



Determining the Relationship Between Phosphorylation and Bindings of Nedd4-1 to AMPA Receptors

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Abstract

Synaptic plasticity has been studied for decades, but what is unknown is the particular mechanisms that drive the brain's ability to strengthen and weaken synapses. Ionotropic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) insertion or removal from the synapse's surface has been shown to modulate the strength of that synapse. The literature also shows that Nedd4-1, an E3 ligase, is vital for the internalization and degradation of surface proteins through ubiquitination. One of Nedd4-1's targets is the AMPA receptor. Overexpression of Nedd4-1 leads to less surface AMPA receptors, a possible symptom of Alzheimer's Disease and other neurological disorders. This study will help to illuminate the role of metabotropic glutamate receptors and phosphorylation in the trafficking of Nedd4-1. We will use immunostaining and confocal imaging to see if DHPG, a metabotropic glutamate receptor (mGluR) group I agonist, can mimic redistribution of Nedd4-1 caused by glutamate and glycine treatment. We will then use immunoprecipitation in HEK cells co-transfected with serine 845 (S845) mutants of GluR1 and HA-tagged Nedd4-1 in order to ascertain the relationship between Nedd4-1 binding to AMPARs and phosphorylation of AMPARs. If DHPG mimics glutamate and glycine, this could be the mechanism by which glutamate drives Nedd4-1 redistribution and possibly internalization of AMPARs. We also expect to see that S845A, the phospho-dead mutant, shows an increase in binding with Nedd4-1 and that S845D, the phosphomimetic mutant, shows a decrease. This data may show a relationship in which, the increase of phosphorylation of S845 leads to the decrease of Nedd4-1 bindings to S845 and vice versa. Together, these results will uncover some of the mechanisms driving synaptic plasticity and possibly some neurological disease pathologies.

