

Recording γ -secretase activity in living mouse brains

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 https://en.wikipedia.org/wiki/Open_access

 Copyright information

Reviewed Preprint

Published from the original preprint after peer review and assessment by eLife.

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Reviewed preprint version 1

April 26, 2024 (this version)

Sent for peer review

February 19, 2024

Posted to preprint server

January 31, 2024

Abstract

γ -Secretase plays a pivotal role in the central nervous system. Our recent development of genetically encoded Förster resonance energy transfer (FRET)-based biosensors has enabled the spatiotemporal recording of γ -secretase activity on a cell-by-cell basis in live neurons *in culture*. Nevertheless, how γ -secretase activity is regulated *in vivo* remains unclear. Here we employ the near-infrared (NIR) C99 720-670 biosensor and NIR confocal microscopy to quantitatively record γ -secretase activity in individual neurons in living mouse brains. Intriguingly, we uncovered that γ -secretase activity may influence the activity of γ -secretase in neighboring neurons, suggesting a potential “cell non-autonomous” regulation of γ -secretase in mouse brains. Given that γ -secretase plays critical roles in important biological events and various diseases, our new assay *in vivo* would become a new platform that enables dissecting the essential roles of γ -secretase in normal health and diseases.

eLife assessment

Hou and colleagues describe the use of a previously characterized FRET sensor for use in determining gamma secretase activity in the brain of living mice. In an approach that targeted the sensor to neurons, they observe patterns of fluorescent sensor readout suggesting clustered regions of secretase activity. These results once validated would be **valuable** in the field of Alzheimer's Disease research, yet further validation of the approach is required, as the current evidence provided is **inadequate** to support the conclusions.

Introduction

γ -Secretase is an intramembrane aspartyl protease complex responsible for the proteolytic processing of a wide range of transmembrane proteins (Güner and Lichtenthaler, 2020 ) such as Notch receptors (De Strooper et al., 1998 ) and APP (De Strooper et al., 1999 ). γ -Secretase is ubiquitously expressed in various tissues and plays multifunctional roles in essential biology and diseases. For instance, the mice lacking presenilin (PSEN) 1 and 2, the catalytic component of γ -secretase (Wolfe et al., 1999 ) are nonviable due to abnormal neurogenesis and vasculature and skeletal formation (Shen et al., 1997 ; Wong et al., 1997 ). Notch knockout (KO) mice

recapitulate the lethal phenotypes of the PSEN1/2 KO (Swiatek et al., 1994 [\[1\]](#); Huppert et al., 2000 [\[2\]](#)), suggesting the essential role of γ -secretase-mediated Notch processing in normal development. Aberrant Notch processing also causes several types of cancer (Allenspach et al., 2002 [\[3\]](#); Aster et al., 2017 [\[4\]](#); McCaw et al., 2021 [\[5\]](#)) as well as a chronic skin inflammatory disease - Hidradenitis suppurativa (HS) (Wang et al., 2010 [\[6\]](#); Wang et al., 2020 [\[7\]](#)).

γ -Secretase plays an essential role in the central nervous system. Conditional knockout of PSEN1 and 2 (Saura et al., 2004 [\[8\]](#)) or other components of γ -secretase in excitatory (Tabuchi et al., 2009 [\[9\]](#); Acx et al., 2017 [\[10\]](#); Bi et al., 2021 [\[11\]](#)), as well as inhibitory neurons (Kang and Shen, 2020 [\[12\]](#)), leads to age-dependent neuronal loss in adult mice. Mutations in PSEN genes lead to early-onset familial Alzheimer's disease (AD) (Sherrington et al., 1995 [\[13\]](#); Levy-Lahad et al., 1995 [\[14\]](#)) and frontotemporal dementia (FTD) (Raux et al., 2000 [\[15\]](#); Deraut et al., 2004 [\[16\]](#)). Some PSEN mutations are linked to epilepsy (Mann et al., 2001 [\[17\]](#); Velez-Pardo et al., 2004 [\[18\]](#)). γ -Secretase is also responsible for the generation of β -amyloid (A β) peptides (De Strooper et al., 1999 [\[19\]](#)), the accumulation of which in the brain parenchyma is one of the pathological hallmarks of AD. Altogether, it is highly plausible that changes in γ -secretase activity could be ultimately linked to the development and progression of neurodegenerative diseases such as AD.

Our recent development of the genetically encoded C99 YPet-TurquoiseGL (C99 Y-T) biosensor has, for the first time, allowed recording γ -secretase activity over time, on a cell-by-cell basis, in live neurons *in culture*. Using the C99 Y-T biosensor, we uncovered that γ -secretase activity is heterogeneously regulated among mouse cortex primary neurons (Maesako et al., 2020 [\[20\]](#)). Nevertheless, to investigate the role of γ -secretase in-depth, an assay that permits the investigation of the dynamics of γ -secretase in more physiological conditions *in vivo* would be required. In the conventional γ -secretase activity assay, APP or Notch1-based recombinant substrates and isolated γ -secretase-rich membrane fractions from rodent/human brains are incubated *in vitro*, followed by the detection of either A β peptides or the intracellular domain fragment (Hoke et al., 2005 [\[21\]](#); Kakuda et al., 2006 [\[22\]](#); Szaruga et al., 2015 [\[23\]](#)). Though the assay is highly sensitive, it only reports γ -secretase activity in a bulk population of cells *ex vivo*, and neither provides spatiotemporal nor cell-by-cell information about the γ -secretase activity in live cells *in vivo*.

