kDa, respectively[2].

In addition to the specific ~250 kDa and ~210 kDa bands, Buonarati *et al.* found that the rabbit polyclonal FP1 antibody that they had generated detected a major ~150 kDa protein in brain lysates, but that the ~150 kDa band was also detected in $Ca_v1.2$ knockout tissue (see Fig. 2A). The commercial rabbit polyclonal ACC-003 antibody, purchased from Alomone Labs, also detected non-specific proteins of ~130 kDa and ~180 kDa in brain lysates. None of the non-specific ~130 kDa, ~150 kDa and ~180 kDa bands were immunoprecipitated by the FP1 antibody (see Fig. 2B).

These data conclusively establish that the FP1 and ACC-003 antibodies recognized non-specific proteins in brain lysates. But – emphasizing further the need to validate the exact source of antibodies – Buonarati *et al.* obtained (from Sigma) samples of the antibody that Michailidis *et al.* had used to detect the putative ~150 kDa hemi-channel fragment. They found that the Sigma antibody recognized ~130 kDa and ~180 kDa non-specific bands in brain lysates, just like the ACC-003 antibody from Alomone. Further research indicated that the Sigma and ACC003 antibodies were raised to an identical epitope, and that Sigma has been distributing the antibody generated by Alomone Labs (without openly disclosing this!).

All of this said, Buonarati *et al.* found that their FP1 antibody could immunoprecipitate small amounts of an ~150 kDa protein that was detected by the rabbit polyclonal CNC1, FP1 and ACC-003 antibodies if the immunoblots were over-exposed, but was absent in samples isolated from $Ca_v1.2$ null tissue. Notably, this $Ca_v1.2$-specific ~150 kDa protein was estimated to contribute ≤1% to the total ~150 kDa immunoreactivity detected when using the FP1 antibody to immunoblot brain lysates. Moreover, they estimated that the specific ~150 kDa protein constitutes no more than 1% of the total $Ca_V1.2$ immunoreactivity.

Open questions

The detection of only very low levels of a specific ~150 kDa $Ca_v1.2$ fragment by Buonarati *et al.* failed to support the substantial levels of mid-chain proteolysis of $Ca_v1.2$ previously reported by Michailidis *et al.* We should note that the immunoblots shown by Michailidis *et al.* to identify the putative ~90 and ~150 kDa hemi-channel fragments also detected several other bands, and that none of the proteins recognized in their immunoblots were rigorously validated (e.g. by immunoblotting $Ca_v1.2$ null tissue). Moreover, the accuracy of the estimated sizes of their fragments is unclear because the acrylamide concentration used in their gels and their source of molecular weight markers were not reported.

Despite major technical issues with their analyses, Michailidis *et al.* reported that "mid-chain proteolysis" was significantly increased following LTCC activation in brain slices and in brain lysates from older (>6 months) mice. Since Buonarati *et al.* probed for the putative ~150 kDa hemi-channel fragment only in samples from 6–12 week old rodents without LTCC activation, it remains theoretically possible that the levels of this $Ca_v1.2$ fragment are increased in older animals and after activating LTCCs, perhaps due to mid-chain proteolysis. This issue also may be further clarified by extending the studies of Buonarati *et al.* to probe for the putative ~90 kDa N-terminal hemi-channel using rigorously validated antibodies. Alternatively, it may be fruitful to use mass spectrometry-based proteomics methods to carefully analyze immunoprecipitated calcium channel complexes for these fragments, with parallel analysis of appropriate control complexes isolated with the same antibodies but from knockout animal tissues.

Conclusion

In summary, Buonarati *et al.* describe a rigorous set of very carefully controlled studies using thoroughly validated antibodies. In this regard, this is an exemplary paper that should be used as a valuable teaching tool to illustrate the controls needed to rigorously validate all antibodies before use in immunoblotting studies. The use of knockout tissue as a negative control is the strongest way to validate antibody specificity, not only for immunoblotting but also for immunohistochemical studies or immunoprecipitation-based analyses.

The data obtained by Buonarati *et al.* convincingly demonstrate that there are two major $Ca_v1.2$ proteins (~250 and ~210 kDa) in the brain samples that were analyzed. The ~210 kDa fragment appears to be generated by proteolytic removal of an ~30–40 kDa fragment from the C-terminal domain. Most importantly, their data provide little, if any, support for the idea that $Ca_v1.2$ LTCCs undergo the substantial levels of mid-chain proteolysis previously suggested by Michailidis *et al.* Furthermore, Buonarati *et al.* found no evidence to support the existence of an ~75 kDa C-terminal fragment described by Gomez-Ospina *et al.*[3].

However, despite the many strengths of the vastly superior immunoblotting studies reported by Buonarati *et al.* that allowed for clear and convincing interpretations, it is very hard to "prove" a negative and it remains a formal possibility that additional mid-chain proteolysis of $Ca_v1.2$ is more prevalent under other conditions.

Editorial Note

This evaluation was originally written in 2019, as a contribution to an earlier pilot evaluation project. It is now published as one of the inaugural Landmark evaluations as it remains an exemplar for meticulous reexamination of published data, and the points made here remain pertinent to the conduct of scientific research today.

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1. Landmark

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