Spring 5-13-2017

Exploring the Role of Dynclli1 in the Trafficking of GLUA2 Containing AMPA Receptors

Leah Esposito
Carroll College, Helena, MT

Follow this and additional works at: https://scholars.carroll.edu/lifesci_theses
Part of the Molecular and Cellular Neuroscience Commons

Recommended Citation
Esposito, Leah, "Exploring the Role of Dynclli1 in the Trafficking of GLUA2 Containing AMPA Receptors" (2017). Life and Environmental Sciences Undergraduate Theses. 5.
https://scholars.carroll.edu/lifesci_theses/5

This Thesis is brought to you for free and open access by the Life and Environmental Sciences at Carroll Scholars. It has been accepted for inclusion in Life and Environmental Sciences Undergraduate Theses by an authorized administrator of Carroll Scholars. For more information, please contact tkratz@carroll.edu.
Exploring the Role of Dyn1li1 in the Trafficking of GLUA2 Containing AMPA Receptors

Honors Thesis

Department of Life and Environmental Science

Helena, Montana

Leah M.J. Esposito

4-28-17
SIGNATURE PAGE

This thesis for honors recognition has been approved for the
Department of **Life & Environmental Sciences**.

[Signature]
Director
5.1.2017
Date

[Signature]
Reader
4/26/17
Date

[Signature]
Reader
4/26/17
Date
Acknowledgements

I would like to thank my mentor Stefanie Otto-Hitt for the unending support and expertise she has given me throughout my time on this project. I would also like to thank my lab partners Mark Barnett and Natalie Oberding for their mentoring and assistance. Finally I would like to thank Dr. Gerald Shields and my readers, Dr. Brandon Sheafor and Dr. Jessica McManus, for providing constructive criticism and insight. This research was supported by grant funds provided by the MJ Murdock Charitable Trust.

Abstract

AMPA receptors are glutamate receptors that are found in the post-synaptic region of a neuron. They are one of the main receptors that are responsible for excitatory signaling in the central nervous system. The trafficking of these receptors is an area of knowledge that is not well developed, but is essential to a complete understanding of these receptor proteins. My research was focused on exploring the role that Dynclil1 has in the trafficking of GluA2-containing AMPARs. In order to investigate this role, the gene encoding Dynclil1 was amplified via PCR, cloned into a mammalian expression vector and transfected into HEK293 cells and NT2 neurons. Live labeling studies and Co-immunoprecipitation experiments were performed to determine association with GluA2 and surface expression. Immunostaining experiments were performed to view Dynclil1 localization. Although the quantifiable results were statistically insignificant, further experimentation is needed. In conclusion, the role that Dynclil1 plays in the trafficking of GluA2-containing AMPARs remains unclear.
Intro

Neurons and glia are the principle cells that make up the nervous system of every vertebrate. Neurons are responsible for communication between the brain and the rest of the body by way of electrical and chemical signaling. An electrical signal is created when positive ions outside of the cell make their way into the cell by way of proteins called ligand gated ion channels. The strength of the electrical signal, often called synaptic plasticity, is dependent upon the number of receptors that are available to bind to a neurotransmitter (Turrigianno 2008). The ability of neurons to control the strength of their connectivity is the basis of adaptive behaviors and memory formation (Burke and Barnes 2006).

Glutamatergic neurotransmission is responsible for most of the excitatory signaling that occurs throughout the human nervous system (Esteban 2008). One of the most prominent glutamate receptors in the central nervous system is the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid) receptor (AMPAR) (Chater and Goda 2014). AMPARs are part of a class of receptors known as iGluRs (ionotropic glutamate receptor) (Chater and Goda 2014), meaning that with its ligand, glutamate bound AMPARs act as ion channels (Chater and Goda 2014). Four protein subunits, namely GLUA1-4, combine in a dimer of dimers formation to generate hetero-tetrameric AMPARs (Hollman and Heinemann 1994). GLUA2 is of interest because it is the most abundant subunit within the differing types of AMPA receptors (Esteban 2008), and because receptors containing this subunit are impermeable to Ca\(^{2+}\) ions while receptors lacking it are Ca\(^{2+}\) permeable (Greger et al. 2003).
The insertion and extraction of AMPARs to and from the plasma membrane of a neuron is regulated by neuronal activity (Anggono and Huganir 2012). A change in the number and makeup of AMPARS mediates LTP (Long-Term Potentiation) and LTD (Long-Term Depression) (Malenka and Bear 2004). The general steps of trafficking AMPARs are the exocytosis of AMPARs at the dendrites, the anchoring of AMPARs to the post-synaptic membrane and the endocytosis of AMPARs for degradation or recycling (Haering et al. 2014). Transmembrane AMPA receptor regulatory proteins (TARPs) are a major class of protein that are known to be involved in the maturation and trafficking of AMPARs (Tomita et al. 2004). Stargazin is a member of the TARPs family that was discovered in a mutant mouse lacking functional AMPARs (Esteban 2008). It has been well characterized and is very specific to AMPARs and no other iGluRs (Chen et al. 2003). Along with TARPs, other proteins play roles in the trafficking of AMPA subunits including proteins that bind to the PDZ domain of the C-terminus (GRIP and PICK1) (Henley and Wilkinson, 2013), chaperone proteins, and anchoring proteins (N-cadherin) (Henley and Wilkinson, 2013). Despite the classification of these AMPAR-interacting proteins, there is still little known about the complex array of proteins that are involved in the trafficking of AMPARs (Henley and Wilkinson 2013).