In order to overcome these critical shortcomings and fulfill the detection capability of γ -secretase activity *in vivo*, we have recently developed a near-infrared (NIR) analog: C99 720-670 biosensor and validated the reporter probe *in culture* (Houser et al., 2020 [\[24\]](#)), since the NIR spectral region exhibits greater depth penetration and minimal absorption, and this spectral region exhibits low autofluorescence. In this study, we employed AAV delivery of the C99 720-670 biosensor and NIR confocal microscopy to monitor γ -secretase activity in intact mouse brains. This study, for the first time, reports successful recording of γ -secretase activity on a cell-by-cell basis in live neurons *in vivo*, as opposed to an existing assay in a bulk population of cells *ex vivo*. Moreover, it highlights the feasibility of NIR confocal microscopy for monitoring biological events in live mouse brains. In agreement with the previous study *in vitro*, we uncovered that γ -secretase activity is differently regulated in individual neurons in live mouse brains *in vivo*. Although the effect size is modest, we also found a statistically significant correlation between γ -secretase activity within a neuron and the activity of γ -secretase in neighboring neurons, suggesting a potential “cell non-autonomous” regulation of γ -secretase activity in mouse brains. The new *in vivo* imaging platform would help to better understand the spatiotemporal regulation of γ -secretase and its consequences in normal health and disease-relevant conditions.

Materials and Methods

Ethics

Experimental procedures were in compliance with the NIH guidelines for the use of animals in experiments and were approved by the Massachusetts General Hospital Animal Care and Use Committee (2003N000243).

Adeno-associated virus (AAV)

Preparation of the AAV-hSyn1-C99 720-670 was performed as described previously (Maesako et al., 2022 [\[1\]](#)). Briefly, the cDNA of the C99 720-670 biosensor (Houser et al., 2020 [\[2\]](#)) was sub-cloned into a pAAV vector containing human Synapsin 1 promoter and WPRE sequences (Maesako et al., 2017 [\[3\]](#)). The plasmid sequence was verified by the MGH DNA core. The packaging into viruses (AAV2/8 stereotype) was performed at the University of Pennsylvania Gene Therapy Program vector core (Philadelphia, PA) (4.95×10^{13} GC/mL). The AAV-hSyn1-C99 720-670 was injected into the somatosensory cortex of 4-6 months old C57BL/6 male mice.

Craniotomy

Craniotomy surgery was performed based on previously described methods (Kuchibhotla et al., 2008 [\[4\]](#)) with minor adjustments. Briefly, C57BL/6 mice were anesthetized using 1-1.5% isoflurane. A ~ 3.5 mm circular section of the skull over the right hemisphere of the brain was surgically removed. The dura mater was left intact during the craniotomy procedure. A 4 mm cover glass was then positioned over the exposed brain area and secured with a mixture of dental acrylic and cyanoacrylate glue. Post-surgery, mice received buprenorphine and Tylenol for pain relief over 3 days. The mice were left to recover for at least 3 weeks before proceeding with the imaging experiments.

NIR confocal microscopy

A diode laser at 640 nm wavelengths was used to excite the C99 720-670 biosensor. Fluorescence emission from mRFP670 (donor) and mRFP720 (acceptor) in the detection range: 670 ± 10 nm and 750 ± 50 nm, respectively, was detected by the high sensitivity-spectral detector, equipping cooled GaAsP photomultiplier on an Olympus FV3000RS confocal microscope. A x25 objective (NA = 1.05) was used for the image acquisition (512×512 pixels, Zoom x1). To obtain Z-stack images, the image acquisition was started at the first appearance of vasculature near the brain surface and continued at 2 μ m step sizes up to approximately 60-80 sections.

Image processing, quantification, and statistical analysis

For quantification, the 3D ImageJ Suite plugin (Ollion et al., 2013 [\[5\]](#)) in Fiji was used to automatically segment neurons expressing the C99 720-670 biosensor. In image processing, top-hat filtering was first applied to remove uneven background illumination (filter size: $r = 7$) as described previously (Netten et al., 1997 [\[6\]](#); Gué et al., 2005 [\[7\]](#)). Then, median filtering was used to remove noise from the images (filter size: $r = 3$) (Huang et al., 1979 [\[8\]](#)). After the removal of the background and noise, 3D iterative thresholding (Gul-Mohammed et al., 2014 [\[9\]](#)) using the MSER criteria (Matas et al., 2004 [\[10\]](#)) was adapted to draw ROIs on the neuronal cell bodies (300-10000 voxels). Lastly, the morphological opening operation was applied to remove wrongly assigned ROIs (filter size: $r = 2$) (Meyer and Beucher, 1990 [\[11\]](#)). In order to determine γ -secretase activity on a cell-by-cell basis, the donor (mRFP670) and acceptor (mRFP720) fluorescence intensities in ROIs were measured, and the acceptor over donor emission ratios (i.e., 720/670 ratios) were calculated. Then, the distance between an ROI and other ROIs was measured using Scikit-learn (Pedregosa et al., 2012 [\[12\]](#)) in Python, which was used to identify neighboring neurons (e.g., 2, 5, 10 closest neurons,

neurons within a 20 μm radius). In the statistical analysis, the Pearson correlation coefficient was measured to determine if the 720/670 ratios in a neuron significantly correlate with the 720/670 ratios of neighboring neurons, which was performed using Scipy (Virtanen et al., 2020) in Python. $P < 0.05$ was considered statistically significant. The sample size was calculated based on previous correlation analysis in our *in vitro* studies (Houser et al., 2021; Maesako et al., 2022) and was estimated to include approximately $n = 200 - 250$ neurons/region-of-interests (ROIs) per animal. Pseudo-color images corresponding to the 720/670 ratios were generated in MATLAB (MathWorks, Natick, MA).

Results

Expression of the C99 720-670 biosensor in the somatosensory cortex of live mice

We have recently developed genetically encoded FRET-based biosensors that, for the first time, allow quantitative recording of γ -secretase activity on a cell-by-cell basis in cultured neurons (Maesako et al., 2020; Houser et al., 2020). The principle of the biosensors is that the C-terminus of APP C99, an immediate substrate of γ -secretase, is fused to the FRET donor and acceptor fluorescent proteins with a flexible linker, and the sensing domains are stabilized near the membrane by connecting to a membrane-anchoring domain. The proteolytic processing of C99 within the biosensor by endogenous γ -secretase results in a change in the proximity and/or orientation between the donor and acceptor fluorophores, which can be recorded as altered FRET efficiency (Fig. 1A). Of note, we ensured that the fusion of the donor/acceptor fluorescent proteins and the anchor domain does not significantly affect the cleavage efficiency of C99 by γ -secretase by evaluating the processing efficiency between the cells expressing C99 FLAG and our FRET probe (Maesako et al., 2020; Houser et al., 2020). To elucidate the cell-by-cell regulation of γ -secretase activity in live mouse brains *in vivo*, we administrated an AAV that packages the C99 720-670 biosensor into the somatosensory cortex of 4-6 months old C57BL/6 mice implanted with cranial window for imaging (Fig. 1B). 3-4 weeks post AAV injection, we successfully detected the neurons expressing the C99 720-670 sensor using a confocal microscope with single-photon excitation at 640 nm wavelength and emission at 700-800 nm range (Fig. 1C and D). Notably, the fluorescence signal from the C99 720-670 biosensor could be detected from the brain surface to approximately 100 μm depth (Fig. 2).