Methods and Materials

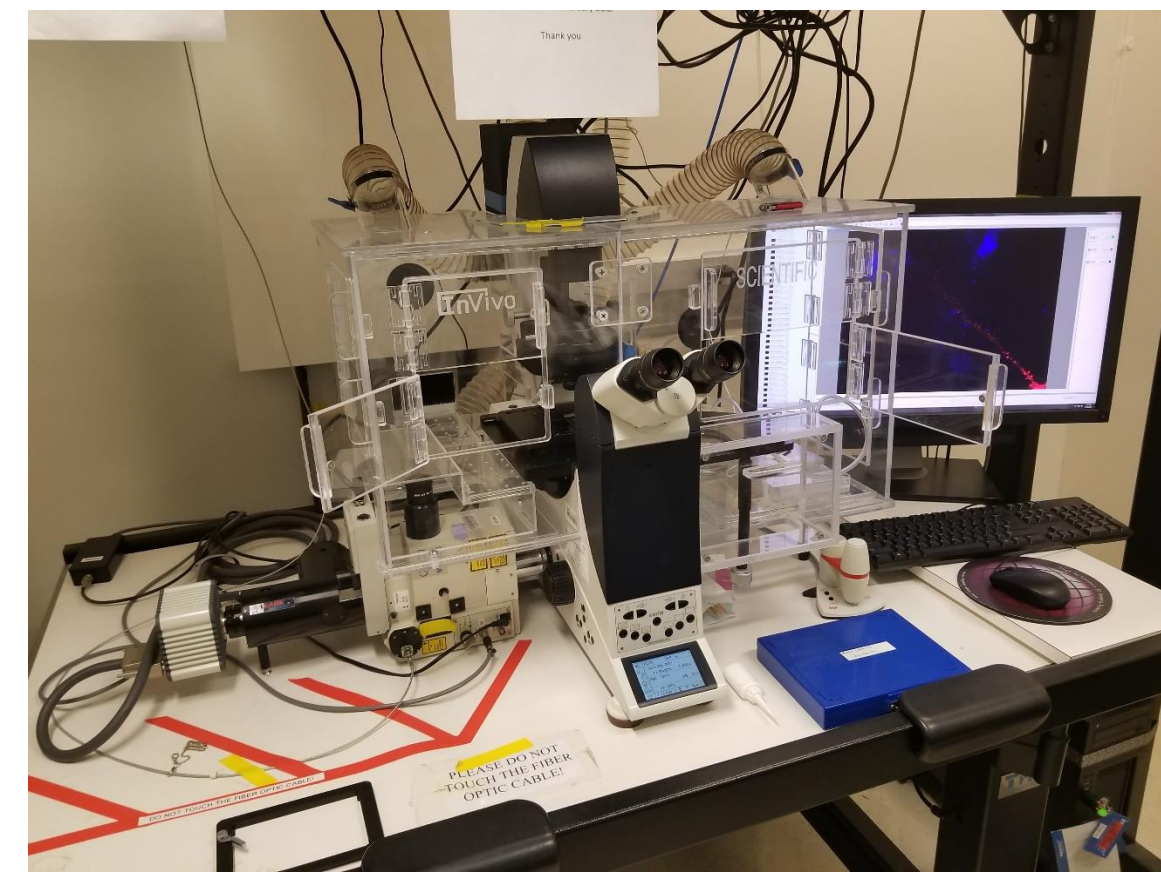


Fig 1 The confocal microscope used in the Immunostaining lab for imaging

In the immunostaining lab, 12 wells (each 10 cm) were used. The solutions consisted of 4 wells of 10 μ L glutamate mixed with 5 μ L of glycine and 35 μ L of media, 4 wells of 2.5 μ L of DHPG mixed with 47.5 μ L of media, and 2.5 μ L of DMSO (Control) with 47.5 μ L of media. In order to preserve the cell, 4% paraformaldehyde was used for 10 minutes. In addition, 2% BSA in PBS-MC and 0.25% Triton X were used to block and permeabilize the cell. GFP rabbit, MAP2 chicken and HA mouse primary antibodies would then be added at 1:1000 in 300 μ L of 2% BSA and incubated at 4 $^{\circ}$ C. The 300 μ L secondary antibody would later be added in 2% BSA at a 1:1000 dilution and incubate at room temperature. After rinsing with PBS-MC, the slides would be able to be mounted and imaged.



Fig 2. Western Blot filled with solution

The solutions in this experiment included S845A, S845D, Wild-Type, IgG, control and mCherry. This experiment was testing S845a(phospho-dead) and S845D (phosphomimetic), the controls include Wild-Type(S845), IgG(antibody control), control(contained all the HEK cell's natural proteins) and mCherry(used in order to control Nedd4-1 expression). First, we transfected the cells with rat AMPA DNA using the mutants: S845A, S845D and Wild-Type. The PEI was used to permeabilize the cells so that the DNA can enter the cell. Then, the cells were lysed so that all the proteins would be released (HEK cells). Then, 10% of the total volume for Total Lysate was removed to serve as a control to the second primary antibody. The 90% of the cells were kept at -20 $^{\circ}$ C overnight. 2 solutions were made, one to dilute the antibody and one to dilute the normal serum for IgG control(the solution was diluted due to the cost of primary antibody). 1 μ L of antibody and normal serum diluted 1:10 in lysis buffer. Then, we added 10 μ L of diluted antibody mix to each sample and IgG control serum to the IgG tube. The primary antibodies and proteins then incubated overnight. After, we prepared Protein A agarose- 25 μ L per sample into 1.5mL tube- and we centrifuged the solution at 6000 rpm for 2 minutes. The agarose beads were washed in 200 μ L of lysis buffer. Then, we added 200 μ L of rinsed Protein A agarose in lysis buffer to each sample. After, the beads were then left to incubate with the primary antibody. Later, 50 μ L of Sample Buffer was added to each tube of agarose in order to give a negative charge to each sample.

