Validation of a commercial antibody to detect endogenous human nicastrin by immunoblot [version 1; peer review: 1 approved with reservations]

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Abstract

Nicastrin (NCSTN) is a transmembrane glycoprotein that is part of the gamma-secretase complex. Gamma-secretase is a protease complex that cleaves type-I single-pass transmembrane proteins. There are many potential substrates for this complex, including NOTCH receptors and amyloid precursor proteins (APP). There are a number of commercial antibodies to nicastrin, but they do not agree on expected peptide size. We confirmed the specificity of a C-terminal binding rabbit anti-human antibody from Sigma-Aldrich (#N1660) using wildtype HEK293 cells and HEK293 cells deleted for nicastrin. The wildtype cells showed a prominent band at approximately 110 kDa. We confirmed this larger than expected sized was due to glycosylation by treating the lysate with peptide-N-glycosidase F (PNGase F), which reduced the band to less than 75 kDa. These data suggest that this polyclonal is specific for nicastrin and can detect endogenous levels of protein.

Keywords

nicastrin, gamma secretase, polyclonal, human, western blot

This article is included in the Antibody Validations gateway.
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Author roles: Mesa RA: Investigation, Writing – Original Draft Preparation, Writing – Review & Editing; Roberson EDO: Conceptualization, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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Introduction
The γ-secretase complex is a multi-subunit, intramembrane protease (reviewed1). It cleaves type-I single-pass transmembrane proteins within their transmembrane domain. This can lead to the release of an intracellular and an extracellular domain that may perform other functions. Examples include the cleavage of amyloid precursor protein (APP) to produce amyloid beta and the cleavage of activated NOTCH receptors to release their intracellular domain for translocation to the nucleus2.

Gamma-secretase is composed of several proteins, including a presenilin protease (PSEN1 or PSEN2), the presenilin enhancer gamma-secretase subunit (PEN2), an anterior pharynx-defective 1 protein (APH1A or APH1B), and nicastrin (NCSTN)3. Nicastrin acquires extensive N-linked glycosylation during its maturation4,5. The three-dimensional structure of human gamma-secretase shows that the heavily glycosylated ectodomain of nicastrin forms a horseshoe-like clamp on the extracellular portion of the complex6,7. It is thought that NCSTN may help control substrate selectivity. There are multiple commercial antibodies for NCSTN available, but they do not agree on the expected product size. We validated one commercial polyclonal antibody (#N1660; Sigma-Aldrich) using HEK293 wildtype and nicastrin knockout cells.

Methods
Antibody details
We used a commercially available rabbit anti-human IgG polyclonal antibody that targets human nicastrin (#N1660; Sigma-Aldrich; RRID:AB_477259) which is has performed well in some previous publications8,9. The antibody was raised against Uniprot nicastrin peptide Q92542 (709 amino acid total size). The polyclonal was generated by challenging rabbits with a synthetic peptide corresponding to the C-terminal cytoplasmic domain of nicastrin (peptides 693-709) fused with keyhole limpet hemocyanin as an adjuvant.

The technical documentation claims this subsequence is identical to the matching region of nicastrin in mouse. However, aligning Q92542 to the primary mouse nicastrin peptide sequence (NP_067620.3) with Clustal Omega10 actually shows 1 mismatch (94.1% identity; Figure 1). It’s unclear if this discrepancy is due to changes to either the human or mouse peptide sequence for the most common isoform over time as the references have been updated.

We used a mouse anti-human beta actin monoclonal antibody (#AB6276; Abcam, Cambridge, MA, USA; RRID:AB_2223210) as a loading control. The details of all primary and secondary antibodies are summarized in Table 1.

Cell lines and culture
We purchased the Human Embryonic Kidney cell line (HEK293) from the ATCC (CRL-1573). We cultured all cells at 37°C and 5% CO2. For culture media, we used Dulbecco’s Modified Eagle’s Media (DMEM; Gibco, Thermo-Fisher Scientific, #11965-084) supplemented with 5% Fetal Bovine Serum (FBS; Gibco, Thermo-Fisher Scientific, #26140-079), 1% HEPES (Corning, #25-060-CI), 100 U/mL penicillin / streptomycin (Gibco, Thermo-Fisher Scientific, #15140-122), and 2 mM glutamine (Corning, #25-005-CI).

