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Investigating the Role of Olfm1 in the Trafficking of GluR2-containing AMPA receptors

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SIGNATURE PAGE

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Investigating the Role of Olfm1 in the Trafficking of GluR2-containing AMPA receptors

Honors Thesis

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Abstract

Neurons have the ability to either increase or decrease the strength of their connections with other neurons. This property is known as synaptic plasticity and is thought to influence higher cognitive functions such as learning and memory. Decreases in cognitive abilities as well as neurological disorders can be attributed to decreased synaptic plasticity. The variable expression of GluR2-containing AMPARs has been linked to changes in synaptic plasticity. The mechanism governing the trafficking of GluR2-containing AMPARs to synapses is not fully understood and was the main focus of my study. I hypothesized that Olfm1 (Olfactomedin 1) plays a key role in the trafficking of GluR2-containing AMPARs to the synapse. HA-tagged Olfm1 was successfully cloned into the mammalian expression vector pcDNA3.1. Western blot analysis verified Olfm1 protein expression in both transfected HEK293 cells as well as endogenous expression in NT2 neurons. GluR2-GFP protein expression was seen in transfected HEK293 cells, but we were unable to confirm endogenous expression in the NT2 neuron cultures. Co-immunoprecipiation experiments were also performed to study the interaction of Olfm1 and GluR2, but the tests remain inconclusive. Therefore, further testing is needed in order to accept or reject my hypothesis that Olfm1 plays a role in the trafficking of GluR2-containing AMPARs.

Introduction

AMPAR Background

Multiple ion channels, scaffolding molecules, neurotransmitter receptors and signal transduction regulators contribute to the proper functioning of the nervous system (Esteban, 2008). Membrane trafficking and regulation of neurotransmitter receptors, specifically at the post-synaptic terminal, become especially important in synaptic function and contribute to the increase or decrease of synaptic plasticity (Esteban, 2008). Most excitatory synapses in the brain are mediated by the neurotransmitter glutamate which is released into the synaptic cleft. Glutamate activates its receptors on postsynaptic neurons, causing an influx of ions in the postsynaptic cell (Hall and Ghosh, 2008). Throughout the nervous system, specifically at excitatory synapses, there are three types of glutamate receptors: AMPAR (α-Amino-3-hydroxy-5-Methyl-4-isoxazolPropionic Acid), NMDA (N- Methyl-D-Aspartate) and Kainite (Esteban, 2008). There are varying concentration levels of these receptors at different stages of development (Hall and Ghosh, 2008). In early development, the majority of glutamate receptors in the brain are NMDA receptors, which negatively regulate the expression of AMPARs (Hall and Ghosh, 2008). As the brain matures, NMDA receptors begin to activate AMPARs and subsequently increase their concentration in the adult brain (Esteban, 2008). Therefore, the mechanism of AMPAR trafficking and regulation may vary depending on the stage of brain development (Esteban, 2008).

AMPARs are synthesized in the endoplasmic reticulum of the soma and transported to the Golgi body for further modification (Greger and Esteban, 2007). Following modification, AMPARs are actively transported to the dendritic spines in order to be incorporated into the synapses via microtubule machinery (Hall and Ghosh, 2008). AMPARs are formed from various combinations of GluR(1-4) subunits into heteromeric tetramers (Greger and Esteban, 2007). It is thought that different variations of the subunits of the AMPAR tetramers affect the size of the postsynaptic response, although the mechanism underlying this affect remains unclear (Greger and Esteban, 2007). My research focuses on AMPARs, specifically ones that contain the GluR2

subunit, which regulate the majority of fast excitatory currents and play a critical role in the strength of the synaptic response (Hall and Ghosh, 2008). The underlying mechanism involving the trafficking of AMPARs into and out of synapses, and the consequential effect on synaptic plasticity, remains unclear and is the focus of my research project.

Synaptic Plasticity and AMPARs

Neurons have the capability to either increase or decrease the strength of their communication with other neurons; a property known as synaptic plasticity. Synaptic plasticity is thought to influence higher cognitive functions such as learning and memory (Anggono and Huganir, 2012). Many factors alter synaptic plasticity including the efficiency of neurotransmitter release via the pre-synaptic neuron and modifications to the post-synaptic neurotransmitter receptor (Anggono and Huganir, 2012). Neurological disorders, such as Alzheimer's Disease or Schizophrenia, may be due to decreased synaptic plasticity (Esteban, 2008). AMPARs can alter synaptic plasticity through their trafficking into and out of synapses via endocytic and exocytic events regulated by Rab proteins (Greger and Esteban, 2007). At synapses, Long-Term Depression (LTD) occurs when AMPARs are internalized (endocytosis) due to low levels of neuronal activity, thereby decreasing synaptic plasticity (Anggono and Huganir, 2012). Alternatively, Long-Term Potentiation (LTP) corresponds with increased AMPAR functioning, and a subsequent increase in synaptic plasticity (Anggono and Huganir, 2012). Discovering the regulation of GluR2-containing AMPARs is essential in order to better understand the mechanisms behind synaptic plasticity and perhaps gain some insight into neurological disorders that are caused by decreased synaptic plasticity.

