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ANTIBODY VALIDATION ARTICLE

REVISED Validation of a commercial antibody to detect endogenous human nicastrin by immunoblot [version 2; peer review: 2 approved, 1 approved with reservations]

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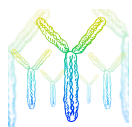
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Abstract

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**.

Open Peer Review

Reviewer Status

	Invited Reviewers		
	1	2	3
version 2 (revision) 10 Jan 2020	 report		 report
version 1 29 Jul 2019	 report	 report	

- Satoru Funamoto** , Doshisha University, Kyoto, Japan
- Lucia Chavez-Gutierrez**, VIBKU Leuven Center for Brain & Disease Research, Leuven, Belgium
- Jongkyun Kang**, Harvard Medical School, Boston, USA

Any reports and responses or comments on the article can be found at the end of the article.

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REVISED Amendments from Version 1

The major change in this revision is the addition of a blot showing the antibody works with murine-derived protein as well. Otherwise we have revised the manuscript to alter some language and include important references suggested by the reviewers. There is also new underlying data for this figure that has been uploaded to FigShare.

Any further responses from the reviewers can be found at the end of the article

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 maturation^{4,5}, though the glycosylation may not be required for typical cleavage activity⁶. 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 complex^{7,8}. It is thought that NCSTN may help control substrate selectivity⁹. Understanding the role of nicastrin in gamma-secretase has been challenging.

