Exploring the Role of Olfm1 in the Trafficking of GluR2- containing AMPA Receptors

Honors Thesis

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Abstract

The interactions between post-synaptic receptors and pre-synaptic molecules are responsible for coordinating numerous human activities such as thought, behavior, memory, learning and homeostasis. Important excitatory post-synaptic receptors that alter strength of connections between pre- and post-synaptic neurons are α-Amino-3-hydroxy-5-Methylisoxazole-4-Propanoic Acid (AMPA) receptors (AMPARs). Understanding the molecular mechanisms that govern AMPAR trafficking to synapses and effects on synaptic plasticity are critical to understanding disease and normal neurological processes. The purpose of this research was to identify GluR2-interacting proteins that play important roles in AMPAR trafficking and to characterize the functional significance of AMPARs. The transmembrane protein Olfactomedin 1 (Olfm1) isoform 1, also called Noelin 1, was studied to determine the role that it plays in AMPAR trafficking at synapses. Unfortunately, cloning complications prevented a definitive conclusion on the nature of Olfm1's role in AMPAR trafficking.

Introduction

General Background

The brain is one of the most complex and least understood organs in the human body (Brenner and Sejnowski, 2011). Therefore, analysis on the brain and its function has become the holy grail of molecular biology research (Abbott, 2013). The human brain is comprised of billions of cells called neurons. Neurons communicate at specialized structures called synapses in a process termed synaptic transmission. Each synapse is made up of at least two neurons, one of which, the pre-synaptic neuron, sends information, and the other, post-synaptic neuron, receives the information. A synapse is

formed when the axon of a pre-synaptic neuron contacts the dendrites of the post-synaptic neuron (Campbell, 2010).

Although the transmission of information in the brain is attained through electrical activity, the actual communication between neurons is achieved through chemical signaling. When an action potential reaches a synapse, calcium channels in the neuronal membrane are opened which increases the calcium ion concentration in the pre-synaptic terminal. This allows chemical neurotransmitters to be released into the synaptic cleft. These neurotransmitters diffuse across the synaptic cleft to protein receptors that are embedded in the membrane of the post-synaptic neuron. Neurotransmission can either increase the possibility of the post-synaptic neuron firing an action potential and is considered excitatory or it can reduce the likelihood of an action potential being generated and is considered inhibitory. The interactions between post-synaptic receptors and neurotransmitters are responsible for coordinating numerous human activities such as thought, behavior, memory, learning and homeostasis (Campbell, 2010).

AMPAR Background

A well-known example of an excitatory post-synaptic receptor with rapid kinetics is the α-Amino-3-hydroxy-5-Methylisoxazole-4-Propanoic Acid receptor (AMPAR; Greger and Esteban, 2007). AMPARs are just one of the three kinds of ionotropic glutamate receptors that are present at a majority of synapses, the other two being N-Methyl-D-Aspartate (NMDA) receptors and kainite receptors (Esteban, 2008). Soon after birth in mammals most of the ionotropic glutamate receptors at excitatory synapses are NMDARs (NMDA receptors). It is not until further postnatal development that AMPARs become more prevalent at excitatory synapses (Esteban, 2008). AMPARs bind the amino

acid neurotransmitter glutamate (Keifer and Zheng, 2010). The binding of glutamate to AMPARs results in an increased excitation of the postsynaptic neuron. Furthermore, AMPARs play a key role in regulating synaptic plasticity, a term used in neuroscience to describe the phenomenon of altering, whether increasing or decreasing, synaptic strength, over time (Malinow and Malenka, 2002).

Synaptic plasticity can be modified at the pre or post-synaptic neuron by altering the effectiveness of neurotransmitter release by the presynaptic neuron or modifying the number, type or characteristics of post-synaptic receptors. Both are effective means of altering synaptic plasticity (Anggono, 2012). Long-Term Potentiation (LTP) is a phenomenon that occurs when synapses are stabilized over an extended period of time and the synaptic strength increases (Hall and Ghosh, 2008). In contrast, when the efficacies of synapses are decreased, the synaptic strength is also decreased and is referred to as Long-Term Depression (LTD; Hall and Ghosh, 2008). In general, LTP requires increases in AMPAR function at synapses while LTD involves the removal of synaptic AMPARs by endocytosis (Shepherd and Huganirl, 2007).

Synaptic plasticity and the ability to alter synaptic strength are believed to be at the foundation of learning and memory (Keifer and Zheng, 2010). Along with influencing learning and memory, synaptic plasticity is crucial for neural development (Abbott and Nelson, 2000). Therefore, modifications in synaptic plasticity have been associated with neurological disease processes such as Alzheimer's disease, Schizophrenia and Down syndrome (Esteban, 2008). In fact, Alzheimer's disease involves the suppression of AMPAR trafficking to synapses leading to cognitive decline and memory deficits (Keifer and Zheng, 2010). Therefore, understanding the molecular mechanisms that govern

AMPAR trafficking to synapses and effects on synaptic plasticity are critical to understanding disease and normal neurological processes.

AMPARs consist of the following four protein subunits: GluR1 (GluA1, GRIA1), GluR2 (GluA2, GRIA2), GluR3 (GluA3, GRIA3), and GluR4 (GluA4, GRIA4). The four subunits have very similar extra-cellular and transmembrane regions, but differ significantly with respect to their unique intracellular C-termini (Anggono and Huganir, 2012). Furthermore, the mRNA of each subunit can be alternatively spliced to create longer or shorter C-terminal domains that distinguish an unmodified GluR2 subunit from a GluR2 subunit that has a long C-terminal domain (GluA2L; Anggono and Huganir, 2012). These subunits combine to form a GluR2 heterodimers (Mayer, 2005). The preferential incorporation of GluR2 into the heterotetrameric proteins causes the proteins containing this subunit to have different physical properties such as impermeability to Ca^{2+} (Anggono and Huganir, 2012).

The subunit composition of AMPARs plays a critical role in when and how AMPARs are trafficked (Greger and Esteban, 2007). For instance, the composition of AMPARS at the synapse varies depending on the stage of development. Specifically, early in postnatal development GluR4-containing receptors are delivered to the synapse. At around two weeks, there is a turnover and GluR4-containing receptors are replaced by GluR2-containing receptors (Esteban, 2008). Furthermore, AMPAR trafficking can be constitutive or activity dependent. In constitutive trafficking, there is the continuous cycling of GluR2-GluR3 containing AMPARs at synapses that is independent of synaptic activity. This mechanism is responsible for maintaining synaptic strength (Malinow and Malenka, 2002). The alternative trafficking pathway is activity dependent and occurs

during synaptic plasticity. The subunits involved in this process include, GluR1, GluR2L and GluR4 (Greger and Esteban, 2007). The regulated delivery of AMPARs to synapses occurs during LTP and results in an overall increase the amount of synaptic AMPARs (Greger and Esteban, 2007). Although both activity-dependent and constitutive trafficking are important mechanisms for the delivery of AMPARs to synapses, very little is known about the intracellular components that control these processes (Greger and Esteban, 2007).

A majority of AMPARs undergo synthesis in the endoplasmic reticulum and are glycosylated in the Golgi located within the soma (Shepherd and Huganirl, 2007). Once synthesized, AMPARs must be transported from the cell body to dendritic spines, the location of most excitatory synapses (Esteban, 2008). Therefore, the molecular mechanisms that are involved in this long distance transport of AMPARs to synapses have been of interest to our lab and other researchers. For example, it has been hypothesized that transport of AMPARs occurs through the ATP-dependent activity of kinesin and dynein motor proteins, both of which transport AMPARs along microtubules (Shepherd and Huganirl, 2007). However, a microtubular cytoskeleton is absent in dendritic spines. Therefore, organelles containing AMPARs eventually reach the end of the microtubular track and must transfer the AMPARs to a cytoskeleton composed of actin in order for the AMPARs to be deposited into synapses (Esteban, 2008). The exact molecular mechanisms that facilitate the movement of AMPARS along the actin-cytoskeleton remain elusive although scaffolding proteins such as 4.1N and RIL have been found to be key players in lateral diffusion (Esteban, 2008).

Transmembrane proteins known to associate with AMPARs and regulate their surface expression are scarce in the literature. Transmembrane AMPAR Regulatory Proteins (TARPs) are required for the surface expression of AMPARs. Stargazin, a protein primarily expressed in the cerebellum, was the first TARP to be identified and since then there have only been four other transmembrane proteins that affect the cell surface expression of AMPARs, including γ-3, γ-4, γ-7 and γ-8 (Esteban, 2008). Transmembrane AMPAR regulatory proteins play two critical roles in the surface expression of AMPARs: 1) altering the expression of TARPs change AMPAR expression at the extra-synaptic surface in granule cells and 2) TARPs help link Membrane-Associated GUanylate Kinase (MAGUK) scaffolding proteins to AMPARs at synapses thereby improving their stability (Esteban, 2008).

Other proteins that interact with AMPARs include two homologous Glutamate Receptor Interacting Proteins (GRIPs) GRIP1 and GRIP2 (Anggono and Huganir, 2012). GRIP1 and GRIP2 interact with the C-terminal domains of GluR2/3 subunits and target AMPARs to synapses. Although it has been demonstrated that GRIP1 and GRIP2 are vitally important for several forms of synaptic plasticity, the mechanisms by which they regulate AMPAR membrane trafficking remains elusive (Anggono and Huganir, 2012). Another protein that associates directly with the C-terminal domains of GluA2/3 subunits is Protein Interacting with C-Kinase 1 (PICK1) (Anggono and Huganir, 2012). PICK1 is required for hippocampal and cerebellar LTD through its involvement in the endocytosis of GluR2-containing AMPARs (Makuck *et al.*, 2011). Although this is not an exhaustive list of all the proteins involved in the highly coordinated trafficking of AMPARs at the

synapse, it does serve as a starting place for further investigation of other proteins that associate with AMPARs during trafficking (Anggono and Huganir, 2012).

The AMPARs containing GluR1/2 subunits are the most prevalent in hippocampal pyramidal neurons followed by AMPARs containing GluR2/3 (Anggono and Huganir, 2012). Therefore, the focus of my research was to identify GluR2-interacting proteins that play an important role in AMPAR trafficking and to characterize the functional significance of AMPARs. As mentioned above, the trafficking of AMPARs is important for synaptic plasticity which influences important aspects of human physiology including learning, memory, and various disease states that impair proper cognitive functioning (Keifer and Zheng, 2010).

Overview of Project Aims

The primary purpose of my research was to identify and characterize novel proteins that regulate the stability of synapses in the brain and the trafficking of AMPARs containing the GluR2 subunit. Our lab examined candidates that were potentially novel-GluR2 interacting proteins involved in AMPAR trafficking at the synapse by thoroughly examining a GluR2 interactome screen that Dr. Stefanie Otto-Hitt performed in Dr. Anirvan Ghosh's lab at UC San Diego (unpublished data; in collaboration with Dr. Jeff Savas and Natalie Shanks, UCSD). Using mass spectrometry, 420 proteins that had a potential interaction with GluR2 were identified. Public databases such as UniProt and PubMed were used to find proteins whose structure and cellular location (i.e. expression in the nervous system) provided evidence of a potential interaction with GluR2 and therefore could be involved in AMPAR trafficking at synapses. Furthermore, proteins

with known associations with AMPARs, such as Stargazin (Chen *et al.*, 2000), Cornichon (Schwenk *et al.*, 2009), and CAMPK44 (von Engelhardt *et al.*, 2012) indicated that the mass spectrometry experiment was successful and would likely lead to the discovery of other novel proteins that interact with AMPARs.