Identifying a Candidate Protein

Synthesis and recruitment of the appropriate pre and post-synaptic proteins are necessary for the formation, maturation, and stabilization of synapses (Hall and Ghosh, 2007). Due to the unknown mechanisms of the trafficking of AMPARs, it was my interest to investigate the role of the GluR2-interacting proteins in the trafficking of GluR2 containing AMPARs. Shanks *et al.*

(2012) composed a list of candidate proteins that potentially could interact with the GluR2 subunit using interactome data, Co-Immunoprecipitation techniques and Mass Spectrometry. Our lab cross-referenced the published list with public databases (i.e. Uniprot and PubMed) in order to identify proteins that play a role in the trafficking of GluR2-containing AMPARs. From this list my predecessor, Shelby Kramer, chose the candidate protein Olfactomedian 1 (Olfm1) to study. Olfm1 is a transmembrane glycoprotein found in *Homo sapiens* (Nakaya *et al*., 2013). While the exact function of Olfm1 remains unknown, it is expressed in the brain and may play a role in neural crest development (Uniprot, 2014). I hypothesize that Olfm1 plays a role in the trafficking of GluR2-containing AMPARs at synapses.

Materials and Methods

Methods Overview

In order to test my hypothesis, my first objective was to clone HA-tagged Olfm1 (HA-Olfm1) into a mammalian expression vector. To confirm protein expression of the inserted Olfm1 construct, transfected cells (from two different cell lines) were analyzed by Western Blot Analysis and Immunostaining procedures. These methods were also used to verify that the cell lines were able to express transfected GluR2 for future analysis. Lastly, a co-immunoprecipitation protocol was used to test my hypothesis that Olfm1 plays a role in the trafficking of GluR2-containing AMPARs.

Maintaining Cell Lines

My experimental protocols utilized two cell lines: Human Embryonic Kidney 293 (HEK293; ATCC Catalog No. CRL-1573) and NTERA-2 cl.D1 (NT2; ATCC Catalog No. CRL-1973). The HEK293 cells were grown in culture media (88% DMEM high glucose media, 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin, and 1% Glutamax; Gibco) in a 10cm tissue culture dish and were passaged using 0.5ml of trypsin-0.53mM EDTA (Gibco). The cells were incubated at 37^{o}C with 5% CO_2. The NT2 cells were maintained in growth media (90% DMEM,

10% FBS; Gibco) in 10cm tissue culture dishes and passaged by mechanically dislodging the cells from the culture dish. The cells were incubated at 37°C with 5% CO_2.

The NT2 cell line was utilized due to its ability to be differentiated into neurons by treating the cells with retinoic acid and mitotic inhibitors. The NT2 differentiation protocol by Tegenge *et al*. (2011) was used. The NT2 cells were prepared for differentiation by passaging them into a petri dish as suspension cultures. After a 24-hour incubation at 37°C with 5% CO_2, the cells were collected into 50ml conical tubes and were centrifuged for 5 minutes at 200g using a Sorvall Legend RT Centrifuge (Thermo Electron Corporation). Following centrifugation, the cell media was aspirated off and replaced with differentiation media (90% DMEM, 10% FBS, 1% 10μM retinoic acid). The cells were maintained and incubated in suspension petri dishes for two weeks at 37°C with 5% CO_2 while performing differentiation media changes every other day. After the two-week period, the suspension culture was plated into 12 well tissue culture dishes for Western Blot and Immunostaining analysis using the protocol of Cheung *et.al.* (1999). The NT2 neuron cells were maintained in NT2 neuron media containing mitotic inhibitors (90% DMEM, 10% FBS, 1% 10μM retinoic acid, 1% 10μM Urd, 1% 10μM Fudor, .01% 100μM AraC), which was changed every other day.

The HEK293 cell line was utilized based on being easily transfected and robust. NT2 neuronal culture viability was time sensitive and required much maintenance, therefore the HEK293 cell line was used for transfection and CoIP experiments prior to performing the same experiments on the NT2 neuronal cells.

