Cloning a genetically encoded pH sensitive fluorescent AMPA-type glutamate receptors subunit fusion protein (pHRed-GluA1) to study the internalization and subsequent degradation of AMPARs by the lysosome

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Abstract

The purpose of this study was to monitor internalization and endocytic sorting of AMPA receptors in membrane-bound compartments, particularly endosomes and lysosomes. In this experiment, a version of GFP-GLUA1 was created in order to serve as an indicator of delivery to the lysosome. In order to monitor the trafficking route of glutamate receptors, pHRed was cloned onto GLUA1 by means of restriction enzymes: AGE1 and BSRG1 using a Polymerase Chain Reaction. By ligating pHRed, a protein with a pH of 4, onto GLUA1, the acidic lysosome can be detected in the lumen, thereby observing the movement of surface receptors in endocytic sorting.

Background

The billions of neurons that compose the brain allow for processes and transmissions of electrochemical signals that can dictate daily activities and control the actions and responses of humans in various situations. Between the two nerve cells exists a synapse, which is the means in which a neuron can communicate to other neurons through chemical or electrical signals. Communication takes place between a neurotransmitter and its receptors. In excitatory synaptic transmission, the neurotransmitter glutamate released from one cell and bound to the AMPA receptor on the receiving cell. One way synaptic plasticity is achieved is to add or remove the number of AMPARs at the membrane which will strengthen or weaken the synapse, respectively. Here we are creating a version of GLUA1 (a subunit of the AMPARs) that will fluoresce at low pH indicating its internalization and delivery to the lysosome for degradation and thus weakening of the synapse.

Growing Bacteria

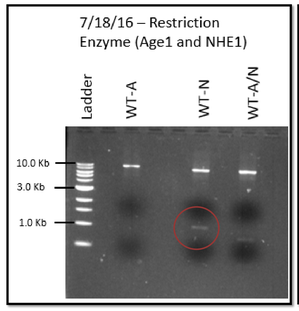
A process called streaking is used in order to grow bacteria colonies overnight. After one day, bacteria is picked from the petri dish and placed in a 37 degree Celsius LB + a solution with carbenicillin. The entire solution was then placed in the shaker room overnight.

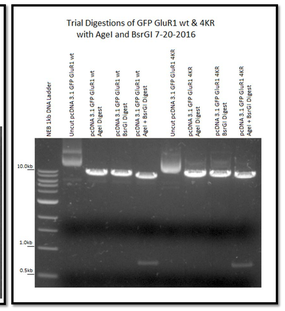
Purification of DNA

Samples of Wild Type DNA and Mutant 4KR DNA were purified in order to isolate DNA and eliminate the amount of contaminants from the samples, thereby limiting erroneous results. The initial step in DNA extraction is cell lysis, in which the structure of the cell is disrupted and the cell's soluble DNA is exposed. The lipids and proteins that compose the cell membrane are then degraded. Subsequently, an RNase is used to remove the RNA. In order to separate the DNA from the liquid solution and accumulate, isopropanol is added to the solution and centrifugation is performed. Once the DNA is precipitated, it is re-suspended in water. The isolated wild type versus mutant DNA was observed under a 1% agarose solution prepared by gel electrophoresis, a process that uses an electric field to allow the DNA to migrate towards the attractive positive electrode, that separates the bands by size.

Restriction Enzyme Digestion

A restriction enzyme digestion was prepared in order to cut DNA generating compatible ends that can eventually be ligated together. Initially, a digestion using the restriction enzyme NHE1 was carried out; however, the NHE1 cut the DNA sequence twice. Nevertheless, it was soon discovered that BSRG1 was the correct restriction enzyme that cut the sequence appropriately; thus, the two restriction enzymes used to cut the DNA sequence were AGE1 and BSRG1, which cut at A/CCGGT and T/GTACA respectively. Restriction enzymes were used to recognize specific sequences of nitrogenous bases in DNA in order to cut the DNA at those certain restriction sites. Once cutting the DNA at the appropriate restriction sites, fragments were produced and eventually separated by gel electrophoresis; the image can be observed be. low

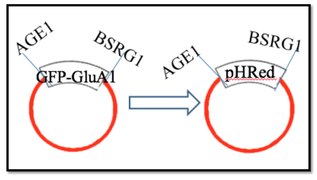




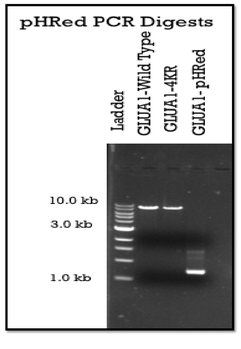
The two images here represent the gel that was ran, one with Age1 and NHE1 which proved that NHE1 cannot be used in the experiment because it cut twice (shown in the red circle). Therefore, looking back at the sequences the decision to use BsrG1 and Age1 was because they do not repeat. This proved this with the second gel image.