The protein complex Dynein is of interest because it may be involved in the trafficking of AMPARs to the synapse (Shanks et al. 2012). Dynein is a large motor protein made up of many subunits that are responsible for trafficking proteins along the microtubules of the cytoskeleton (Banks et al. 2011). The delivery of AMPA receptors to synapses requires Dynein-dependent or Kinesin-dependent transport.
of AMPAR vesicles (Correia et. al. 2008). One of the subunits that makes up the Dynein complex is Dynein light-intermediate chain 1 (Dync1li1) (Banks et al. 2011), which binds to cargos including Pericentrin and Na+ channels (Tynan et al. 2000). A mutant mouse with a point mutation in the Dync1li1 gene showed increased anxiety and altered neuronal development, indicating that the protein is involved in neurological function in some way (Banks et al. 2011). Dync1li1 also interacts with GLUA2 (Shanks et al. 2012).

Researching the role that Dync1li1 plays in the trafficking of AMPA receptors would further the scientific community’s understanding of this complicated process. Furthermore, discoveries that shed light on these processes could result in the advancement of medical treatment for degenerative neurological diseases. For my research project, I hypothesized that Dync1li1 plays a role in the trafficking of AMPARs at the neuronal synapse. I investigated this hypothesis through (1) co-immunoprecipitation assays characterize the interaction between GluA2 and Dync1li1, (2) live labeling and immunostaining analyses to determine surface expression and localization of Dync1li1 and GluA2 within cultured neurons, and (3) RNAi knockdown of Dync1li1 in Caenorhabditis elegans to analyze its effect on AMPAR-mediated behaviors.

**Materials and Methods**

**Cell Culturing and Maintenance**

Maintenance of cell cultures provided cells for experimentation. Human embryonic kidney 293 (HEK293) cells and undifferentiated human NT2 cells were
maintained throughout the research. Both cell types were removed from liquid nitrogen and grown on 10cm Corning cell culturing dishes in their respective media. Cell culturing dishes were kept in an incubator at 37°C and 5% CO₂.

HEK 293 cells were grown in 12 ml of growth media consisting of Eagles Minimum Essential Medium (EMEM) and 10% Fetal Bovine Serum. The cells were allowed to divide with regular media changes until they reached 90% confluency on the culturing dishes. The media was then suctioned off and replaced with 2 ml of trypsin. The cells, in trypsin, were placed in the incubator for 5 minutes, and 10 ml of growth media were added. The cells were then mechanically dislodged by pipetting. Three milliliters of solution were then added to 9 ml of fresh media in a separate dish.

NT2 cells were grown in 12 ml of DMEM/F12 media supplemented with 10% FBS and 1% Penicillin/Streptomysin. These cells were allowed to divide with regular media changes until they reached 90% confluency. The media was then suctioned off and replaced and the cells were mechanically dislodged with a sterile scraper and pipetting. Three milliliters of this solution was then added to 9 ml of fresh media in a separate dish.

The NT2 differentiation to neurons was performed using methodology outlined in Cheung et al. (1999) and Tengage et al (2011). Five petri dishes of undifferentiated NT2 cells were cultured in NT2 media that was supplemented with retinoic acid (1:1000). Every other day the cells were collected in two 50 ml conical vials and pelleted in order to change the media. The cells would then be redistributed to five new petri dishes. After two weeks, the neurons were plated on
12 well dishes at a 1:2 dilution for immunostaining and 1:1 for Western blotting. Before the cells were plated, coverslips were placed in one of the 12 well dishes for future immunostaining. The dishes were allowed to coat in a solution containing 8.8 ml of PBS, 1 ml of 0.1% gelatin, 100 µl of PDL (polyDlysine, Invitorgen), and 100 µl of Laminin (Invitrogen) overnight. Regular media changes were performed every other day using Neuron media. This media consisted of NT2 media, retinoic acid at 1:1000 dilution, cytosine arabinofuranoside (AraC; Sigma) at a 1:10,000 dilution, fluorodeoxyuridine (FUdR; Sigma) at 1:1,000 dilution, and Uradine (Urd; Sigma) at 1:1,000 dilution. After two weeks the neurons were mature enough for experimentation.