Cloning Olfm1 into a Mammalian Expression Vector

The Olfm1 clone was obtained from Open BioSystems, with the intent of cloning it into the mammalian expression vector, pcDNA 3.1 (Invitrogen). Primers were made for a PCR

reaction, which included the HA-tag and Kozack sequence for the c-terminal of Olfm1. The PCR reaction was 50µL total including: 100ng Olfm1 DNA template (Open BioSystems), 5µL 10X PCR buffer (TOPO Kit; Invitrogen), 0.5µL dNTPs (TOPO Kit; Invitrogen), 1µM of primers (Invitrogen), 1µL of Plantimun *Taq polymerase* (Invitrogen), and the addition of water to final volume. The PCR conditions were as follows: 94^{o}C/ 4 minutes; 30 cycles of 94 ^{o}C/30 seconds, 58 ^{o}C/30 seconds, 72 ^{o}C/ 3 minutes; 72 ^{o}C/5 minutes; 4 ^{o}C/∞. Following PCR, Orange G dye was added to the samples (1µL of Orange G for every 5µL of sample) and they were electrophoresed on a 1% agrose gel (Bio-Rad) containing SYBR® Safe DNA Gel Stain (Invitrogen) at 100 Volts. The gels were visualized using a UV trainsilluminator to confirm if the PCR successfully generated the HA-Olfm1 construct. The DNA was then extracted from the gel using QIAquick® Gel Extraction Kit (QIAGEN).

A restriction digest was performed on the purified Olfm1 DNA extracted from the gel. The restriction digest was carried out in two different reactions: (A) Olfm1 and (B) pcDNA 3.1 vector. Reaction A contained: 3µL 10 X Buffer 2 (New England Biolabs), 3µL10X BSA (New England Biolabs), 1µL Hind III (restriction enzyme; New England Biolabs), 1µL Not I (restriction enzyme; New England Biolabs), 12µL water, and 10µL HA-Olfm1 DNA. Reaction B contained: 3µL 10 X Buffer 2 (New England Biolabs), 3µL 10X BSA (New England Biolabs), 1µL Hind III (restriction enzyme; New England Biolabs), 1µL Not I (restriction enzyme; New England Biolabs), 20µL water, and 2µL pcDNA3.1 vector (Invitrogen). The negative control contained nuclease free water (Ambion) instead of Olfm1 PCR DNA. The reactions were incubated for two hours at 37 ^{o}C. Then 1µL calf intestinal alkaline phosphatase (CIP) enzyme (New England Biolabs) was added to Reaction B. The reactions were left to incubate at the same conditions for another hour. The reactions were then PCR purified using QIAquick® PCR Purification Kit (QIAGEN) using the provided protocol.

Ligation reactions were performed in order to insert the HA-Olfm1 gene into the

mammalian expression vector, pcDNA 3.1 (Invitrogen). All reactions contained 1µL 10X T4 DNA Ligase Buffer (New England Biolabs), 0.5µL T4 Ligase (New England Biolabs), 2µL CIP-treated pcDNA 3.1 vector, 1.5µL water, and 5µL of DNA (water for negative control). The reactions were incubated overnight at 16 ^{o}C.

Following the overnight incubation, 2µL of the ligation reactions were transformed into a vial of One Shot Chemical Competent *E. coli* cells (TOP10; Invitrogen) and incubated on ice for 20 minutes. The reaction was then heat shocked at 42 ^{o}C for 30 seconds and immediately transferred to ice. Two hundred-fifty microliters of SOC Media (Invitrogen) were added to the cells which were then incubated at 37 ^{o}C in the MaxQ* 4450 Benchtop Orbital Shaker (Thermo Scientific) for one hour at 200 rpm. Fifty microliters from each transformation were added to a pre-warmed LB/Ampicillin plates. The plates were incubated overnight at 37 ^{o}C.

Transformed bacterial cell colonies were selected to verify the insertion of the HA-Olfm1 gene into the pcDNA 3.1 vector. To confirm insertion, 1.5µL of 1000x concentrated ampicillin antibiotic (EMDBiosciences), 1.5mL of LB broth (Sigma-Aldrich) and the selected bacteria colony were combined into a glass test tube, using flame sterilization techniques, and incubated overnight at 37^{o} C on the MaxQ* 4450 Benchtop Orbital Shaker (Thermo Scientific) at 200 rpm. Following the incubation, the DNA was purified using a QIAprep Spin Miniprep Kit (QIAGEN), in order to perform the 10µL diagnostic restriction digest reactions to confirm that HA-Olfm1 construct had ligated to the vector. The reaction consisted of: 1µL of 10X Buffer 2 (New England Biolabs), 1µL 10X BSA (New England Biolabs), 0.2µL HindIII/Not I (New England Biolabs), 2.6µL water, and 5µL Miniprep DNA. The diagnostic digest reactions were then electrophoresed on a 1% agrose gel (Bio-Rad Systems) containing SYBR® Safe DNA Gel Stain (Invitrogen) for 60 minutes at 100 volts. A UV transilluminator was used to visualize the DNA constructs and confirm that the HA-Olfm1 gene was indeed cloned into pcDNA 3.1.