HEK293 NCSTN knockout line
We used a HEK293 NCSTN knockout line we had previously generated using CRISPR/Cas9 genome-editing12. Briefly, we synthesized our single-guide RNA as an IDT gBlock and cloned

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Table 1. Details of the primary and secondary antibodies.

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<th>Antibody (Ab)</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
<th>RRID</th>
<th>Lot number</th>
<th>Ab species</th>
<th>Ab type</th>
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<td>N1660</td>
<td>AB_477259</td>
<td>076M4843V</td>
<td>rabbit</td>
<td>polyclonal</td>
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<tr>
<td>Anti-beta Actin (AC-15)</td>
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<td>ab6276</td>
<td>AB_2223210</td>
<td>GR181659-16</td>
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<td>monoclonal</td>
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<td>Goat Anti-Rabbit IgG</td>
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<td>R115</td>
<td>N/A</td>
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<td>(H&amp;L)-HRPO</td>
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<tr>
<td>Goat Anti-Mouse IgG</td>
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<td>(H&amp;L)-HRPO</td>
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</tbody>
</table>

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Figure 1. Human / mouse nicastrin alignment. Shown is a partial alignment between human and mouse nicastrin. The highlighted area represents the peptides use for generation of the polyclonal antibody. Asterisks represent a matching amino acid between the two sequences, and spaces are mismatches.
it into the pCR-Blunt TOPO vector. We co-transfected the single-guide RNA vector along with humanized Cas9 (RRID: Addgene_43861) into HEK293 cells, plated to single colonies, and screened for deleted clones by sequencing (Sequence Read Archive project PRJNA268374) and RT-qPCR (Data available from figshare, see source data13). Full methodology for RT-qPCR is provided in the supplementary material of Cao et al.12

Protein extraction
Reagent details can be found in Table 2 and Table 3. We harvested cells at ≥90% confluence and pelleted them by centrifugation at 4°C and 400 xg for 5 minutes. We washed the cell pellet three times in 10 mL of cold phosphate buffered saline (PBS). We then added 300 µL of cold lysis buffer (50 mM Tris-HCl pH 8.0, 2 mM EDTA, 150 mM NaCl, 1.0% NP-40, and 1.5% protease inhibitor cocktail) and lysed the cells with constant agitation for 30 minutes at 4°C. We removed insoluble debris by centrifugation for 15 minutes at 4°C and 10,400 xg. We determined the concentration of the cleared lysates using a Pierce BCA assay kit (#23227). We stored the lysates in aliquots at -80°C until further use.

Enzymatic deglycosylation
We used peptide-N-glycosidase F (PNGase F; #P0704S; New England Biolabs, Ipswich, MA, USA) to remove N-linked sugars. We denatured about 50 µg of protein in glycoprotein denaturing buffer (included with NEB kit; 0.5% SDS, 40 mM DTT) at 100°C for 10 minutes, and then incubated the lysate with PNGase F for 3 hours at 37°C, according to the manufacturer’s instructions. We treated a control in parallel under the same conditions, but omitted the PNGase F enzyme.

Immunoblotting
We denatured the protein lysate by boiling for 5 minutes in Laemmli sample buffer (5% β-mercaptoethanol). We resolved the proteins on precast 7.5% polyacrylamide gels (Mini-protein TGX; Bio-Rad, Hercules, CA, USA) after loading approximately 20 µg of lysate. We used the Precision Plus Dual-Color Standard as a molecular weight marker (Bio-Rad, Hercules, CA). We prepared PVDF membranes (0.45 µm) by incubating 2 minutes in 100% isopropanol, washing in Milli-Q water for 2 minutes, and equilibrating in transfer buffer for 10 minutes. We transferred separated proteins to the PVDF membrane in transfer buffer without methanol at 200 mA for 2 hours. We blocked the membrane by incubating in blocking buffer (TBST with 5% skim milk powder) for 1 hour at room temperature with gentle rocking. We probed the membrane using primary antibodies to nicastrin (1/1000) and beta actin (1/5000) diluted in blocking buffer overnight at 4°C with gentle rocking. We removed excess unbound antibody by rinsing the membranes 5 times for 10 minutes each in TBST. We blocked the membrane by incubating in blocking buffer (TBST with 5% skim milk powder) for 1 hour at room temperature with gentle rocking. We removed excess unbound antibody by rinsing the membranes 5 times for 10 minutes each in TBST. We used the Supernignal West Pico Chemiluminescent Substrate reagent (ThermoFisher, Waltham, MA) to detect secondary antibodies.