Of the 420 potential candidates, I hypothesized that the transmembrane protein Olfactomedin 1 (Olfm1) isoform 1, also called Noelin 1, plays a role in AMPAR trafficking at synapses. The gene for Olfm1 encodes a 485 amino acid glycoprotein and is found on human chromosome 9 (UniProt, 2013). The exact function Olfm1 is yet to be discovered; however, it is expressed abundantly in the brain (UniProt, 2013). Functionally, Olfm1 has been proposed to control the production of neural crest cells by the neural tube and may be peripherally associated with the AMPAR complex, although this claim is not based on experimental findings (UniProt, 2013). Olfm1promotes neuronal growth after axon damage in zebrafish (*Denio rerio)* through the interaction with the Nogo A receptor (NgR1) complex (Nakaya *et al.*, 2012) and also serves as a modulator of Wnt signaling in zebrafish (Nakaya *et al.*, 2008). Furthermore, in The Association for Research in Vision and Ophthalmology's (ARVO) 2013 Annual Meeting Abstracts, it was revealed that the GluR2 subunit of AMPARs was expressed in and co-localized with Olfm1in the Retinal Ganglion Cells (RGCs) of mice (Unpublished data, http://www.arvo.org/webs/am2013/abstract/sessions/124.pdf). Even though the exact nature of the interaction was not elaborated on, this finding did provide further precedence for the study of Olfm1 in AMPAR trafficking. The discovery of an interaction between Olfm1and GluR2 subunits in AMPARs would be significant, because this protein would likely be involved in AMPAR trafficking and regulating synaptic

plasticity, processes known to influence learning, memory and cognitive functioning (Anggono and Huganir, 2012).

To test my hypothesis that Olfm1plays a role in AMPAR trafficking at synapses, three experimental approaches were taken. The first included the co-transfection of an HA-tagged Olfm1 construct with GluR2 and GluR1 in the Human Embryonic Kidney 293 (HEK293) cells. The GluR2 and GluR1 subunits dimerize in HEK293 cells to form a heterotetrameric protein. Through the use of immunohistochemistry, Olfm1's interaction with AMPARs could be validated. The second experimental approach involves designing an siRNA against Olfm1to knockdown its expression and determine if this plays a role in the expression of GluR2 on the cell surface. The final experimental approach that investigates Olfm1's effects on GluR2 trafficking includes the overexpression of Olfm1in NT2 neuron cultures to determine if increasing the expression of Olfm1directly correlates to the increased expression of GluR2 at synapses (Otto-Hitt, 2013).

Although elucidation of the above aims would be a tremendous feat, further experiments would need to be performed at the organismal level. Combining the nematode, *Caenorhabditis elegans (C. elegans)*, as a model organism along with Olfm1gene, knockdown experiments can be performed to determine the effects Olfm1 has on AMPAR trafficking *in vivo.* Furthermore, any behavioral changes that result from the knockdown of Olfm1 can be studied using techniques that involve chemosensory processes (Hart *et al*., 1995) and nose touch responses in the *C. elegans* (Maricq *et al.*, 1995). The experiments performed at the organismal level are crucial, because they will allow us to determine if Olfm1knockdown in the worms via RNAi has similar effects on AMPAR trafficking in the siRNA experiments in cell culture.

Although I was unable to successfully clone the HA-tagged Olfm1construct, I did succeed in establishing key experimental protocols that will be valuable to the advancement of this project and the elucidation of Olfm1's role in GluR2 trafficking. For example, I succeeded in helping to establish infallible protocols for Western Blot, Co-immunoprecipitation, cell culture transfection and co-transfection, and NT2 Neuron/HEK293 cell co-culture synapse assays. Furthermore, I played a pivotal role in identifying two additional novel protein candidates, Adcy2 and Cpt1c, that may also play important roles in AMPAR trafficking.

Materials and Methods

Cell Lines and *C. elegans* strains

Human Embryonic Kidney 293 (HEK293) cells were one of the two cell lines used in my experimental procedures (ATCC Catalog No. CRL-1573). This is a well-established cell line that was cultured by transforming HEK primary cell cultures with sheared pieces of Adenovirus 5 (AD5) DNA (Graham and Smiley, 1977). HEK293 cells were suitable for my research purposes because they can be easily transfected and harvested to determine the expression of proteins (Graham and Smiley, 1977). Furthermore, Shaw *et al.* (2002) demonstrated that HEK293 shares similarities with neurons including the production of neurofilament (NF) subunits NF-L, NF-M, NF-H, and α-internexin.

The second cell line used was NTERA-2 cl.D1 (NT2) cells (ATCC Catalog No. CRL-1973). This is a pluripotent teratocarcinoma cell line that can be differentiated into

neurons by treating the cells with retinoic acid (Paquet-Durand *et al.*, 2003). However, this is a lengthy process that can take anywhere from 42 to 54 days (Paquet-Durand *et al.*, 2003). Therefore, I used an alternative method that produced post-mitotic NT2 neurons in as few as 24 to 28 days (Paquet-Durand *et al.*, 2003). The fully differentiated NT2 neurons express proteins that are critical for neurogenesis including Microtubule-Associated Protein 2 (MAP2) and Tau proteins (Zhang and Dong, 2012). Additionally, these cells express glutamate receptors, including AMPARs, the focus of my research (Paquet-Durand *et al.*, 2003).

The *C. elegans* wild-type strain and a culture kit were obtained from Carolina Biological Supply.

Maintaining Cell Lines and *C. elegans*

The HEK293 cells were examined under an inverted light microscope (Zeiss Primo Vert) to detect the presence of any bacterial contamination before cell passage. The culture medium (88% DMEM high glucose media, 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin, and 1% Glutamax; Gibco) was aspirated off and 0.5 ml of trypsin-0.53 mM EDTA (Gibco) was added to the 10 cm dish. Next, 10 ml of new culture medium was added and the cells were mechanically dislodged by pipetting up and down. Depending on the desired density, the appropriate volume of cells was transferred to 10 cm culture dishes containing fresh culture medium. The culture dishes were incubated at 37°C with 5% CO_2.

The NT2 cells were maintained by Dr. Stefanie Otto-Hitt in complete growth media (90% DMEM, 10% FBS) in an undifferentiated state as an adherent culture on 10 cm tissue culture grade dishes. Each week the cells were passaged to maintain an

undifferentiated stock. The old culture media was aspirated from the dishes and was replaced with 10 ml of fresh culture media. Cells were then mechanically dislodged from the dish with a cell scraper and resuspended. One ml of the cell solution was added to the tissue culture dishes containing 10 ml of complete media. The undifferentiated cultures were maintained at 37°C with 5% CO_2.

To generate neurons from the undifferentiated cell stocks, NT2 cells were first passaged as described above, but instead of being plated on tissue culture grade dishes they were plated as suspension cultures in petri dishes. Twenty four hours after plating, the cells were collected from petri dishes and added to 50 ml conical tubes. The cells were centrifuged for 5 min at 200g using a Sorvall Legend RT Centrifuge (Thermo Electron Corporation). After centrifugation, the media was removed and replaced with complete media supplemented with 1% 10μM retinoic acid (Sigma). The cells were maintained as a suspension for two weeks with media changes performed every other day.

After two weeks, the suspension cultures were plated on 12 well tissue culture dishes for Western Blot and Immunostaining analysis.

The 12 well tissue culture dishes were coated with Poly-D-Lysine (PDL, Millipore) and Matrigel (BD Biosciences) following the manufacturer's protocol. Poly-D-Lysine was added to the wells and incubated for 30 min at 37°C after which the wells were washed three times with sterile tissue culture water. Following the removal of the last wash, the wells were treated with Matrigel solution for 30 min at 37°C. The Matrigel solution was removed from the wells and the 12 well dishes were dried at room temperature in the tissue culture hood for 15 min before plating the NT2 cells. Prior to

the coating procedure above, glass coverslips (18mm, Neuvitro) were added to each well so the cells could be analyzed by immunostaining. After coating the dishes, the two week old NT2 cell suspensions were centrifuged for 5 min at 200g and the media was aspirated off. The remaining cell pellet was resuspended in complete growth media supplemented with 1% 10μM retinoic acid (Sigma) and mitotic inhibitors (1μM Cytrabine, 10μM Floxuridine, and 10μM Uridine; Sigma) and were plated at varying densities on the pre-coated 12 well dishes. The differentiating cultures were maintained for another 2 weeks as an adherent culture with media changes performed every other day.

The protocols found in the Carolina culture kit were used to maintain the wild type *C. elegans* stock cultures. The various aspects of maintaining the worms included: medium preparation, *Escherichia coli (E. coli)* food stock preparation, adding the *E. Coli* food source, and inoculating the seeded plate with *C. elegans.* To prepare the medium, the Nematode growth agar (Carolina) was melted in a boiling water bath for approximately 30 min. The bottle was cooled to approximately 45°C and the bench top was disinfected with 70% ethanol. Using aseptic technique, agar was poured into five petri dishes after flaming the mouth of the agar bottle. The petri dishes sat on the bench top to solidify while the *E. coli* stock culture was prepared. Two nutrient broth tubes containing 5 ml of growth medium were inoculated with 0.2 ml of *E. coli* using sterile technique. The cultures were incubated at 37°C for 24 hours. The first culture was used as an original stock for future cultures of *C. elegans* and the second stock was used for the initial culturing of the *C. elegans.* After the agar petri dishes were poured and the *E. coli* stock culture was prepared, 1 ml of the *E. coli* inoculum culture was spread on the nematode growth agar using aseptic technique.

Before I inoculated the seeded plate with *C. elegans*, the original *C. elegan* stock plate was observed under a dissecting microscope (Swift M28 Zoom Stereo) to find an area with a high worm density. Using aseptic technique, the agar was cut into 1 cm^3 blocks that contained the *C. elegans.* The block was transferred face down to the petri dish that contained the *E. coli* food source. The worms were kept at room temperature, approximately 20°C, and their life cycle was observed as the *C. elegans* developed from larvae to adults in three days. Eventually, the plates would become saturated with reproductively mature worms and were transferred to new agar plates containing *E. coli* as the feeder host using aseptic technique (Carolina).

Approaches to Cloning Olfm1into Mammalian Expression Vectors

Gene cloning

Investigation into the interaction between GluR2 and Olfm1began with the cloning of an HA-tagged Olfm1construct into a mammalian expression vector that would allow for the production of Olfm1protein in cell culture. Four methods of cloning were attempted: 1) amplifying Olfm1from a cDNA library and cloning into the pcDNA 3.1 expression vector; 2) amplifying Olfm1from an OpenBiosystems clone using SuperTaq; 3) amplifying Olfm1from an OpenBiosystems clone using iProof and including the Kozak sequence; 4) blunt-end cloning Olfm1into the pRK5 mammalian expression vector.

I first attempted to isolate Olfm1's cDNA using reverse transcription from human whole brain RNA (Ambion). Two reactions were set-up; the first was the reverse transcript reaction and the second served as a negative control. In both PCR tubes 1µg of total RNA (Ambion) was added to 2 µl of Oligo(dT) (Ambion) and 9 µl nuclease-free

water (Ambion). The PCR tube was heated at 75°C for 3 minutes and then placed on ice. After being placed on ice, the remaining reverse transcript components were added to each reaction tube: 2 µl 10x Reverse Transcript buffer (Ambion), 4 µl dNTP mix (Ambion), and 1µl RNase inhibitor (Ambion). Additionally, the reverse transcript reaction was supplemented with 1 µl MMLV-RT+ (Ambion) while the negative control reaction was supplemented with 1 µl nuclease free water (Ambion). The PCR tubes were incubated for one hour at 44°C, followed by a 10 min incubation at 92°C to activate the Reverse Transcriptase enzyme.

After cDNA was generated using the reverse transcription procedure, a Polymerase Chain Reaction (PCR) was performed to generate an N- and C-terminal human influenza HemAgglutinin (HA) tag construct for cloning into the pcDNA 3.1 (-) vector (Invitrogen). An HA epitope tag was used to create recombinant Olfm1 protein that can be recognized by an antibody specific to the HA tag. This recognition would allow for the verification of expression of Olfm1 protein in cell culture. The HA tag consists of the following nucleotides: 5' TAT CCA TAT GAC GTT CCA GAT TAC GCT 3'. Olfm1 is a transmembrane protein and therefore the N-terminal tag had to be inserted after the signal peptide that is cleaved during post-translational processing. To generate the Olfm1 N-terminal HA tag construct (HA-Olfm1), four different primers were designed and ordered from Integrated DNA Technologies (IDT):

Primer 1- 5'*AAA* GCG GCC GCA **ATG CCA GGT CGT TGG AGG TGG** 3'
Primer 2- 5' *AAA* AAG CTT CTA CAA CTC GTC GGA GCG CAT GAC 3'
Primer 3- 5' TAT CCA TAT GAC GTT CCA GAT TAC GCT CTC CTC AGC CTC CTC TTC CTC 3'
Primer 4- 5' AGC GTA ATC TGG AAC GTC ATA TGG ATA CTT CCG GGC CGG GTG CAT GTC 3'

Primer Regions are as follows:

Nucleotide overhang for restriction enzyme
NotI restriction enzyme site plus filler adenine nucleotide (New England Biolabs)
Signal Peptide of Olfm1
HindIII restriction enzyme site (New England Biolabs)
Reverse complement of stop codon
Reverse complement of 3' coding sequence
HA tag (top strand)
5' end of coding sequence after signal peptide
Reverse complement of HA tag
Signal peptide bottom strand

Three rounds of PCR were necessary to generate the HA-Olfm1 construct. The first round of PCR generated two different DNA fragments: one with a single-stranded HA tag sequence downstream from the signal peptide of Olfm1and the second with the single-stranded HA tag upstream from the remainder of the Olfm1coding sequence, downstream from the signal peptide. The second round of PCR created double stranded PCR fragments from the first round of PCR products. Finally, the third round of PCR joined the fragments from the second PCR together to produce a complete Olfm1construct with the HA tag inserted between the signal peptide sequence and the remainder of the coding sequence.