Automatic segmentation of neurons expressing the C99 720-670 biosensor

In image processing (Fig. 3A), the background fluorescence was first subtracted, and the noise was reduced in the images using median filtering to perform the automatic segmentation of individual neurons. Then, an initial set of ROIs was automatically created on all cellular structures in the images using 3D iterative thresholding. Next, the intensities of the donor (miRFP670) and acceptor (miRFP720) fluorescence were measured, and the acceptor over donor emission ratios (i.e., 720/670 ratios) were calculated for each ROI. Of note, we uncovered that those ROIs contained non-specific autofluorescent objects whose morphology clearly differed from neurons (Fig. 4A). Therefore, morphological filtering was first applied to exclude the non-specific objects from ROIs. Moreover, we analyzed the scatter plot of 720/670 ratios (Y-axis: miRFP670 emission, X-axis: miRFP720 emission) and found that the autofluorescent objects have a significantly lower 720/670 ratio compared to neurons expressing C99 720-670 (Fig. 4B). We also confirmed the identity of the autofluorescent objects by imaging using the same microscope settings in mice without AAV-hSyn1-C99 720-670 injection and found the same population of non-specific objects with low 720/670 ratios is present. Therefore, in the segmentation procedure, we excluded the ROIs exhibiting 720/670 ratios below a set threshold value as 1.5.

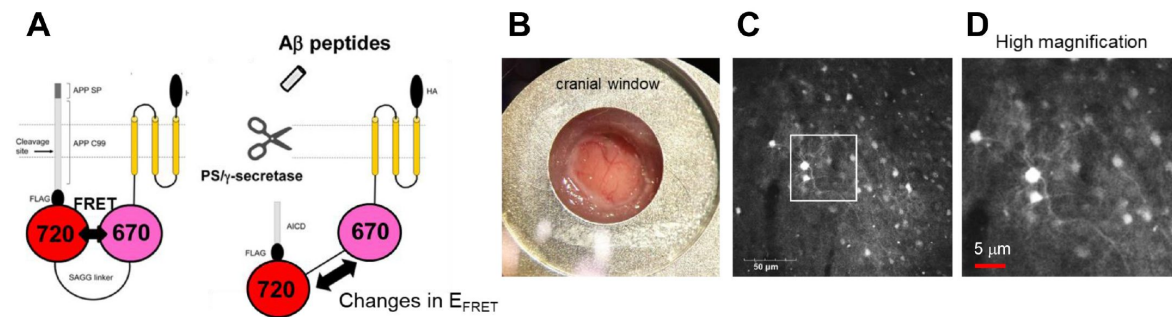


Figure 1.

Expression of the C99 720-670 biosensor in the living mouse brain

(A) A schematic presentation of the C99 720-670 biosensor. (B) A cranial window was implanted on the top of the brain for image acquisition. (C) Extensive expression of the C99 720-670 biosensor in the somatosensory cortex was verified by confocal microscopy *in vivo*. Scale bar: 50 μm . (D) A high magnification image corresponding to the square in Fig. 1C. Scale bar: 5 μm .

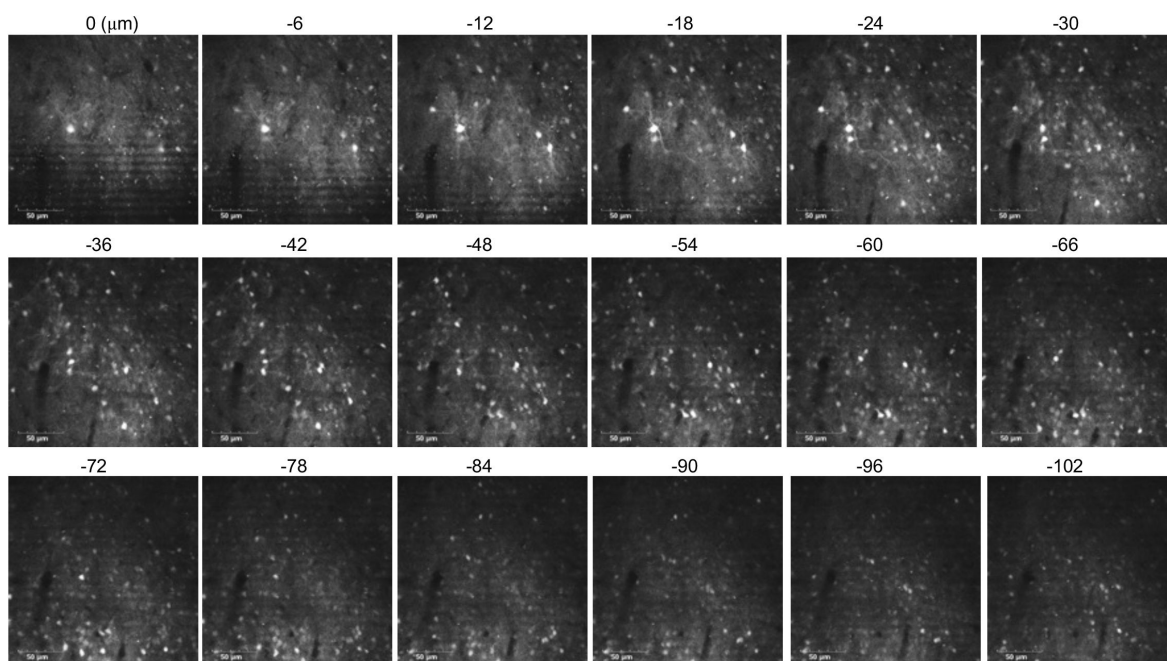


Figure 2.

