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Exploring the Role of Dctn2 in the Trafficking of GluR2- containing AMPA Receptors

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Honors Thesis

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Abstract

AMPA receptors are post-synaptic receptors that play a role in thinking and learning. Although their function is understood, the trafficking and regulation of AMPA receptors in neurons remains unclear. The goal of my research was to determine whether Dynactin Subunit 2 (Dctn2) affects the trafficking of AMPA receptors through the regulation of the AMPA receptor subunit, GluR2. To accomplish this, Dctn2 was amplified via PCR, ligated into the mammalian expression vector pcDNA 3.1, and transfected into Human Embryonic Kidney (HEK) 293 cells, NT2 cells and neurons. Immunostainings and Co-immunoprecipitations were performed on the transfected cells to determine protein localization and if Dctn2 associates with GluR2. Unfortunately, the immunostainings and co-immunoprecipitation assays did not yield definitive results, thereby preventing a clear understanding of the role of Dctn2 in AMPA receptor trafficking.

Introduction

Even though there are many types of receptors in the post-synaptic region of the dendrite, γ-Amino-3-hydroxy-5-Methylisoxazole-4-Propionic Acid (AMPA) receptors are of interest due to their key role in excitatory neurotransmissions within the central nervous system (Esteban, 2008). AMPA receptors are hetero-tetramer protein structures assembled by various pairings of the protein subunits GluR1, GluR2, GluR3, and GluR4 with GluR2 seemingly the most abundant subunit within membrane bound receptors (Esteban, 2008). Once assembled within the endoplasmic reticulum (Esteban, 2008), AMPA receptors can be placed within the post-synaptic membrane or removed to adjust

the number of receptors present for binding (Brown *et al*., 2007). The adjustment of the levels of AMPA receptors allows for an increase or decrease in the frequency of excitatory signals which in turn affect the degree of synaptic strength between two neurons (Brown *et al*. 2007). This regulation encompasses the phenomenon within neurons known as synaptic plasticity (Esteban, 2008). Synaptic plasticity refers to the increase or decrease in AMPA receptors at the synapse and the subsequent increase or decrease in the strength of the signals they produce. Furthermore, changes in synaptic plasticity have direct effects on learning and memory (Brown *et al*. 2007).

The first described protein capable of influencing the trafficking, of GluR2-containing AMPA receptors was Stargazin (Esteban, 2008). Four other proteins referred to as γ-3, γ-4, γ-8 (Tomita *et al*., 2003) and γ-7 (Kato *et al*., 2007) have also been shown to be involved in the trafficking of AMPA receptors and are collectively referred to as TARPs (Transmembrane AMPAR Regulatory Proteins; Esteban, 2008). With the discovery of TARPs, the search for other proteins having similar effects on GluR2 trafficking began.

Overview and Aim:

The purpose of my research was to identify novel proteins that affect the trafficking of GluR2-containing AMPA receptors. The GluR2 subunit was used to determine a proteins trafficking ability due to its high abundance within membrane bound receptors (Esteban, 2008). To date, very few proteins have been shown to influence AMPA receptor trafficking. In a study by Shanks *et al*. (2013), co-immunoprecipitation coupled with mass spectrometry analysis was used to generate a list of 421 proteins that

interact with GluR2. The data set published in their study was used to select candidates for my project.

Dynactin subunit 2 (Dctn2) was selected as a strong candidate due to its high levels of association with GluR2. Dctn2 is a subunit of Dynein, a motor protein within the cell that functions by binding to proteins and transporting them to the minus ends of the microtubules (Splinter *et al*., 2012). Dctn2 is unique in that it is involved in both mitotic processes through the recruitment of proteins to the centrosome and in immunoregulation through its involvement in presenting MHCII complexes into the cell membrane (Uniprot, 2014).