**Cloning Dynclli1 and Preparation of Constructs**

Preparation of constructs was required in order for transfection and translation of the Dynclli1 gene to occur in living mammalian cells. *Escherichia coli* cultures that had been previously transformed with a plasmid containing Dynclli1 were ordered from OpenBio Systems. These *E. coli* were spread on Lysogeny Broth (LB) plates containing a 1:1000 dilution of kanamycin and were allowed to grow overnight at 37°C. The next day, one colony was selected and grown in LB containing kanamycin at 37°C and 200rpm of shaking. The following day, this culture was used to prepare a DNA miniprep using the QUIAGEN miniprep kit. The DNA concentration was then found using spectrophotometry.

Primers (Figure 1) were designed specifically for the Dynclli1 gene region of the plasmid:
PCR was then performed to isolate the coding region of Dync1li1 (Table 1 and Figure 2):

Table 1. Reagents for PCR of Dync1li1

<table>
<thead>
<tr>
<th>Reagent</th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dync1li1 DNA template</td>
<td>0.7 (500 ng)</td>
</tr>
<tr>
<td>10x PCR Buffer (Invitrogen)</td>
<td>5</td>
</tr>
<tr>
<td>50 µM DNTPs (Invitrogen)</td>
<td>1</td>
</tr>
<tr>
<td>Forward primer (IDT)</td>
<td>1</td>
</tr>
<tr>
<td>Reverse Primer (IDT)</td>
<td>1</td>
</tr>
<tr>
<td>50 µM MgSO₄ (Invitrogen)</td>
<td>2</td>
</tr>
<tr>
<td>Nuclease Free H₂O (BioRad)</td>
<td>40.3</td>
</tr>
</tbody>
</table>
After completion of the PCR, gel electrophoresis was used to check for amplification of Dyn1li1. A 1 Kb ladder was used as a standard on a 1% agarose gel infused with one microliter of SYBR safe fluorescent stain. Fifteen microliters of the PCR reaction were loaded into the gel alongside 7µl of ladder. The gel was run at 100V until completion. After conformation of amplification, PCR purification was performed using a QUIAGEN PCR purification kit.

A restriction digest (Table 2) was then performed on the original plasmid containing Dyn1li1 and a vector digest was performed on the mammalian expression vector pcDNA3.1:

**Table 2.** Reagents for restriction digest of Dyn1li1 and pcDNA3.1 vector

<table>
<thead>
<tr>
<th>Reagent</th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x buffer B3 (New England Bio Systems)</td>
<td>3</td>
</tr>
<tr>
<td>10x BSA (New England Bio Systems)</td>
<td>3</td>
</tr>
<tr>
<td>Purified Dyn1li1 DNA (for restriction digest) / pcDNA 3.1 vector DNA (for vector digest)</td>
<td>10</td>
</tr>
<tr>
<td>REI restriction enzyme (New England Bio</td>
<td>1</td>
</tr>
</tbody>
</table>
After setup and addition of all of the reagents to separate PCR tubes, the reactions were incubated at 37°C for two hours. One microliter of CIP (Calf-intestinal Alkaline Phosphatase) was then added to the vector digest to prevent re-ligation. The reactions were incubated for another hour.

A ligation reaction (Table 3) was then run using the two restriction digests:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₄ DNA ligase 10x buffer (New England Bio Systems)</td>
<td>1</td>
</tr>
<tr>
<td>T₄ DNA ligase (New England Bio Systems)</td>
<td>0.5</td>
</tr>
<tr>
<td>pcDNA 3.1 restriction digest</td>
<td>2</td>
</tr>
<tr>
<td>Dynclli1 restriction digest</td>
<td>5</td>
</tr>
<tr>
<td>Nuclease free H₂O (BioRad)</td>
<td>1.5</td>
</tr>
</tbody>
</table>

After addition of reagents to PCR tubes, the reaction was incubated at 16°C overnight.

A transformation was then performed using TOP 10 competent cells (Invitrogen). Two microliters of ligation reaction were added to thawed aliquots of cells. The cells were allowed to incubate on ice for 30 minutes and then placed in a
42°C water bath for 30 seconds for heat shock treatment, after which they were immediately transferred to ice. Super Optimal broth with Catabolite repression (SOC) (250 µl) (Invitrogen) was added to the cells and they were allowed to incubate at 37°C while shaking at 200 rpm for one hour. The cell solution (100 µl) was then added to pre-warmed LB plates infused with ampicillin. After overnight incubation at 37°C, fifteen colonies were selected and grown in 5ml of LB broth and 5 µl of ampicillin. These cultures were incubated overnight at 37°C while shaking at a rate of 200 rpm.