Verifying Gene Expression

Western Blot analysis was used to verify that the inserted HA-Olfm1 construct was expressing the HA-Olfm1 protein. This was accomplished by first transfecting the HEK293 cells with the HA-Olfm1 constructs. The transfection protocol was as follows: six microliters of FuGene® (Promega) was added to 94μL of DMEM high glucose media (Gibco) in an Eppendorph tube and incubated for five minutes at room temperature. One microgram of the DNA construct was added and was incubated for 20 minutes at room temp. The transfection mixture was added to a single well of a 12 well dish containing 1mL of roughly 70% confluent HEK293 cells. The positive control, HA-tagged Neuronal Pentatraxtin Receptor (HA-NPR; Anirvan Ghosh Lab, UCSD), was transfected in the same manner as HA-Olfm1. The transfected HEK293 cells were incubated at 37^{o}C with 5% CO_2 for 24-48 hours. After incubation, the transfected cells were denatured by adding 100μL of previously boiled sample buffer (95% Laemmli Sample Buffer (Bio-Rad) 5% β-Mercaptoethanol (GBiosciences)) to each transfected well. The samples were transferred into their own respective Eppendorf tubes and were boiled for five minutes. The denatured samples were loaded into a Mini-PROTEAN® Precast Gel (Bio-Rad) and electrophoresed at 125 Volts in 1X western running buffer (10X Tris/Glycine/SDS buffer; Bio-Rad). The gel was transferred to a nitrocellulose membrane (Bio-Rad) for 60 minutes at 90 Volts using 1X transfer buffer (10X Tris/glysine; Bio-Rad). The membrane was immersed in blocking solution (2% non-fat milk, 1XTTBS (1X TBS, 0.01% Tween 20 (Bio-Rad)) for 30 minutes on a rocker. The membrane was blotted with primary antibody, mouse anti-HA (Covance), at a 1:1000 dilution in blocking solution overnight on a rocker at 4^{o}C. Following the primary antibody incubation, the membrane was washed for three 10-minute periods with 1XTTBS. The membrane was blotted at room temperature with the secondary antibody, goat anti-mouse-HRP (Bio-Rad) using a 1:1000 dilution in blocking solution on a rocker. The membrane was washed for 10-minute periods: twice with 1XTTBS and once with 1XTBS. The

colorimetric system was used to visually quantitate the Western and the steps were as follows (for a single membrane): 9 mL deionized water, one milliliter of 1X HRP color development buffer (Bio-Rad Immun-Blot Assay Kit), 60 µL of HRP color reagent B (Bio-Rad Immun-Blot Assay Kit) and 2mL of HRP color reagent A (Bio-Rad Immun-Blot Assay Kit) were added to the membranes. The membranes were incubated at room temp, on a rocker, for 30 minutes in the dark until the bands became visible. White light transillumination (Gel Logic 1500 Imaging System) was used to image the membranes.

Western blot analysis was also used to detect other proteins. GFP-tagged GluR2 (GluR2-GFP; Anirvan Ghosh Lab, UCSD) was also transfected in HEK293 cells using the same protocol described above. The primary and secondary antibodies used for GluR2-GFP were mouse anti-GFP (Santa Cruz Biotechnology) and goat anti-mouse-HRP (Bio-Rad), respectively. The NT2 Neurons were analyzed for the following endogenously expressed proteins: Olfm1 [primary antibody 1:100 dilution: rabbit anti-Olfm1 (AbCam); secondary 1:1000 dilution: goat anti-rabbit-HRP (Bio-Rad)], Synapsin [primary antibody: rabbit anti-synapsin (Millipore); secondary antibody 1:1000 dilution: goat anti-rabbit-HRP (Bio-Rad)], Tau [primary antibody, 1:1000 dilution: mouse anti-tau (Millipore); secondary antibody 1:1000 dilution: goat anti-mouse-HRP (Bio-Rad)], and GluR2 [primary antibody, 1:500 dilution, mouse anti-GluR2 (Millipore); secondary antibody 1:1000 dilution: goat anti-mouse-HRP (Bio-Rad)].