Having been previously identified as a receptor transporter (Uniprot, 2014), Dctn2 appeared to be an excellent candidate for studies on its potential role in the trafficking of GluR2-containing AMPA receptors. To determine whether Dctn2 plays a role in GluR2 trafficking, my research involved cloning Dctn2 into the mammalian expression vector, pcDNA3.1 for overexpression studies. Immunostaining was also performed on cultured neurons derived from differentiated NT2 cells to determine where Dctn2 was localized. Co-immunoprecipitations (Co-IPs) were performed to verify that GluR2 and Dctn2 interact as previously reported in the study by Shanks *et al*. (2012). Due to the role of Dctn2 in binding cargo for transportation along microtubules and the transporting of MHCII receptors to the cell surface, I hypothesized that Dctn2 plays a role in the trafficking of GluR2-containing AMPA receptors. If my hypothesis was correct, a band would appear at the 50 KDa (Uniprot, 2014) marker in the Co-IP assay, indicating an association between Dctn2 and GluR2. Furthermore, using immunostaining, I would expect to see positive expression and binding of Dctn2 with GluR2, indicated by the

appearance of yellow puncta. If my hypothesis is false, I would expect to see no band in my Co-IP assay and only red and green fluorescence in my immunostainings. Overall, the purpose of this research is to fill a gap in are understanding of the proteins within the brain and the mechanisms by which they act.

Materials & Methods

Tissue Culturing and Maintenance

Human Embryonic Kidney 293 cells (HEK293) and undifferentiated NT2 cells were maintained weeks in advance for the future transfection of Dctn2. HEK293 cells were removed from liquid nitrogen storage and placed on a 10cm Corning culture plate with 12 mL of HEK293 culturing solution consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin, and 1% Glutamax. HEK293 cells were maintained at 37°C with 5% CO_2. The cells were observed using an inverted light microscope (Zeiss Primo Vert) to determine the degree of confluence. Once the cells reached 90% confluency, the cells were split by aspirating off the media and applying 1 mL of trypsin. Cells were incubated at room temperature for two minutes before adding 1 mL of HEK293 media and pipetting the solution to mechanically dislodge cells. On a separate tissue culture plate, 9 mL of fresh HEK293 media was added to 3 mL of cell solution from the previous passage.

NT2 cells were removed from liquid nitrogen storage and cultured on Corning culture plates using 12 mLs of NT2 media consisting of DMEM – F12 supplemented with 10% FBS, and 1% Penicillin/Streptomycin. NT2 cells were incubated under the

same conditions as the HEK293 cells. When cells reached 90% confluency, the media was aspirated off and replaced with 12 mL fresh media. A sterile scraper was then used to mechanically dislodge the cells. The cells were then resuspended by pipetting. In a new culturing dish containing 9 mL of fresh NT2 media, 3 mL of the cell suspension from the previous plate was added for a total volume of 12 mL. The new cultures were placed in the incubator at 37°C with 5% CO_2 and examined every day.

Neurons were produced using the method outlined by Cheung et al (1999) and Tengage *et al*. (2011). Neurons were prepared by culturing five petri dishes of NT2 cells with 12 mL of Neuron Media (DMEM-F12, 10% FBS, 1% Streptomycin/Penicillin) and fresh retinoic acid at a concentration of 1:1000. Media switches were performed every other day by pelleting the cells and resuspending in new media. After two weeks, neurons were plated at a 1:10 concentration for immunostaining and 1:1 concentration for protein analysis. Neurons were incubated in fresh neuron media, switched every other day, containing the mitotic inhibitors 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 of maturing, neurons formed visible axonal and dendritic projections, showing that they were mature enough for immunostaining.

Cloning Dctn2

E. coli that had previously been transformed with a plasmid containing the Dctn2 gene were obtained through Open Biosystems**.** The cells were spread on a selective LB plate containing chloramphenicol at 25 mg/ml and cultured overnight in a 37°C

incubator. A colony was then selected at random and cultured overnight at 37°C with shaking at 200 rpm in 5 mL of LB broth containing chloramphenicol (25 mg/ml). Following incubation, 1 mL of inoculated broth was extracted and the cell contents pelleted in a 1.5 mL microcentrifuge tube at 5,000 rpm for five minutes. A DNA miniprep was prepared from the pelleted cells using the QIAGEN miniprep kit, beginning from the pellet resuspension in P1 buffer step and ending with eluting the DNA with 30 µL of elution buffer. The concentration of DNA was determined using a spectrophotometer.

Forward and reverse primers were designed to specifically amplify the coding region of the Dctn2 gene. The primers consisted of a two nucleotide overhang, a restriction site, a start codon, the human influenza Hemagglutinin (HA) coding region, and 19–21 base pairs that complemented the template strand of Dctn2 (**Figure 1**).

