*Clayton Seitz, Cellular Neurobiology Final Exam*

**To permanently label engram neurons of one specific memory event with ChR2, a “fos-CreER” line is often used, and AAV is often injected into the brain area of interest to achieve Cre-dependent ChR2 expression.**

**Please explain why a “fos” promoter instead of the “fos” coding sequence is used in the “fos-CreER” line.**

The fos promoter is thought to bind transcription factors in response to neural activity, for example during memory formation. By preserving this promoter and replacing the coding sequence with CreER, they are able to express CreER and, in turn, Cre-dependent ChR2 in response to neural activity during memory formation.

**Please explain why CreER instead of Cre has to be used in such studies.**

CreER is a fusion between Cre and a mutant form of ligand binding domain of estrogen receptor that keeps the Cre protein in the cytoplasm via interactions between ER and Hsp90. CreER entry into the nucleus can be controlled using drug treatment and without tight control over CreER entry, the experimenter can be certain that ChR2 is only being expressed in cells involved in the formation of memory of one specific event.

**Please explain how Cre-dependent ChR2 expression is achieved.**

A stop codon with loxP sites on either side is placed upstream of a ChR2 gene which is then excised in the presence of Cre. Thus, ChR2 is only expressed in the presence of Cre.

**Can another transgenic line be used to achieve Cre-dependent ChR2 expression – without using AAV? Why (or why not)?**

BAC transgenic is commonly used for replacing a coding sequence with the desired gene thereby allowing expression of the gene of interest to be under the control of the existing promoter. However, we want ChR2 expression to be Cre-dependent which, currently, would only be possible if we use a Cre recombinase dependent AAV.

**Explain how “tracing the relationship between input and output (TRIO)” was achieved in the published study in Beier et al. Cell 162: 622.**

Tracing the relationship between input and output (TRIO) is a process used to classify populations of cells based on which brain regions they receive their inputs from and which brain regions to which they project their outputs. This group used the Cre-lox and Flp-FRT system to achieve input output tracing.More specifically, TRIO was achieved by injecting CAV-FLExloxP-Flp which expressed a Cre dependent Flp recombinase into two subdivisions of the ventral striatum (lateral or medial nucleus accumbens), medial prefrontal cortex, and the amygdyla which had been identified previously. They also injected Flp-dependent AAV-CAG-FLExFRT-TC which expresses the avian EnvA envelope glycoprotein fused with mCherry and AAV-CAG-FLExFRT-G (Cre dependent rabies glycoprotein) into the VTA. G-deleted rabies virus expressing GFP (RVdG) was then injected into the VTA, being dependent on existing expression of TC and G for successful infection. In other words, the expression of GFP by the rabies virus can only occur in cells with existing TC and G expression which is, in turn, dependent on the retrograde transport of CAV-FLExloxP-Flp. In this way, the authors can perform output tracing - the only cells expressing GFP are those that output to one of the four brain regions where CAV-FLExloxP-Flp was injected. As for the input tracing, the rabies virus can propagate to VTA inputs in a retrograde fashion after being activated by VTA neurons i.e. GFP expression can be achieved in VTA inputs and, in utlimately they have established an input output relationship.

**From homology screening, you have identified a previously unknown protein that is expressed in mammalian neurons with significant overlap in amino acid sequence with the SNARE protein syntaxin. Describe two experiments that could help demonstrate whether this novel protein is involved in vesicle docking and/or fusion.  Please include diagrams of expected results.**

I will design one experiment that will show if this protein is involved in vesicle docking and another that will show if this protein is involved in vesicle fusion. These experiments together will show whether this protein is required for neither, one, or both.