Immunostaining NT2 Neurons

To visually confirm expression of endogenous neuronal proteins (Tau, Synapsin, and MAP2) and to view their spatial relationship to endogenous Olfm1, an immunostaining procedure was performed on the NT2 neuron cultures. A 12-well NT2 neuron dish plated specifically for immunostaining (see Maintaining Cell Lines), was first fixed with 4% Paraformaldehyde (PFA; Sigma) for 15 minutes (500 µL/well). The fixed cell coverslips were then washed three times for

five minutes each with 1X phosphate buffered saline (PBS) pH 7.4 (Gibco). Immunostaining blocking buffer (PBS (Gibco), 3% BSA (Calbiochem), 0.1% TritonX-100(Roche)) was added to the wells and incubated on a rocker at room temperature for 20 minutes. Primary antibodies [rabbit anti-Olfm1 (AbCam) 1:50 dilution, mouse anti-tau (Millipore) 1:1000 dilution, rabbit anti-synapsin (Millipore) 1:1000 dilution, chicken anti-MAP2 (Millipore) 1:5000 dilution] were added in immunostaining blocking buffer to their respective wells and were incubated on a rocker at room temperature for two hours. The cells were washed three times for five-minute periods with immunostaining blocking buffer. The light sensitive secondary antibodies [Alexa Flour® 488 goat anti-rabbit (Invitrogen), Alexa Flour® 488 goat anti-mouse (Invitrogen), Alexa Flour® 488 goat anti-rabbit (Invitrogen), Alexa Flour® 543 goat anti-rabbit (Invitrogen)] were diluted in immunostaining blocking buffer (1:1000) and incubated in the dark for 60 minutes on a rocker at room temperature. The cells were washed twice for five-minute periods using immunostaining-blocking buffer and once with 1X PBS (Gibco) in addition to DAPI/Hoeschst stain. The coverslips were mounted using Flouromount G (SouthernBiotech) onto microscope slides (Thermo Scientific).

Co-immunopreciptiation

In order to accept or reject my hypothesis suggesting that Olfm1 plays a role in the trafficking of GluR2-containg AMPARs, a co-immunoprecipitation (CoIP) protocol was followed. HEK293 cells were first plated into a 12 well dish and left in the incubator at 37^{o}C overnight. Following the overnight incubation, the 3 plates were transfected with the following proteins: (1) Experimental: 1µg of HA-Olfm1 + 1µg of GluR2-GFP; (2) Negative Control: 1µg of HA-Olfm1 + 1µg of GluR2-GFP; (3) Positive Control: 1µg of Mycllrtm2 + 1µg of GluR2-GFP. The transfected HEK293 cells were left in the incubator at 37^{o}C for 24 hours. The antibody-bound protein A/G bead (Santa Cruz Biotechnology) complex was prepared by adding 500µL RIPA Buffer (10X RIPA (Cell Signaling Tech), 9mL of nuclease free water and 100µL Protease

Inhibitor Cocktail solution (Cell Signaling Tech)), 25µL of protein A/G beads and 1µg of antibody per CoIP experiment to an Eppendorf tube on ice. The two antibodies used were GFP (experimental) and IgG (negative control). The beads were incubated with rotation at 4^{o}C for 45 minutes. The beads were then centrifuged for 2 minutes/2,000 rpm/room temperature. The buffer was removed and 500µL of RIPA buffer was added to each Eppendorf tube and mixed gently. The beads were centrifuged again following the same criteria as the first spin. Following the second spin, the buffer was removed and enough RIPA was added to the beads so that each CoIP sample would receive 25µL of antibody bead solution. The antibody-bound beads were kept on ice until it was ready to be used.

After the 24-hour transfection period, the cells were put on a flat bed of ice. The media was removed from the plates and briefly washed with ice-cold 1X PBS. Five hundred microliters of RIPA buffer were added to each dish and left to incubate at 4^{o}C for 40 minutes. Following the 40-minute incubation, the cells were collected into Eppendorf tubes and centrifuged for 20-minutes/13,000 rpm/4^{o}C. The cell lysates were then transferred into pre-chilled Eppendorf tubes on ice (carefully in order to not disturb the cell pellet). Twenty-five microliters of the cell lysate was transferred into separate Eppendorf tubes. These were the input samples (making sure our protein had been transfected into the cell). Twenty-five microliters of the prepared antibody A/G beads (either GFP or IgG) were added to the remaining cell lysates and were rotated overnight at 4^{o}C.

Following the overnight incubation, the samples were centrifuged for 1 minute/1,000 rpm/4^{o}C. The supernatant was removed carefully in order not to disturb the antibody-protein bead complex. Three hundred microliters of RIPA buffer were added to the beads and mixed gently. It was once again centrifuged, using the same criteria as above. This step was repeated once using RIPA buffer and again using ice-cold 1X PBS. After the final wash, as much of the PBS was removed as possible without disturbing the antibody-protein bead complex. Twenty five

microliters of 2X Sample Buffer (950µL Laemlli, 50µL β-Mercaptoethanol) was added to each Eppendorf tube (including the input samples) and boiled for 5 minutes. The denatured protein samples were immediately processed for western blot analysis (Note: Can be stored at -20^{o}C for future analysis).