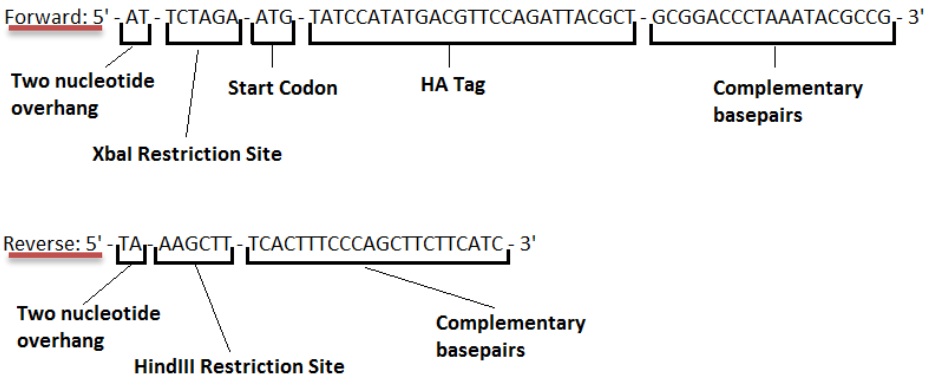
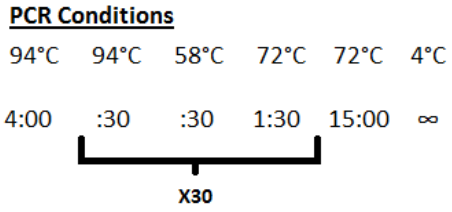


Figure 1. Primer Designs for Dctn2. Forward primer contains HA Tag sequence for translation on the N-Terminal.

A 50 µL PCR was then prepared containing 2 µL (~1ng) of the miniprep DNA template, 5 µL of 10X PCR buffer (Invitrogen), 0.5 µL of 50 mM dNTP (Invitrogen), 5 µL of forward and 5 µL of reverse primers, 1 µL of Super Taq Polymerase (Invitrogen)

and nuclease free H_2O (BioRad) to 50 µL (31.5 µL). The reaction was then placed in the following conditions:



After PCR completion, 30 µL of the PCR reaction was mixed with 6 µL of Orange G loading dye. The 36 µL sample was run alongside 7 µL of a 1 Kb plus ladder using gel electrophoresis. A 1% agarose gel infused with 1 µL of SYBR Safe DNA gel stain (Invitrogen) per 10 mL agarose solution was used for visualization of DNA bands under UV light. The DNA band was then excised using a razor blade and weighed at 0.12 grams. Using the QIAGEN gel extraction kit and the protocol provided, the Dctn2 DNA band was extracted and purified.

Two separate restriction digests were then performed on 10 µL of purified Dctn2 DNA and 10 µL of mammalian expression vector pcDNA3.1. Each reaction consisted of 3 µL of 10x buffer 2 (Supplier), 3 µL of 10x BSA (New England Bio Systems), 1 µL of the restriction enzyme XbaI (New England Bio Systems), 1 µL of the restriction enzyme HindIII (New England Bio Systems), nuclease free H_2O to 20 µL, and the addition of 10 µL of either pcDNA3.1 or purified Dctn2 DNA to each reaction. The two reactions were incubated at 37°C for two hours. After two hours, 1 µL of CIP (Calf-intestinal Alkaline Phosphatase) was added to the reaction containing the vector DNA to cleave the 5' phosphates and prevent re-ligation. Both reactions were then incubated for an additional hour.

A 10 µL ligation reaction was performed following the restriction digests to ligate the Dctn2 DNA into the pcDNA3.1 vector. The reaction consisted of 5 µL of digested DNA, 2 µL of digested vector, 1.5 µL of nuclease free H_2O, 1 µL of DNA ligase buffer, and 0.5 µL T4 DNA ligase. After vortexing, the reaction was left to incubate at room temperature for 10 min and then stored at 4°C.