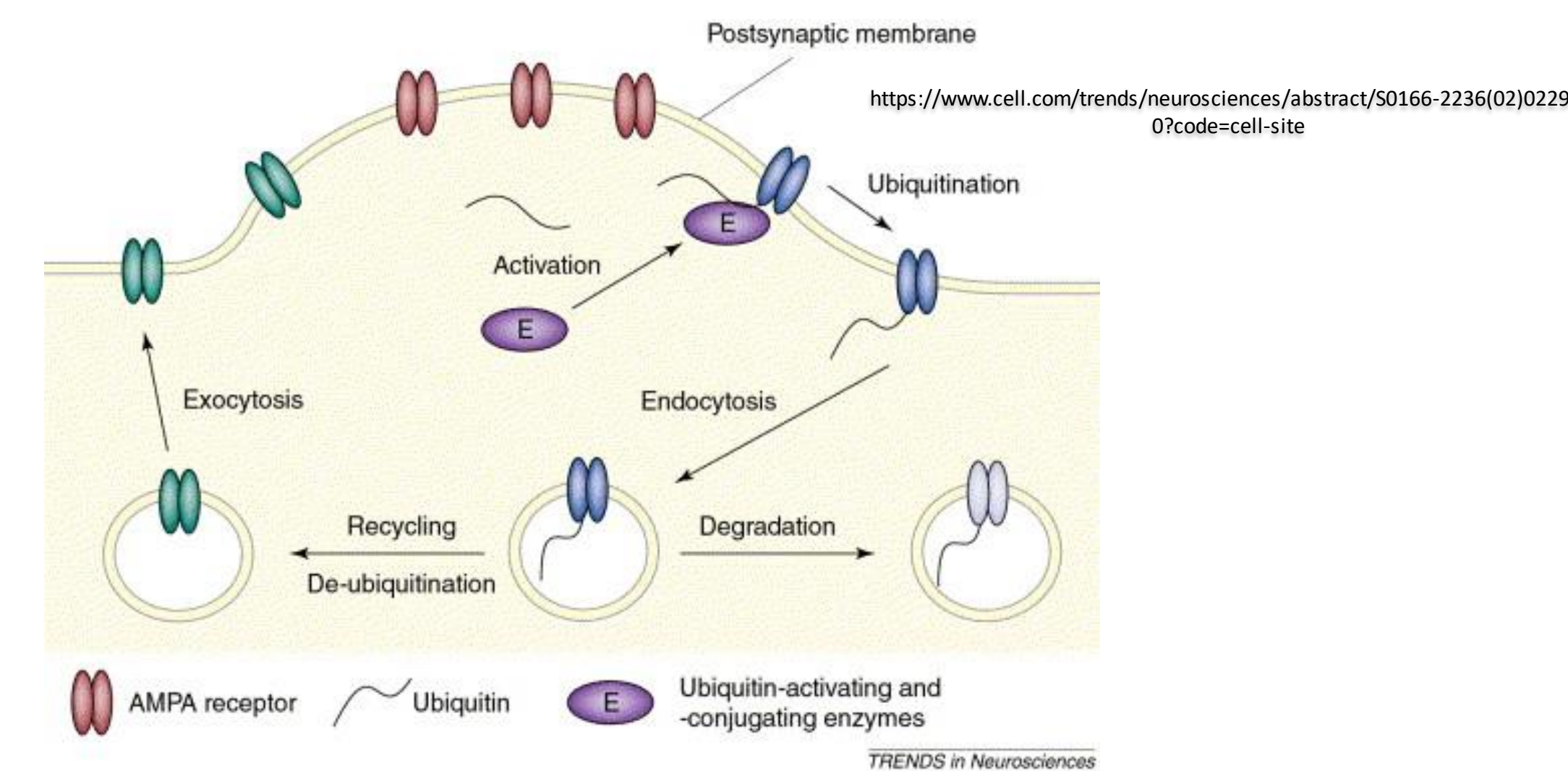


Fig 3. Ubiquitination process in AMPA receptors

In order to image the solutions an 8% gel was made. The gel was made of stacking gel on top of the running gel. The negative electrode was on top and positive electrode was at the bottom in order to pull the solutions downwards. The proteins in the gel were later transferred to a blot paper. The blot paper was incubated with our second primary antibody and our secondary antibody and then imaged.