Results

Gene Cloning and Protein Expression in HEK293 cells

In order to test my hypothesis that Olfm1 plays a role in the trafficking of Glu-R2 containing AMPARs, my first objective was to clone the C-terminal HA-tagged Olfm1 construct into a mammalian expression vector using the following methods: PCR amplification, ligation and transformation into the pcDNA3.1 vector and finally analysis using diagnostic digest and Western Blot protocols. The PCR amplification of the HA-Olfm1 construct obtained from Open BioSystems was successful (Figure 1). Ligation and transformation of the PCR amplified HA-Olfm1 into the mammalian expression vector, pcDNA3.1, was validated by diagnostic digest (Figure 2).

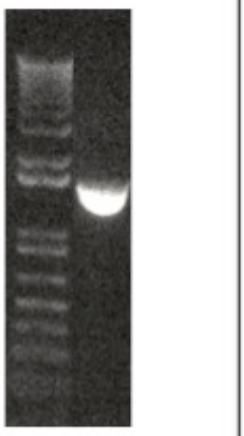

Figure 1. PCR Amplification of C-terminal HA-Olfm1. Lane 1 Trackit Kb Plus DNA Ladder (Invitrogen), Lane 2 C-terminal HA-Olfm1. Lane 2 band is approximately at 1403 nucleotides, which correctly corresponds with the HA-Olfm1 gene location.

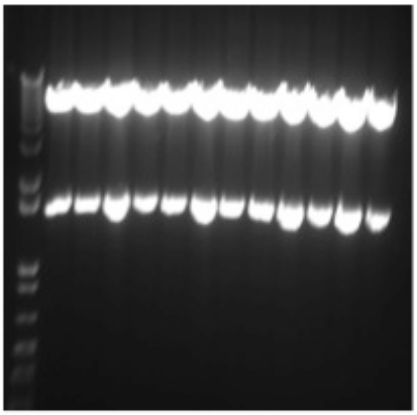

Figure 2. Diagnostic Digest with HindIII/Not1 restriction enzymes. Lane 1 Trackit Kb DNA ladder (Invitrogen), Lane 2-13 transformed HA-Olfm1 into the pcDNA3.1 vector. All 12-transformation colonies contained the HA-Olfm1 construct as seen by the correct corresponding band at 1403 nucleotides. This indicates that the HA-Olfm1 construct successfully ligated into the vector.

Western blot analysis demonstrated that the cloned constructs were expressing the HA-Olfm1 protein in transfected HEK293 cells at its respective band of 55 kDa–75 kDa (Figure 3). GluR2 was also successfully transfected into HEK293 cells and analyzed via western blot (Figure 4).

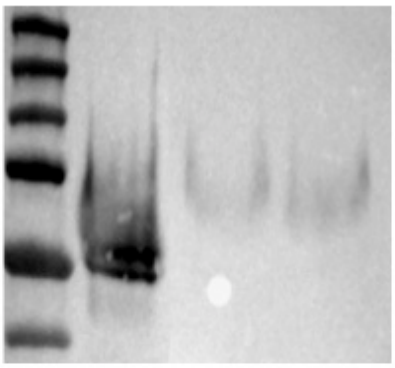

Figure 3. Western Blot Analysis on HA-Olfm1. Lane 1 Ladder (Precision Plus Protein™ Dual Color Standards Bio-Rad), Lane 2 HA-NPR (positive control), Lane 3-4 HA-Olfm1 protein. Mouse anti-HA primary antibody and goat anti-mouse-HRP secondary antibody were used to visualize binding via the colorimetric system.

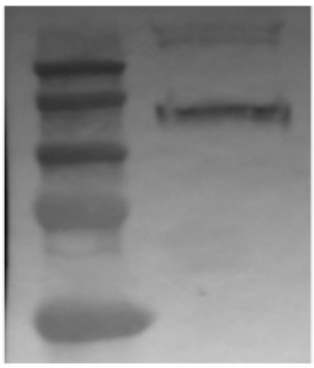

Figure 4. Western Blot Analysis of GluR2 in HEK293 cells. Lane 1 Ladder (Precision Plus Protein™ Dual Color Standards Bio-Rad), Lane 2 GluR2. GluR2 can be visualized at its appropriate band, 102 kDa.

