Case Study: Module 5, Small Molecule and Genetic Probes (Part 2)

Methods in Neurobiology

Overview

In this assignment you will be paired with another student and ask to analyze his work from Part 1 (Module 4). In this assignment the student can include techniques learned in Module 5.
This is a 20-point assignment.

Instructions

Once paired with another student, you will have to review your coworker's project illustrated in Part 1. The review will have to include:

1. An initial sentence/small paragraph to summarize the project you are reviewing.

Then answer the following questions using at least 1-2 sentences/bullet points:

- Do you think that their research plan is scientifically sound and appropriate? Why?
- Is there another experimental strategy that could answer to their scientific question? If yes, illustrate your method/s. If no, explain why.
- Please include references. Your work should be 1-2 page max (not including citations).

Few examples follow.

Example One

Part 1: Question/Biological Problem
Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset neurodegenerative disorder characterized by the progressive loss of motor neurons in the brain and spinal cord, which culminates in paralysis and death within a few years from diagnosis. New technologies for gene mapping have enabled the identification of nearly 30 genes in ALS pathogenesis[1]. The first gene discovered associated with familiar forms of ALS is SOD1. To date, over 180 different mutations have been described in SOD1 gene whose function is still unknown. One of the earliest and most common mutations discovered is a glycine to alanine substitution in position 93 of the protein.

Biological Question:
My aim is to investigate the role of SOD1 G93A in the accumulation of toxic oxygen species in cells.

Research Model: To study the function of mutant SOD1, this gene has been stably transfected in human neuroblastoma cell line.

- To see how expression of mutant copy of SOD1 affects cell physiology, a genetic probe named HyPer[2] developed to measure intracellular level of H2O2 and therefore production of ROS, would be transiently transfected in the cytoplasm of SOD1 G93A - expressing cells or in a control cell line carrying an empty vector.
- HyPer is based on the fluorescence properties of YFP, whose original sequence has been mutated to be sensitive to ROS accumulation. HyPer has two excitation peaks with maxima at 420 nm and 500 nm, and one emission peak with maximum at 516 nm. Upon exposure to H2O2, the excitation peak at 420nm decreases proportionally to the increase in the peak at 500 nm, allowing ratiometric measurement of H2O2.
- After 2 days cells will be visualized using a confocal laser scanner microscope and images at different wavelengths will be recorded. The decrease of absorption at 420 nm of the probe will be indicative of an increased of intracellular concentration of H2O2.

- If mutant SOD1 impacts the functioning of the redox machinery of the cell directly or by inhibiting endogenous wild-type SOD1, variations in the accumulation of ROS amount will be detected compared to control cells.

1. Beckman JS, Estévez AG, Crow JP, Barbeito L. Superoxide dismutase and the death of motoneurons in ALS. Trends Neurosci. 2001 Nov;24(11 Suppl):S15-20. doi: 10.1016/s0166-2236(00)01981-0. PMID: 11881740
2. HyPer is a product sold by Evrogen.
http://evrogen.com/products/HyPer/HyPer_Detailed_description.shtml

Part 2

- To investigate the role of mutant SOD1 in the accumulation of toxic oxygen species (ROS) in human neuroblastoma cells, Ms B. plans to express a genetic probe named HyPer, developed to measure intracellular level of H2O2 in the cytoplasm of SOD1 G93A- expressing cells or in a control cell line carrying the empty vector. Recordings through live cell imaging using this probe will measure the content of H2O2 in cells. If mutant SOD1 is part of the redox machinery of cells or its expression has a dominant effect on wild-type SOD1, I expect that cells carrying the mutant form will show a higher level of cytoplasmic H2O2 compared to control cells.
- The use of optical genetic indicators to measure the content of H2O2 is an extremely precise method that combines imaging with biochemistry and allows accurate determination of H2O2 content in live cells.
- Another way that could be employed to measure the amount of ROS and oxidative stress would be to measure mitochondria function through the employ of a mitochondria specific indicator. Uncoupling of the respiratory chain in mitochondria is one of cellular sources of ROS. In this way a probe that senses perturbation of mitochondria function can measure indirectly production of ROS.
- In order to measure toxic oxygen species, human neuroblastoma cells expressing mutant SOD1 and control cells will be treated with JC-1[1], a small molecule fluorescent ratiometric indicator, specific for detecting mitochondria membrane potential.
- JC-1 dye exhibits potential-dependent accumulation in mitochondria, indicated by a concentration dependent–fluorescence emission shift from the monomer (green, ~529 nm) that is prominent at lower dye concentrations, to aggregates (red, ~590 nm), which are formed as the dye concentration increases. In case of accumulation of ROS and mitochondria uncoupling, there is a progressive loss of red J-aggregate fluorescence and cytoplasmic diffusion of green monomer fluorescence. Consequently, mitochondria depolarization is indicated by a decrease in the red/green fluorescence intensity ratio.
- Thus SOD1 G93A-neuroblastoma cells and controls can be stained with JC-1, that is cell permeant and the amount of mitochondria dysfunction can be measured by imaging.
- The use of JC-1 is per se' an indirect method to measure ROS level.