Results for Immunostaining and Immunoprecipitation

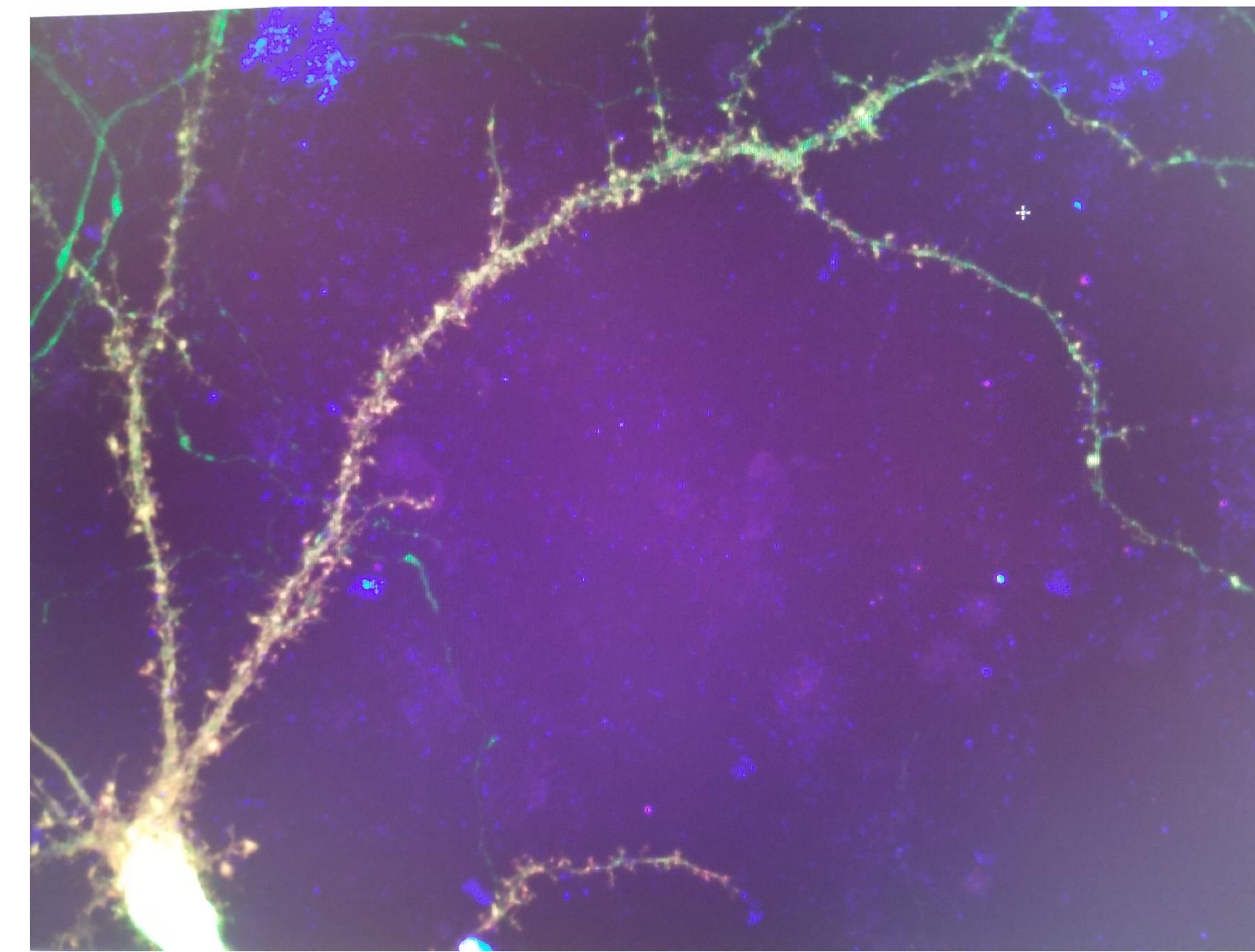


Fig 4. Neuron with stained HA-tag attached to Nedd4-1

