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Article

Relationship between p62 and learning behavior in male and female mice deficient in hippocampal folliculin

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SUMMARY

Alzheimer's disease is a degenerative disease characterized by loss of memory, loss of cognitive function, and functional impairment with associated neuropsychological symptoms. Sequestosome 1 (referred to henceforth as p62) is a ubiquitin-binding scaffold protein that transports ubiquitinated targets to autophagosomes for degradation. Folliculin (FLCN) is a protein that affects diverse signaling pathways involving lysosome biogenesis, a cellular process increasingly relevant to neurodegenerative diseases like Alzheimer's disease. p62 was among several proteins that had not been studied regarding FLCN manipulation in the dorsal hippocampus between sexes in young C57BL6/J wild-type mice. We hypothesized that if FLCN and p62 are functionally connected, then reducing FLCN may have downstream effects on p62 protein levels, an effect we examined in both female and male mice. To this end, we used western blots to measure p62 levels in the samples from a previous study that used shRNA against FLCN in female and male mice (N = 28, 13 males, 15 females) in the dorsal hippocampus. Our p62 data was analyzed with a two-way analysis of variation (ANOVA), sex (male, female) x folliculin (control, shRNA), to determine the effects of the independent variables on p62 expression. We found that p62 regressed with folliculin expression levels. Other studies indicate that p62 impacts memory function; however, our results show there is no evidence of a functional connection between FLCN and p62 in young wild-type mice. Our work may therefore impact future p62 research and does not preclude the possibility that p62 may affect cognitive functioning in diseased and/or aged models.

INTRODUCTION

Alzheimer's disease is the fifth leading cause of death in adults older than 65 in the United States (1). In 2020, the estimated total cost for the treatment was about $305 billion, and that number is expected to go up by an additional $1 trillion as the population grows older (1). Researchers have been working for decades to try to find the cause of sporadic Alzheimer's in the hopes of finding a cure. Alzheimer's Disease is a complex neurodegenerative disorder without a known etiological cause. Autophagic and lysosomal dysfunction have been heavily linked to Alzheimer's Disease and are suggested to be the earliest known pathological hallmark of the disease (2). Folliculin (FLCN) is involved in autophagic/lysosomal processes (3), yet little is known about how it functions or interacts with other key proteins in the dorsal hippocampus, a brain region involved in learning and memory that degenerates early in Alzheimer's progression. FLCN controls many aspects of autophagy; among them may include sequestosome 1 (p62) (4, 5). Autophagy is a catabolic process in eukaryotic cells that bring cytoplasmic components and organelles to the lysosomes for digestion (6). Lysosomes are specialized organelles that break up macromolecules, allowing the cell to reuse the materials (6). p62 is a ubiquitin-binding scaffold protein that acts as a receptor for ubiquitinated targets to transport to autophagosomes for degradation (7). Relationships have been found between dysregulation of p62 and neurodegenerative disorders, including Alzheimer's Disease (8). Studies have shown that p62 expression and cytoplasmic p62 protein are reduced in the frontal cortex of Alzheimer's patients (9). Whether sex impacts interactions between FLCN and p62 in the hippocampus has yet to be determined. If differences can be identified between male and female FLCN and p62 interactions those differences could help take research down an uncharted path.

Our previous but currently not published work focused on FLCN's impact on memory functions and related molecular pathways in female and male mice. Of further interest was whether FLCN manipulation would have any effect on p62 expression. Given that both FLCN and p62 have roles in lysosomal processes and that p62 has been more directly linked to neurodegenerative disease, we wanted to test whether there was a functional connection between these two proteins (7). We hypothesized that if FLCN and p62 are functionally connected, then reducing FLCN may have downstream effects on p62 protein levels, effects that may vary based on sex. Neither FLCN manipulation nor sex affected p62 levels. These results remain useful for researchers currently studying p62 and could affect future p62 studies. Furthermore, these results do not preclude the possibility that p62 affects other aspects of dorsal hippocampal function, such as cognitive functioning, in diseased aged models.