TOP10 competent cells (Invitrogen) were then transformed using the ligation product. Two microliters of the ligation product was added to an aliquot of TOP10 cells and placed on ice for 30 minutes. Cells were then heat shocked by placing them in a 42°C water bath for 30 seconds. The cells were then placed on ice and 250 µL of S.O.C. Medium (Invitrogen) was added. The tube was placed on a shaker to incubate at 37°C and 200 rpm for one hour. After the one hour incubation, 100 µL of cells were spread on a pre-warmed ampicillin infused LB plate and incubated at 37°C overnight. After 24 hours, twelve colonies were selected and placed in separate culture tubes containing 5 mL of LB broth with 5 µL ampicillin. Inoculated culture tubes were placed on an incubated shaker overnight at 37°C/200 rpm.

The following day, 12 minipreps were prepared using the previously used QIAGEN miniprep kit. Samples of the extracted DNA were tested for the presence of a proper gene ligation through the use of a 10 µL diagnostic digest and were analyzed using a Gel Logic imager set to Trans UV. Each diagnostic digest consisted of 5 µL of miniprep DNA, 2.6 µL of nuclease free H_2O, 1 µL of 10x buffer 2, 1 µL of 10x BSA, and 0.2 µL of the restriction enzymes XbaI and HindIII. The digests were placed in a thermocycler to incubate at 37°C for 1 hour. After incubation, 2 µL of orange G loading dye was added to each digest and the contents of each was loaded, alongside 7 µL of 1

Kb plus ladder, into wells of a 1% agarose gel for electrophoresis at 100 volts. All positive miniprep DNAs were stored a 4°C. The miniprep DNA concentrations were determined using a spectrophotometer.

NT2/HEK293 Transfection

HEK293 and NT2 cells were plated on a 12 well plate for transfections; 6 wells were seeded with HEK 293 and 6 wells with NT2 cells. Both HEK293 and NT2 cells were transfected using FuGene (Promega). For HEK 293 cell transfection, 94 µL of DMEM solution was added to a 1.5 mL Eppendorf tube along with 6 µL of FuGene. For NT2 cell transfection, 94 µL of DMEM-F12 solution was added to a 1.5 mL Eppendorf tube along with 6 µL of FuGene. Each was incubated at room temperature for five minutes. After incubation, 1 µg of miniprep Dctn2 DNA was added to each solution, mixed well, and incubated at room temperature for 20 minutes. The contents of each tube were then added dropwise to individual wells of the previously seeded 12 well plate. Cells were then allowed to incubate for two days to build up levels of Dctn2 protein for later analysis.

Proteins were then harvested by pipetting off the media and adding 100 µL of freshly boiled sample buffer to each well. The sample buffer containing 50 µL Laemmli (BIO-RAD) and 50 µL β-mercaptocethanol (Gibco) was mixed well and placed in boiling water for 3 minutes before being added to the cells. After the sample buffer was applied, a 100 µL pipet tip was cut off at the end to form a larger bore. The cells were then scraped using the pipet tip to make a lysate that was thick in consistency and was collected into a 1.5 mL Eppendorf tube. The samples were then allowed to cool and 40µL

was loaded into a mini-PROTEAN® TGX™ gel (BIO-RAD) along 15 µL of Precision Plus ladder (BioRad) for polyacrylamide gel electrophoresis. The inner and outer chambers were filled with 1x Tris/Glycine/SDS Buffer (BIO-RAD) and the gel was run at 125 volts until the lower band of the ladder reached the bottom of the gel. The gel was then transferred to a nitrocellulose/filter paper sandwich (BIO-RAD) in a transfer buffer solution consisting of 100 mL 10x Tris/Glycine Buffer (BIO-RAD), 100 mL pure methanol, and 800 mL deionized water. The transfer was run at 90 volts for one hour.

The western blot was then removed and placed in a blocking solution consisting of 2 grams of blotting-grade blocker (BIO-RAD) in 100 mL of a 0.01% Tween20/1x TBS solution. The blot was incubated at room temperature for 30 min while on a rocker table. After 30 minutes, the blocking solution was poured off and 3 mL of blocking solution was added with the primary mouse anti-HA antibody (Covance) at a 1:1000 dilution (3µL). The blot was then placed on a rocker table and incubated overnight at 4°C.