Gamma-secretase can cleave many substrates without nicastrin, though nicastrin does help to exclude some substrates via steric hindrance¹⁰⁻¹². 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, St. Louis, MO, USA; RRID:AB_477259) which has performed well in some previous publications^{13,14}. 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 Omega^{15,16} 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.

```

Human Q92542 FLIASKELELITLVGFGILIFSLIVTYCINAKADVLFFIAPREPGAVSY 709
Mouse NP_067620.3 FLIASKELEFITLIVGFSTLVFSLIVTYCINAKADVLFVAPREPGAVSY 708
*****  ***  ***  * ********** *****

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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 used for generation of the polyclonal antibody. Asterisks represent a matching amino acid between the two sequences, and spaces are mismatches.

Table 1. Details of the primary and secondary antibodies.

Antibody (Ab)	Manufacturer	Catalog Number	RRID	Lot number	Ab species	Ab type
Anti-Nicastrin	Sigma	N1660	AB_477259	076M4843V	rabbit	polyclonal
Anti-beta Actin (AC-15)	abcam	ab6276	AB_2223210	GR181659-16	mouse	monoclonal
Goat Anti-Rabbit IgG (H&L)- HRPO	Leinco Technologies	R115	AB_2810875	0117L320	goat	polyclonal
Goat Anti-Mouse IgG (H&L)- HRPO	Jackson ImmunoResearch	115-035-003	AB_10015289	129457	goat	polyclonal

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-editing¹⁷. Briefly, we synthesized our single-guide RNA as an IDT gBlock and cloned 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 data¹⁸). Full methodology for RT-qPCR is provided in the supplementary material of Cao *et al.*¹⁷

Cell line 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 ×g 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 ×g. 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.

Mouse liver protein extraction

We received a snap-frozen mouse liver (2 month-old C57BL/6 mouse) from the Alfred Kim lab, which had been obtained according to their approved IACUC protocol. We minced the liver into pieces and homogenized in ice-cold lysis buffer (5 mM Tris-HCl pH 8.0, 250 mM sucrose, 5 mM EDTA, 1.5% protease inhibitor cocktail) using a Wheaton tissue grinder. We then passed the solution through a QIAshredder spin-column (Qiagen #79656) to facilitate more complete lysis. We spun cellular debris out of solution by a 5 minute spin at 5,000 ×g. We then precipitated membrane-enriched fragments by spinning for 5 minutes at 11,000 ×g (4°C), then spinning the supernatant for an additional 1 hour at 4°C and 11,000 ×g. We then extracted proteins from the membrane pellet by resuspending in buffer containing 2% (v/v) Triton X-100 and incubating on ice for 30 minutes. Any remaining unlysed material was pelleted with a 1 hour, 4°C, 11,000 ×g spin. We determined the protein concentration of the lysate using the Pierce BCA assay kit. We stored lysates in aliquots at -80°C until blotting.

Table 2. Details of Cell lysis reagents.

Reagent	Manufacturer	Catalog Number
1M Tris-HCl pH 8	Corning	46-031-CM
0.5M EDTA pH8	Corning	46-034-CI
5M NaCl	Sigma	S5150
Surfact-Amps NP-40 Detergent Solution	Thermo Scientific	85124
Protease Inhibitor Cocktail	Sigma	P8340
Sucrose	Sigma	S1888
Triton X-100	Sigma	T8787

Table 3. Details of SDS-PAGE / Immunoblotting reagents.

Protocol Steps	Reagents	Manufacturer	Catalog Number
Protein concentration measurement	Pierce BCA Protein assay kit	Thermo Scientific	23227
Cell lysate preparation	2x Laemmli sample buffer β-mercaptoethanol	Biorad Sigma	161-0737 M3148
Electrophoresis	7.5% Mini-PROTEAN TGX gel, 10wl, 30 μl 10X Tris/Glycine/SDS Buffer	Biorad Biorad	4561023 161-0732
Immunoblotting	Immobilon-P PVDF Membrane (0.45 μm) 2-Propanol 10X Tris/Glycine Buffer 10X TBST	Millipore Sigma Biorad EZ BioResearch	IPVH08100 190764 161-0734 S-1012
Chemiluminescence reaction	SuperSignal West Pico Chemiluminescent Substrate	Thermo Scientific	34080

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 buffer. The anti-mouse and anti-rabbit secondary antibodies were both conjugated to horseradish peroxidase (**HRP**). We incubated the membranes with secondary antibody (1/7000) in blocking buffer for 1.5 hours at room temperature, followed by washing 5 times for 10 minutes each in TBST. We used the Supersignal West Pico Chemiluminescent Substrate reagent (ThermoFisher, Waltham, MA) to detect secondary antibodies.

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^{19,20}). The loading controls for the wildtype replicates and knockout replicates all show the expected band for actin (**Figure 2B**, underlying data^{19,20}), 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 (KADVLFIAPREPGAVSY) 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^{-9} to 7×10^{-11}). It is therefore unclear if this band is from a non-specific contaminant in the antibody, a

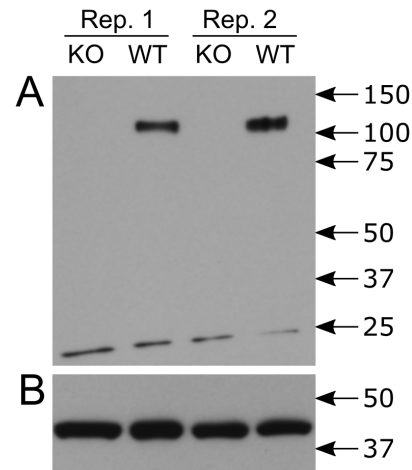