In the first round of PCR there were two 50 μL reactions: Reaction A and Reaction B. Each reaction contained 5 μL 10x Complete PCR Buffer SUPERTaq™ (Ambion), 2.5 μL 10 mM PCR dNTP Mix SUPERTaq™ Kit (Ambion), 37.6 μL nuclease-free water (Ambion), 2.5 μl cDNA generated in the reverse transcription reaction, and 0.4 μL SUPERTaq enzyme (Ambion). In addition, reaction A contained 1 μL 10 μM Primer 1 (IDT) and 1 μL 10 μM Primer 4 (IDT). Additional components of Reaction B included 1μL 10 μM Primer 3 (IDT) and 1 μL 10 μM Primer 2 (IDT).

The Bio-Rad thermocycler conditions were set at 98°C for 30 sec for the initial denaturation, then 25 cycles of 98°C for 10 sec, 58°C for 30 sec, and 72°C for 3 min, and the final extension was 72°C for 5 min and then held at 4°C. After the PCR, a QIAquick® PCR Purification Kit (QIAGEN) was used according to the manufacturer's protocol to purify the DNA for the second round of PCR.

In the second round of PCR there were two 50 μL reactions: Reaction A2 and Reaction B2. The components of both reactions included 5 μL 10x Complete PCR Buffer SUPERTaq™ (Ambion), 1 μL 10 mM PCR dNTP Mix SUPERTaq™ Kit (Ambion), 36.1 μL nuclease free water (Ambion), 2.5 μL 10 μM Primer 1 (IDT), and 0.4 μL SUPERTaq enzyme (Ambion). In addition, Reaction A2 contained 5 μL template DNA from round one Reaction A and Reaction B2 contained 5 μL template DNA from round one Reaction B. The Bio-Rad thermocycler conditions were set at 98°C for 30 sec for the initial denaturation, then 10 cycles of 98°C for 10 sec, 58°C for 30 sec, and 72°C for 3 min, and the final extension was 72°C for 5 min and then held at 4°C. After the PCR, a QIAquick® PCR Purification Kit (QIAGEN) was used according to the manufacture's protocol to purify the DNA to be used for the third round of PCR.

In the third round of PCR there was one 45 μL reaction. The components of this reaction include: 5 μL 10X Complete PCR Buffer SUPERTaq™ (Ambion), 1 μL 10 mM PCR dNTP Mix SUPERTaq™ Kit (Ambion), 18.6 μL nuclease free water (Ambion), 10 μL template DNA from Reaction A2, 10 μL template DNA from Reaction B2 and 0.4 μL SUPERTaq enzyme (Ambion).

The Bio-Rad thermocycler conditions were set at 98°C for 30 sec for the initial denaturation, then 5 cycles of 98°C for 10 sec, 58°C for 30 sec, and 72°C for 3 min, and the final extension was 72°C for 5 min. Following these five cycles, 2.5 μL of Primer 1 and 2.5 μL of Primer 2 were added to the 45μL reaction. Following this addition, the Bio-Rad thermocycler conditions were set at 98°C for 30 sec for the initial denaturation, then 20 cycles of 98°C for 10 sec, 58°C for 30 sec, and 72°C for 3 min, and the final extension was 72°C for 5 min and then held at 4°C.

To generate the C-terminal HA tagged Olfm1construct (Olfm1-HA) one round of PCR was used with the following primers (IDT).

Primer 1- 5'*AAA* GCG GCC GCA ATG CCA GGT CGT TGG AGG TGG 3'
Olfm1 Reverse C-term-HA- 5' *CCG* AAG CTT CTA AGC GTA ATC TGG AAC GTC ATA TGG ATA CAA CTC GTC GGA GCG GAT GAC GTG 3'

The Regions are as follows:

Nucleotide overhang for restriction enzyme
NotI restriction enzyme site plus filler Adenine nucleotide (New England Biolabs)
Signal Peptide of Olfm1
HindIII restriction enzyme site (New England Biolabs)
Reverse complement of stop codon
Reverse complement of HA tag
Reverse complement coding sequence from 3' end of Olfm1after stop codon

To generate the Olfm1-HA construct there were two 50 μL reactions: Reaction A and its control. Each reaction contained 5 μL 10x Complete PCR Buffer SUPERTaq™ (Ambion), 2.5 μL 10 mM PCR dNTP Mix SUPERTaq™ Kit (Ambion), 37.6 μL free water (Ambion), 2.5 μl cDNA generated in the reverse transcription reaction, 1 μL 10 μM Primer 1 (IDT), 1 μL 10 μM Olfm1Reverse C-term-HA Primer (IDT), and 0.4 μL SUPERTaq enzyme (Ambion). Reaction A's control contained 2.5 μl of nuclease free water (Ambion) instead of the cDNA. The Bio-Rad thermocycler conditions were set at

94°C for 4 min for the initial denaturation, then 30 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 3 min, and the final extension was 72°C for 5 min and then held at 4°C.

After the PCR reactions for the HA-Olfm1 and Olfm1-HA were complete, 5 μL of HA-Olfm1 and 5 μL of Olfm1-HA along with their controls were electrophoresed on a 1% agarose gel (Bio-Rad Systems) containing SYBR® Safe DNA Gel Stain (Invitrogen) at 100 volts for approximately 60 min. The gel was visualized using a UV transilluminator (Gel Logic 1500 Imaging System) to see if the PCR was successful.

Unfortunately, I was having difficulties optimizing the PCR for cloning. Therefore, I ordered the Olfm1 clone from Open BioSystems and used the SuperTaq enzyme to clone it into the mammalian expression vector pcDNA 3.1 (Invitrogen).

The PCR protocol is identical to the procedure above except 10 μL plasmid DNA (Open BIOsystems) was used in place of the 2.5 μL of cDNA from the reverse transcription reaction and the volume of nuclease free water (Ambion) was adjusted to a final volume of 50 μL. After the PCR reactions for HA-Olfm1 and Olfm1-HA were complete, 5 μL of HA-Olfm1 and 5 μL of Olfm1-HA along with their controls were electrophoresed on a 1% agarose gel (Bio-Rad Systems) containing SYBR® Safe DNA Gel Stain (Invitrogen) at 100 volts for approximately 60 min. The gel was visualized using a UV transilluminator (Gel Logic 1500 Imaging System) to see if the PCR was successful, and it was.

After purifying the remaining 45 μL PCR samples from the second cloning method (QIAquick® PCR Purification Kit) a restriction digest was performed on the HA-Olfm1 and Olfm1-HA constructs. The reaction conditions for the restriction digest were as follows:

Three 30 μL reactions were set up: Reaction A, which contained HA-Olfm1, Reaction B which contained Olfm1-HA and Reaction C which contained the pcDNA 3.1 (-) vector (Invitrogen). All three reactions contained 3 μL NEBuffer 2, (New England Biolabs), 3 μL 10x Purified BSA (New England Biolabs), 1 μL NotI restriction enzyme (NewEngland Biolabs), and 1 μL HindIII restriction enzyme (NewEngland Biolabs). Additionally, Reaction A contained 10 μL of HA-Olfm1 from the PCR and 12 μL nuclease free water (Ambion), Reaction B contained 10 μL of Olfm1-HA from the PCR and 12 μL nuclease free water (Ambion) and reaction C contained 5 μL pcDNA 3.1- (Invitrogen) and 17 μL nuclease free water (Ambion).

The restriction digests were incubated at 37°C for 4 hours after which 1 μL Calf Intestinal alkaline Phosphatase (CIP) enzyme (New England Biolabs) was added to Reaction C. The three reactions were incubated for an additional hour at 37°C. After this incubation a QIAquick® PCR Purification Kit (QIAGEN) was used according to the manufacturer's protocol to purify DNA from the restriction digest reaction.

After the restriction digest, three 10 μL Ligation reactions were set-up to insert the HA-Olfm1 and Olfm1-HA genes into the into the mammalian expression vector pcDNA 3.1 (Invitrogen). All three reactions contained 1 μl 10x Buffer for T4 DNA Ligase (New England Biolabs), 0.5 μL T4 Ligase (New England Biolabs), 2 μL of the pcDNA 3.1 (-) vector digest and 1.5 μL nuclease free water (Ambion). In addition, Reaction A contained 5 μL HA-Olfm1 DNA from the restriction digest reaction, Reaction B contained 5 μL Olfm1-HA DNA from the restriction digest reaction, and

Reaction C contained 5 μL nuclease free water (Ambion) and served as the negative control. These three reactions were incubated at 16°C for approximately 16 hours.

After the 16 hour incubation, the ligation reactions were transformed into 50 μL of a DH5α bacteria strain (Invitrogen) using heat shock transformation. More specifically, 2 μL of Reactions A, B, and C were added to the competent DH5α bacteria cells and were incubated on ice for 30 min. The cells were then heat shocked for 30 sec in a 42°C water bath after which they were incubated on ice for 2 min. Following this incubation, 950 μL of SOC Media (Invitrogen) was added to the transformed bacteria cells over a flame for sterilization purposes. The bacteria cells were then incubated at 37°C on a MaxQ* 4450 Benchtop Orbital Shaker (Thermo Scientific) for 1 hour at 200 rpm. During this time, three LB/Ampicillin (100μg ampicillin/mL LB) plates were placed in the incubator at 37°C. After the transformation incubation, 150 μL of the transformed bacteria cells in the SOC media were plated on the LB/Ampicillin plates using a glass spreader and aseptic technique. The plates were then incubated at 37°C for approximately 12 hours.

Bacterial colonies from the transformation experiment were then selected to see if they contained the Olfm1 clone. To accomplish this, 1.5 μL of 1000x concentrated ampicillin antibiotic (EMDBiosciences) was added to 1.5 mL of LB broth (Sigma-Aldrich) in a glass test tube over a flame for serialization purposes. Four colonies were picked from the Olfm1 N-terminal tagged transformed bacteria plate and four colonies were picked from the Olfm1 C-terminal tagged transformed bacteria plate. The test tubes containing the media were incubated at 37°C on the MaxQ* 4450 Benchtop Orbital Shaker (Thermo Scientific) at 200 rpm for approximately 16 hours.

DNA from the eight 1.5 mL cultures (4 HA-Olfm1 and 4 Olfm1-HA) was purified using a QIAprep Spin Miniprep Kit (QIAGEN). After eluting 30 μL of the DNA from the bacteria cultures, diagnostic restriction digests were performed to make sure that the pcDNA 3.1 vector had successfully ligated the Olfm1 PCR constructs. Four diagnostic restriction digest reactions, were set up for the HA-Olfm1 construct as followed: 1 μL NEBuffer 2, (New England Biolabs), 1 μL 10x Purified BSA (New England Biolabs), 5 μL of the HA-Olfm1 from the minprep purification, 0.2 μL NotI restriction enzyme (NewEngland Biolabs), 0.2 μL HindIII restriction enzyme (NewEngland Biolabs), and 2.6 μL nuclease free water (Ambion). Four diagnostic restriction digest reactions were also set up for the Olfm1-HA construct in the same manner. The diagnostic digests were incubated at 37°C for two hours. After the incubation, 10 μL of each diagnostic digest was electrophoresed on a 1% agarose gel (Bio-Rad Systems) containing SYBR® Safe DNA Gel Stain (Invitrogen) at 100 volts for approximately 60 min. The gel was visualized using a UV transilluminator to detect those miniprep DNAs that contained the Olfm1construct.