RESULTS

We analyzed mouse brain samples from the previous but not published yet FLCN study by western blots to protein expression levels of p62 and FLCN to determine whether FLCN reductions change the levels of p62 in the brain and if there was a difference between the male and female p62 levels. To determine whether the differences were significant, we used a 2-way analysis of variation (ANOVA). There was no significant interaction ($F_{1,24} = 0.83$, $p = 0.37$). There was no main effect for shRNA treatment ($F_{1,24} = 0.40$, $p = 0.53$). There was no main effect for sex ($F_{1,24} = 0.40$, $p = 0.54$). (**Fig. 1A**)

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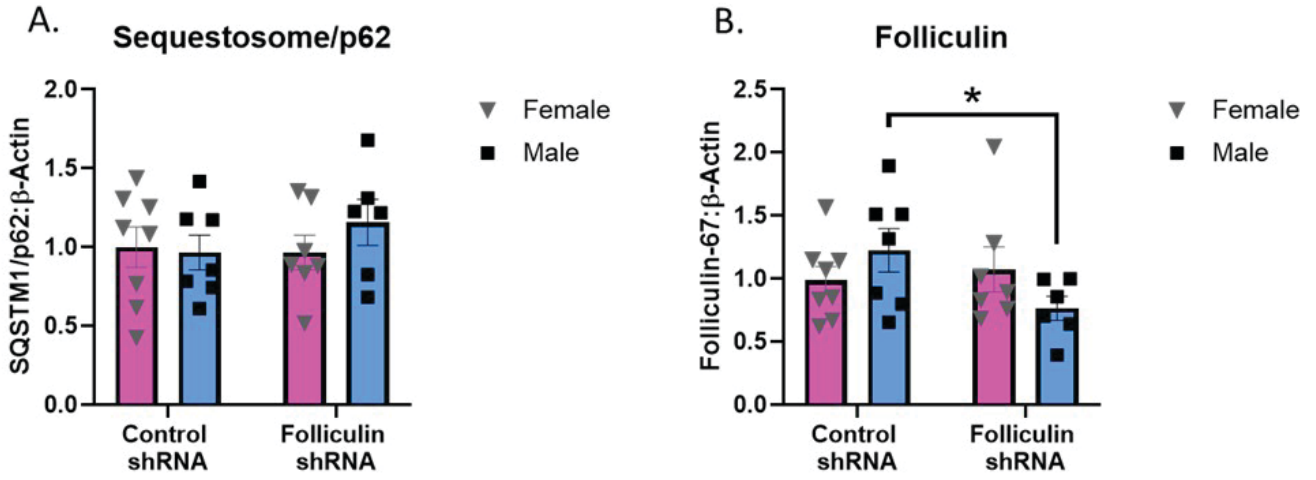

Figure 1: Statistical Analysis of p62 and Folliculin. (A) p62 levels in the hippocampus are unaffected by sex and/or folliculin shRNA. (B) Folliculin was significantly reduced by FLCN shRNA in male mice only.

Control shRNA and FLCN shRNA having the same p62 levels and for females and males having the same hippocampal levels.

The statistical analysis consisted of a 2-way ANOVA. There was a trending interaction ($F_{1,24}$ = 3.502, p = 0.07). There was no main effect for shRNA treatment ($F_{1,24}$ = 1.658, p = 0.21). There was no main effect for sex ($F_{1,24}$ = 0.06, p = 0.81). Using an *a priori* Fisher's LSD test to compare control shRNA to FLCN shRNA, we see a difference between males who received the control shRNA versus males who received the FLCN shRNA (p = 0.04; **Fig. 1B**). With a chosen α = 0.05, we rejected the null hypothesis in which control shRNA and FLCN shRNA had the same FLCN levels.

Additional statistical analyses were performed on these results using previously acquired memory data to evaluate whether p62 levels correlated with any other experimental outcomes. Using regression statistics, we tested whether p62 expression levels were dependent on FLCN expression levels on either FLCN or p62. We plotted the regression lines for the data between p62 vs. FLCN (**Fig. 2**), with each sex x shRNA variable analyzed individually within each comparison. Regression analysis determining whether p62 levels correlated with FLCN levels showed there were no significant effects in control females (F-ctrl, R^2 = 0.28, $F_{1,5}$ = 2.31, p = 0.18). There was no main effect in FLCN shRNA females (F-flcn, R^2 = 0.22, $F_{1,5}$ = 1.40, p = 0.29). There was no main effect in control males (M-ctrl, R^2 = 0.07, $F_{1,5}$ = 0.38, p = 0.56). There was no main effect in FLCN shRNA males (M-flcn, R^2 = 0.01, $F_{1,4}$ = 0.03, p = 0.88); additionally, we found the slopes are not significantly different from one another ($F_{3,20}$ = 1.02, p = 0.41).