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**Table 2. Details of Cell lysis reagents.**

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<th>Reagent</th>
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<td>0.5M EDTA pH8</td>
<td>Corning</td>
<td>46-034-Cl</td>
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<tr>
<td>5M NaCl</td>
<td>Sigma</td>
<td>S5150</td>
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<tr>
<td>Surfact-Amps NP-40 Detergent Solution</td>
<td>Thermo Scientific</td>
<td>85124</td>
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<td>Protease Inhibitor Cocktail</td>
<td>Sigma</td>
<td>P8340</td>
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**Table 3. Details of SDS-PAGE / Immunoblotting reagents.**

<table>
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<th>Protocol Steps</th>
<th>Reagents</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
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<td>Protein concentration</td>
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<td>Thermo Scientific</td>
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<tr>
<td>measurement</td>
<td>Cell lysate preparation</td>
<td>Biorad Sigma</td>
<td>161-0737 M3148</td>
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<tr>
<td>Electrophoresis</td>
<td>7.5% Mini-PROTEAN TGX gel, 10wl, 30 µl 10X Tris/Glycine/SDS Buffer</td>
<td>Biorad Sigma</td>
<td>4561023 161-0732</td>
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<td>Immunoblotting</td>
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<td>Millipore Sigma</td>
<td>IPV/H08100 190764</td>
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<td>2-Propanol</td>
<td>10X Tris/Glycine Buffer</td>
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<td>10X TBST</td>
<td>Chemiluminescence reaction</td>
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<td></td>
<td>SuperSignal West Pico Chemiluminescent Substrate</td>
<td>Thermo Scientific</td>
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Results

The nicastrin polyclonal binds to endogenous nicastrin in HEK293 extracts

We collected protein lysates from wildtype HEK293 cells and HEK293 NCSTN knockouts. The manufacturer provided example blots were derived from HEK293 cells, but used an overexpression construct. In wildtype HEK293 cell lysates, a single, strong band at ~110 kDa can be seen on the blot, and this band is missing in the nicastrin knockout line lysates (Figure 2A, underlying data\textsuperscript{14,15}). The loading controls for the wildtype replicates and knockout replicates all show the expected band for actin (Figure 2B, underlying data\textsuperscript{14,15}), supporting that the loss of the nicastrin band is specific to the knockout and not a loading error. It is worth noting that despite a low background, the nicastrin blots showed an approximately 25 kDa band in both wildtype and knockout lysates. We searched the protein sequence used to develop the antibody (KADVLFIAPEPAGAVSY) with protein blast using the Homo sapiens non-redundant peptide database automatically adjusted for short queries, but only matches to nicastrin had a reasonable e-value (2×10\textsuperscript{-9} to 7×10\textsuperscript{-11}). It is therefore unclear if this band is from a non-specific contaminant in the antibody, a similar peptide that is poorly annotated in the non-redundant protein database, or a nicastrin degradation product.

The larger than expected band size for nicastrin is due to glycosylation

The nicastrin antibody documentation lists the expected fragment size as approximately 110 kDa, and this band size was confirmed on our blots. However, calculating the fragment size of human nicastrin protein sequence Q92542 using Expasy tools\textsuperscript{16} gives an estimated 78.4 kDa size for the nascent fragment and a reduced 75.2 kDa size after cleavage of the signal peptide. We hypothesized this discrepancy might be due to glycosylation.

We tested this hypothesis by first treating the lysates PNGase F, which will release asparagine-linked oligosaccharides. This reduced the molecular weight of the nicastrin band to less than 75 kDa (Figure 3A, underlying data\textsuperscript{17,18}) without affecting the actin band (Figure 3B underlying data\textsuperscript{17,18}). This phenomenon of a smaller than expected nicastrin band has been observed previously\textsuperscript{19}. It is possible that a longer signal sequence than expected is cleaved from the nascent peptide, or the charge profile of the polypeptide affects its migration.