Figure 2. Immunoblot of endogenous nicastrin. A. The NCSTN antibody binds to endogenous levels of protein in wildtype (WT) HEK293 cells with a band at ~110 kDa. The band is absent in NCSTN knockout (KO) cells. Both replicates show an unidentified band at 25 kDa. **B.** The actin antibody shows the expected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

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**²¹ 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^{22,23}) without affecting the actin band (**Figure 3B** underlying data^{22,23}). This phenomenon of a smaller than expected nicastrin band has been observed previously^{6,24}. It is possible that a longer signal sequence than expected is cleaved from the nascent peptide. Given that detailed information is available for the signal cleavage of nicastrin⁹, a more likely explanation might be that the charge profile of the polypeptide affects its migration.

The antibody binds to endogenous mouse nicastrin

As noted above, there were mismatches between the sequence used to generate the antibody and the mouse sequence for nicastrin. It was possible that this mismatch was enough to reduce the effectiveness of this antibody in mouse extracts. We extracted protein from frozen mouse liver to test this possibility. We were able to confirm the presence of a band of the expected size in the mouse extracts (**Figure 4**, underlying data²⁵). The same small, non-specific band was present in these blots as well.

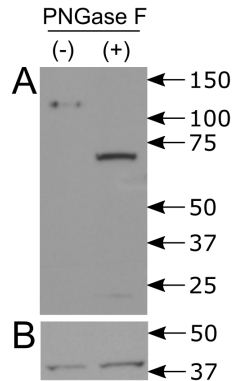


Figure 3. Nicastrin immunoblot with PNGase F treatment. **A.** In lysates untreated with PNGase F (-), the expected ~110 kDa band is present. With PNGase F treatment (+), the band regresses to less than 75 kDa. **B.** In both PNGase treated and untreated lysates, the beta actin band is unchanged.

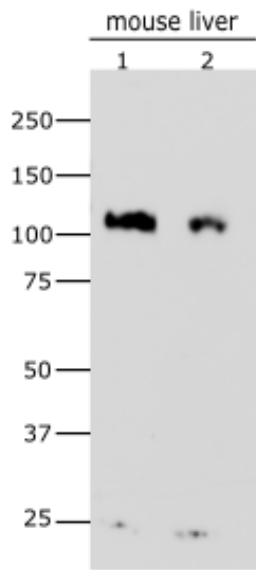


Figure 4. Immunoblot of murine nicastrin. Blot showing the results for 35 µg (1) or 25 µg (2) of mouse membrane protein lysate. The expected ~110 kDa band for mature nicastrin is present, as is the non-specific band present in most blots at < 25 kDa. These data suggest the antibody works as well for murine nicastrin as it does for human nicastrin.

Conclusion

We tested by immunoblot an anti-nicastrin antibody using HEK293 cell lysates and mouse liver extracts. 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. The antibody functions for both endogenous human and mouse

protein. 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.

Data availability

Source data

Home sapiens HEK293 NCSTN knockout by Cas9, Accession number: [PRJNA268374](https://doi.org/10.6084/m9.figshare.7578539.v1)

Figshare: HEK293 nicastrin knockout RT-qPCR. <https://doi.org/10.6084/m9.figshare.7578539.v1>¹⁸

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](https://creativecommons.org/licenses/by/4.0/) (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>¹⁹

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. <https://doi.org/10.6084/m9.figshare.8952953.v1>²⁰

This project contains the following underlying data:

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

Figshare: NCSTN antibody validation - actin antibody in HEK293 knockout line after PNGase treatment. <https://doi.org/10.6084/m9.figshare.8952983.v1>²²

This project contains the following underlying data:

- AntibodyValidation_NCSTN_PNGase_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>²³

This project contains the following underlying data:

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

Figshare: NCSTN antibody validation - NCSTN antibody with murine liver protein. <http://www.doi.org/10.6084/m9.figshare.11541864.v1>²⁵

This project contains the following underlying data:

- SuppFig5_MouseNCSTN.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).

Acknowledgements

We thank Professor Matthias Voss (Kiel University, Kiel, Germany) and Professor Yasuomi Urano (Doshisha University, Kyoto, Japan) for suggestions and sharing protocols. We thank the Alfred Kim lab for their kind gift of a mouse liver to test nicastrin binding in mice lysates.

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Version 2

Reviewer Report 15 April 2020

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Jongkyun Kang

Department of Neurology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

This article validated several commercial antibodies against Nicastrin protein which is one of the gamma-secretase complex. Authors used different cell types and organisms to validate antibodies for the specificity and sensitivity.

As other reviewers pointed out, this article has less interesting since other papers already showed the specificity of several antibodies in human or mouse cell line or tissues. Furthermore, postmodification mechanism of Nicastrin by glycosylation was also well established.