To determine any baseline differences in FLCN and to confirm the efficacy of the shRNA reduction, we FLCN in the hippocampus via immunohistochemistry (IHC; **Fig. 3**). IHC confirms reduction of FLCN expression at the infusion site in the hippocampal Cornu Ammonis (CA1) region.

DISCUSSION

We did expect FLCN knockdown to affect p62 expression, however, the results showed that p62 has no significant relationship with sex or FLCN. Both p62 and FLCN interact with multiple protein targets related to autophagy and lysosomal

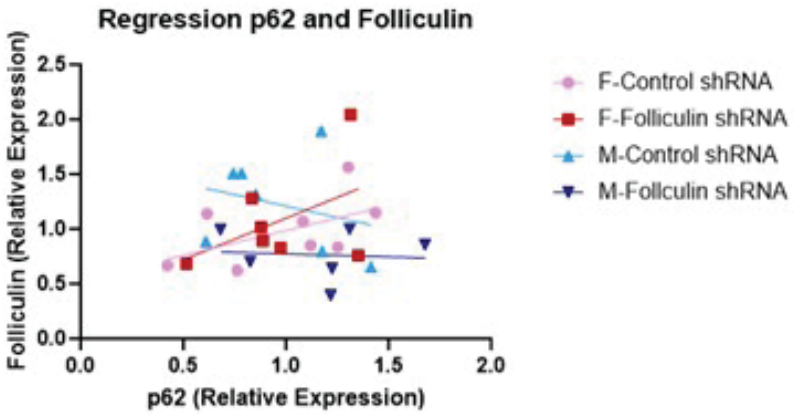

Figure 2: Regression p62 and FLCN Graph Data. This graphic models the regression lines of our data based on the folliculin and p62 levels. Females with control shRNA had a slope of 0.45 and a R^2 of 0.28. Females with folliculin shRNA had a slope of 0.76 and a R^2 of 0.22. Males with control shRNA had a slope of -0.41 and a R^2 of 0.07. Males with folliculin shRNA had a slope of -0.05 and an R^2 of 0.01.

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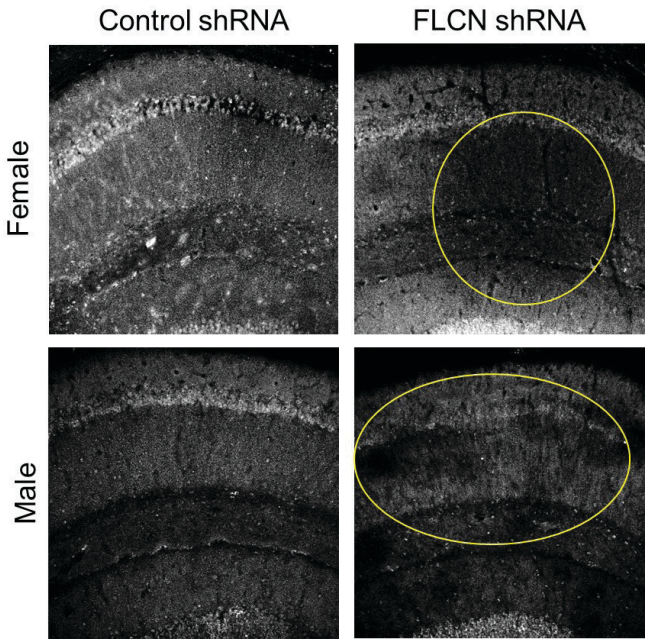

Figure 3: Immunohistochemistry of CA1 of the Dorsal Hippocampus. Representative images from mice that received either control shRNA or one targeting folliculin that was directed to the CA1 region of the hippocampus. Highlighted areas (yellow circles) in the FLCN shRNA group are commensurate with reduced folliculin expression within the targeted CA1 region.