Fifteen DNA Minipreps were made from each of these cultures and diagnostic digests (Table 4) were run on all fifteen minipreps to check for success of the ligation:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x buffer 3 (New England Bio Systems)</td>
<td>1</td>
</tr>
<tr>
<td>10x BSA (New England Bio Systems)</td>
<td>1</td>
</tr>
<tr>
<td>XBA I restriction enzyme (New England Bio Systems)</td>
<td>0.2</td>
</tr>
<tr>
<td>NOT I restriction enzyme (New England Bio Systems)</td>
<td>0.2</td>
</tr>
<tr>
<td>Nuclease free H_{2}O (BioRad)</td>
<td>2.6</td>
</tr>
<tr>
<td>Miniprep DNA</td>
<td>5</td>
</tr>
</tbody>
</table>

The reactions were incubated at 37°C for one hour in a thermocycler. After incubation, 2 µl of orange G loading dye were added to each reaction. The digests
were then run on a 1% agarose gel infused with SYBR safe stain next to a 1 KB plus ladder at 100V until completion. All minipreps that showed successful ligation were stored at 4°C. DNA concentrations were measured using spectroscopy.

**Transfection of HEK293 Cells and Western Blotting**

Transfection was required in order for cells to express target proteins at elevated levels. HEK 293 cells were transfected using a PEI protocol that was developed by Dr. Megan Williams of the University of Utah. One milliliter of a 0.2% gelatin solution was added to each well in a 12 well cell-culturing dish. The gelatin was allowed to coat the wells at room temperature for 15 minutes. The gelatin was then removed by suction and the plate was allowed to air dry for another 15 minutes. HEK 293 cells were split into the 12 well dish. The following day the media was changed one hour before the transfection was performed. For each DNA transfection, a solution of 50 µl of Opti-MEM and 5 µl PEI (Polyethylenimine) was made in an Eppendorf tube. DNA (500ng) was then added and the tubes were vortexed and spun down for a five to ten seconds. They were allowed to incubate at room temperature for 10 minutes. The transfectant (50 µl) was added to one of the 12 wells of HEK 293 cells. The cells were incubated at 37°C and 5% CO₂ for 6 hours. The media was then suctioned off and replaced with fresh HEK 293 media. The cells were then left for two days at 37°C and 5% CO₂ to allow for an increased amount of the Dync1li1 protein to be translated.

Sample buffer consisting of 5% β- mercaptoethanol and 95% Laemmli sample buffer was made and boiled for 3 min. The media was then pipetted off of
the transfected cells and replaced with 100 μl of sample buffer per well. The cells were then scraped and mixed into the sample buffer to create a thick lysate. The lysate was then pipetted into an Eppendorf tube and boiled for 5 minutes after which 30 μl of it was loaded into a mini-PROTEAN® TGX™ gel for polyacrylamide gel electrophoresis. A precision Plus ladder (15 μl) was used as a standard. The gel was run in 1x Tris/Glycine/SDS buffer at 125 V until the ladder reached the bottom of the gel. The protein on the gel was then transferred to nitrocellulose paper using a filter paper and sponge sandwich run in a buffer consisting of 10x Tris/Glycine buffer, 100 mL pure methanol, and 800 mL deionized water at 90 V for one hour.

The nitrocellulose paper was then allowed to sit in blocking solution consisting of two grams of blotting-grade blocker, and 100 mL of a 0.01% Tween20/1x TBS solution for half an hour. The primary antibody, mouse anti-HA (Pierce Biotechnology), was then added at a 1:10000 dilution and the membrane was allowed to incubate overnight at 4°C on a rocker table. The following day, the membrane was washed in 0.01% Tween20/1x TBS three times at 10 minutes each. Blocking solution and the secondary HRP goat anti-mouse antibody (BioRad), at a 1:10000 dilution, were then added to the membrane. It was allowed to rock at room temperature for one hour. The membrane was then washed three times in 0.01% Tween20/1x TBS for 10 minutes each and washed once in 1x TBS for 10 minutes. Opti-4CN diluent solution consisting of 1 part Opti-4CN diluent, 9 parts DI H₂O and 0.2 parts Opti-4CN substrate was then added to the membrane. This allowed the visualization of the HA-tagged proteins on the membrane after about 10 minutes of rocking at room temperature.
Co-Immunoprecipiation Assay

HEK 293 cells were split into a six well dish that had been coated with a 0.2% gelatin solution. Each well was co-transfected with a GLUA2 construct and either Dyncl1li1, Stargazin, or NPTXR. Stargazin acted as the positive control being a known interactor with GLUA2. NPTXR acted as the negative control being known not to interact with GLUA2. The cells were transfected twice using the PEI transfection method discussed above. The two transfections were performed one day apart.

The lysis buffer was prepared by adding 10 µl of Protease Cocktail Inhibitor to each ml of Pierce IP lysis buffer needed. The entire Co-IP experiment was conducted in a 4°C walk-in refrigerator. On a flat bed of ice, the media was taken off of the transfected cells and replaced with 300 µl/well of lysis buffer. The cells incubated on a rocker for five minutes. The lysates were then collected in Eppendorf tubes and spun in a centrifuge for ten minutes at 11495 rpm. The supernatant was then collected and added to the prepared beads.