To obtain large quantities of the DNA constructs from the positive Olfm1clones following the diagnostic digest, midiprep cultures were prepared. To prepare the midiprep cultures of the constructs, 25 ml of LB broth (Sigma-Aldrich) was added to 125 ml autoclaved Erlenmeyer flask in front of a flame using sterile technique. Next, 25 μL of 1000x concentrated ampicillin antibiotic (Sigma) was added to the LB broth. Following this addition, 0.5 mL of the miniprep culture that contained the HA-Olfm1 construct was added to the broth and 0.5 mL of miniprep culture that contained the Olfml-HA construct was added to the LB broth. These cultures were incubated at 37°C on the 4450 Benchtop

Orbital Shaker (Thermo Scientific) at 200 rpm for approximately 16 hours. The Plasmid Plus Midi Kit (Qiagen) was used according to the manufactures protocol to elute 200 μL of Olfm1 N-terminal tagged DNA construct and 200 μL of Olfm1C-terminal tagged DNA construct. This DNA was used to transfect the HEK293 cells and validate expression.

Blunt Cloning: Using pRK5 backbone and PCR constructs with Kozak sequence

Unfortunately, cloning in the previous manner did not prove to be effective as discussed below in the Western Blot analysis. Furthermore, our previous constructs did not contain the Kozak sequence, a six nucleotide initiator that precedes the AUG start codon and enhances translation in mammalian cells (Kozak, 1987). For my third cloning method, I included the Kozak sequence in my PCR primers to help enhance the translation of Olfm1. Additionally, after submitting my Olfm1clones for sequencing (Genewiz), I found that there were errors in the coding sequence of Olfm1. In order to reduce the number of errors apparently generated by the SuperTaq enzyme used above, I used the high fidelity iProof enzyme (Bio-Rad) which has an error rate that is 50-fold lower than that of SUPERTaq™ (Ambion). The Olfm1construct generated using this new procedure was cloned into the mammalian expression pcDNA 3.1 vector using the methods described above. Unfortunately, Western Blot analysis of the Olfm1 clones generated from this method of cloning did not reliably express protein as discussed below in the Western Blot section.

For my fourth and final cloning attempt, I used the iProof DNA polymerase and primers from method three that contained the Kozak sequence. For this set of

experiments I cloned my PCR constructs into a different mammalian expression vector, pRK5, using blunt cloning (Anirvan Ghosh Lab, UCSD). The blunt cloning procedure is as follows:

First, digested PCR constructs were obtained. The PCR protocol is identical to the procedures described above, except primer 1 contained the Kozak sequence and a Bio-Rad iProof PCR kit was used.

Primer 1- 5'*AAA* GCG GCC GCAGCC ACC ATG CCA GGT CGT TGG AGG TGG 3'
The Regions are as followed:
Nucleotide overhang for restriction enzyme
NotI restriction enzyme site plus filler Adenine nucleotide (New England Biolabs)
Kozak Sequence
Signal Peptide of Olfm1

To prepare the pRK5 plasmid for the insertion of HA-Olfm1 and Olfm1-HA, a sequential digest was performed on HA-NPTXR in the pRK5 plasmid (Anirvan Ghosh Lab, UCSD) to remove the HA-NPTXR construct from the pRK5 plasmid. Two reactions, one containing the HA-NPTXR construct and the other serving as a control, were set up as followed: 3 μL NEBuffer 4, (New England Biolabs), 15 μL HA-NPTXR construct, 0.6 μL EcoR1 restriction enzyme (NewEngland Biolabs), and 11.4 μL nuclease free water (Ambion). The control contained nuclease free water (Ambion) in place of the EcoR1 restriction enzyme. The digests were incubated at 37°C for one hour. A QIAquick® PCR Purification Kit (Qiagen) was used according to the manufacturer's protocol to purify the DNA for the second restriction digest. Again, two reactions were set up, one containing the HA-NPTXR construct and the other serving as a control. The reactions were set up as follows: 3 μL NEBuffer 4, (New England Biolabs), 3 μL 10x Purified BSA (New England Biolabs), 23.4 μL of the HA-NPTXR construct digested with EcoR1, and 0.6 μL of the XbaI restriction enzyme (NewEngland Biolabs). The

control again contained nuclease free water (Ambion) in place of the XbaI restriction enzyme. The second restriction digest was incubated at 37°C for one hour. After the incubation, 15 μL of the second restriction digest and its control were electrophoresed on a 1% agarose gel (Bio-Rad Systems) containing SYBR® Safe DNA Gel Stain (Invitrogen) at 100 volts for approximately 60 min. The gel was visualized using a UV transilluminator to ensure that the NPTXR gene was cut out of the pRK5 vector. After electrophoresing the DNA from the sequential digest, 30 μL of DNA from the gel was extracted using a QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturer's protocol to be used for blunt cloning HA-Olfm1 and Olfm1-HA PCR constructs into the pRK5 plasmid.

The PCR constructs containing the Kozak sequence and the pRK5 vector from the digest described above were prepared for ligation by generating blunt ends using a Klenow enzyme as follows. Three 30 μL reactions set up: Reaction A which contained HA-Olfm1 and Kozak sequence that had been digested with the restriction enzymes Not1 and HindIII (NewEngland Biolabs), Reaction B which contained Olfm1-HA and Kozak sequence that had been digested with restriction enzymes Not1 and HindIII (NewEngland Biolabs) and Reaction C which contained the pRK5 vector that was obtained from the sequential digest and gel extraction.

Reactions A and B both contained 3 μL NEBuffer 2, (New England Biolabs), 1 μL 2 mm PCR dNTP Mix (Bio-Rad), 1μL Klenow enzyme (New England Biolabs), and 2 μL nuclease free water (Ambion). Additionally, Reaction A contained 23 μL of HA-Olfm1 and Kozak sequence from the PCR digest and Reaction B contained 23 μL of Olfm1-HA and Kozak sequence from the PCR digest. Reaction C contained 3 μL

NEBuffer 2, (New England Biolabs), 1 μL 2 mm PCR dNTP Mix (Bio-Rad), 1μL Klenow enzyme (New England Biolabs), and 25 μL of the PRK5 plasmid from the sequential digest and gel extraction described above. The three reaction mixtures were incubated at room temperature for 30 min after which they were heat inactivated for 20 min at 70°C. Next, reaction mixtures A and B were placed on ice, while 1 μL of CIP enzyme (New England Biolabs) was added to reaction C and incubated at 37°C for one hour.

Three 10 μL ligation reactions were set-up to insert the HA-Olfm1 and Olfm1-HA constructs into the into the pRK5 vector. All three reactions contained 1 μl 10x Buffer for T4 DNA Ligase (New England Biolabs), 0.5 μL T4 Ligase (New England Biolabs), and 2.5 μL of CIP-ed pRK5 vector. Additionally, Reaction A contained 6 μL HA-Olfm1 DNA from the PCR digest and treatment with Klenow and Reaction B contained 6 μL Olfm1-HA DNA from the PCR digest and treatment with Klenow, and Reaction C, the negative control, contained 6 μL nuclease free water (Ambion). The three reactions were incubated at 16°C for approximately 16 hours.

The ligation reactions were then transformed into 50 μL DH5α bacteria cells (Invitrogen) using the heat shock transformation procedure identical to that described above except 5 μL of DNA was added to the competent DH5α bacteria cells instead of 2 μL.

Lacking colonies to proceed from the first attempt at blunt cloning, I made the following changes to the experimental procedure for two more attempts at blunt cloning. First, I ran out 45 μL of the sequential restriction digest to get higher concentration of the

pRK5 backbone after gel extraction. Also, in the second attempt at blunt cloning I discovered that although the PCR with iProof enzyme would leave blunt ends, the ends contained no 5' phosphate group which is crucial for the construct to be ligated into the pRK5 vector. A third and final round of blunt cloning was attempted making the following changes: 1) add 2 μL of CIP to the pRK5 vector rather than 1 μL, 2) PCR purify the CIP-treated pRK5 vector before doing the ligation reaction, 3) treat both the PCR digests and the pRK5 backbone with Klenow (New England Biolabs), 4) Learn more about the pRK5 vector by allowing it to self-ligate and digest it with Kpn1 (New England Biolabs) to ensure that the enzyme will only cut the vector once.

The protocol I used to ligate the digested PCR constructs and pRK5 backbone was identical to that described above. However, in the second and third attempt I was able to generate miniprep DNAs using the procedure in the gene cloning section beginning on page twenty-four.

DNA was obtained from the miniprep cultures using the QIAquick Miniprep kit (Qiagen) according to the manufacturer's protocol and diagnostic restriction digests were performed to ensure that the pRK5 vector had taken up the Olfm1 constructs in the correct orientation. The diagnostic restriction digest reactions were set up for the HA-Olfm1 and Olfm1-HA constructs as followed: 1 μL NEBuffer 1, (New England Biolabs), 1 μL 10x Purified BSA (New England Biolabs), 5 μL of HA-Olfm1 from the minprep, 0.2 μL KpnI restriction enzyme (New England Biolabs) and 2.6 μL nuclease free water (Ambion). Diagnostic restriction digest reactions were also set up for Olfm1- HA construct in the same manner except 5 μL of Oflm1-HA from the minprep was used. The diagnostic digests were incubated at 37°C for approximately 12 hours. Ten μL of each

diagnostic digest was electrophoresed on a 1% agarose gel (Bio-Rad Systems) containing SYBR® Safe DNA Gel Stain (Invitrogen) at 100 volts for approximately 60 min. The gel was visualized using a transilluminator.

Western Blot Analysis on Clones

Western Blot analysis was used to test whether the HA-tagged Olfm1 constructs generated using the four cloning strategies above expressed Olfm1 protein. First, HEK293 cells were transfected with the Olfm1constructs that were validated by diagnostic digest. To transfect the HEK293 cells, 12 µL of FuGene® (Promega) was added to 188 µL of DMEM high glucose media (Gibco®). After the mixture was incubated at room temperature for 5 min, 2 µg of HA-Olfm1 construct was added. Next, 200 µL of the transfection mixture was added to 60 mm cell dishes containing 70% confluent cells. The same protocol was followed for the Olfm1-HA construct, HA-tagged Neuronal Pentraxin Receptor (NPTXR), which served as a positive control for the experiment, and GluR1-GFP and GluR2-GFP constructs (Anirvan Ghosh Lab, UCSD), whose expressions were being validated for further experiments. The transfected cells were incubated at 37°C with 5% CO_2 for 48 hours.

After this incubation, a Western Blot analysis was performed to detect the presence of proteins expressed from the DNA constructs listed above. First, protein from the transfected HEK293 cells was collected and denatured by adding 200 µL of hot sample buffer (95% Laemmli Sample Buffer (Bio-Rad) 5% β-Mercaptoethanol (GBiosciences)) to the cells in the 60 mm dishes. The HEK293 samples were placed in 1.5 mL Eppendorf tubes and boiled for 5 min. Next, 30 µL of the samples were loaded

into 10% SDS-PAGE protein gels and electrophoresed in a Bio-Rad electrophorator set at 90 Volts for approximately 30 min and then ran at 120 Volts until the samples were within 0.5 centimeters of the bottom of the gel. The Western Running Buffer used was 1x SDS Running Buffer (3 g Tris, 14.4 g glycine, 1 g SDS per liter of solution). After the gels were electrophoresed, the gel was transferred to a nitrocellulose membrane for 60 min at 100 Volts (Bio-Rad). The transfer buffer used was 1x Transfer buffer (3 g Tris, 14.4 g glycine, 100 ml methanol per 1 liter solution). Following the transfer, the membrane was incubated in 2% non-fat milk containing 1xTTBS with 0.01% Tween 20 for 30 min. The first membrane was blotted with mouse anti-HA antibody (Covance) at a 1:1000 dilution to detect the presence of HA-Olfm1 and Olfm1-HA proteins generated from the cloned constructs and the HA-NPTXR protein (Anirvan Ghosh Lab, UCSD). The second membrane was blotted with a mouse anti-GFP antibody (Santa Cruz Biotechnology) at a 1:1000 dilution to detect the expression of the GluR1-GFP and GluR2-GFP proteins. These membranes were incubated in antibody solution for approximately 12 hours at 4°C on a rocking platform.