**Experiment 1:** Gene Editing & Electron Microscopy

Diagram

Description automatically generatedFirst, I would introduce a mutation into the wild-type gene of interest via homologous recombination in mice. This would involve synthesis of a targeting vector with appropriate sequences for positive-negative selection e.g. neomycin resistance cassette and HSV thymidine kinase gene. Alternatively, it may be possible to develop siRNA that would silence the translation of mRNA transcripts of the target protein. In any case, it would be necessary to generate a number of blots to confirm the desired expression profile had been achieved. Following genomic or transcriptomic modifications, I would prepare tissue samples for analysis via electron microscopy. Electron micrographs could be used to count the average number of docked vesicles in wild type versus mutant samples and therefore determine if the sequence identified using homology screening it involved in vesicle fusion. I would expect that, since syntaxin is thought to bind to synaptobrevin during vesicle docking, there would be a reduction in the number of docked vesicles observed in the mutant

**Experiment 2:** Measuring Post-Synaptic Current

A picture containing sky, text

Description automatically generatedUsing the same mutant mouse line discussed above, I would utilize a patch-clamp experiment to measure the response of post-synaptic neurons to action potentials induced at the pre-synaptic neuron. Any notable impairment of the production of post-synaptic currents in mutant mice with respect to wild-type mice would suggest that expression of the sequence under consideration is important for fusion of synaptic vesicles and is potentially an alternate form of the syntaxin protein. Under the hypothesis that this protein is an alternate form of syntaxin, I would expect to see a reduction in the magnitude of the post-synaptic current along with a decreased signal to noise ratio.

**You recently discovered a compound extracted from a tropical plant that improves memory. Based upon the growing body of evidence demonstrating that memory is in part mediated by changes in synaptic strength, you set out to explore how this substance might alter excitatory synaptic transmission in the hippocampus. First, you’ll need to come up with a catchy name for the molecule – in anticipation of its possible use in the clinic. Second, outline a hypothesis regarding the effects of this molecule on excitatory synapses and describe three experiments that will provide mechanistic insights into the effects of this drug on synaptic strength. Be clear about the proposed experiments, including enough detail to ensure that the experiment is feasible. Be sure to explain strengths and limitations of your approaches.**

**Molecule Name**: Remembrin

In principle, Remembrin could operate at several stages of the putative pathway for synaptic plasticity. I will hypothesize that Remembrin operates early in this pathway by increasing release probability of glutamate exocytosis thereby enhancing synaptic plasticity at excitatory synapses. I will assume that I already have enough evidence to show that that I don’t need to design an electrophysiology experiment that shows increased postsynaptic response after treatment with Remembrin.

**Experiment 1:** Measuring EPSPs

The first step in showing that Remembrin affects synaptic plasticity is to measure changes in excitatory post-synaptic potentials after LTP induction in the presence and absence of Remembrin. More specifically, we can use patch-clamp techniques to measure post-synaptic membrane voltage before and after high frequency stimulation applied to pre-synaptic neuron (LTP induction). If Remembrin is involved in synaptic plasticity, we would expect to see an increase in the average amplitude of EPSPs after treatment and LTP induction.

**Experiment 2:** Measuring pre-synaptic glutamate release

Enhanced plasticity could occur via the release of higher amounts of glutamate into the synapse. To determine if Remembrin acts at this stage, I would attempt to measure changes in glutamate release after drug treatment. One method of precisely measuring glutamate in the synapse would be to use a fluorescence glutamate reporter with sub micromolar resolution. LTP could be induced in cultured hippocampal neurons with and without treatment with Remembrin while simultaneously acquiring fluorescent signal of such a reporter. Note that this experiment is limited in that it doesn’t tell us the origins of elevated glutamate section. For example, quantal analysis defines several parameters of interest here: the readily releasable pool of vesicles, probability of vesicle fusion, and quantal content. This experiment does not tell us which is modified but it is a reasonable choice given our broad hypothesis.