The following day, the membrane was washed three times in a 0.01% Tween20/1x TBS solution at 10 minutes per wash. Blocking solution was then applied to the membrane with the secondary HRP goat anti-mouse antibody (BIO-RAD) at a 1:1000 dilution and incubated at room temperature for 1 hour on a rocker table. After 1 hour, the secondary antibody solution was poured off and the membrane was washed twice with 0.01% Tween20/1x TBS solution and once with 1x TBS solution at 10 min/wash. Following the washes, a colorimetric protein assay was performed using the Immuno-Blot HRP Colorimetric kit (BIO-RAD) and the provided procedure. The colorimetric kit allowed for the visualization of the binding of the mouse anti-HA antibody to the HA protein tag on the Dctn2 protein.

Immunostaining NT2 Neurons

Immunostainings were performed on neurons derived from NT2 cells. Fully mature neurons were produced on non-gelled cover slips in 6 wells of a 12-well plate, using the previously outlined method. Once neurons reached maturity the neuron media was pipetted off and 500 µL of 4% paraformaldehyde (PFA, Sigma) solution was carefully placed on the cells and allowed to incubate for 15 minutes, on a rocker table, to fix the cells on to the coverslip for the future washes. A rocker table was used for all future steps in the immunostaining protocol. The PFA was then removed and each well was washed three times, five minutes per wash, with 500µL of 1x Phosphate Buffered Saline (PBS) pH 7.4 (GIBCO). After the washes, 500 µL of a blocking solution consisting of PBS, 3% Albumin Bovine Serum (CALBIOCHEM), and 0.1% Triton x-100 (Roche) was placed in each of the wells and incubated at room temperature for 20 minutes. The blocking solution was pipetted off of the neurons and 500 µL of fresh blocking solution was placed on the cells along with the primary antibody, mouse anti-Dctn2 (R&D Systems), at concentrations of 4 µg/mL, 8 µg/mL, or 16 µg/mL in order to determine the optimal antibody concentration for future experiments. Chick anti-Map2, Chick anti-Synapsin, and Chick anti-Tau primary anitbodies were also applied to individual cell cultures to determine where Dctn2 localizes and to visualize dendrites, synapses, and axons respectively. The cells incubated in the primary antibody solution for two hours at room temperature. After two hours, the cells were washed three times, five minutes per wash, with 500 µL of blocking solution to remove antibodies that had bound to proteins non-specifically. A secondary antibodies, 488 nm fluorescing goat anti-mouse (Life Technologies) and 543nm goat anti-chick (Life Technologies) were added to

fresh blocking solution at a concentration of 1:1000. The secondary antibody solution was placed on the cells at 500 µL per well and allowed to incubate in the dark, for one hour. The cells were washed twice with 500 µL of blocking solution per wash and once with a solution consisting of PBS and Hoescht stain at a concentration of 10 µL/mL. Each wash was carried out for five minutes. The plate was removed from the rocker and the coverslips were mounted on supefrost-Plus Microscope Slides (Thermo Scientific) using Fluoromount-G (Electron Microscopy Sciences). Slides were allowed to cure overnight before visualizing.

Co-Immunoprecipitation Assay

Co-immunoprecipitations (Co-IPs) to look at the interaction between GluR2 and Dctn2 were performed on HEK293 cells. Prior to the Co-IPs, HEK293 cells were grown in a 6-well dish (Thermo Scientific) and transfected with 2 µg of HA-Dctn2 and GFP-GluR2 using miniprep DNA, FuGene reagent, DMEM and the provided protocol. Two hundred microliters of transfectant was added drop wise to each well. Cells were incubated for two days post-transfection to allow for the production of a sufficient amount of protein for the Co-IP. After two days, RIPA lysis buffer (Cell Signaling) was prepared from a 10x stock solution. RIPA was diluted from a 1mL 10x solution to a 1x solution by diluting it with 9 mL of nuclease-free water. After diluting, 100 µL of 100x protease inhibitors (Cell Signaling Tech) was added to the 10mL RIPA solution. RIPA was then stored on ice for further use.