After the incubation with primary antibody, the membranes were washed three times for 10 min in 1xTTBS with 0.01% Tween 20. After the three washes, the secondary antibody Goat Anti-Mouse-HRP (Bio-Rad) was added to the nitrocellulose membranes at a 1:1000 dilution in 2% non-fat milk containing 1xTTBS with 0.01% Tween 20. The membranes were incubated for 1 hour at room temperature with the secondary antibody. After this incubation, the secondary antibodies were washed off with two 10 min washes in 1x TTBS and one 10 min wash in 1x TBS. For color development of a single membrane, 60 µl of HRP color reagent B and 1 ml 1x HRP color development buffer was

added to 9 ml deionized water at room temperature. Immediately prior to color development, 2 ml of color reagent A was added to the solution (Bio-Rad Immun-Blot Assay kit). The membranes were incubated in color development solution for approximately thirty minutes or until bands became visible. The membranes were imaged using a white light transilluminator (Gel Logic 1500 Imaging System).

Western Blot Analysis on NT2 Neurons

To insure that the NTERA-2 (ATCC cl.D1 [NT2/D1]) cell line had been successfully differentiated into neurons, a Western Blot analysis was performed to detect the expression of proteins that are characteristic of neurons. The first protein was Brain Derived Neurotropic Factor (BDNF), which plays a role in regulating synaptic structure and function at glutamatergic synapses (Keifer and Zheng, 2010). The second protein was Tau, which is abundant in neurons and primarily functions to stabilize axonal microtubules (Zhang, 2012). The third protein was Synapsin 1, a phosphoprotein that is involved in neurotransmitter release at synapses (Camilli and Greengard, 1983). The final protein needed to ensure that neurons had been successfully differentiated was GluR2.

The protocol used for the Western Blot analysis is identical to the protocol above and the following are the concentrations of primary antibodies used to detect the expression of each of the proteins of interest: 1) rabbit anti-BDNF antibody (Millipore) at a 1:1000 dilution, 2) mouse anti-Tau (Millipore) at a 1:1000 dilution, 3) rabbit anti-Synapsin 1 antibody (Millipore) at a 1:1000 dilution 4) mouse anti-GluR2 antibody (Millipore) at a 1:500 and 1:50 dilution. When the 1:50 dilution of primary antibody was performed for GluR2, the membrane was incubated at room temperature for approximately 18 hours rather than at 4°C for approximately 12 hours.

Immunostaining NT2 Neurons

To validate the expression of neuronal proteins MAP2, Tau, Synapsin 1 and GluR2 in the NTERA-2 (NT2) cultures the following immunostaining protocol was followed. Differentiated NT2 neurons grown in 12 well dishes with glass coverslips were rinsed briefly with 1x Phosphate Buffered Saline (PBS) pH 7.4 (GIBCO). Next, the cells were fixed for 20 minutes in 500 μL of 4% Paraformaldehyde (Sigma). On a VWR Mini Blot Mixer, three 5 min washes were performed on each well with PBS. Next, the cells were blocked on the VWR Mini Blot Mixer for 30 min in 500 μL of blocking solution (1x PBS, 500 μL Triton X-100 (Roche), 1.5g Albumin Bovine Serum (CALBIOCHEM)). The cells were incubated in sets of four with 250 μL primary antibody in blocking solution at the following dilutions: 1) chicken anti-MAP2 antibody (Millipore) at a 1:5000 dilution 2) mouse anti-Tau antibody (Millipore) at a 1:1000 dilution 3) mouse anti-GluR2 antibody (Millipore) at a 1:500 dilution 4) rabbit anti-Synapsin 1 (Millipore) at a 1:1000 dilution. The 12 well dishes with the primary antibody solutions were incubated for approximately 12 hours at 4°C on a rocking platform.

The primary antibody solution was removed and the cells were washed three times for 5 min in 500 μL blocking solution. The secondary antibodies I used are sensitive to light, so the cells were incubated in the dark for 1 hour in secondary antibody solution. The primary antibodies were detected by the following secondary antibodies at a 1:1000 dilution in blocking solution: 1) the Alexa Flour® 488 goat anti-chicken antibody (Invitrogen) was used on cells probed with the chicken anti-Map2 antibody, 2) the Alexa Flour® 488 goat anti-mouse antibody (Invitrogen) was used on cells probed

with mouse anti-Tau antibody and the mouse anti-GluR2 antibody, 3) the Alexa Flour® 488 goat anti-rabbit antibody (Invitrogen) was used on cells probed with rabbit anti-Synapsin 1 antibody. The cells were washed three times for 5 min in 500 μL blocking solution in the dark. A final wash was performed in 500 μL PBS containing 0.5 μL Hoechst stain (Sigma) for 5 min in the dark. The coverslips were mounted onto SP Supefrost-Plus Microscope Slides (Thermo Scientific) using Fluoromount G (Electron Microscopy Sciences).

Co-Immunoprecipitation Assays and Immunoprecipitation Assay

Co-Immunoprecipitation (Co-IP) is a technique that can be used to detect protein-protein interactions by using a specific antibody for a target protein which allows for the isolation of any proteins that are bound to the target protein (Thermo Scientific). For my research purposes, GluR2 was the target protein and I hypothesized that Olfm1 would be bound to the target protein in HEK293 cells. Co-IP experiments were carried out using the following constructs: 1) HA-Cpt1c (a fellow researcher's hypothesized GluR2 interacting protein), 2) Olfm1-HA (my hypothesized GluR2 interacting protein) 3) N-terminal Myc-tagged Lrtm2 (Anirvan Ghosh Lab, UCSD: served as a positive control as it is known to be involved in GluR2 expression at synapses (de Wit et al. 2009), and 4) N-terminal Myc-NR1(Anirvan Ghosh Lab, UCSD: served as a negative control because it is a NMDA receptor subunit and should not interact with the GluR2 subunit of AMPARs (Esteban, 2008). Finally, an ImmunoPrecipitation (IP) assay was performed on the GluR2-GFP construct (Anirvan Ghosh Lab, UCSD) to detect the expression of GluR2 in NT2 cells.

Several 60 mm tissue culture dishes were plated with 1 ml of 293 cell solution in 2 ml of 293 cell media (88% DMEM high glucose media, 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin, and 1% Glutamax; Gibco). To determine if there was an interaction between HA-Cpt1c and Olfm1-HA and the GluR2 subunit of the AMPA receptors, HEK293 cells were co-transfected with 4 μg of DNA. To transfect the cells, 12 μL of FuGene® (Promega) was added to 188 μL of DMEM high glucose media (Gibco®). The mixture was incubated at room temperature for 5 min. After the incubation, 2 μg of HA-Cpt1c construct and 2 μg of the GluR2-GFP DNA was added to the DMEM FuGene mixture. This was done in the same manner for the Olfm1-HA construct, for the positive control myc-Lrrtm2, and the negative control myc-NR1. After a 20 min incubation at room temperature, 200 μL of each transfection mixture was added to a 60 mm dish that contained HEK293 cells. The cells were then incubated at 37°C with 5% CO_2 for 24 hours.

RIPA lysis buffer was prepared using a 10x RIPA Stock (Cell Signaling Technology Cat# 9806). A 1 mL aliquot of RIPA buffer was thawed briefly at room temperature and then 9 mL of -free water (Ambion) was added to the 15 mL conical tube and placed on ice. Next, 100 μL of 100x Protease Inhibitor Cocktail solution (Cell Signaling Technology Cat# 5871) was added to the RIPA buffer on ice.

The mouse anti-GFP antibody (Santa Cruz Biotechnology) was bound to the Protein in A/G Plus-Agarose Beads (Santa Cruz Biotechnology) by combining 500 μL RIPA buffer, 25 μL of protein A/G beads, and 1 μg of the GFP antibody for each IP experiment in an ice cold 1.5 mL Eppendorf tube. The Protein A/G Beads containing the antibody were incubated on a rotating platform for 45 min at 4°C.

On a flat bed of ice, the HEK293 cell media (88% DMEM high glucose media, 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin, and 1% Glutamax; Gibco) was removed from the 60 mm dish containing the co-transfected HEK293 cells and the cells were washed briefly with ice-cold 1xPBS (Gibco). Next, 500 μL of RIPA buffer was added to each dish on ice and then incubated on a rocking platform for 40 min at 4°C. After this incubation, the cells were collected in Eppendorf tubes on ice and centrifuged at 13,000 rpm for 20 min at 4°C. During the centrifugation, the Protein A/G Beads containing the GFP antibody were centrifuged at 2,000 rpm for 2 min at room temperature and the RIPA buffer was removed. Next, 500 μL of RIPA was added to the Eppendorf tubes and the beads were washed by tapping the tubes on the bench top. A second centrifugation of the Protein A/G Beads containing the GFP antibody and RIPA buffer was performed at 2,000 rpm for 2 min at room temperature. Then RIPA buffer was added to the Protein A/G Beads so that each Co-IP experiment would receive 25μL and the tube was placed on ice.

After the 20 min centrifugation at 13,000 rpm at 4°C, the cell lysates were carefully transferred to pre-chilled Eppendorf tubes on ice to avoid disturbing the pellet. Next, 40 μL of the cell lysate from each sample was transferred to a second set of pre-chilled Eppendorf tubes and kept on ice. These samples served as the INPUT samples or positive control samples. To the remainder of the cell lysates, 25 μL of the prepared A/G Protein Beads was added to the Eppendorf Tubes and rotated on Labquake rotator at 4°C for approximately 12 hours. The INPUT samples were stored at 4°C along with the RIPA buffer.

The samples were centrifuged at 4°C for 1 min at 1000 rpm. Next, the supernatant was removed and 300 μL of RIPA was added to the beads and tapped to mix. This mixture was centrifuged at 4 °C for 1 min at 1000 rpm after which the supernatant was again removed. A second wash with 300 μL of RIPA buffer was followed by a wash with 300 μL 1x PBS (Gibco) using the same conditions.

The 1x PBS was carefully removed and 25 μL of 2x Sample Buffer (950 μL Laemmli Sample Buffer (Bio-Rad) + 50 μL β-Mercaptoethanol (GBiosciences)) was added to each tube and boiled for 5 min. Thirty μL of each sample was added to a 10% SDS-PAGE gel and a Western Blot analysis was carried out under the same conditions as above with the following changes: 1) the membrane that contained only the input samples was probed with mouse anti-GFP antibody (Santa Cruz Biotechnology) at a 1:1000 dilution, 2) the membrane that contained the positive and negative input samples and their corresponding CoIP samples were probed with mouse anti-Myc antibody (Santa Cruz Biotechnology) at a 1:1000 dilution, 3) the membrane that contained the HA-Cpt1c and Olfm1-HA inputs and CoIP experiments was probed with mouse anti-HA antibody (Covance) at a 1:1000 dilution. The secondary antibody used was goat anti-Mouse-HRP (Bio-Rad) at a 1:1000 dilution in 2% non-fat milk containing 1xTTBS with 0.01% Tween 20. For color development, a Bio-Rad Immun-Blot Assay kit was used according to the manufacturer protocol as described above.

An IP assay served as a more sensitive way to detect the presence of the GluR2 subunits as opposed to Western Blot analysis in the NT2 cells. The IP protocol to determine whether the differentiated NT2 neurons were expressing the GluR2 subunit was identical to the Co-IP procedure described above with the following changes: 1)

mouse anti-GluR2 antibody (Millipore) was bound to the Protein in A/G Plus-Agarose Beads (Santa Cruz Biotechnology), 2) The membrane that contained the INPUT samples and IP samples was blotted with mouse anti-GluR2 antibody (Millipore) at a 1:500 dilution, 3) The secondary antibody goat Anti-Mouse-HRP (Bio-Rad) was added to probe for the mouse anti-GluR2 antibody.