Z-section images of the brain expressing the C99 720-670 biosensor.

The C99 720-670 biosensor fluorescence signal was detected at approximately 100 μm depth from the brain surface. Scale bar: 50 μm .

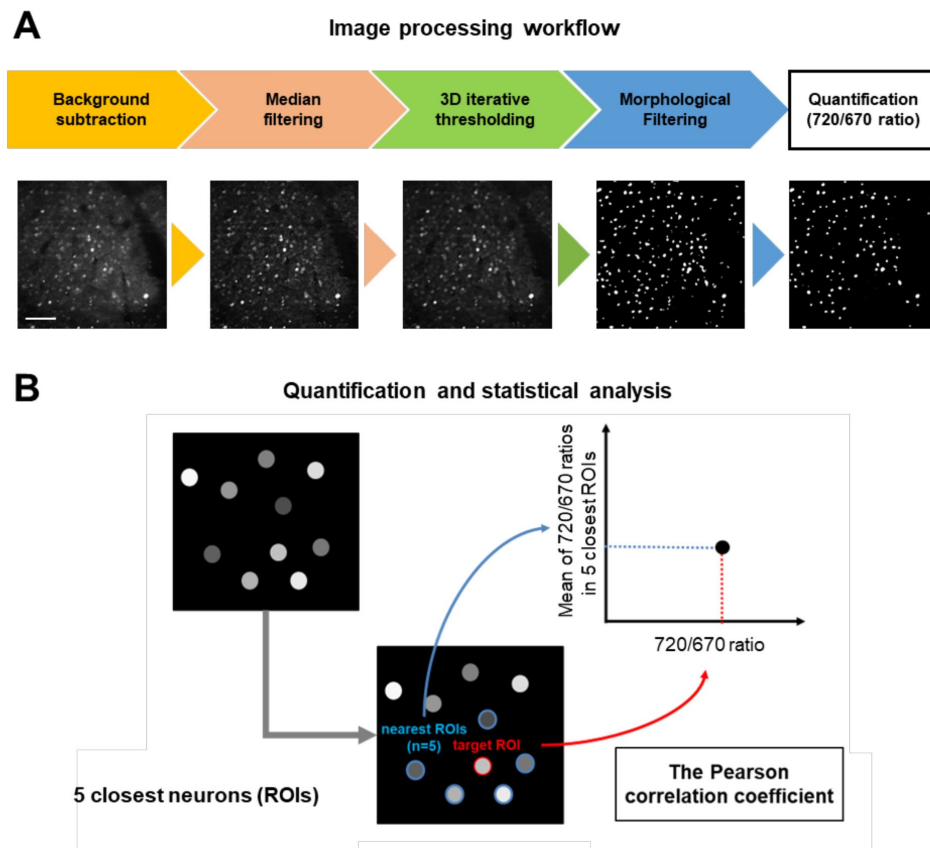


Figure 3.

Imaging processing workflow and data analysis

(A) Before measuring the acceptor over donor emission ratios (i.e., 720/670 ratios) on a cell-by-cell basis, which reports γ -secretase activity in individual neurons, four-step image processing steps were applied: 1) background subtraction, 2) median filtering, 3) 3D iterative thresholding, and 4) morphological filtering. Scale bar: 50 μm . **(B)** To elucidate the relationship between γ -secretase activity and those in neighboring neurons, the distance between neuron and neuron was first determined, then identified each neuron's five closest neurons, and calculated the average 720/670 ratio of the five neighboring neurons. The Pearson correlation coefficient between each neuron's 720/670 ratio and the average ratio of the five neighboring neurons was calculated.

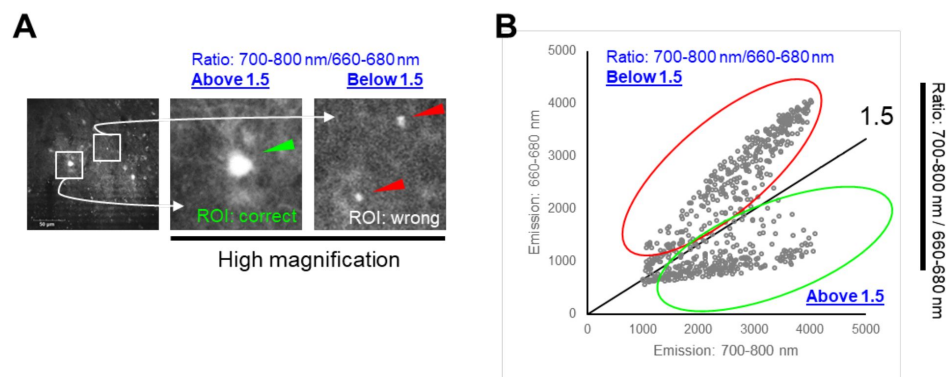


Figure 4.

Identification and removal of auto fluorescent objects

(A) Our four-step segmentation approach still could not perfectly remove wrongly assigned ROIs, such as shown in the right panel (red arrowheads). However, these ROIs displayed higher 660-680 nm emissions and thus significantly lower 720/670 ratios (below 1.5). (B) A scatter plot supported our observation: two populations of ROIs displaying 720/670 ratios above 1.5 and the ratios below, later of which were excluded from our data analysis.