1. JC-1 is a product sold by Thermofisher Scientific
https://www.thermofisher.com/us/en/home/life-science/cell-analysis/cell-viability-and-regulation/apoptosis/mitochondria-function.html

Example Two

Part 1: Question/Biological Problem

Recently, the efficacy of antidepressant agents has been a matter of controversy. The selective serotonin reuptake inhibitors (SSRIs), such as citalopram and fluoxetine (Prozac), are currently considered to be a first-line therapeutic tool for the treatment of depression, despite the fact that less than 40% of treated patients respond to this type of

antidepressant[1]. SSRIs were thought to moderate depressive symptoms by mainly enhancing the availability of synaptic serotonin (5-HT). However, several findings support the contention that certain SSRIs can block other monoamine transporters, such as those implicated in noradrenergic transmission.

Biological Question:
Our aim is to test if fluoxetine, a SSRI that blocks serotonin reuptake, has a similar effect on the noradrenergic system.

Research Model:
Use mouse brain slices from the locus coeruleus (LC).

Research Plan

- To test if fluoxetine can increase stimulation of LC neurons, a mouse model expressing a noradrenalin optical indicator in LC will be used. Briefly wild-type mice will be injected with a viral vector for targeted expression in LC of GRAB-NE, a genetic probe sensitive to the level of norepinephrin (NE). Since this probe is specifically sensitive to NE, there is no need to use a specific promoter for selective expression of the probe in a particular type of neuron, since only neurons carrying NE receptors will be activated.
- GRAB-NE[2] is a modular protein composed of an EGFP domain fused to a NE receptor domain able to bind NE. Once this neurotransmitter binds, a rearrangement in the protein causes deprotonation of EGFP and fluorescence emission.
- Mouse brain slices containing LC will be obtained from the mouse line described above and release of NE upon stimulation with fluoxetine or in control conditions will be measure in vivo using laser scanner microscopy.
- If fluoxetine inhibits NE reuptake as it does with serotonin, our method will allow to measure an increase of NE availability at the synapse.

1. Celada P, Puig M, Amargós-Bosch M, Adell A, Artigas F. The therapeutic role of HT1A and 5-HT2A receptors in depression. *J Psychiatry Neurosci*. 2004;29(4):252-265.
2. Feng, J., Zhang, C., Lischinsky, J.E., Jing, M., Zhou, J., Wang, H., Zhang, Y., Dong, A., Wu, Z., Wu, H., Chen, W., Zhang, P., Zou, J., Hires, S.A., Zhu, J.J., Cui, G., Lin, D., Du, J., Li, Y. 2019 A Genetically Encoded Fluorescent Sensor for Rapid and Specific In Vivo Detection of Norepinephrine. Neuron 102(4):745-761.e8.

Part 2:

- To test if fluoxetine, a SSRI that blocks serotonin reuptake, has a similar effect on the noradrenergic system, Mr. A is using an optical indicator of release of NE, that has been targeted via viral delivery to brain slices containing LC, a brain area with high number of noradrenergic neurons.
- This approach, although may present some technical difficulties in achieving widespread expression of GRAB-NE in all NE neurons, has the advantage to measure directly in vivo the amount of NE release due to neuronal firing after fluoxetine administration.
- Another way to measure the effect of fluoxetine in vivo, would be to measure the synchronization of the adrenergic network activity through live imaging and Ca^{2+} signaling.
- To do that brain slices from wild-type mice have to be loaded with a Ca^{2+} sensitive dye such as Fura2[1], that allows accurate measurements of the intracellular Ca^{2+} concentration through the ratio between the probe excitation spectra at 340/380 nm.
- If fluoxetine inhibits reuptake of NE at the synapsis, increased excitation of downstream neurons will be measured as an increased Ca^{2+} fluorescence.
- This method, although is an indirect measure of noradrenergic activity, has the advantage of eliminating the use of virus for gene delivery in organotypic brain slices.

1. Fura2 is a compound sold by Thermofisher Scientific. https://www.thermofisher.com/us/en/home/industrial/pharma-biopharma/drug-discovery-development/target-and-lead-identification-and-validation/g-protein-coupled/cell-based-second-messenger-assays/fura-2-calcium-indicator.html