Mouse anti-GFP (Santa Cruz Biotechnology) and mouse anti-IgG (Santa Cruz Biotechnology) antibodies were bound to protein A/G beads (Santa Cruz Biotechnology)

by adding 500 µL RIPA solution, 25 µL of protein A/G, and 1 µg of the respective antibody to 1.5 µL centrifuge tubes. Protein A/G beads bound to IgG antibody would serve as a negative control, while protein A/G beads bound to mouse anti-GFP antibody would serve as the experimental by binding to the GFP tagged GluR2 and the associated proteins. Each would later serve to perform a single Co-IP assay. The tubes were incubated end-over-end on a rotator at 4°C for 45 minutes. During the incubation, cell lysates from the HEK293 cells were collected.

To obtain cell lysates, the HEK293 cell plate was placed on ice and the media was removed. The cells were washed briefly with ice cold 1x PBS. Cells were placed on a rocker and 500 µL of RIPA was pipetted onto each dish and incubated at 4°C for 40 minutes. Cells were collected in Eppendorf tubes on ice and centrifuged at 13,000 rpm for 20 minutes at 4°C. While the cells were spinning, the antibody bound protein A/G beads were centrifuge at 2,000 rpm for 2 minutes at room temperature. The supernatant was removed from the beads and was replaced with 500 µL RIPA solution. Beads were mixed by tapping and placed on ice until needed.

After the cell lysates were done centrifuging, the supernatants from each sample were carefully removed and placed in pre-chilled Eppendorf tubes. More pre-chilled Eppendorf tubes were prepared and 20 µL of lysate was removed from each sample and placed in the chilled tubes to serve as our positive controls. Half of the remaining cell lysates received 25 µL of the GFP antibody bound protein A/G bead solution and the remaining half IgG antibody bound protein A/G beads. Lysates mixed with bead solutions were rotated end-over-end overnight at 4°C.

The following day, the samples were centrifuged at 1,000 rpm for one minute at 4°C and the supernatant was removed. Next, 300 µL RIPA solution was added to each sample and mixed by tapping. This wash was performed twice and was followed by one more wash using ice-cold PBS instead of RIPA. After the final wash and centrifugation, as much PBS was removed as possible using a micropipette and 25 µL of 2x sample buffer, consisting of 950 µL Laemlli and 50 µL β-mercaptoethanol, was added to each sample pellet as well as to the lysates serving as inputs. All samples were boiled for five minutes at 100°C and loaded into a mini-PROTEAN® TGX™ gel (BIO-RAD) for western blot analysis as previously described using primary antibody mouse anti-HA (Covance) and secondary antibody HRP goat anti-mouse (BIO-RAD), both diluted to 1:1000.

Results

PCR amplification of Dctn2 displayed a strong signal at the proper band length indicating successful amplification using the primers and PCR conditions discussed earlier (Figure 2).

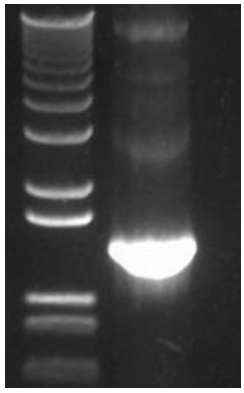


Figure 2. Dctn2 PCR Amplification. PCR contents ran on a 1% agarose gel at the proper band width of approximately 1200 bp.

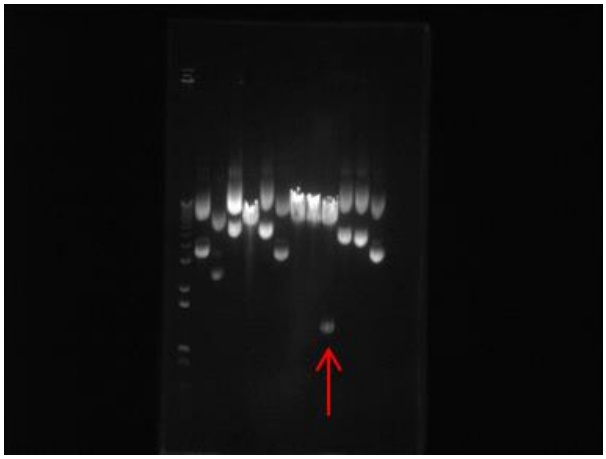


Figure 3. Diagnostic Digest of Dctn2 using restriction enzymes Xba1 and HindIII. Top10 cell colony in lane 9 (red arrow) depicts proper ligation of Dctn2 insert into pcDNA 3.1.