The SureBead preparation was performed during the 10-minute lysate spin. The beads were thoroughly resuspended in their solution and 100 µl of the bead solution was pipetted into one 1.5ml Eppendorf tube for every CO-IP reaction. The beads were then magnetized and the supernatant was discarded. The beads were washed with 1 ml of 0.1% Tween20/PBS three times. Mouse anti HA (Pierce Biotechnology), (5µl) and 200 µl of 0.1%Tween20/PBS were added to the beads. They were allowed to incubate at 4°C in a rotator. The beads were then magnetized
and the supernatant was discarded. The beads were washed with 1 ml 0.1% Tween20/PBS three times.

After the final wash, 500 µl of the cell lysate was added to the beads. The beads were allowed to rotate overnight at 4°C. The following day, the tubes were spun down for five to ten seconds and magnetized. They were washed three times with 1000 µl 0.1% Tween20/PBS and transferred to a new tube. The beads were magnetized and the supernatant was removed and replaced with 40 µl of sample buffer. The tubes were incubated in the sample buffer at 70°C for 10 minutes after which the beads were magnetized and the supernatant was collected and used in a Western blot. The primary antibody used in the Western blot for the Co-IPs was rabbit anti-GFP (Invitrogen) to probe for the visualization of the GFP tagged GLUA2 construct.

**Live Labeling of HEK293 cells**

HEK 293 cells were split into a 12 well dish that contained gelatin-coated coverslips in each well. The cells were transfected with GLUA1 and GLUA2 constructs in order for AMPA receptor complexes to be formed. The cells were then transfected with Dynclli1, Stargazin, or NPTXR constructs. These transfections were performed a day apart.

The media was then removed from the cells and was replaced with rabbit anti-GFP antibody (Invitrogen) diluted at 1:500 in Live Labeling buffer, which consisted of 10 ml EMEM, 500µl of 0.5 M HEPES, and 0.05g BSA. The cells were placed on ice and allowed to incubate in the buffer for 50 minutes at 4°C on a rocker
The remainder of the experiment was carried out at room temperature on the rocking table. The cells were washed three times with LL buffer for 5 minutes each. They were then fixed in a 4% (paraformaldehyde) PFA solution for 15 minutes. Three five minute washes with PBS were then performed. Blocking buffer consisting of 50ml PBS, 1.5g BSA, and 500µl TritonX-100, was added to each well and the cells were incubated for 20 minutes. Mouse anti HA antibody, diluted at 1:1000 in blocking buffer, was added to the cells. They were allowed to block in this solution for two hours.

The cells were then washed three times at five minutes each with blocking buffer. The secondary antibodies, 488 nm fluorescing goat anti-rabbit and 543 nm fluorescing goat anti-mouse (Life Technologies), were added to the cells at a dilution of 1:1000 in blocking buffer. This solution was allowed to incubate for one hour in the dark. The cells were then washed twice with blocking buffer for five minutes each and then once with a solution containing PBS and Hoechst stain at a concentration of 10 µl/ml PBS for five minutes. The coverslips were then removed from the 12 well dish and mounted on slides using Fluoromount-G. The slides were allowed to dry in the dark overnight before visualizing.

The cells were visualized using a Nikon OptiPhot 200 fluorescence microscope. Fluorescence levels on the images were obtained using ImageJ software. These levels were then divided by the cell area, resulting in a percent surface expression. A mean percent surface area was taken for each group along with standard error. A two sample t-test was performed as well in order to check for significant variances between different groups.
Transfection of NT2 Neurons

NT2 neurons that had been cultured using the protocol mentioned above were used in this experiment. For each DNA transfection, 125 µl of Opti-MEM medium was mixed with 10 µg of DNA and 6 µl of Liptofectamine 2000 reagent (Invitrogen). These mixtures were allowed to incubate for 5 minutes after which they were added to the cells. The cells were allowed to incubate for 2 days before further experimentation.

Immunostaining of NT2 neurons

NT2 neurons that had been cultured using the protocol mentioned above, were used in this experiment. This entire experiment was performed at room temperature on a table rocker. The media was pipetted off of the mature neurons and replaced with a 4% PFA solution. The cells were allowed to incubate for 15 minutes, after which three five-minute washes with PBS were performed. Blocking buffer consisting 50ml PBS, 1.5g BSA, and 500µl TritonX-100, was added to each well and the cells were incubated for 20 minutes. Mouse anti HA antibody (Pierce Biotechnology) diluted at 1:1000 in blocking buffer was added to the cells. Primary antibodies chick anti-synapsin, chick anti-Tau, and chick anti-Map2 (Abcam) were added in order to visualize synapses, axons and dendrites, respectively. They were allowed to block in this solution for two hours.

The cells were washed three times at five minutes each with blocking buffer. The secondary antibodies, 488 nm fluorescing goat anti-mouse and 543 nm
fluorescing goat anti-chick (Life Technologies), were added to the cells at a dilution of 1:1000 in blocking buffer. This solution was allowed to incubate for one hour in the dark. The cells were then washed twice with blocking buffer for five minutes each and then once with a solution containing PBS and Hoechst stain at a concentration of 10 µl/ml PBS for five minutes. The coverslips were then removed from the 12 well dish and mounted on slides using Fluoromount-G. The slides were allowed to dry in the dark overnight before visualizing.