“Cell non-autonomous” regulation of γ -secretase in mouse brains

To elucidate how the γ -secretase activity is regulated on a cell-by-cell basis *in vivo*, we first measured the distance between pairs of neurons, then identified each neuron's five closest neurons, and calculated the average 720/670 ratio of the five neighboring neurons. Then, the Pearson correlation coefficient was measured to determine if there is a linear correlation between the 720/670 ratio (as a measure of γ -secretase activity) in each neuron and the average ratio of the five neighboring neurons (**Fig. 3B**). Intriguingly, we found that neighboring neurons likely exhibited similar 720/670 ratios (i.e., “clustering” of the neurons displaying similar 720/670 ratios) (**Fig. 5A**). Indeed, while the effect is modest, unbiased quantification and statistical analysis showed a significant linear correlation between the 720/670 ratio in each neuron and the average ratio in five neighboring neurons (**Fig. 5B**). Such a statistically significant positive correlation was also detected in two other mice (**Fig. 5C** and **5D**), suggesting that γ -secretase activity in a neuron may positively correlate with the degree of activity in its neighboring neurons. We also ensured that the 720/670 ratio was positively correlated with the average 720/670 ratio of the two and ten neighboring neurons (**Fig. 6A-C**). To further corroborate these findings, we determined the average 720/670 ratio of neurons within a 20 μ m radius and examined the correlation between the 720/670 ratio in each neuron and the average 720/670 ratio in neighboring neurons (**Fig. 7A**). We found a significant linear correlation between the 720/670 ratio in each neuron and the average ratio of neurons within 20 μ m radius in three independent mice (**Fig. 7B-D**), further evidencing neurons displaying similar 720/670 ratios co-localize in mouse brains. Furthermore, we examined if inhibition of γ -secretase can cancel the clustering of the neurons exhibiting similar levels of γ -secretase activities. As such, we subcutaneously administrated a potent γ -secretase inhibitor: DAPT (Dovey et al., 2001). We found that DAPT dose-dependently increased APP C-terminal fragments (APP CTFs), immediate substrates of γ -secretase. In contrast, full-length APP levels were not significantly altered (**Fig. 8A**), suggesting that DAPT inhibits γ -secretase in the brain. Then, we administrated 100 mg/kg DAPT into mice expressing the C99 720-670 biosensor, performed confocal microscopy, adapted the same imaging processing, and performed correlation analysis between the 720/670 ratio in each neuron and the average ratio of the five neighboring neurons or the neurons within 20 μ m radius. Notably, there was no significant correlation in DAPT-administrated mice (**Fig. 8B-E**), suggesting that the positive correlation between the 720/670 ratio in each neuron and the average ratio in neighboring neurons is canceled by the inhibition of γ -secretase and thus the positive correlation is dependent on γ -secretase activity. Collectively, these results strongly indicate that γ -secretase activities are synchronized in neighboring neurons, and γ -secretase activity may be “cell non-autonomously” regulated in living mouse brains.

Discussion

The brain is one of the tissues in which γ -secretase complexes play vital roles. For example, γ -secretase generates A β peptides (De Strooper et al., 1999), which are accumulated in AD brains. Recent clinical trials show that antibodies against A β can slow cognitive decline in AD (van Dyck et al., 2023; Sims et al., 2023). Moreover, γ -secretase is also responsible for maintaining neuronal survival (Saura et al., 2004; Tabuchi et al., 2009; Acx et al., 2017; Bi et al., 2021). However, little is known about how endogenous γ -secretase activity is spatiotemporally regulated in the intact brain. In the present study, we recorded γ -secretase activity in individual neurons in a living mouse brain by employing AAV-mediated gene delivery to express the NIR range γ -secretase reporter: C99 720-670 biosensor (Houser et al., 2020). Using NIR confocal microscopy, we uncovered that γ -secretase activity influences the activity of γ -secretase in neighboring neurons, suggesting a potential “cell non-autonomous” regulation of γ -secretase in mouse brains.

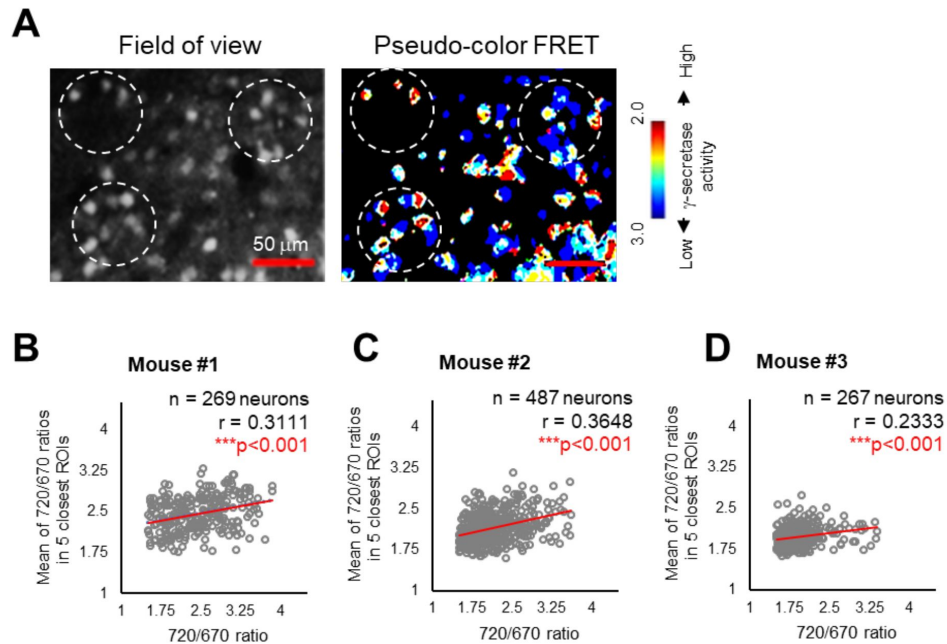


Figure 5.