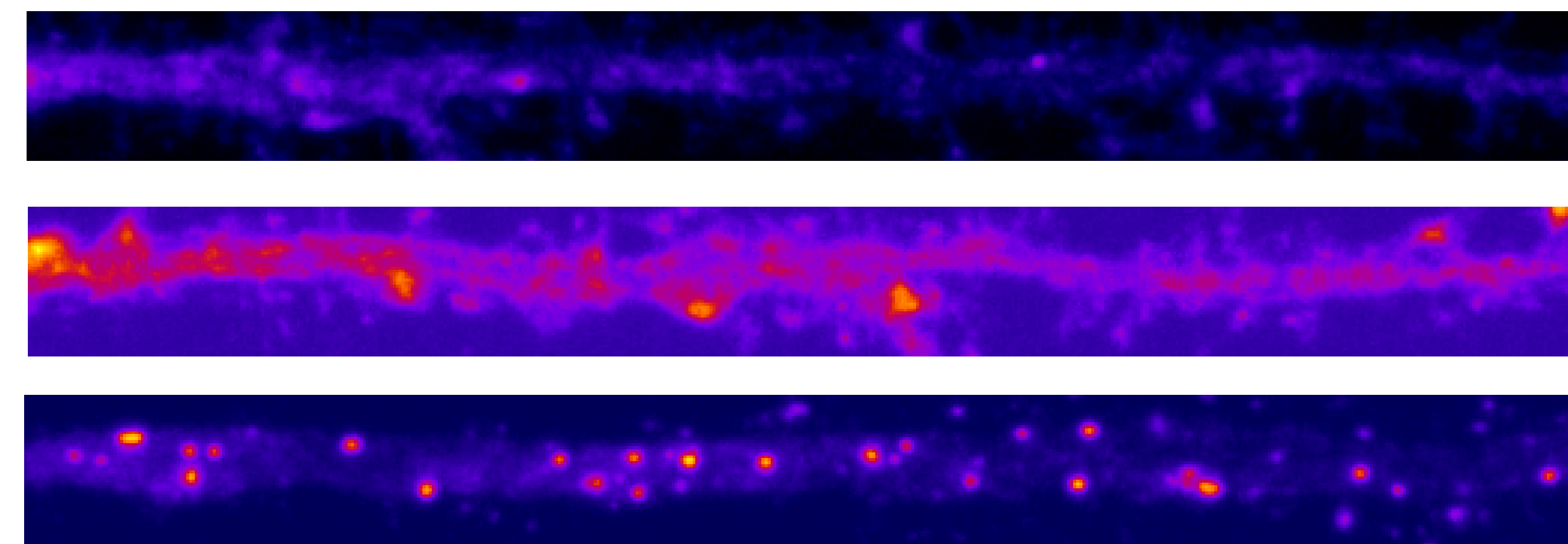


Fig 5. Single Dendrites treated with (top to bottom): DMSO, DHPG, and Glutamate with Glycine