**Culturing of C. elegans**

*C. elegans* were cultured using the methodology of Eisenmann (2005). Nematode Growth Medium was prepared in order to make agar plates for worm growth. A mixture of 3g NaCl, 17g Agar, 2.5g peptone and 975ml of RO H2O was autoclaved. After the solution was cool, 1ml of 5mg/ml cholesterol in ethanol, 1 ml of 1 M MgSO4, and 25 ml of 1M KPO4 were added. The solution was then poured into petri dishes and allowed to solidify. These plates were stored at room temperature.

Bacterial growth media was then made to culture *E. coli* (OP50 strain) as a food source for the worms. The growth media consisted of 2.5 g Bacto-tryptone, 1.25g Bacto-yeast, 1.25g NaCl and enough RO H2O to bring the total volume to 200 ml. The pH of the solution was then adjusted to 7 using 1M NaOH, and autoclaved. A single colony of *E. coli* OP50 from a previously streaked plate was used to aseptically inoculate the broth. The liquid culture was stored at 4°C.

The OP50 (100 µl) liquid culture was pipetted onto the center of an NGM plate using sterile technique. The culture was spread using a sterile glass rod and
the plate was allowed to incubate at room temperature overnight. The worms were then thawed from their storage at -80°C on an NGM plate spread with OP50 *E. coli*. The worms were allowed to grow and multiply for a week in a 37°C incubator.

The worms were transferred to new plates once a week using the chunking method, which involves cutting a chunk of agar about two centimeters square in area out of an old plate and placing it on a new agar plate. A dissection microscope was used to visualize the worm field. A section of the plate that was well covered with worms was selected and a square piece of agar was cut out of that area using a sterile scalpel. This chunk was then transferred to a new NGM plate.

**RNA extraction from *C. elegans***

Two plates of worms were rinsed with 5 ml of M9 buffer into two 15 ml conical vials in order to collect worms for extraction. The worms were then homogenized by drawing them up through a syringe. The vials were then spun at 3,000 rpm in order to pellet the worms. The supernatant was taken off and 1 ml of Trizol was added to each tube. The mixture was then transferred to two Eppendorf tubes. The tubes were allowed to incubate at room temperature for five minutes after which 0.2 ml of chloroform was added. The tubes were vortexed vigorously for 15 seconds. The tubes were then centrifuged at 4°C for 15 minutes at 3000 rpm. Three hundred microliters of the aqueous phase were carefully transferred to separate Eppendorf tubes. Isopropanol (0.5ml) was added to each tube and they were allowed to incubate on ice for 10 minutes. The samples were then centrifuged for 10 minutes at 10,000 rpm at 4°C. After the spin, a white pellet formed.
supernatant was removed and the pellet was washed with 1ml of 75% ethanol solution but not fully resuspended. The solution was spun again for 5 minutes at 5,000 rpm at 4°C. The ethanol was then removed and the pellet was allowed to air dry for 10 minutes at room temperature. The pellet was resuspended in nuclease free water and incubated at 50°C for 10 minutes in order for it to dissolve back into solution. The samples were stored at -80°C. RNA concentration was determined using a spectrophotometer.

**RT-PCR of C. elegans RNA**

The gene homolog to the human Dync1li1 gene was found in C. elegans and primers were designed for that gene:

Forward: AT-TCTAGA-ATG-CCACCAACTGCGCAAC
Reverse: TA-GGTACC-TTA-TGCATCCTGTCGCCGGG

Four reactions were set up to produce many cDNA copies from multiple mRNA templates. Reaction A acted as the experimental condition. It contained C. elegans RNA and the enzyme reverse transcriptase (RT), which would use the primers and RNA to reverse transcribe the mRNA into DNA. Reaction B acted as a positive control. It contained Gapdh (Glyceraldehyde 3-phosphate dehydrogenase) RNA and the RT enzyme. Reaction C acted as a negative control. It contained C. elegans RNA and no enzyme. Reaction D acted as a second negative control to check the purity of the reagents. It contained all of the reagents but no RNA. Two µg of RNA, one µl of Oligio DT primers or random primers, and enough nuclease free H2O to bring the final volume to 12 µl was added to each PCR tube. The tubes were
allowed to incubate at 65°C for five minutes in a thermocycler. The tubes were then placed on ice and spun briefly. 5x reaction buffer (5 µl), one µl of Ribolock RNase inhibitor, two µl of 10 µM dNTP mix, and one µl of RevertAid RT enzyme (ThermoScientific) were added to each reaction. For reaction #3, nuclease free H₂O was added instead of RevertAid RT enzyme. These were mixed and allowed to incubate at 25°C for five minutes, then at 42°C for one hour, and then at 70°C for five minutes.