A potential "cell non-autonomous" regulation of γ -secretase in mouse brains

(A) A representative image showing the expressions of the C99 720-670 biosensor (Field of view) and a pseudo-color image corresponding 720/670 ratios (Pseudo-color FRET). Scale bar: 50 μ m. **(B-D)** Scatter plots showing the 720/670 ratio in individual neurons (X-axis) and the average Mean of the 720/670 ratio in five neighboring neurons in three independent mice. The number of neurons, correlation coefficient (r), and p-value are shown. Pearson correlation coefficient. *** p < 0.001

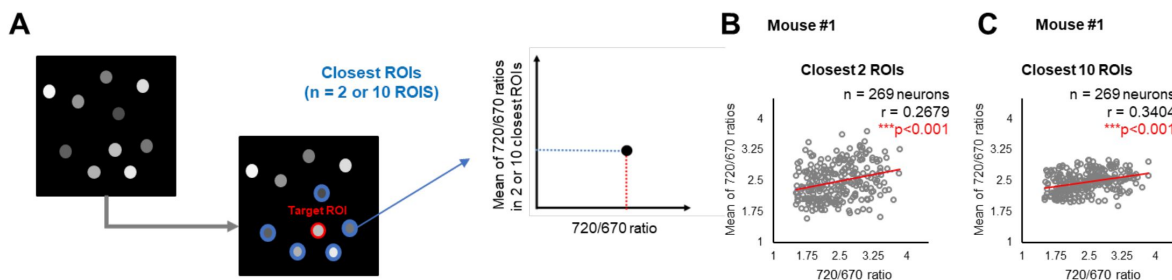


Figure 6.

Validation #1 - A potential "cell non-autonomous" regulation of γ -secretase in mouse brains

(A) The two or ten closest neurons were identified, and the average 720/670 ratio of the two or ten neighboring neurons was calculated and plotted. **(B)** We verified a significant positive correlation between the 720/670 ratio and the average ratio of two and **(C)** ten closest neurons. The number of neurons, correlation coefficient (r), and p-value are shown. Pearson correlation coefficient. *** p < 0.001

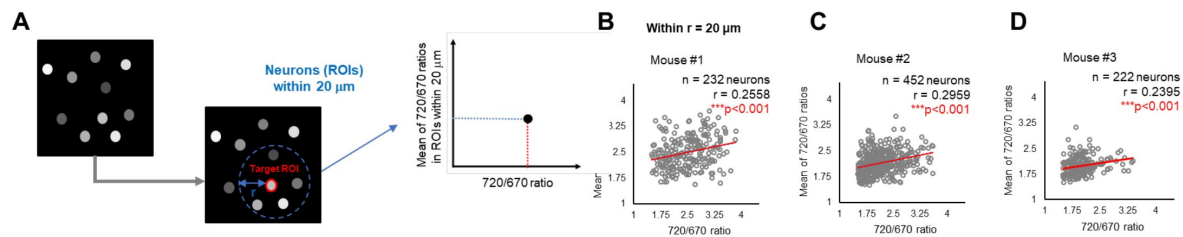


Figure 7.

Validation #2 - A potential “cell non-autonomous” regulation of γ -secretase in mouse brains

(A) The average 720/670 ratio of neurons within a 20 μ m radius was calculated and plotted. (B-D) There was a significant positive correlation between the 720/670 ratio and the average ratio of neurons within a 20 μ m radius in three independent mice. The number of neurons, correlation coefficient (r), and p -value are shown. Pearson correlation coefficient. *** $p < 0.001$

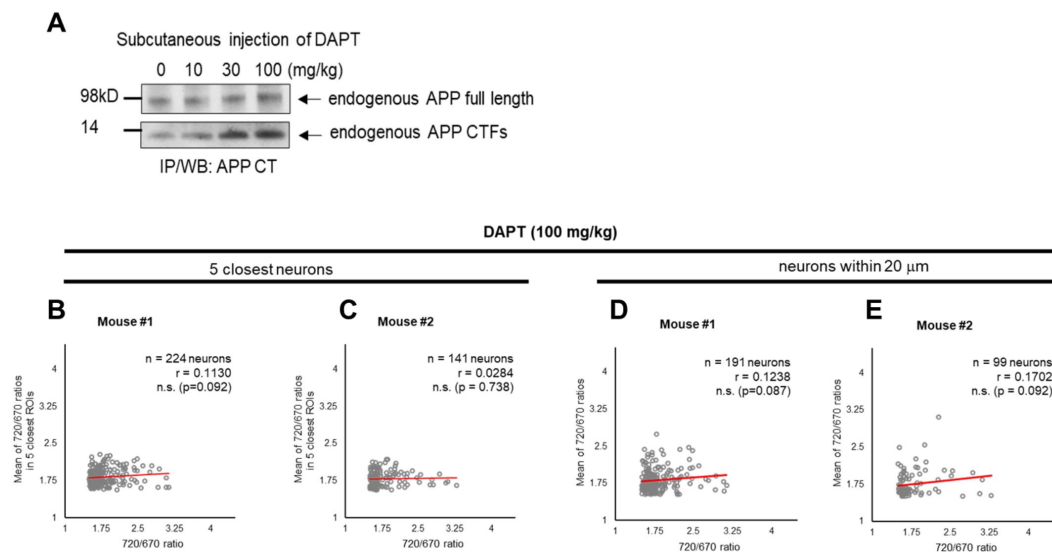


Figure 8.

γ -Secretase inhibition cancels the “cell non-autonomous” regulation

(A) The dose-dependent accumulation of endogenous APP C-terminus fragments (APP-CTFs) by subcutaneous administration of DAPT evidences the inhibition of γ -secretase activity in mouse brains. (B and C) Neither significant correlation between the 720/670 ratio and the average ratio of the five closest neurons, nor (D and E) the average ratio of neurons within a 20 μ m radius. The number of neurons, correlation coefficient (r), and p -value are shown. Pearson correlation coefficient. n.s. not significant

Whether the γ -secretase activity is similarly or differently regulated among neurons remains elusive. A previous study employing microengraving technology reported that individual neurons generate and secrete different levels of A β peptides (Liao et al., 2016). In the same line, our FRET-based biosensors have allowed “visualizing” that γ -secretase activity is heterogeneously regulated on a cell-by-cell basis in primary neurons (Maesako et al., 2020). Furthermore, the cell-by-cell heterogeneity in γ -secretase activity was further verified using 1) unique multiplexing FRET analysis in which the processing of two different substrates (e.g., APP vs. Notch1) by γ -secretase can be simultaneously measured in the same cell (Houser et al., 2021), and 2) multiplexed immunocytochemistry in which intracellular A β is detected on a cell-by-cell basis (McKendell et al., 2022). Our new study further adds that each neuron exhibits a distinct level of γ -secretase activity in intact mouse brains.