NT2 Neuron/HEK293 Cell Co-Culture Synapsin1 and Synaptotagmin 1Assay

To visualize synapses made by NT2 neurons onto cells expressing synapse recruiting proteins, a co-culture assay was used. In this assay, fully differentiated NT2 neurons are co-cultured with HEK293 cells that express synapse recruiting proteins. The existence of the mature synapses was monitored using antibodies against proteins present at these synapses. To test whether this assay was feasible with our culture system, HEK293 cells were transfected with either Flag-tagged NLG (neuroligin) or pBOS-GFP using the protocols described above and the co-cultures were immunostained to look for expression of the following proteins: 1) flag-tagged NLG (Neuroligin) (Anirvan Ghosh Lab, UCSD) which served as a positive control, because it is a protein that mediates the formation and maintenance of synapses between neurons (Ripley *et al.*), 2) pBOS-GFP (Anirvan Ghosh Lab, UCSD) which served as a negative control because it expresses the non-synaptogenic GFP protein. Therefore, HEK293cells expressing the GFP protein should not be capable of forming synapses with NT2 neurons, 3) Synapsin 1, a phosphoprotein that is involved in neurotransmitter release at synapses (Camilli and Greengard, 1983), 4) Synaptotagmin-1 a calcium sensing protein that is involved in neurotransmitter release by linking the membranes between synapses (Seven *et al.*, 2013).

In 60 mm cell culture dishes, 1 mL of cell solution was added from a 100% confluent HEK293 parent dish and was incubated at 37°C with 5% CO_2 for 24 hours. The dishes were then transfected with 2 μg of either Flag-NLG DNA or 2μg of pBOS-GFP DNA. To transfect the cells, 12 μL of FuGene® (Promega) was added to 188 μL of DMEM high glucose media (Gibco®). The mixtures were incubated at room temperature for 5 min, after which 2 μg of Flag-NLG DNA was added to one DMEM-FuGene mixture and 2 μg of pBOS-GFP was added to a second DMEM-FuGene mixture. Next, 200 μL of the transfection mixture was added to 60 mm cell dishes. The transfected cells were incubated at 37°C with 5% CO_2 for 24 hours.

Next, 0.5 mL Trypsin-0.53 mM EDTA (Gibco) was added to the 60mm cell dishes drop wise and allowed to sit at room temperature for approximately five minutes so that the cells dislodged from the cell dish. After five minutes, 2 mL of HEK293 culture media (88% DMEM high glucose media, 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin, and 1% Glutamax; Gibco) was added to the dish and the cell solution was transferred to a 15 mL conical tube and centrifuged at room temperature for 5 min at 200xg using a Sorvall Legend RT Centrifuge (Thermo Electron Corporation). Then the supernatant was removed and the cell pellet was re-suspended in 1 mL of NT2 cell culture media (90% DMEM, 10% FBS). Finally, 100 μL of 293 cell solution was added to fully differentiated NT2 neurons plated on glass coverslips in a 12 well dish. The NT2 neuron/transfected HEK293 cell co-culture was incubated at 37°C with 5% CO_2 for 24 hours.

An immunostaining assay was performed on the NT2 Neuron/transfected HEK293 cell co-culture cells using the same procedure described above, with the

following change: the cells were incubated in primary antibody solution containing two antibodies in 250 μL of blocking solution at a 1:1000 dilution. The first combination was rabbit anti-Synapsin 1 antibody (Millipore) and mouse anti-FLAG antibody. The next combination was rabbit anti-Synapsin 1 antibody (Millipore) and mouse anti-GFP antibody (Santa Cruze Biotechnology). The third combination was rabbit anti-Synaptotagmin 1 antibody (Millipore) and mouse anti-FLAG antibody. The final combination was rabbit anti-Synaptotagmin 1 antibody (Millipore) and mouse anti-GFP antibody (Santa Cruze Biotechnology).

The secondary antibody used to probe for Synapsin 1 and Synaptotagmin 1 was Alexa Flour® 546 goat anti-rabbit antibody (Invitrogen). The secondary antibody used to probe for the GFP and Flag-Nlg was Alexa Flour® 488 goat anti-mouse antibody (Invitrogen).

Results

Gene Cloning Results

-Amplification of Olfm1from a cDNA library and cloning into pcDNA 3.1 expression vector

The conversion of human whole brain RNA to cDNA was successful using the RT-PCR method described above. However, I was unable to successively isolate the cDNA encoding Olfm1 using PCR amplification (Figure 1, lane 3). Therefore, I had to find an alternate way to clone Olfm1 into the mammalian expression vector pcDNA 3.1.

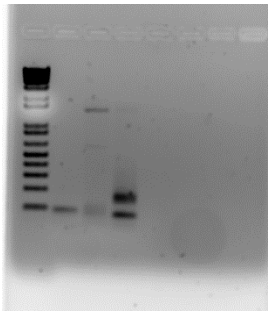

Figure 1. Amplification of N-terminal HA-tagged Olfm1 using RT-PCR. Lane 1 Trackit 1 Kb Plus DNA Ladder (Invitrogen), Lane 2 N-terminal HA tag with clean PCR from Round 2A, Lane 3 N-terminal HA tag with clean PCR reaction from Round 2B, Lane 4 N-terminal HA tag Olfm1 PCR Round 3

-Amplification of Olfm1from an OpenBiosystems clone using SuperTaq DNA Polymerase

PCR amplification of HA-Olfm1 and Olfm1-HA from the OpenBiosystems clone was validated using gel electrophoresis as indicated by bands at 1403 base pairs (Figure 2, lane 2 and lane 4).

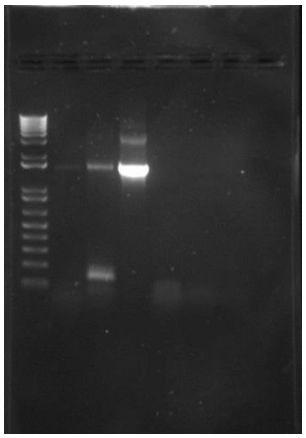

Figure 2. Amplification of N- and C- terminal HA-tagged Olfm1. Lane 1 Trackit 1 Kb Plus DNA Ladder (Invitrogen), Lane 2 HA-Olfm1 with clean PCR from Round 2, Lane 3 HA-Olfm1 with unclean PCR reaction from Round 2, Lane 4 Olfm1-HA construct, Lane 5 Round 1 Reaction A control, Lane 6 Round 1 Reaction B control, Lane 7 Olfm1-HA construct control

The miniprep DNAs that contained the Olfm1construct were detected using gel electrophoresis (Figure 3). The bands at approximately 1403 nucleotides corresponding

to the Olfm1gene containing the HA-tag in lanes 5-9 and there was a band at approximately 5428 nucleotides representing the pcDNA3.1 (-) plasmid in lanes 2-9.

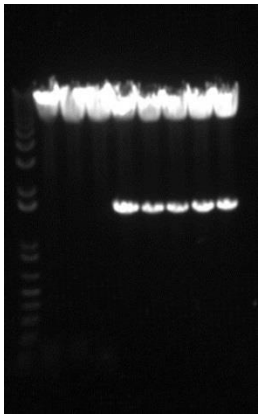

Figure 3. Diagnostic Digest of Olfm1 clones from SuperTaq cloning experiment. Lane 1 Trackit 1 Kb Plus DNA Ladder (Invitrogen), Lanes 2-5 HA-Olfm1, Lanes 6-9 Olfm1-HA

-Amplification of Olfm1 from the OpenBiosystems clone using iProof and including the Kozak sequence

PCR amplification of HA-Olfm1 and Olfm1-HA from the OpenBiosystems clone using the iProof high fidelity DNA polymerase enzyme was validated using gel electrophoresis as indicated by bands at 1403 base pairs (Figure 4 lane 4 and Figure 5 lane 2). The miniprep DNAs that contained the Olfm1construct were detected using gel electrophoresis (Figure 6). The bands at approximately 1403 nucleotides correspond to the Olfm1gene containing the HA-tag in lanes 5, 6, 8, and 9 and there was a band at approximately 5428 nucleotides representing the pcDNA3.1 (-) plasmid in lanes 2-15 (Figure 6).

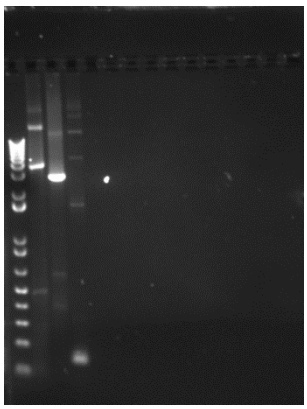

Figure 4. Amplification N- terminal HA-tagged Olfm1. Lane 1 Trackit 1 Kb Plus DNA Ladder (Invitrogen), Lane 2 HA-Adcy2 (a second colleagues target protein), Lane 3 HA-Cpt1c, Lane 4HA-Olfm1, Lane 5 negative control

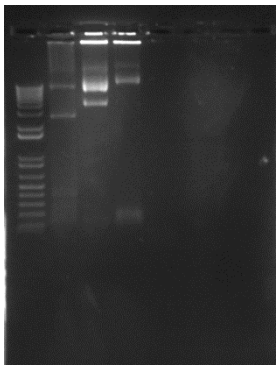

Figure 5. Amplification C- terminal HA-tagged Olfm1. Lane 1 Trackit 1 Kb Plus DNA Ladder (Invitrogen), Lane 2 Olfm1-HA, Lane 3 Cpt1c-HA, Lane 4 Adcy2-HA (PCR did not work), Lane 5 negative control

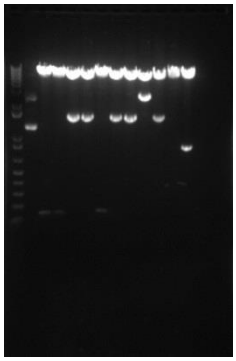

Figure 6. Diagnostic digest of Olfm1 clones from iProof cloning experiment. Lane 1 Trackit 1 Kb Plus DNA Ladder (Invitrogen), Lanes 2-5 HA-Olfm1with Kozak sequence, Lanes 6-9 Olfm1-HA with Kozak sequence, Lanes 10-13 Cpt1c-HA with Kozak sequence

-Blunt cloning of Olfm1into the pRK5 mammalian expression vector

Lane 2 displays the results of the sequential digest on HA-NPTXR containing the pRK5 plasmid digested with EcoR1 and XbaI restriction enzymes. The top band at approximately 4000 bp corresponds to the pRK5 plasmid, while the lower band at approximately 1650 bp corresponds to the HA-NPTXR gene (Figure 7). The top band in lane 2 was extracted from the gel and used for further cloning steps.

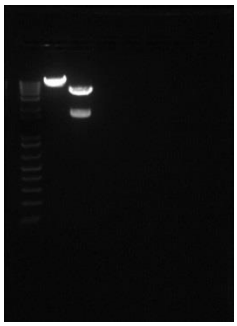

Figure 7. Sequential digest of HA-NPTXR in the pRK5 plasmid. Lane 1 Trackit 1 Kb Plus DNA Ladder (Invitrogen), Lane 2 control containing the undigested pRK5 plasmid, Lane 3 HA-NPTXR construct digested with EcoR1 and XbaI restriction enzymes

As noted above, there were no colonies to pick for further cloning steps from the first round of blunt cloning. There were colonies from the second attempt and the results for the diagnostic digests on the miniprep DNAs from these colonies are shown in Figure 8. The Olfm1 PCR constructs were not ligated into the pRK5 vector because the iProof enzyme left blunt ends that did not contain a 5’ phosphate group. For T4 ligase to form a phosphodiester bond there must be a 5’ phosphate group on the Olfm1 construct so that it can be joined to the 3’ hydroxyl group of the mammalian expression vector.

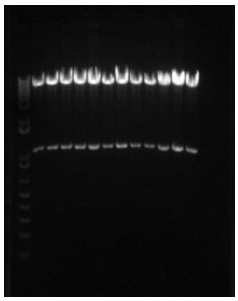

Figure 8. Diagnostic Digest with KpnI restriction enzyme. Lane 1 Trackit 1 Kb Plus DNA Ladder (Invitrogen), Lanes 2-7 Oflm1-HA digest with Kpn1 that was ligated at 16°C overnight, Lanes 8-13 Olfm1-HA digest with Kpn1 that was ligated at room temperature for two hours.

Results of the diagnostic digests on the miniprep DNAs from the third attempt at blunt cloning (Figure 9). The miniprep DNA in lane 14 was kept to test its expression in HEK293 cells.