Despite less novelty, this article would be helpful for the community who have a plan to perform a Western analysis on HEK293 cell line which is major experimental condition of this paper.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

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: Molecular biology, Biochemistry, Neuropathology

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.

Reviewer Report 31 January 2020

<https://doi.org/10.5256/f1000research.24258.r58489>

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Satoru Funamoto 

Laboratory of Neuropathology, Graduate School of Life and Medical Sciences, Doshisha University, Kyoto, Japan

This reviewer thinks that this manuscript is sufficient for the F1000Research.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

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.

Version 1

Reviewer Report 18 September 2019

<https://doi.org/10.5256/f1000research.21724.r51771>

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Lucia Chavez-Gutierrez

VIBKU Leuven Center for Brain & Disease Research, Leuven, Belgium

The authors validated a commercially available rabbit polyclonal anti-Nicastrin antibody from Sigma-Aldrich (#N1660) in immunoblot analysis, using as inputs total lysates from wildtype and Nicastrin knockout HEK293 cell lines. The N1660 antibody, generated against a synthetic peptide corresponding to the C-terminal cytoplasmic part of human Nicastrin (693-709), identified a band at ~110 kDa only in the wild type lysate and another band at ~ 25 kDa in both wildtype and knockout lysates. The data does not clarify whether the low-molecular weight signal is a non-specific band or a nicastrin-derived peptide.

Treatment with PNGase F to remove all N-linked oligosaccharide chains, reduced the molecular weight of the nicastrin band to less than 75 kDa, without affecting the loading control actin band. The observed shift in molecular weight is supported by previous observations (Herreman A. *et al.*, 2003¹) and the authors should acknowledge this study.

Other Points:

- The authors should test the N1660 antibody against mouse Nicastrin.
- “It is thought that NCSTN may help control substrate selectivity.” The ‘substrate receptor’ model (Shah *et al.*, 2005²) has been challenged by independent studies (ChávezGutiérrez *et al.*, 2008³; Zhao *et al.*, 2010⁴), and it is not supported by available high resolution structural data for the gamma secretase complex (Bai *et al.*, 2015⁵). More recent studies indicate that the ectodomain of NCT has both passive and active roles in gamma secretase-mediated proteolysis. On one hand, it restricts the access of substrates presenting large ectodomains by steric hindrance (Bolduc *et al.*, 2016⁶). On the other, it has an active role in the regulation of the sequential proteolysis of APP and response to gamma secretase modulators (GSMs); thus, nicastrin modulates A β length.
- “This phenomenon of a smaller than expected nicastrin band has been observed previously. 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.” Shah *et al.*, 2005² provides information about the precise signal peptide sequence cleaved from the nascent NCT polypeptide.

References

1. Herreman A, Van Gassen G, Bentahir M, Nyabi O, et al.: gamma-Secretase activity requires the presenilin-dependent trafficking of nicastrin through the Golgi apparatus but not its complex glycosylation. *J Cell Sci.* 2003; **116** (Pt 6): 1127-36 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Shah S, Lee SF, Tabuchi K, Hao YH, et al.: Nicastrin functions as a gamma-secretase-substrate receptor. *Cell.* 2005; **122** (3): 435-47 [PubMed Abstract](#) | [Publisher Full Text](#)
3. Chávez-Gutiérrez L, Tolia A, Maes E, Li T, et al.: Glu(332) in the Nicastrin ectodomain is essential for gamma-secretase complex maturation but not for its activity. *J Biol Chem.* 2008; **283** (29): 20096-105 [PubMed Abstract](#) | [Publisher Full Text](#)
4. Zhao G, Liu Z, Ilagan MX, Kopan R: Gamma-secretase composed of PS1/Pen2/Aph1a can cleave notch and amyloid precursor protein in the absence of nicastrin. *J Neurosci.* 2010; **30** (5): 1648-56 [PubMed Abstract](#) | [Publisher Full Text](#)
5. Bai XC, Yan C, Yang G, Lu P, et al.: An atomic structure of human γ -secretase. *Nature.* 2015; **525** (7568): 212-217 [PubMed Abstract](#) | [Publisher Full Text](#)
6. Bolduc DM, Montagna DR, Gu Y, Selkoe DJ, et al.: Nicastrin functions to sterically hinder γ -secretase-substrate interactions driven by substrate transmembrane domain. *Proc Natl Acad Sci U S A.* 2016; **113** (5): E509-18 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Partly

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: Biochemistry, Intramembrane proteolysis, Alzheimer's Disease

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.

Author Response 08 Jan 2020

Elisha Roberson, Washington University, St. Louis, USA