Although increasing evidence suggests γ -secretase activity is heterogeneously regulated on a cell-by-cell basis, little is known about the molecular mechanism(s) underlying such heterogeneity. Given that there are two isoforms of PSEN (i.e., PSEN1 and PSEN2) and three Aph1 (Aph1a, Aph1b, and Aph1c) in rodents, six different γ -secretase complexes can be expressed by the same cells. While the PSEN1 knockout mice displayed a lethal phenotype (Shen et al., 1997; Wong et al., 1997), the PSEN2 knockout revealed no severe phenotypes (Herreman et al., 1999). Furthermore, Aph1a knockout mice show a lethal phenotype, whereas those lacking Aph1b or Aph1c survive into adulthood (Serneels et al., 2005). These studies suggest that different γ -secretase complexes exhibit distinct γ -secretase activity. In this sense, the spatiotemporal expression of different γ -secretase complexes could explain the cell-by-cell basis heterogeneity in γ -secretase activity. On the other hand, previous studies utilizing pharmacological agents that bind to the active form of γ -secretase demonstrated that only a small portion of PSEN might be engaged in the active γ -secretase complex (Lai et al., 2003; Placanica et al., 2009). If this is the case, different equilibrium between the active and inactive γ -secretase could be another possible explanation of the heterogeneity in cellular γ -secretase activity.

Interestingly, we also uncovered not drastic but a statistically significant positive correlation between the 720/670 ratio and those ratios in neighboring neurons (Fig. 5–7), suggesting that neurons with similar levels of γ -secretase activity form a cluster. Of note, this positive correlation in the γ -secretase activities is canceled by pharmacological inhibition of γ -secretase (Fig. 8). Although these results implicate that γ -secretase activity “propagates” from neuron to neuron, the underlying mechanism remains elusive. Interestingly, a recent study shows that A β 42 exerts product feedback inhibition on γ -secretase (Zoltowska et al., 2023), suggesting that secreted A β 42 may be one of the negative regulators of γ -secretase. Furthermore, it is reported that hypoxia-inducible factor-1 alpha (HIF-1 α), which is activated under hypoxia (Semenza et al., 1991; Semenza et al., 1992), regulates γ -secretase activity (Villa et al., 2014; Alexander et al., 2022). Therefore, local oxygen concentration may be linked to the clustering of neurons exhibiting similar levels of γ -secretase activity.

Conditional knockout of γ -secretase in the adult neurons displays synaptic dysfunctions, neuroinflammation, and neuronal loss in an age-dependent manner (Saura et al., 2004; Tabuchi et al., 2009; Acx et al., 2017; Bi et al., 2021). It is plausible that γ -secretase may function as “a membrane proteasome” (Kopan and Ilagan, 2004), responsible for the degradation of over 150 different substrate stubs to maintain proper membrane homeostasis, which may be critical for neuronal survival. Notably, a conditional knockout strategy has also elucidated the critical roles of γ -secretase beyond the CNS, i.e., in other tissues, *in vivo*. For instance, specific PSEN1 knockout in hematopoietic progenitors demonstrated the importance of PSEN1/ γ -secretase in developing and sustaining leukemia (Habets et al., 2019). Although GSIs were discontinued in the clinical trials of AD, their inhibiting effects on Notch signaling have led to the repurposing of GSIs as anticancer drugs (reviewed in McCaw et al., 2021). Furthermore, epidermis-specific Nicastrin conditional knockout allowed the identification of IL-36a as a key inflammatory cytokine involved in the malfunction of the skin barrier in the pathogenesis of HS (Yang et al., 2020). However, it is

difficult to determine by existing tools how endogenous γ -secretase activity is spatiotemporally regulated and how the altered γ -secretase activity contributes to the disease progression. These dynamic processes in living cells can be directly monitored using our FRET-based imaging assays.

In conclusion, this study provides a new imaging prototype to better understand the regulation of γ -secretase and its consequences in living mice *in vivo*. We have recorded γ -secretase activity on a cell-by-cell basis in mouse brains and found that neighboring neurons exhibit similar levels of γ -secretase activity, suggesting that γ -secretase influences the activity of γ -secretase in surrounding neurons.

Conflict of interest statement

The authors declare no conflicts of interest with the contents of this article.

Acknowledgements

This work was funded by the National Institute of Health grants AG79838 (M.M.)

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Reviewer #1 (Public Review):

Summary:

In their paper, Hou and co-workers explored the use of a FRET sensor for endogenous g-sec activity in vivo in the mouse brain. They used AAV to deliver the sensor to the brain for neuron specific expression and applied NIR in cranial windows to assess FRET activity; optimizing as well an imaging and segmentation protocol. In brief they observe clustered g-sec activity in neighboring cells arguing for a cell non-autonomous regulation of endogenous g-sec activity in vivo.

Weaknesses:

Overall the authors provide a very limited data set and in fact only a proof of concept that their sensor can be applied in vivo. This is not really a research paper, but a technical note. With respect to their observation of clustered activity, the images do not convince me as they show only limited areas of interest: from these examples (for instance fig 5) one sees that merely all neurons in the field show variable activity and a clustering is not really evident from these examples. Even within a cluster, there is variability. With r values between 0.23 to .36, the correlation is not that striking. The authors herein do not control for expression levels of the sensor: for instance, can they show that in all neurons in the field, the sensor is equally expressed, but FRET activity is correlated in sets of neurons? Or are the FRET activities that are measured only in positively transduced neurons, while neighboring neurons are not expressing the sensor? Without such validation, it is difficult to make this conclusion.

Secondly, I am lacking some more physiological relevance for this observation. The experiments are performed in wild-type mice, but it would be more relevant to compare this with a fadPSEN1 KI or a PSEN1cKO model to investigate the contribution of a gain of toxic function or LOF to the claimed cell non-autonomous activations. Or what would be the outcome if the sensor was targeted to glial cells?