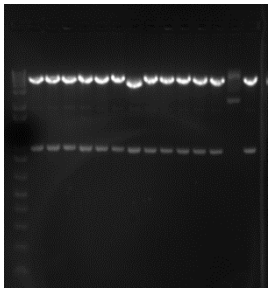

Figure 9. Diagnostic Digest with KpnI restriction enzyme. Lane 1 Trackit 1 Kb Plus DNA Ladder (Invitrogen), Lanes 2-13 Olfm1-HA digest with Kpn1 20 µl reaction, Lanes 14-15 Olfm1-HA digest with Kpn1 10 µl reaction

Western Blot Analysis on Clones

Western blot analysis of HEK293 cells transfected with Olfm1 did not validate the generation of successful clones containing the Kozak sequence that were produced using the iProof enzyme (Figure 10). The weak signal detected at approximately 55 kDa in lane 6 from the positive control HA-NPTXR suggests that the concentration of protein was not high enough when the cells were harvested.

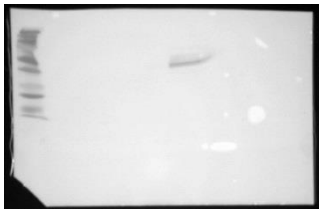

Figure 10. Western Analysis on Clones containing the Kozak Sequence. Lane 1 Ladder (Precision Plus Protein™ Dual Color Standards Bio-Rad), Lane 2 HA-Cpt1c, Lane 3 Cpt1c-HA, Lane 4 HA-Olfm1, Lane 5 Olfm1-HA, Lane 6 HA-NPTXR

After another cloning attempt using primers with the Kozak sequence and the iProof enzyme, my colleague's N-terminal construct protein was detected at approximately 78 kDa in transfected HEK293 cells (Figure 11, Lane 2). The positive control HA-NPTXR was also detected at approximately 55 kDa (Figure 11, Lanes 6-7).

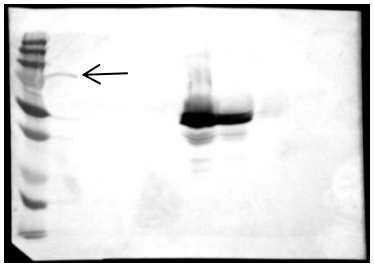

Figure 11. Western Analysis on Clones containing the Kozak Sequence. Lane 1 Ladder (Precision Plus Protein™ Dual Color Standards Bio-Rad), Lane 2 HA-Cpt1c, Lane 3 Cpt1c-HA, Lane 4 HA-Olfm1, Lane 5 Olfm1-HA Lanes 6-7 HA-NPTXR construct detected at approximately 55 kDa, Lane 8 HA-Adcy2, Lane 9 Adcy2-HA

Western analysis also detected Olfm1 protein at approximately 54 kDa in transfected HEK293 cells (Figure 12, Lane 5). This protein contained the C-terminal HA tag and was generated using primers with the Kozak sequence and the iProof enzyme. The positive control HA-NPTXR was also detected at approximately 55 kDa (Figure 12, Lanes 8-9).

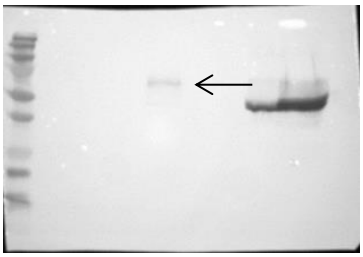

Figure 12. Western Analysis on Clones containing the Kozak Sequence. Lane 1 Ladder (Precision Plus Protein™ Dual Color Standards Bio-Rad), Lane 2 HA-Cpt1c, Lane 3 Cpt1c-HA, Lane 4 HA-Olfm1, Lane 5 Olfm1-HA, Lane 6 HA-Adcy2, Lane 7 Adcy2-HA, Lanes 8-9 HA-NPTXR protein

The expression of GluR2-GFP in HEK293 cells was validated using Western Blot analysis by probing the membrane with mouse anti-GFP (Santa Cruz Biotechnology) and a band at 102 kDa was detected (Figure 13, Lane 3).

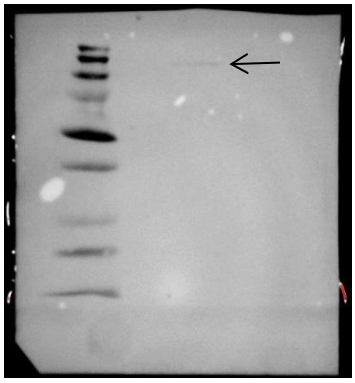

Figure 13. Western Analysis on GluR2-GFP. Lane 1 Ladder (Precision Plus Protein™ Dual Color Standards Bio-Rad), Lane 2 GluR1-GFP, Lane 3 GluR2-GFP

Western Blot Analysis on NT2 Neurons

The culturing of NT2 neurons using the above protocol was validated by the detection of the following proteins: 1) Tau protein detected as a series of 5 bands between 52-68 kDa(Figure 14, Lanes 2-5); 2) BDNF protein detected at approximately 50 kDa

(Figure 15, Lanes 2-5); 3) Synapsin I protein detected at 77 and 80 kDa (Figure 16, Lanes 2-5).

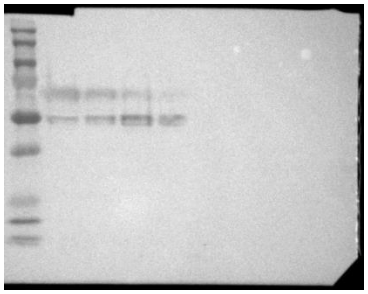

Figure 14. Western Blot analysis of NT2 Neurons plated at different densities shows expression of Tau Protein. Lane 1 Ladder (Precision Plus Protein™ Dual Color Standards Bio-Rad), Lane 2 cells at 250 μL density, Lane 3 cells at 500 μL density, Lane 4 cells at 750 μL density, Lane 5 cells at 1 mL density

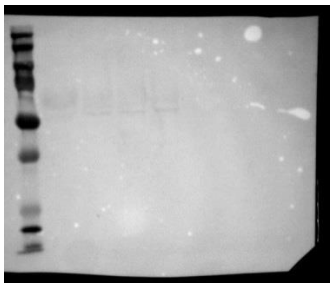

Figure 15. Western Blot analysis of NT2 Neurons plated at different densities shows expression of BDNF. Lane 1 Ladder (Precision Plus Protein™ Dual Color Standards Bio-Rad), Lane 2 cells at 250 μL density, Lane 3 cells at 500 μL density, Lane 4 cells at 750 μL density, Lane 5 cells at 1 mL density

Figure 16. Western Blot analysis of NT2 Neurons plated at different densities shows expression of Synapsin 1. Lane 1 Ladder (Precision Plus Protein™ Dual Color

Standards Bio-Rad), Lane 2 cells at 250 μL density, Lane 3 cells at 500 μL density, Lane 4 cells at 750 μL density, Lane 5 cells at 1 mL density

Immunostaining NT2 Neurons

Immunostaining results validated the expression of the MAP2 neuronal proteins (Figure 17).

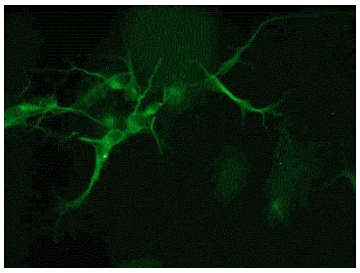

Figure 17. Map 2 staining of NT2 Neurons plated at 250 μL density

Co-Immunoprecipitation Assays and Immunoprecipitation Assay

Results of the Co-immunoprecipitation showed nonspecific binding and lack of bands detected by Western Blot analysis. No bands were detected in the input samples probed with mouse anti-GFP antibody (Santa Cruz Biotechnology; Figure 18). Although bands were detected in the Western analysis probed with mouse anti-myc antibody (Santa Cruz Biotechnology, Figure 19), the bands were not at the correct molecular weight and identical bands were present in the membrane probed with mouse anti-HA (Covance) antibodies (Figure 20) indicating non-specific binding. In the CoIP with Olfm1-HA no bands were detected on either the input membrane probed with mouse anti-GFP antibody (Santa Cruz Biotechnology) or the membrane probed with mouse anti-HA (Covance). Finally, the GluR2 protein was not detected in a membrane probed with mouse anti-

GluR2 likely due to interference of heavy and light chains of the antibody (Millipore;

Figure 21, Lanes 2, 4, and 6).

Figure 18. CoIP Input Samples. Lane 1 Ladder (Precision Plus Protein™ Dual Color Standards Bio-Rad), Lanes 2-3 GluR2-GFP and myc-Lrrtm2 positive control, Lanes 4-5 GluR2-GFP and myc-NR1 negative control, Lanes 6-7 GluR2-GFP and HA-Cpt1c

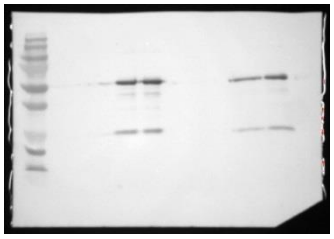

Figure 19. CoIP mouse anti-Myc. Lane 1 Ladder (Precision Plus Protein™ Dual Color Standards Bio-Rad), Lanes 2 and 3 GluR2-GFP and myc-Lrrtm2 positive control Input samples, Lanes 4 and 5 GluR2-GFP and myc-Lrrtm2 positive control CoIP samples, Lanes 6 and 7 GluR2-GFP and myc-NR1 negative control Input samples, Lanes 8 and 9 GluR2-GFP and myc-NR1 CoIP Samples

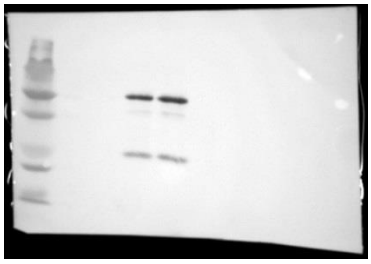

Figure 20. CoIP mouse anti HA antibody. Lane 1 Ladder (Precision Plus Protein™ Dual Color Standards Bio-Rad), Lanes 2 and 3 GluR2-GFP and HA-Cpt1c Input samples, Lanes 4 and 5 GluR2-GFP and HA-Cpt1c CoIP samples

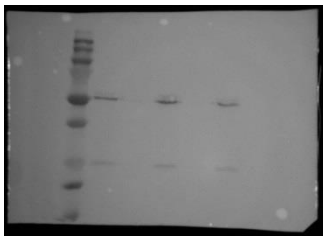

Figure 21. CoIP to detect GluR2 expression in NT2 neurons. Lane 1 Ladder (Precision Plus Protein™ Dual Color Standards Bio-Rad), Lanes 2, 4, 6 anti-GluR2 CoIP samples, Lanes 3, 5, 7 input samples from CoIP

NT2 Neuron/HEK293 Cell Co-Culture Synapsin 1 and Synatotagmin 1 Assay

Synapses between NT2 neurons and HEK293 cells were visualized on those HEK293 cells expressing the synapse recruiting protein NLG (Figure 22). No synapses were visualized on HEK293 cells expressing the negative control GFP, a non-synaptogenic protein (Figure 23).

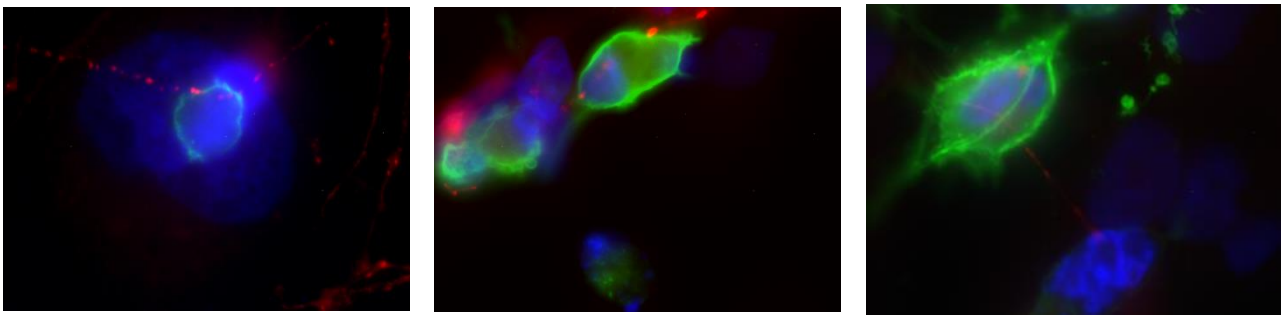

Figure 22. NT2 neurons in contact with NLG- expression HEK293 cells form synapses. The nuclei of all cells (blue). Expression of the presynaptic protein Synapsin 1 by NT2 neurons (red). Expression of NLG by HEK293 cells (green).