Verifying Endogenous Expression in NT2 Neurons

The next objective was to verify endogenous expression of Olfm1 and GluR2, in addition to endogenously expressed presynaptic markers for synapses and axonal microtubule formation: Synapsin and Tau. Western blot analysis on the NT2 neuron cultures showed endogenous expression of Olfm1, Synapsin and Tau (Figure 5), but did not show endogenous expression of GluR2.

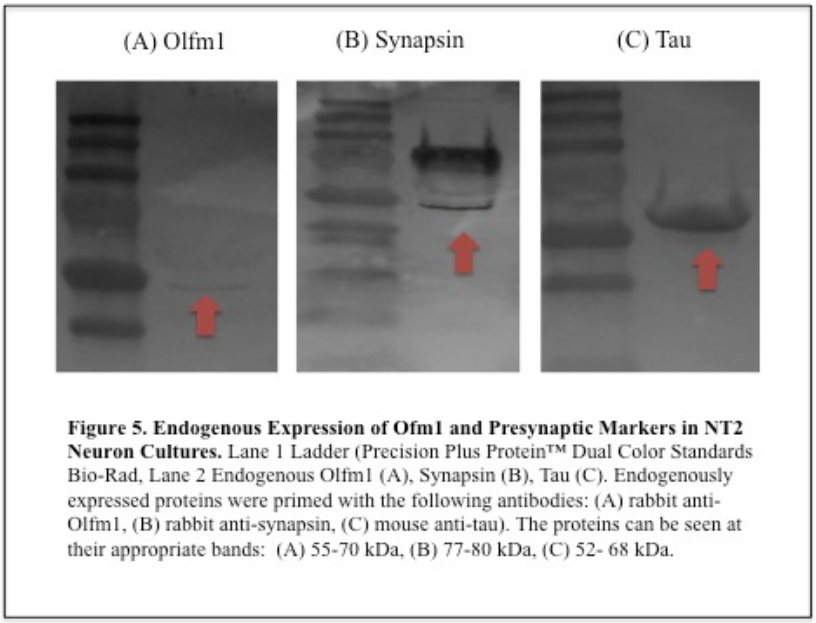

Figure 5. Endogenous Expression of Ofm1 and Presynaptic Markers in NT2 Neuron Cultures. Lane 1 Ladder (Precision Plus Protein™ Dual Color Standards Bio-Rad, Lane 2 Endogenous Olfm1 (A), Synapsin (B), Tau (C). Endogenously expressed proteins were primed with the following antibodies: (A) rabbit anti-Olfm1, (B) rabbit anti-synapsin, (C) mouse anti-tau). The proteins can be seen at their appropriate bands: (A) 55-70 kDa, (B) 77-80 kDa, (C) 52- 68 kDa.

Immunostaining NT2 Neuron Results

Immunostaining visually showed the expression of endogenous neuronal proteins and their spatial relationship with endogenously expressed Olfm1 (Figure 6).