Ligation of Dctn2 insert into the mammalian expression vector pcDNA 3.1 was successful but displayed a low probability of proper insertion as depicted by the one positive sample out of the 12 analyzed by diagnostic digest (Figure 3). HEK 293 cells that were transfected with the miniprep DNA from the positive sample showed expression of Dctn2 by western blot analysis (Figure 4).

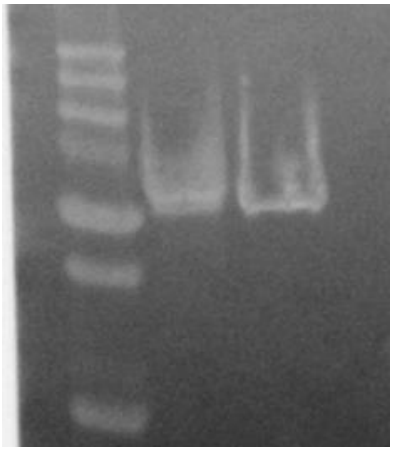


Figure 4. Western Blot in HEK293 Cells. The Precision Plus ladder (lane 1), the positive control, HA-NPR (lane 2), and N-terminal HA-tagged Dctn2 (Lane 3) both displayed clear bands indicating strong expression in HEK293 cell lines when selected using a MS α HA antibody.

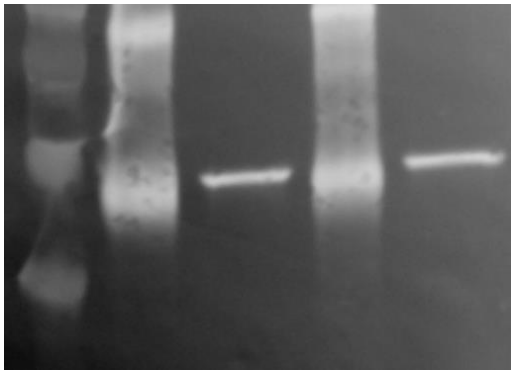
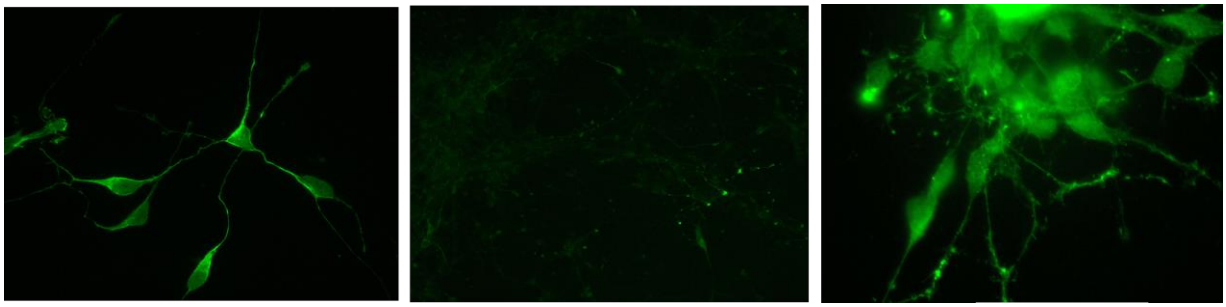


Figure 5. Co-IP of Dctn2 and GluR2. Western blot of the Co-IP results for Dctn2-GluR2 interaction. Lanes 2 & 4 display non-specific binding of the 2° antibody to the agarose bead. Precision Plus ladder (lane 1), IgG (lane 2), IgG input (lane 3), GFP (lane 4), GFP input (lane 5).

Positive expression of Dctn2 was confirmed by visualizing the protein bands in lanes 3 and 5 (Figure 5). Lanes 2 and 4 failed to produce bands that would indicate a positive interaction between Dctn2 and GluR2. Instead, smears appeared in the lanes containing both the IgG bound agarose beads and the GFP bound agarose beads.