**Experiment 3:** Trafficking of AMPA receptors

The previous experiment determines if Remembrin induces changes to the post-synaptic cell. On the other hand, we may also want to determine if the drug affects synaptic plasticity by changing the post-synaptic density of AMPA receptors. To target only receptors in the post-synaptic membrane, we could use a chemical labeling technique, avoiding genetic manipulations if possible. Depending on experimental complications, a fluorescent dye to be covalently bound to AMPA receptors in the same cells in which LTP was induced. Barring spectral overlap, this would allow performing experiment two and three simultaneously. As with the previous experiment this would not tell us how Remembrin affects AMPA density in the post-synaptic membrane. However, under our hypothesis, we would expect to observe no change and this experiment would be sufficient to remove this as a possibility.

**You are recording synaptic transmission in a tissue slice preparation from brainstem where you expressed channelrhodopsin in a nucleus that sends dense axonal projections to the nucleus solitaries, a key center for adrenergic signaling and autonomic control. With the knowledge that this synaptic input likely influences adrenergic signaling in the CNS, you are particularly interested in examining the nature of this synaptic connection onto these adrenergic neurons.**

**Describe experiments that would help you understand the excitatory vs inhibitory effects of fast ion channel effects that you observe following activation of the synaptic terminals with 473 nm light flashes.**

We can design paired pulse experiments that can be used alongside the existing optogenetic tools. For example, we might glue an optical fiber to the glass pipet and record the post-synaptic current of adrenergic neurons in the nucleus solitaries in response to activation of channelrhodopsin in the nucleus with 473 nm light. Since it is widely believed that residual calcium in the presynaptic cell increases release probability of synaptic vesicles containing neurotransmitter, comparing the amplitudes of EPSCs in the post-synaptic membrane could reveal inhibitory or excitatory effects.

**Along with the rapid effects on membrane potential, you also note that high frequency stimulation results in a hyperpolarization of the neurons that persists for >20 min after stimulation. This persistent voltage effect involves a decrease in input resistance for the postsynaptic neuron. Illustrate the data that demonstrates the change in input resistance and present a hypothesis to explain the underlying mechanisms.  Outline 2-3 experiments that would provide insight into how this persistent change is induced and maintained.  It is important that you consider and incorporate intracellular along with intercellular mechanisms in your answer.**

Diagram

Description automatically generatedSince this effect is occurring over such long-time scales, it is unlikely that there is a presynaptic mechanism such as a change in the release probability of inhibitory neurotransmitter. Rather, it is more likely that a post-synaptic mechanism is at play which could be an intracellular change in the postsynaptic cell e.g., modification of post-synaptic receptors. Intercellular mechanisms such as reduced reuptake of inhibitory neurotransmitter which would result in continuous activation of receptors; however, it is highly unlikely that neurotransmitter remains in the synaptic cleft for this long due to diffusion. Since the voltage effect lasts >20min, I hypothesize that tetanic stimulation induces long lasting changes to post-synaptic ion channels such that input resistance is reduced and there is a long lasting hyperpolarization.

**Experiment 1:** Paired-Pulse Ratio

The first experiment will involve the measurement of the EPSCs in response to paired pulses of 473nm light on the nucleus expressing channelrhodopsin. The relative amplitude of EPSCs can be compared to determine excitatory or inhibitory characteristics of these axonal projections to the nucleus solitaries. For example, a reduction of the amplitude of the second EPSC induced by optogenetic stimulation would reveal an inhibitory effect. On the other hand, an increase in the amplitude would suggest an excitatory effect. In any case, it would be necessary to design the optogenetic stimulation at the short time scales required by these fast ion channels.

**Experiment 2:** Channel Blockers

The purpose of this experiment is to determine whether or not this lasting voltage effect is due to changes in ion channels in the post-synaptic cell. Since we already know that there is a decrease in input resistance at the post-synaptic cell, we can try to determine which channels are responsible for this change by measuring EPSCs before and after tetanic stimulation in the presence of various channel blockers. This would aid in resolving the EPSC into its different components and would potentially show which channels are modified by tetanic stimulation. However, this wouldn’t elucidate the origins of such modifications, but it would provide insight into the nature of the long-lasting hyperpolarization effect. Results could determine future experiments that analyze structural changes, genetic effects, etc.