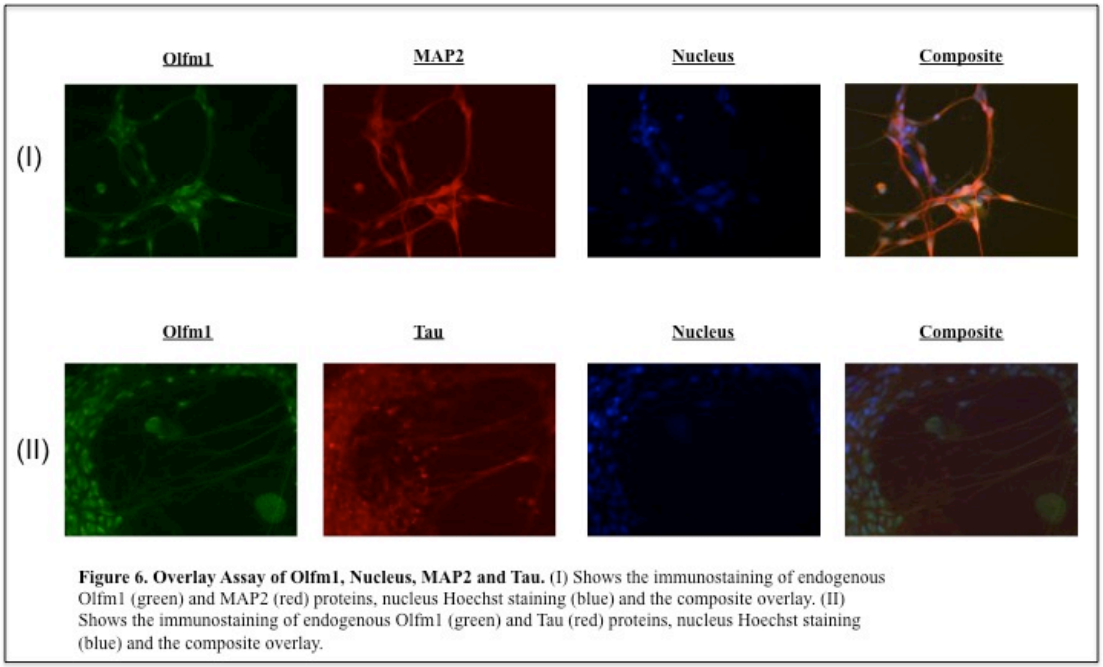

Figure 6. Overlay Assay of Olfm1, Nucleus, MAP2 and Tau. (I) Shows the immunostaining of endogenous Olfm1 (green) and MAP2 (red) proteins, nucleus Hoechst staining (blue) and the composite overlay. (II) Shows the immunostaining of endogenous Olfm1 (green) and Tau (red) proteins, nucleus Hoechst staining (blue) and the composite overlay.

Co-immunoprecipitation

The results for the CoIP remain inconclusive at this point in time and the protocol is currently being improved. The second objective was to use western blot analysis to verify the expression of HA-Olfm1 and GluR2-GFP in HEK293 cells and the endogenous expression of Olfm1, GluR2, Tau and Synapsin in NT2 neuron cultures.

Discussion

For my research project, the first objective was to clone HA-tagged Olfm1 into the mammalian expression vector, pcDNA3.1. This was followed by the verification of the expression of HA-Olfm1 and GluR2-GFP in HEK293 cells and the endogenous expression of Olfm1, GluR2, Tau and Synapsin in NT2 neuron cultures using western blot analysis. In addition, Olfm1 localization in NT2 neurons was assessed using an immunostaining protocol. Lastly, a CoIP protocol was to test the potential interaction of Olfm1 and GluR2.

The first objective was to clone HA-tagged Olfm1 into pcDNA3.1. The HA-tag was inserted into the Olfm1 construct so visual expression of the protein could be observed, since an Olfm1 specific antibody was not available at the time. The HA-tag does not interfere with protein expression and its antibody (monoclonal antibody HA.11) is extremely specific (Covance). The Kozak consensus sequence was also included into the Olfm1 construct to ensure translation initiation and efficiency (Life Technologies). Using this protocol, the diagnostic digest showed consistent positive results that the Olfm1 gene had indeed been successfully cloned into the mammalian expression vector, pcDNA3.1 (Figure 2).

The second objective was to use western blot analysis to verify the expression of HA-Olfm1 and GluR2-GFP in HEK293 cells and the endogenous expression of Olfm1, GluR2, Tau and Synapsin in NT2 neuron cultures. HA-Olfm1 and GluR2-GFP both showed protein expression in the transfected HEK293 cells. These successful western blot analyses were essential in order to move forward with the CoIP experiment because it demonstrated that both proteins

could be expressed in HEK293 cells. Western blot analyses showing the endogenous expression of Olfm1, Tau and Synapsin were successful (Figure 5), demonstrating that NT2 neuron cultures express markers of mature neurons (Tau and Synapsin) and my candidate protein, Olfm1. Unfortunately we could not verify endogenous expression on GluR2 in NT2 neurons, but this does not mean that GluR2 is not present. Problems may have arisen from the GluR2 antibody not working properly, or from our not using enough of the antibody to detect expression of GluR2. The GluR2 antibody is very expensive and we did not have enough funds to continually restock this antibody during the trial and error parts of the experiment. However, it is important that we verify endogenous GluR2 expression in the NT2 neuronal cells, so that CoIPs can be performed in this cell line.

In addition to determining whether Olfm1 was being expressed by NT2 neurons, I also wanted to see where Olfm1 was localizing in the neurons. From the immunostaining and overlay images, it seems that Olfm1 is localizing near or in the nucleus (Figure 6). It is difficult to explain why it is localizing there and there are published accounts of its localization within the cell (Uniprot, 2015). More experiments need to be performed in order to confirm the nuclear localization of Olfm1 and its function.

In order to verify that Olfm1 interacts with GluR2, a CoIP protocol was used. Unfortunately, the CoIP experiments I have carried out to date have not been successful and I am currently troubleshooting the procedure. In the future, the Pierce Antibody Clean up Kit (Thermoscientific) and Pierce Co-Immunoprecipitation Kit (Thermoscientific) will be used in the process of obtaining results. Once consistent results are gathered, knock-down and overexpression experiments of Olfm1 will be performed in the NT2 neuronal cell line. These experiments will assess how either the absence or overexpression of Olfm1 impacts GluR2 expression at the synapse. Until then, I can neither accept nor reject my hypothesis that Olfm1 plays a role in the trafficking of GluR2-containg AMPARs.

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