After the incubations the tubes were removed from the thermocycler and placed on ice. Five more reactions were set up (Table 5). These reactions would amplify the targeted gene area. Instead of the Dynclil1 primers, primers for Act-1, a highly translated protein in C. elegans, were used in order to verify that the extracted RNA was pure.

<table>
<thead>
<tr>
<th>Reaction #1</th>
<th>Reaction #2</th>
<th>Reaction #3</th>
<th>Reaction #4</th>
<th>Reaction #5</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x GoTaq Green Mastermix: 12.5 µl</td>
<td>2x GoTaq Green Mastermix: 12.5 µl</td>
<td>2x GoTaq Green Mastermix: 12.5 µl</td>
<td>2x GoTaq Green Mastermix: 12.5 µl</td>
<td>2x GoTaq Green Mastermix: 12.5 µl</td>
</tr>
<tr>
<td>10 µM Forward Gapdh Primers: 1.5 µl</td>
<td>10 µM Forward Act-1 Primers: 1.5 µl</td>
<td>10 µM Forward Gapdh Primers: 1.5 µl</td>
<td>10 µM Forward Act-1 Primers: 1.5 µl</td>
<td>10 µM Forward Act-1 Primers: 1.5 µl</td>
</tr>
<tr>
<td>10 µM Reverse Gapdh Primers: 1.5 µl</td>
<td>10 µM Reverse Act-1 Primers: 1.5 µl</td>
<td>10 µM Reverse Gapdh Primers: 1.5 µl</td>
<td>10 µM Reverse Act-1 Primers: 1.5 µl</td>
<td>10 µM Reverse Act-1 Primers: 1.5 µl</td>
</tr>
<tr>
<td>Nuclease Free H₂O: 4.5 µl</td>
<td>Nuclease Free H₂O: 4.5 µl</td>
<td>Nuclease Free H₂O: 4.5 µl</td>
<td>Nuclease Free H₂O: 4.5 µl</td>
<td>Nuclease Free H₂O: 4.5 µl</td>
</tr>
</tbody>
</table>

Table 5. Reagents (ThermoScientific) for set up of PCR of target genes in cDNAs
Once all of the reagents had been added to the new PCR tubes, all reactions were vortexed, centrifuged for five to ten seconds, and placed in the thermocycler at the following conditions (Figure 3):

**PCR Conditions**
- 95°C 95°C 58°C 72°C 72°C 4°C
- 2:00 :30 :30 1:00 5:00 ∞

Repeat 31X

**Figure 3. PCR conditions for Act-1 isolation**

The samples were run on a gel in order to check the results. Orange G loading dye was added to 10 µl of the reactions. These were then run on a 1% agarose gel infused with one µl of SYBER safe fluorescent stain in 1x TBE buffer. The gel was run at 90V until the bands reached the bottom of the gel. The gel was then imaged using UV light.

**Results**

The PCR of *Dyn1li1* from the original plasmid was successful. Dyn1li1 is 1.5 Kb long and appears at the correct length on the ladder in high intensity (Figure 4). Insertion of the gene into mammalian expression vector pcDNA3.1 was successful as well with high probability. Nine out of the 12 samples tested were positive for the insert (Figure 5).
Transfection of HEK 293 cells with the construct was a success and positive expression of the Dync1li1 protein was achieved (Figure 6).
The CO-IP results were inconclusive due to background binding of the secondary antibody. The GFP tagged GLUA2 construct is supposed to run at about 125 kDa (Figure 7).

![Figure 7. Co-IP of Dyn1li1 and GLUA2.](image)

Revers transcriptio of Act-1 from the *C. elegans* RNA was successful. A strong signal was displayed at the correct band length (Figure 8).

![Figure 8. Reverse Transcription of Act-1 and GapDH (positive control).](image)
Immunostaining of neuronal proteins Tau (Figure 9b) and Map2 (Figure 9a) were successful, which verified healthy neuron growth and maturation. Transfection of Dync1li1 was not effective. Expression was not strong or well localized.

Live labeling studies indicated slightly elevated expression of GLUA2 puncta in the positive control group, Stargazin, and the experimental group (Dync1li1) when compared to the negative control group (NPTXR) (Figure 10). However, the difference is not statistically significant due to the standard error overlap and p-values of greater than 0.05 in the two variable t-test (Figure 11 & Table 6).
Figure 10a and 10b. GLU A2 puncta showing surface expression in Dyncli1 transfected HEK 293 cells (8a) and Stargazin Transfected HEK 293 cells (8b).

**Surface Expression of GluA2**

<table>
<thead>
<tr>
<th>Percent GluA2 Puncta on Cell Surface of HEK 293 cells</th>
<th>Gene Transfected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dync1li1</td>
</tr>
<tr>
<td></td>
<td>Stargazin</td>
</tr>
<tr>
<td></td>
<td>NPTXR</td>
</tr>
</tbody>
</table>

Figure 11. A graph comparing the surface expression of GLUA2 between groups of cells transfected with Dyncli1 (experimental), Stargazin (positive control), and NPTXR (negative control).