degradation. When FLCN is manipulated, it can affect targets related to TFEB/TFE3 transcription or mTORC1 signal transduction (3). As such, it is important that neuroscientists know which pathways are and are not being affected in the brain. Additionally, showing that p62 has no significant relationship with sex, FLCN, and the ability to learn informs researchers to look at a different pathway relationship that may affect disease progression. Separately, we confirmed by QPCR, Western Blots, and Immunohistochemistry that the shRNA folliculin virus was effective in the CA1 region at reducing FLCN transcripts (**Fig. 3**), and visually, with immunohistochemistry, there is a marked loss in FLCN expression at the infusion site. Western blots can dilute the effects of the virus because the virus was only put into the CA1, whereas IHC allows for a complete picture of the CA1 infusion site. As such, the lack of change in p62 is likely due to a lack of direct interaction with FLCN within the brain within the dorsal hippocampus. The results were specifically from mice exhibiting memory loss after being infected with the FLCN shRNA, which could be why our results show that p62 has no significant correlation to memory loss in these specific mice. In the future, decreasing p62 and measuring changes to the FLCN levels in male vs female mice in relation to memory development would be a logical progression to study next.

Whole dorsal hippocampal protein levels of FLCN in females were not significantly affected by the shRNA. It likely has to do with the dissection of the tissue being of the whole dorsal hippocampus, while the infusion was targeted to the CA1 subregion of the hippocampus. We are conducting additional work to elucidate this effect. Immunohistochemistry strongly supports FLCN shRNA as effectively reducing FLCN within the CA1 subregion. Additionally, it remains novel that few studies examine sex differences in autophagic/lysosomal proteins in the brain.

We are continuing to characterize sex-specific changes in autophagy and lysosomal proteins in Alzheimer's disease models using FLCN and other lysosomal proteins as therapeutic agents. Behavior is always a component of these studies, making it highly dependent on brain area. For these studies, we focus on the dorsal hippocampus as a key brain area that controls special learning and memory and is uniquely susceptible to neurologic diseases.

MATERIALS AND METHODS

All methods and procedures were approved by the Legacy Research Institute IACUC (protocol #123-20) prior to any rodent work occurring. All methods were performed in accordance with guidelines and regulations directed in the Guide for the Care and Use of Laboratory Animals and in full compliance with OLAW in an AAALAC-accredited facility. Mice were maintained on a 12/12 light cycle, lights on at 0730, and given ad libitum access to food and water. Mice were three months old at the time of intrahippocampal surgical infusion of the shRNA constructs. Tissues were taken five weeks post-operation.

Intrahippocampal Administration

A Short Hairpin RNA (shRNA), packaged in a 3rd generation lentivirus designed to interfere with FLCN translation, was administered bilaterally into the hippocampus of male and female C57BL6/J mice. A month later, tissue samples were collected. In brief, intrahippocampal administration was done by anesthetizing mice with isofluorane. A midline incision was made to the skull, and CA1 was located relative to Bregma (AP: -2.0, ML: +/- 1.25, DV: -1.60). 0.5 µL of lentivirus was infused over 10 minutes. Mice were allowed to recover and given Meloxicam tablets (BioServ MD275-0125) for pain. FLCN shRNA was packaged in 3rd generation lentivirus by the Kantor Lab viral core at Duke University in North Carolina.

Western Blots

Western blotting methods have been previously published (10). The whole dorsal hippocampus was collected from the C57BL6/J wild-type mice and stored at -80 °C. They were previously prepared as a 4x preparation with 4x Laemmli Sample Buffer (BioRad #1610747). We used the Criterion Electrophoresis Cell system (BioRad, Cat# 1656001) with 4-12% Bis-Tris gels (BioRad, Cat# 3450124). 1 X MOPS running buffer solution was added to the gel inserts. 10 µL of a molecular weight ladder (BioRad, Cat#1610374) was added to each end of the gels, while 25 µL of the sample, consisting of 40 mg of protein, was added to each of the sample wells of the gels. Gels were run at 200 V until bands reached the end of the gel (~45-60 min).

PVDF membrane (BioRad, Cat#1620184) was activated with methanol and placed in a transfer buffer until it was time to put it on the gel. Cold transfer buffer was used with an ice pack to keep it cold. The machine was set to run at 400 mA for 1 hour. After the transfer was done, blots were rinsed in TBS for five minutes, three times, replacing the TBS each time. Blots were then blocked in 5% milk in 1x TBS-T for 1 hour.

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Primary Antibody Solution

5% BSA with antibody (actin 1:2000; Millipore-Sigma A5441-100UL; FLCN 1:500; ThermoFisher #11236-2-AP). Blots were incubated overnight at 4 °C.