Map2 Tau Synapsin

Figure 6. Immunostaining of the neuronal proteins NT2 neuron cultures. Map2 and Tau are cytoskeletal proteins present in dendrites (Map2) and axons (Tau). Synapsin is responsible for the binding of neurotransmitters to the cytoskeleton

Immunostaining for Dctn2 in NT2 neurons showed non-specific binding of the primary antibody. The region of localization was not clearly defined and was scattered in random patches throughout the slide. Immunostaining of Map2, Tau, and Synapsin

proteins were effective and verified successful neuron growth and proper protein expression (Figure 6).

Discussion

Cloning Dctn2

The cloning of PCR-amplified Dctn2 into the mammalian expression vector, pcDNA3.1, was one of the most critical components of my research project as subsequent experiments depended on its success. As shown in Figure 4, only one of the 12 transformed bacteria cells containing my cloning reaction displayed evidence of working correctly. Generally, however, the diagnostic digests samples that failed for use in future assays would most likely be re-ligated vector lacking the DNA insert, which is not the case here. In looking at the bands, it is clear that DNA had inserted into the vector. However, what caused it to run at so many different base pair lengths was unclear. When cloning Dctn2 into pcDNA3.1 was first attempted, the ligation reaction was placed at 16°C overnight in a thermocycler. Using this method, none of the Dctn2 ligations were successful and even larger inserts had formed. To avoid the formation of 'super inserts', I altered the ligation protocol so that the reactions incubated for only 15 minutes at room temperature before bacterial transformation. The decreased incubation period showed evidence of smaller 'super inserts' and also provided me with bands that were equivalent to my inserts.

Immunostaining of Dctn2

The immunostaining of Dctn2 in NT2 neurons was problematic in that endogenous expression of the protein was not clear and there was strong evidence of non-

specific binding of the antibody to unknown structures. This may be due to a lack of strong endogenous expression of the Dctn2 protein within the NT2 neurons which would then cause the antibodies to bind to non-specific sites on the coverslips. Immunostaining of Dctn2 was predicted to express along cytoskeletal proteins in the form of puncta, but this was not observed. To increase the level of expression, I plan to transfect HA- Dctn2 into the neuron cultures using the transfection reagent Lipofectamine 2000 (Invitrogen). However, Lipofectamine 2000 first needs to be optimized for the NT2 neuron cultures due to the amount of DNA that it requires for transfection and the fact that if used at the wrong concentrations it becomes lethal to the cells. To further remedy this problem I intend to change the primary antibody from mouse anti Dctn2 to mouse anti HA after transfecting using HA- Dctn2. In the western blots previously performed, mouse anti HA was used and displayed a high degree of specific binding. The mouse anti Dctn2 antibody was believed to be more specific for Dctn2 and instead presented a decreased binding affinity for unknown reasons.

Co-Immunoprecipitation Assay

The Co-IP results showed two strong bands in the input samples that corresponded to the positive expression of the Dctn2 protein in transfected HEK293 cells. However, the smudges that were produced in lanes 2 and 4 of Figure 5 indicate a high degree of non-specific binding of the secondary antibody. It is believed that this is caused by the protein A/G agarose bead that is bound to the primary antibody. In the protocol used, the primary antibody remains bound to the protein A/G agarose beads and becomes a sight to which the secondary antibody could easily "stick". Therefore, the desired bands that would indicate the association between Dctn2 and GluR2 may be in

fact present, but may not be visualized due to the distortion that has formed across the lane. To remedy this, a method for the removal of the protein A/G agarose beads from the cell lysate must be further explored. A possible method for removal would be to spin down the solution after boiling the lysate in sample buffer, thereby pelleting the A/G agarose beads. The supernatant could be used for western blot analysis and hypothetically would be protein A/G free.

Conclusion

Due to the inconclusive data produced by the immunostaining and Co-IP experiments, I can neither accept nor reject my hypothesis stating the Dctn2 is involved in the trafficking of GluR2 containing AMPA receptors. However, this research was useful in developing the method used to successfully PCR amplify a gene, ligate it into a vector, and successfully express the gene within mammalian cells. With these methods in place, continued investigation of more proteins from the candidate list can be used so that we can determine their relationship with GluR2. Once confirmed GluR2 interacting proteins have been found, overexpression studies can be performed and their effects on AMPA receptor trafficking and on the cell as a whole, can be determined. This will allow us to better understand the proteins, functions, and mechanisms that AMPA receptors go through in order to promote learning and cognitive functions.

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