**t-Test: Two-Sample Assuming Unequal Variances**

<table>
<thead>
<tr>
<th>Genes</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dyncli1 and Stargazin</td>
<td>0.505</td>
</tr>
<tr>
<td>Dyncli1 and NPTXR</td>
<td>0.522</td>
</tr>
<tr>
<td>Stargazin and NPTXR</td>
<td>0.526</td>
</tr>
</tbody>
</table>

Table 6. Statistical analysis using a two-sample t-test to check for statistically significant differences in surface expression of GLUA2 between experimental group and positive and negative controls. P-value must be less than or equal to 0.05 for the difference to be statistically significant.
Discussion

Cloning and Expression of Dync1li1

The success of the cloning and insertion of Dync1li1 into pcDNA3.1 was essential in order for further experiments to be carried out. In order to be certain of future experimental success, the expression of Dync1li1 within a living cell was required as well. The success of these experiments verifies the validity of the protocols used for cloning, transfection, and Western blotting.

Co-Immunoprecipitation

The Western blot of the CO-IP experiment showed bands running higher than where GLUA2 normally runs. Increased background binding due to insufficient expression of GLUA2 or Dync1li1 could have been the cause of this. If there was not sufficient protein for the anti-GFP antibody to bind to, then they may have bound to other cell debris. The larger bands could have also been caused by insufficient denaturation of GLUA2, which would form coiled structures that run higher than their actual size of 125 kDa. If this is true, then the presence of the high bands in the Dync1li1 input but not in the Co-IP lane could indicate that Dync1li1 does not interact directly with GLUA2. The future direction of this experiment would involve addressing these issues by adjusting the protocol. Some of these adjustments would include increasing the amount of DNA transfected into the cells, increasing the time of the denaturing step and, perhaps, trying a different lysis buffer to lyse the cells less aggressively.
Immunostaining

Immunostaining of neuronal proteins (Map2 and Tau) verify that the culturing techniques outlined in the methods section produced healthy neurons. As for the staining of Dynein1l1, the lipofectamine transfection was not sufficient. Dynein1l1 was thought to appear as puncta along the cytoskeleton and there was no such finding. The future direction of this experiment includes optimizing the lipofectamine transfection protocol by adjusting the ratio of lipofectamine in order to allow for sufficient expression of Dynein1l1 within the neurons and, at the same time, not becoming toxic to the cells.

RT-PCR of *C. elegans* RNA

The success of the reverse transcription of Act-1 showed that the methods were sound and all of the reagents were active for the reverse transcription protocol. This also shows that the RNA extraction protocol produced enough pure RNA for experimentation. Now that the protocols and reagents have been verified, work on isolating the Dynein1l1 homolog in the *C. elegans* RNA can be performed.

Live Labeling

The mean percent of GLU2A puncta on the cellular surface did not vary significantly between any of the groups. There is a slight elevation of GLU2A expression in Stargazin and Dynein1l1 compared to NTXPR, but the difference is not significant. However, Stargazin is a known interactor with GluA2 and causes increased surface expression of GLU2A (Chen et al. 2003). Therefore, there should
have been significant variance between NPR and Stargazin. The fact that Dync1li1 showed close to the same level of expression that Stargazin did is encouraging, and warrants performing further studies. The methods used for measuring fluorescence levels allowed for too much human error and were not as accurate as they need to be. Along with this, the group sizes were not considerably large. Both of these factors were most likely responsible for the insignificance in variance. For future live labeling studies, staining of more cells and a use of more accurate imaging technology would be critical in providing significant data.

**Conclusion**

In conclusion, these results, as a whole, show progress and promise for further experimentation to take place. There is no conclusive data, but the progress is in the right direction. The CO-IP assays need further trouble shooting to acquire meaningful results, but the live labeling experiments show some promise of indicating elevated surface expression of GluA2 in Dync1li1 transfected cells. With further experimentation and collection of more data, those experiments will likely yield significant results. Lastly, the *C. elegans* and immunostaining experiments are not at a point where their results can be analyzed, but further progress will continue and those results will be significant to this project. The area of molecular biology is the basis of all biological processes in a living system. If we understand the small parts of a process that make up its whole, we will ultimately understand the entire system. This will lead to the development of more targeted treatments of diseases and answers to questions that have baffled the scientific community for decades.
References


AMPA receptors into spines during long-term potentiation. Natural Neuroscience 11:457-466.


identify novel AMPA receptor auxiliary subunit, GSG1L. Cell Reports
1(6):590-598.

carcinoma stem cells (NT2) into neurons for neurite outgrowth analysis. Cell

stargazing-like TARPs with cycling AMPA receptors at synapses. Science
303:1508-1511.


Tynan, S., Purohit, A., Doxsey, S., Vallee, R. 2000. Light intermediate chain 1 defines a
functional subfraction of cytoplasmic dynein which binds to pericentrin.