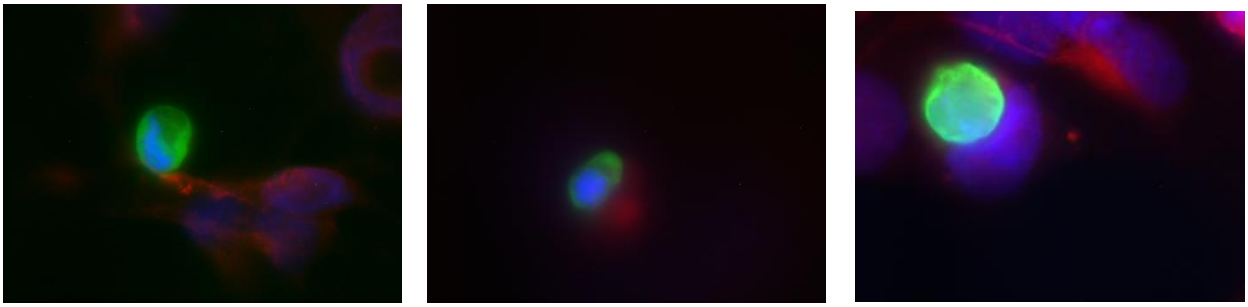

Figure 23. NT2 neurons in contact with GFP- expression HEK293 cells do not form synapses. The nuclei of all cells (blue). Expression of the presynaptic protein Synapsin 1 by NT2 neurons (red). Expression of GFP by HEK293 cells (green).

Discussion

Despite numerous attempts and the implementation of four different cloning protocols, expression of the HA-tagged constructs was not consistently validated by Western blot analysis. Expression of HA-Cpt1c and Olfm1-HA were detected in one series of Western blot experiments (Figures 11 and 12); however, their expression could not be confirmed in subsequent experiments. The four cloning procedures I used to generate an HA-tagged construct were: 1) amplifying OLFM1from a cDNA library and cloning into pcDNA 3.1 expression vector; 2) amplifying Olfm1from the OpenBiosystems clone using SuperTaq; 3) amplifying Olfm1from the OpenBiosystems clone using iProof and including the Kozak sequence; 4) blunt cloning Olfm1into the pRK5 mammalian expression vector.

The first approach to generating an Olfm1 HA-tagged construct was to use human whole brain RNA and perform a Reverse-Transcription Polymerase Chain Reaction (RT-PCR). This is a highly sensitive technique that is used to amplify specific mRNA targets from a complex mixture of mRNAs (Bachman, 2013). I used RT-PCR to amplify Olfm1 mRNA from a pool of human whole brain RNA. Using RT-PCR is beneficial because it allows for amplification of RNA even if it has a relatively low abundance (Bachman, 2013). However, in RT-PCR unintentional multiplex priming amplifies multiple RNAs other than the one of interest. Multiplex priming may have occurred in my experiment as I was unable to isolate my particular gene from the mixture

of cDNAs. A possible resolution in the future would be to isolate the Olfm1 target by designing two sets of primers and performing a nested PCR which would ensure an even higher level of specificity from a complex mixture of cDNA (Bonin, 2011).

In the second cloning procedure, Super-Taq DNA polymerase and primers lacking the Kozak sequence were used. Sequencing indicated that the fidelity of the DNA polymerase enzyme was not high enough to reliably amplify the Olfm1 sequence. If the SuperTaq polymerase only made conservative errors then perhaps these errors would not have been detrimental to the proteins proper processing and eventual expression. Unfortunately, the Super-Taq enzyme was making non-conservative errors, for example substituting polar amino acids for non-polar amino acids, ultimately marking the protein for degradation and preventing detection by Western blot analysis.

A high fidelity iProof enzyme and primers containing the Kozak sequence were ordered with the intention of correcting for both transcription and translation issues respectively. The high fidelity iProof enzyme was chosen because it had an error rate of 4.4×10^{-7} and was less likely to make mistakes during PCR amplification (Bio-Rad). When Western blot analysis did not detect Olfm1 protein, it was thought that perhaps it was because there were difficulties in translation. Therefore, a Kozak sequence was added to Primer 1 to help the ribosome recognize the translational start site and initiate translation (Kozak, 1987). These solutions were successful because Olfm1 expression was validated by Western analysis (Figure 12). Further testing, such as sequencing, needs to be performed to be sure that Olfm1 was successfully cloned into the mammalian expression vector.

The final cloning approach was blunt cloning. Blunt cloning was not my first approach to cloning because although it is versatile, it has many issues. For example, blunt cloning results in fewer colonies because it is 10 to 100 times less effective than cohesive end cloning and also results in colonies that have inserts in the wrong orientation (Sambrook and Russell, 2001). Also, blunt cloning has the potential for the vector to re-ligate before inserting the Olfm1 construct. Despite the potential complications, this method was used because I hypothesized that perhaps the pcDNA 3.1 mammalian expression vector was not favorably expressed by the HEK293 cells.

The pRK5 vector was chosen for blunt cloning because HA-NPTXR was cloned into the pRK5 expression vector and served as my positive control in the Western assays. To use the pRK5 plasmid for the cloning HA-Olfm1 and Olfm1-HA, a sequential digest was performed on HA-NPTXR in the pRK5 plasmid (Anirvan Ghosh Lab, UCSD) using the restriction enzymes EcoR1 and XbaI. The sequential digest and resulting gel extraction allowed me to isolate the pRK5 vector and subsequent treatment with Klenow blunted the ends generated by EcoR1 and Xba1. Next, the PCR amplified Olfm1 was treated with restriction enzymes Not1 and HindIII and was also treated with Klenow to blunt the ends. The T4 ligase enzyme was used to join the blunted Olfm1 HA-tagged construct and the pRK5 vector.

Colonies on agar plates suggested that the ligation process worked; however, difficulty arose when validating the correct orientation of the insertions. Our lab lacked a definitive map of the pRK5 plasmid that was used to clone HA-NPTXR, making it difficult to determine which restriction enzymes were appropriate for a diagnostic digest. An extensive literature review was performed in hopes of obtaining the correct map, but

the exact plasmid was never definitively determined. Therefore, I used a map obtained by Dr. Stefanie Otto-Hitt to perform a diagnostic digest with the Kpn1 restriction enzyme that should cut the construct only once. If the Olfm1 construct was in the correct orientation, gel electrophoresis would detect a band larger than 433 bp, but if it were in the incorrect orientation a band at 1444 bp would be detected. A band at approximately 2000 bp was detected (Figure 9, lane 14). Expression of a potential Olfm1 candidate in HEK293 cells by Western Blot analysis has not yet been performed.

Although Western analysis did not confirm the expression of Olfm1 protein consistently, diagnostic digest experiments suggested that the Olfm1 PCR amplified HA-tagged construct was successfully ligating into the mammalian expression vector. This leads me to believe that perhaps cloning was successful, but potential problems were arising during transcription, translation, or both. There are two possible methods for determining if the transfected HEK293 cells were able to transcribe Olfm1 construct. The first is to perform a Northern Blot analysis. This technique would be used to determine whether the Olfm1 gene is being transcribed in HEK293 cells by probing for the presence of Olfm1 mRNA. The next method is quantitative PCR (qPCR). Performing qPCR on Olfm1-transfected HEK293 cells would allow me to determine if the cells were producing mature mRNA that could be translated into a protein. Designing appropriate primers, qPCR would differentiate between primary and mature mRNA. If the HEK293 cells are producing primary mRNA but not mature mRNA, then the cells are not correctly processing the mRNA into mature mRNA. However, if mature mRNA is detected by qPCR, then the cells are transcribing the Olfm1 construct, but there are subsequent

problems with translation. Quantitative PCR would also reveal if the HEK293 cells are transcribing the Olfm1 gene at all.

In addition to problems with transcription, translational or post-translational issues may have arisen in the HEK293 cells. It is unlikely that there are problems with the cells intracellular machinery, including ribosomes and tRNAs, because the positive control was detected in each Western blot analysis. However, Olfm1 is a recombinant protein containing the HA-tag that is charged. This may lead to issues in cleavage of the signal peptide that target the protein to the membrane or it could cause the protein to improperly fold. These sorts of issues were thought to be corrected for by creating constructs with HA tags at either the N- or C- termini of the protein. Additionally, companies such as Millipore and ClonTech claim that the HA epitope tag is not likely to affect the bioactivity or the distribution of the recombinant protein (ClonTech). Nevertheless, there is a slight possibility that perhaps the HA tag is interfering with the expression of not only Olfm1 protein but my colleagues Cpt1c and Adcy2 proteins. Since I cannot rule out this possibility, further testing needs to be performed. There are a variety of tags that can be used to create recombinant proteins that could be tested. For example, primers could be designed to create Myc-Tag, HAT-tag, FLAG-tag or His-tag.

Western analysis on the NT2 neurons indicate that the culturing method used to produce post-mitotic NT2 neurons was successful. Detection of neuron specific proteins including BDNF, which regulates glutamatergic synapses, Tau, which stabilizes axonal microtubules, and Synapsin 1, a phosphoprotein that is involved in neurotransmitter release at synapses, are all indicative of the successful differentiation of the NT2 cells into mature neurons. Further research is needed to detect the expression of GluR2 protein

in the NT2 neurons. Although Paquet-Durand *et al.* (2003) claimed that their protocol would produce differentiated cells that express glutamate receptors, perhaps AMPARs are not being expressed. Western blot analysis needs to be performed to detect the expression of GluR1, GluR3 and GluR4 proteins to determine if perhaps the differentiated cells do not express GluR2 protein but do express the other components of AMPARs. Alternatively, a dissimilar differentiation protocol could be used that would allow a longer period for the cells to differentiate into neurons, potentially increasing the likelihood of GluR2 containing AMPARs being expressed.

Immunostaining of the NT2 neuron/HEK293 co-culture assays further validated the successful differentiation of the NT2 cells into mature neurons. Synapses between NT2 neurons and HEK293 cells were visualized on those HEK293 cells expressing the synapse recruiting protein NLG and no synapses were visualized on HEK293 cells expressing the negative control GFP, a non-synaptogenic protein. This indicates that future assays with these neurons can be used to elucidate the effect Olfm1 and other GluR2-interacting proteins have on AMPAR trafficking.

Additionally, more effort needs to be invested in troubleshooting the Co-Immunoprecipitation assay that repetitively showed non-specific binding. The non-specific binding shown in Western analysis indicates that the light and heavy chains of the antibody conjugated to the beads were being detected between 25-60 kDa (Thermoscientific Troubleshooting Guide). Several approaches can be taken in the future to lessen the background interference. The first solution would be to try to bind the antibody to different agarose beads such as MagnaBind or IgG Beads (Thermoscientific Troubleshooting Guide). I could also alter the wash buffer by adding a detergent such as

Tween-20 or increase the number of washing steps in the protocol. Additionally, these washes could be alternated with distilled water washes to help reduce the nonspecific binding (Thermoscientific Troubleshooting Guide). Finally, more work needs to be done at the organismal level with the *C. elegans* to determine the behavioral effects that Olfm1 knockdown has on the nematodes. These results would be significant because they would provide further precedence for research on Olfm1 and its role in AMPAR trafficking.

In conclusion, there is insufficient information and data to accept or reject my hypothesis that Olfm1 is involved in AMPAR trafficking at synapses. A novel protein-protein interaction between GluR2 and Olfm1 cannot be ruled out because the proper assays were not successfully performed. The difficulties in cloning proved to be a major set-back in progression of the elucidation of Olfm1's role in AMPAR trafficking. However, my research ruled out numerous ways that cloning can and cannot be performed. Additionally, my research was important because it provided a solid platform for future undergraduates to continue research on the Olfm1 protein and GluR2-containing AMPARs. More research needs be done not only in the areas of molecular biology, but also at the organismal level to see what types of behavioral effects Olfm1 may have in *C. elegans.* Investigation of Olfm1's interaction with GluR2 subunits in retinal neurons at The Association for Research in Vision and Ophthalmology gives further priority and justification for continued research on Olfm1's possible role in AMPAR trafficking.

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