Primary Antibody Solution

5% milk in TBS-T with antibody (both used at 1:1000) was then prepared and incubated with the blots for 1 hour. Chemiluminescent 1:1 ECL (Pierce, #32106) solution was poured onto the blots and then visualized on the BioRad ChemiDoc. The visualized images were analyzed using Image Lab (BioRad, Cat#12012931), and the data was analyzed with GraphPad Prism (v10) software.

Immunohistochemistry was performed on 40 mm brain sections for confirmation of viral efficacy (**Fig. 3**). Sections were permeabilized in PBS+1% Triton X. Antigen retrieval was performed with 2N HCL incubated at 37 °C for 30 minutes. Sections were then blocked in donkey blocking buffer, consisting of 2% normal donkey serum, 0.2% Triton X, and 2% bovine serum albumin in PBS. Sections were then incubated overnight at 4 °C in FLCN antibody (1:100; ThermoFisher #11236-2-AP) in donkey blocking buffer. The next day, the sections were incubated with AlexaFluor 555 (1:350; ThermoFisher #A-21428). Following mounting, sections were visualized on our confocal microscope (Leica TCS SPE-II).

Statistical Analysis

Data was analyzed as a 2-way ANOVA using GraphPad Prism (Version 10).

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REFERENCES

1. Wong, Winston. “Economic burden of Alzheimer disease and managed care considerations.” *Am J Manag Care,* vol. 26, no. 8, Aug. 2020, https://doi.org/10.37765/ajmc.2020.88482.
2. Bordi, Matteo, *et al.* “Autophagy flux in CA1 neurons of Alzheimer hippocampus: Increased induction overburdens failing lysosomes to propel neuritic dystrophy.” *Autophagy,* vol. 12, no. 12, Dec. 2016, https://doi.org/10.1080/15548627.2016.1239003.
3. de Martín Garrido, Natàlia, and Christopher H S Aylett. “Nutrient Signaling and Lysosome Positioning Crosstalk Through a Multifunctional Protein, Folliculin.” *Front. Cell Dev. Biol.*, vol. 8, no. 108, Mar. 2020, https://doi.org/10.3389/fcell.2020.00108.
4. Xia, Qin, *et al.* “Folliculin, a tumor suppressor associated with Birt-Hogg-Dubé (BHD) syndrome, is a novel modifier of TDP-43 cytoplasmic translocation and aggregation”. *Human molecular genetics*, vol. 25, no. 1, Jan. 2016, https://doi.org/10.1093/hmg/ddv450.
5. Zhang, Qi, *et al.* “Suppression of autophagy enhances preferential toxicity of paclitaxel to folliculin-defficient renal cancer cells”. *Journal of experimental & clinical cancer research,* vol. 32, no. 1, Dec. 2013, https://doi.org/10.1186/1756-9966-32-99.
6. Zhang, Nan, Zhao, Ying, "Other Molecular Mechanisms Regulating Autophagy." *PubMed.gov,* vol. 1206, no. 261-271, 2019, https://doi.org/10.1007/978-981-15-0602-4_13.
7. 7. Shacka, John J, *et al.* “The autophagy-lysosomal degradation pathway: role in neurodegenerative disease and therapy.” *Frontiers in bioscience : A Journal and Virtual Library*, vol. 13 718-36. 1 Jan. 2008, https://doi.org/10.2741/2714.
8. 8. Ma, Shifan, *et al.* “SQSTM1/p62: A Potential Target for Neurodegenerative Disease.” *ACS Chemical Neuroscience*, vol. 10, no. 5, Jan. 2019, https://doi.org/10.1021/acschemneuro.8b00516.
9. Salminen, Antero, *et al.* “Emerging role of p62/sequestosome-1 in the pathogenesis of Alzheimer’s disease.” *Progress in Neurobiology,* vol. 96, no. 1, Jan. 2012, https://doi.org/10.1016/j.pneurobio.2011.11.005.
10. Baer, Sadie B., Dorn, Adrianah D., Osborne, DM., (2024). Sex differences in response to obesity and caloric restriction on cognition and hippocampal measures of autophagic-lysosomal transcripts and signaling pathways. *BMC Neurosci,* Jan2; 25(1). https://doi.org/10.1186/s12868-023-00840-1.