Conclusion

We tested by immunoblot an anti-nicastrin antibody using HEK293 cell lysates. Our results show that the antibody is sensitive enough to detect endogenous protein with reasonable specificity. It is able to bind to both glycosylated nicastrin and nicastrin without sugar linkages. It is unclear how well the antibody would work for cell staining due to the non-specific 25 kDa band we observed on nicastrin blots. Based on these data obtained with the protocols described above, we can confirm the utility of this nicastrin antibody for immunoblotting.

Figure 2. Immunoblot of endogenous nicastrin. A. The NCSTN antibody binds to endogenous levels of protein in wildtype (WT) HEK293 cells with a band at ~110 kDa. The band is absent in NCSTN knockout (KO) cells. Both replicates show an unidentified band at 25 kDa. B. The actin antibody shows the expected ~42 kDa band in both replicates of wildtype and knockout cells. Abbreviations: rep., replicate.

Figure 3. Nicastrin immunoblot with PNGase F treatment. A. In lysates untreated with PNGase F (-), the expected ~110 kDa band is present. With PNGase F treatment (+), the band regresses to less than 75 kDa. B. In both PNGase treated and untreated lysates, the beta actin band is unchanged.

Data availability

Source data

Home sapiens HEK293 NCSTN knockout by Cas9, Accession number: PRJNA268374

Figshare: HEK293 nicastrin knockout RT-qPCR. https://doi.org/10.6084/m9.figshare.7578539.v1

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This project contains the following source data:

- knockout_rtqpcr.csv (Raw Ct values of RT-qPCR confirming the knockout (CRISPR-Cas9 mediated) of nicastrin in HEK293 cells.)

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Underlying data
Figshare: NCSTN antibody validation - actin antibody in HEK293 knockout line. https://doi.org/10.6084/m9.figshare.8952968.v1^16

This project contains the following underlying data:

- AntibodyValidation_NCSTN_KO_actin_Ab.svg (TIF image of actin antibody blot stored in a scaleable vector graphic file)

Figshare: NCSTN antibody validation - NCSTN antibody in HEK293 knockout line after PNGase treatment. https://doi.org/10.6084/m9.figshare.8952977.v1^19

This project contains the following underlying data:

- AntibodyValidation_NCSTN_PNGase_NCSTN_Ab.svg
- AntibodyValidation_NCSTN_PNGase_actin_Ab.svg (TIF image of NCSTN antibody blot stored in a scaleable vector graphic file)

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

Grant information
This work was partially supported by the Washington University in St. Louis (WUSTL) Rheumatic Diseases Research Resource-based Center (RDRRC) [P30-AR073752] and the Washington University Institute for Clinical and Translational Sciences [UL1-TR000448].

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements
We thank Professor Matthias Voss (Kiel University, Kiel, Germany) and Professor Yasuomi Urano (Doshisha University, Kyoto, Japan) for suggestions and sharing protocols.

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Open Peer Review

Current Peer Review Status: ?

Version 1

Reviewer Report 06 August 2019
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Satoru Funamoto
Laboratory of Neuropathology, Graduate School of Life and Medical Sciences, Doshisha University, Kyoto, Japan

It is very important to validate commercial antibodies. However this reviewer thinks it would help to enhance the value of this manuscript if authors consider the below:

1. Sigma’s anti-Nicastrin antibody has been already a golden standard for Nicastrin detection. It is not clear how other commercial antibodies do not agree on size. It is very important to show bands detected with several commercial anti-Nicastrin antibodies. This could be very informative.

2. It is already published that PNGase and Endo H treatments altered migration distance of Nicastrin in gels¹.

References

Is the work clearly and accurately presented and does it cite the current literature?
No

Are sufficient details of materials, methods and analysis provided to allow replication by others?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes
Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neuropathology and biochemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Comments on this article

Version 1

Author Response 31 Jul 2019

Elisha Roberson, Washington University, St. Louis, USA

Thanks Anita! That's very helpful.

Eli

Competing Interests: Author

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