For this reviewer it is not clear what resolution they are measuring activity, at cellular or subcellular level? In other words are the intensity spots neuronal cell bodies? Given g-sec activity are in all endosomal compartments and at the cell surface, including in the synapse, does NIR imaging have the resolution to distinguish subcellular or surface localized

activities? If cells 'communicate' g-sec activities, I would expect to see hot spots of activity at synapses between neurons: is this possible to assess with the current setup?

Without some more validation and physiological relevant studies, it remains a single observation and rather a technical note paper, instead of a true research paper.

<https://doi.org/10.7554/eLife.96848.1.sa2>

Reviewer #2 (Public Review):

Summary:

The manuscript by Hou et al is a short technical report which details the potential use of a recently developed FRET based biosensor for gamma-secretase activity (Houser et al 2020) for in vivo imaging in the mouse brain. Gamma-secretase plays a crucial role in Alzheimer disease pathology and therefore developing methodologies for precise in vivo measurements would be highly valuable to better understand AD pathophysiology in animal models.

The current version of the sensor utilizes a pair of far-red fluorescent proteins fused to a substrate of the enzyme. Using live imaging, it was previously demonstrated it is possible to monitor gamma-secretase activity in cultured cells. Notably, this is a variant of a biosensor that was previously described using CFP-YFP variants FRET pair (Maesako et al, iScience. 2020). The main claim and hypothesis for the MS is that IR excitation and emission has considerable advantages in terms of depth of penetration, as well as reduction in autofluorescence. These properties would make this approach potentially suitable to monitor cellular level dynamics of Gama-secretase in vivo.

The authors use confocal microscopy and show it is possible to detect fluorescence from single cortical cells. The paper described in detail technical information regarding imaging and analysis. The data presented in figures 5-8 details analysis of FRET ratio (FR) measurements within populations of cells. The authors claim it is possible to obtain reliable measurements at the level of individual cells. They compare the FR values across cells and mice and find a spatial correlation among neighboring cells. This is compared with data obtained after inhibition of endogenous gamma-secretase activity, which abolishes this correlation.

Strengths:

The authors describe in detail their experimental design and analysis for in vivo imaging of the reporter. The idea of using a far-red FRET sensor for in vivo imaging is novel and potentially useful to circumvent many of the pitfalls associated with intensity-based FRET imaging in complex biological environments (such as autofluorescence and scattering).

Weaknesses:

There are several critical points regarding validation of this approach and concerns with the data presented that must be addressed:

- (1) Regarding the variability and spatial correlation- the dynamic range of the sensor previously reported in vitro is in the range of 20-30% change (Houser et al 2020) whereas the range of FR detected in vivo is between cells is significantly larger (Fig. 3). This raises considerable doubts for specific detection of cellular activity (see point 3).
- (2) One direct way to test the dynamic range of the sensor in vivo, is to increase or decrease endogenous gamma-secretase activity and to ensure this experimental design allows to accurately monitor gamma-secretase activity. In the previous characterization of the reporter (Hauser et al 2020), DAPT application and inhibition of gamma-secretase activity results in

increased FR (Figures 2 and 3 of Houser et al). This is in agreement with the design of the biosensor, since FR should be inversely correlated with enzymatic activity. Here, while the authors repeat the same manipulation and apply DAPT to block gamma-secretase activity, it seems to induce the opposite effect and reduces FR (comparing figures 8 with figures 5,6,7). First, there is no quantification comparing FR with and without DAPT. Moreover, it is possible to conduct this experiment in the same animals, meaning comparing FR before and after DAPT in the same mouse and cell populations. This point is absolutely critical- if indeed FR is reduced following DAPT application, this needs to be explained since this contradicts the basic design and interpretation of the biosensor.

(3) For further validation, I would suggest including in vivo measurements with a sensor version with no biological activity as a negative control, for example, a mutation that prevents enzymatic cleavage and FRET changes. This should be used to showcase instrumental variability and would help to validate the variability of FR is indeed biological in origin. This would significantly strengthen the claims regarding spatial correlation within population of cells.

(4) In general, confocal microscopy is not ideal for in vivo imaging. Although the authors demonstrate data collected using IR imaging increases penetration depth, out of focus fluorescence is still evident (Figure 4). Many previous papers have primarily used FLIM based analysis in combination with 2p microscopy for in vivo FRET imaging (Some examples: Ma et al, Neuron, 2018; Massengil et al, Nature methods, 2022; Diaz-Garcia et al, Cell Metabolism, 2017; Laviv et al, Neuron, 2020). This technique does not rely on absolute photon number and therefore has several advantage sin terms of quantification of FRET signals in vivo. It is therefore likely that use of previously developed sensors of gamma-secretase with conventional FRET pairs, might be better suited for in vivo imaging. This point should be at least discussed as an alternative.

<https://doi.org/10.7554/eLife.96848.1.sa1>

Reviewer #3 (Public Review):

This paper builds on the authors' original development of a near infrared (NIR) FRET sensor by reporting in vivo real-time measurements for gamma-secretase activity in the mouse cortex. The in vivo application of the sensor using state of the art techniques is supported by a clear description and straightforward data, and the project represents significant progress because so few biosensors work in vivo. Notably, the NIR biosensor is detectable to ~ 100 μ m depth in the cortex. A minor limitation is that this sensor has a relatively modest ΔF as reported in Houser et al, which is an additional challenge for its use in vivo. Thus, the data is fully dependent on post-capture processing and computational analyses. This can unintentionally introduce biases but is not an insurmountable issue with the proper controls that the authors have performed here.

The observation of gamma-secretase signaling that spreads across cells is potentially quite interesting, but it can be better supported. An alternative interpretation is that there exist pre-formed and clustered hubs of high gamma-secretase activity, and that DAPT has stochastic or differential accessibility to cells within the cluster. This could be resolved by an experiment of induction, for example, if gamma-secretase activity is induced or activated at a specific locale and there was observed coordinated spreading to neighboring neurons with their sensor.

Furthermore, to rule out the possibility that uneven viral transduction was not simply responsible for the observed clustering, it would be helpful to see an analysis of 670nm fluorescence alone.

<https://doi.org/10.7554/eLife.96848.1.sa0>