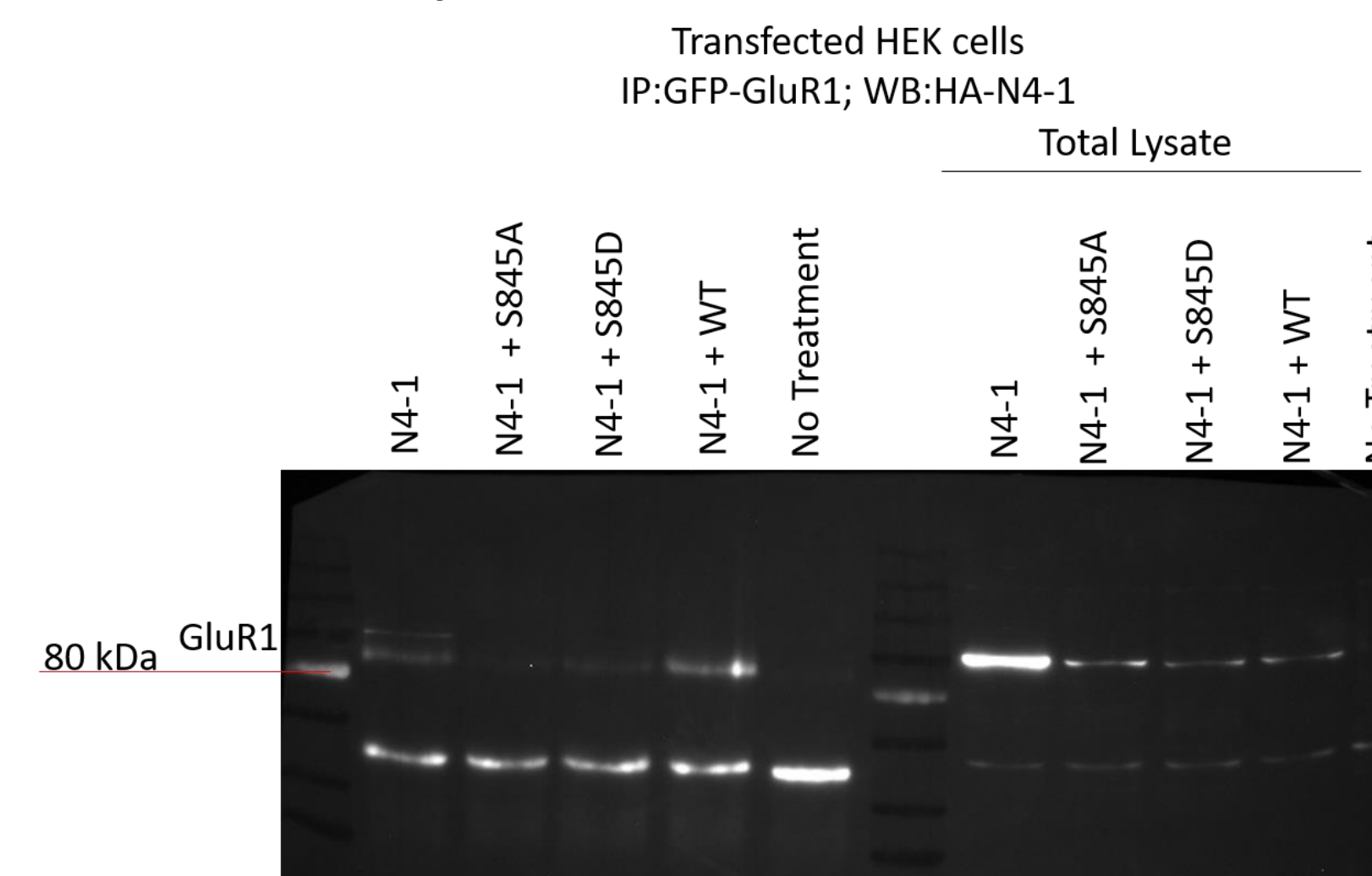


Fig 6 Western Blot Results

Nedd4-1 Redistribution Analysis

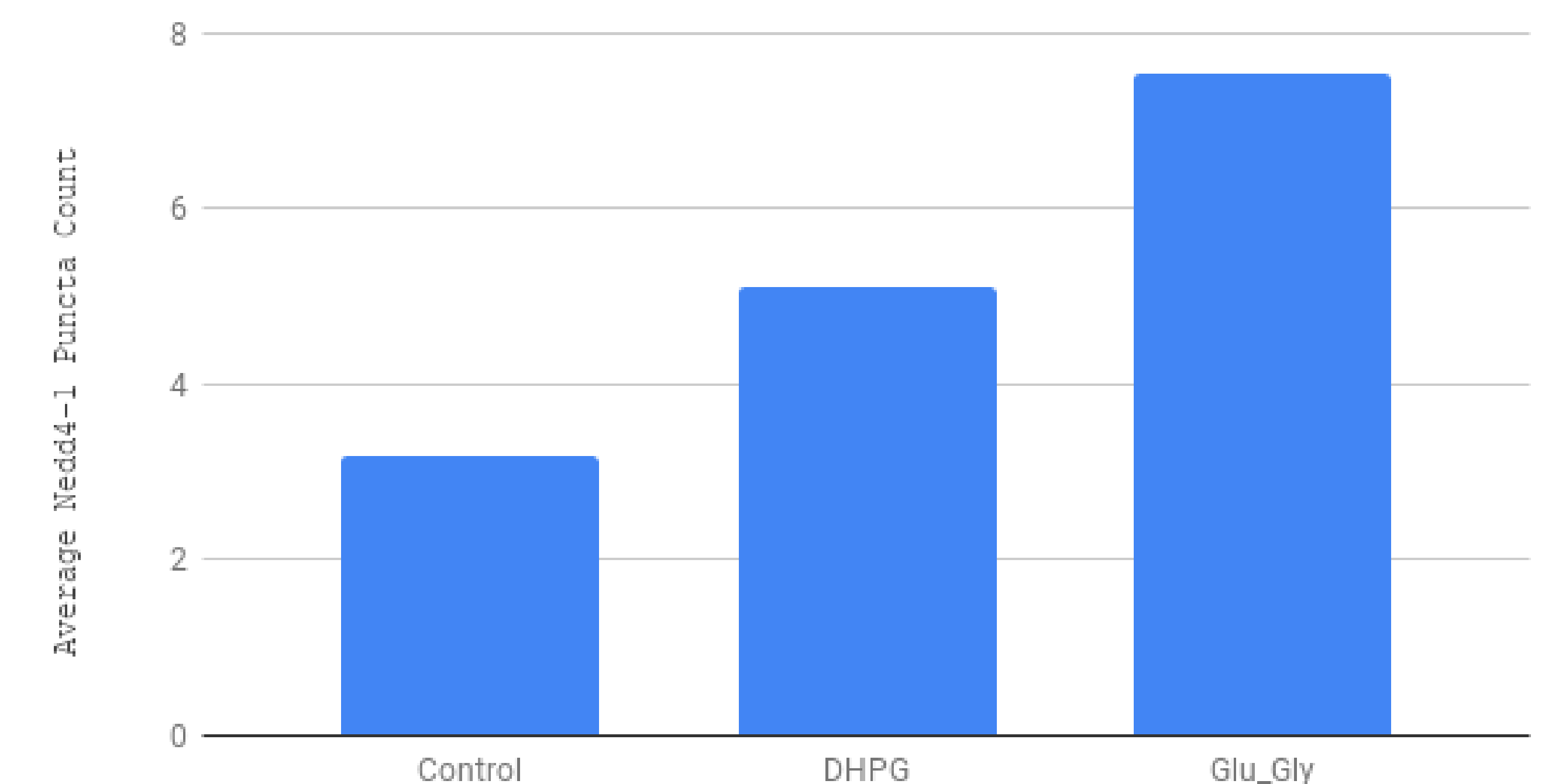


Fig 7 Average Nedd4-1 Puncta Count in a Dendrite

Conclusions and Analysis

According to the immunostaining experiment data, the average puncta count in DHPG is shown to be greater than that of DMSO, but the average puncta count of the glutamate and glycine mixture is greater than that of DHPG. One possible explanation that DHPG showed less puncta in the dendrite could be because there was less glutamate receptors to bind to. DHPG is known to only bind to receptors mGluR1 and mGluR5 while glutamate binds to mGluR1-5, AMPARs, and NMDARs. One factor that may have affected the data could be not giving the DHPG as much time as the glutamate and glycine mixture to bind to mGluR1. Another factor could be since the small sample size which is due to the inefficiency of the virus and the poor cell health. Unfortunately, the immunoprecipitation experiment data looks inconclusive. During the isolation of the AMPAR process, the GFP tags should have only tagged the AMPAR. Instead, since the Nedd4-1 band is visible in our Nedd4-1 alone condition, the GFP tag has failed. In another experiment, only the antibody band was visible. This could be due to not letting the antibody bind to the AMPA receptors overnight. To avoid future errors for the immunoprecipitation experiment, there should be less chances of cross-contamination of DNA or serums, a more sterile technique and giving the constructs more time to express and test the experiments at each time to see where it went wrong.

References

Scudder, S. L., Goo, M. S., Cartier, A. E., Molteni, A., Schwarz, L. A., Wright, R., & Patrick, G. N. (2014, December 10). *Synaptic Strength Is Bidirectionally Controlled by Opposing Activity-Dependent Regulation of Nedd4-1 and USP8*. Retrieved From <http://www.jneurosci.org/content/34/50/16637/tab-figures-data>

Widagdo, J., Jang, S. E., Guntupalli, S., & Anggono, V. (2017, October 12). *Regulation of AMPA Receptor Trafficking by Protein Ubiquitination*. Retrieved July 25, 2018, from <https://www.frontiersin.org/articles/10.3389/fnmol.2017.00347/full>

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