PCR: Cloning Strategy

The polymerase chain reaction (PCR) is a technique used to multiply a single copy or a few copies of a piece of DNA resulting in thousands to millions of copies of a particular DNA sequence. Primers are used, and carefully picked beforehand, due to their crucial role in initiating the PCR reaction, binding any side of the section of DNA that will be copied.

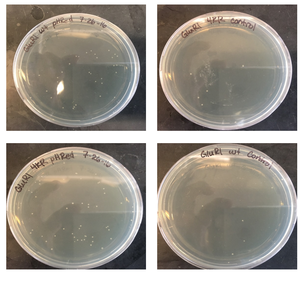


The PCR reaction goes through many heating and cooling steps called denaturing, annealing, and extending, the three main procedures in the heating and cooling phase. Denaturing separates the double stranded DNA through heat, annealing lowers the temperature in order for the primers to attach, and finally extending raises the temperature back again to construct the new strand of DNA.



Once cutting the DNA vector following the polymerase chain reaction, DNA purification was performed through column filters to isolate the DNA. The image to the left displays the results from a PCR gel digestion. GLUA1-Wild Type is contained in the first lane and GLUA1-4KR is seen in the second lane. Both the original GLUA1-Wild Type and the mutant GLUA1-4KR have been digested. The third lane contains solely of the pHRed.

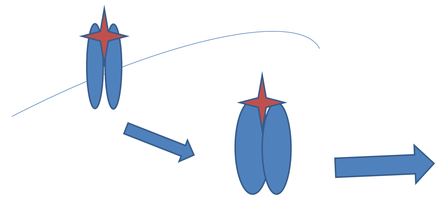
Ligation of pHRed and Transforming it to Bacteria



Ligation is the process of joining two linear fragments of DNA. This is the last step in re-constructing the plasmid, permanently joining the nucleotides together. The T4 DNA ligase enzyme, DNA fragments, and a buffer containing ATP are necessary in order for a ligation reaction to occur. Following the "pasting," the transformed bacteria was plated in order to grow colonies overnight. To the left is an image of the bacteria grown, with the control to help assist in visualizing the accuracy of the ligation.

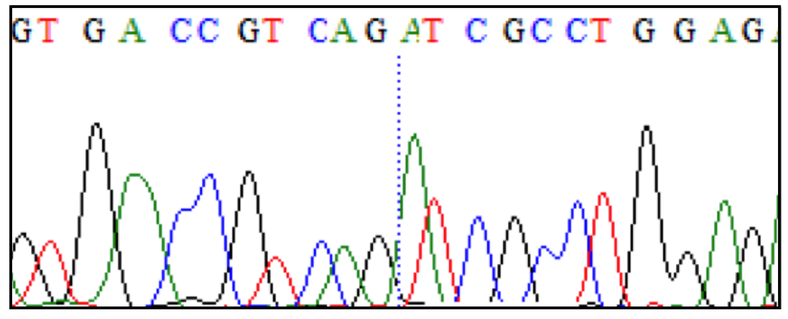
Trafficking of AMPARs to the Lysosome

Internalization and exocytosis account for the trafficking of glutamate receptors, such as AMPA and NMDA. In terms of trafficking to the lysosome for degradation, the use of a pH sensitive fluorescent AMPARs (pHRed-GluA1) which fluoresces at low pH (pH 4), we can track the movement of individual surface receptors as they are internalized and eventually delivered to the lysosome.



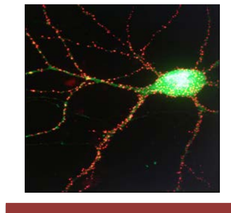
pHRed-GluA1 will floursece at pH4 and thus report when AMPARs have trafficked to the lysosome

Sequences



The chromatogram above shows the sequencing results from the GW1-pHRed-GluA1 fusion DNA we grew. The 30th to 50th bases of the DNA sequence are shown. The lack of 'N's in the sequence, and the fact that the peaks do not overlap prove that the results are as certain as the sequence suggests.

Validation Process



Further research includes a gel digestion in order to identify the clones that have the correct sequence. In addition, sequencing will be performed to prove the procedure was done correctly. Finally, transfecting primary rat dissociated hippocampal neurons is the last step to validate the procedure. In this process genetic material is inserted into mammalian cells, which allows the expression or production of proteins using the cell's own machinery. The image on the right shows what we would have anticipated to see under the microscope.

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