Reviewer question 1: Treatment with PNGase F to remove all N-linked oligosaccharide chains, reduced the molecular weight of the nicastrin band to less than 75 kDa, without affecting the loading control actin band. The observed shift in molecular weight is supported by previous observations (Herreman A. *et al.*, 2003) and the authors should acknowledge this study.

Response: This is a valid point, and have cited the appropriate study. Thanks for the suggestion.

Reviewer question 2: The authors should test the N1660 antibody against mouse Nicastrin.

Response: As requested we used murine protein to test the antibody. This data has been added as Figure 4, and the expected band size was observed, along with the non-specific small molecular weight band that show up in the HEK293 blots.

Reviewer question 3: "It is thought that NCSTN may help control substrate selectivity." The 'substrate receptor' model (Shah *et al.*, 2005) has been challenged by independent studies (ChávezGutiérrez *et al.*, 2008; Zhao *et al.*, 2010), and it is not supported by available high resolution structural data for the gamma secretase complex (Bai *et al.*, 2015). More recent studies indicate that the ectodomain of NCT has both passive and active roles in gamma secretase-mediated proteolysis. On one hand, it restricts the access of substrates presenting large ectodomains by steric hindrance (Bolduc *et al.*, 2016). On the other, it has an active role in the regulation of the sequential proteolysis of APP and response to gamma secretase modulators (GSMs); thus, nicastrin modulates A β length.

Response: These are all excellent points. It is, of course, not possible to exhaustively discuss all the possibilities for nicastrin function in gamma-secretase in the introduction,

as that is more suitable to a full literature review. However, these are points worth at least mentioning, and good references to support the paper. We have revised the paper to point to some of these different evidence sets regarding nicastrin function and have added the suggested references.

Reviewer question 4: "This phenomenon of a smaller than expected nicastrin band has been observed previously. 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." Shah *et al.*, 2005 provides information about the precise signal peptide sequence cleaved from the nascent NCT polypeptide.

Response: Given the work from Shah determining the signal peptide sequence, we have revised this section to point out this information (referencing the paper). We have also reworded to suggest that perhaps the disparity is more likely to be due to conformation / charge profile than additional signal peptide sequence cleavage.

Competing Interests: I have no competing interests to report.

Reviewer Report 06 August 2019

<https://doi.org/10.5256/f1000research.21724.r51767>

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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

1. Herreman A, Van Gassen G, Bentahir M, Nyabi O, et al.: gamma-Secretase activity requires the presenilin-dependent trafficking of nicastrin through the Golgi apparatus but not its complex glycosylation. *J Cell Sci.* 2003; **116** (Pt 6): 1127-36 [PubMed Abstract](#) | [Publisher Full Text](#)

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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?

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Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neuropathology and biochemistry

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Author Response 08 Jan 2020

Elisha Roberson, Washington University, St. Louis, USA

Reviewer question 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.

Response: We agree that a comprehensive screening of multiple antibodies would be fruitful. For this experiment we focused on validating a commonly used antibody to ensure that it was suitable for our future experimental designs. We are considering this project in the future, given that we can generate the necessary funds to purchase the requisite antibodies.

Reviewer question 2: It is already published that PNGase and Endo H treatments altered migration distance of Nicastrin in gels.

Response: It has been previously published in your suggested reference from 2003. We are able to confirm that this effect still persists, and that >15 years later the antibody specificity is still acceptable. We appreciate the suggestion for this reference and have appropriately cited it in the revised version.

Competing Interests: We declare no competing interests.

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

Reader Comment 30 Jul 2019

Anita Bandrowski, RRID, San Diego, USA

I have registered your antibody:

"Goat Anti-Rabbit IgG (H&L)- HRPO

Leinco Technologies

R115N/A

0117L320

goat

polyclonal"

and obtained the following RRID:AB_2810875

Competing Interests: I run the the RRID project.

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