Doctoral Dissertation

Generation of Orthogonal Engineered Synapse Organizers Toward the Development of Electrophysiological Technique with Target Recognition Capability

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Abstract

Exploring the cell and neurons is important for better understanding the physiology of the body system. This investigation is elaborated to convince the recording of biological work in non-invasive techniques with various micro- or nano-electrodes. In these ways, electrophysiology has become one of the best biological methods in ongoing research. Voltage-gated calcium channels are highly selective for calcium and have a broad range of animating or deactivating features. Based on their threshold of voltage-dependent facilitation, they are classified as high and low-voltage-activated channels. Somatic calcium can be recorded to show the activity of action potential. Somatic calcium can trigger gene transcription. N-methyl-D-aspartate is called an ion-based glutamate receptor and can mediate postsynaptic calcium ions in the cortex or pyramidal neurons. Calcium imaging is generally operated for the monitoring of interconnected neurons such as analyzing the circuitry in the cortex. This idea is also applied to recognize synaptically bridged neurons.

Previously, it was the major problem that cell-type specific recording was not possible but with time optical-approach-type electrophysiological techniques now can do this job. This was done by the genetically encoded protein which only detects the specific cell which is matched with their cell type-specific promoter. But it is still a problem for the prob/sharp microelectrode-based electrophysiological techniques to determine cell-type-specific recording. So, using the properties of a synapse organizer we are trying to develop a methodology by which we can soon record cell-type-specific recordings. For the development of prob/sharp microelectrode-based electrophysiology, especially for specific cell recording, we need to develop engineered synapse organizers. It will be helpful to attach specific proteins attached with prob/sharp microelectrode and then we can create a bridge for the specific cell recordings. Synapse organizer properties are helpful to make those types of recordings, but for that, I must use a genetically engineered organizer. For making genetically engineered synapse organizers I used their extracellular part for genetic engineering. Both post and pre-synaptic organizers were used to make genetically engineered synapse organizers. Used various types of protein which are orthogonally attached with the specific protein.

There are several numbers of pre-synaptic organizing proteins. Neurexins (Nrxs) work as presynaptic organizers and are well-known directors of synapse effects thus playing a vital role in gathering and rebuilding structure through performing with many presynaptic and postsynaptic molecules or ligands. Neuroligins (NLs) are the most especially known Nrx partners. Five NL genes are present in human beings, and they are called NL1, NL2, NL3, NL4, and NL5. Through alternative splicing, NL and Nrx make their bonding, and both are controlled by their gene. Nrx and NL furnish trans-synaptic affinity through Ca^{2+}-dependent interlinkages of their substitute spliced outer cellular domains. The principal outer cellular domain of NLs expresses analogous with acetylcholinesterase (AChE) but misses cholinesterase interest and they mediate linking to Nrxs. Over exhibition of AChE, losses amount of β-Nrxs in vivo and cell culture then lessen the evolution of glutamatergic synapses in cell culture, which indicates that may be crosstalk within two or more proteins.

In the orthogonal test, I found GFP-nanobody containing Nrx bounds with GFP and venus protein. mCherry-nanobody contains Nrx bounds with mCherry protein. mCherry contains Nrx bounds with mCherry-nanobody protein. Spot and BC2 contain Nrx bound with BC2-nanobody and spot-nanobody proteins respectively. GFP-containing Nrx showed their existence extracellularly in the dark fluorescence view and can bind with GFP-nanobody. I did a cell-microbead interaction experiment. In the cell-microbeads interaction experiment, I confirmed with a presynaptic marker called an anti-synaptophysin antibody. GFPnull/YFP containing engineered NrxΔ1 bound with GFP-nanobody microbead and confirmed by postsynaptic Anti-FLAG and Rab-3 markers. It was not easy to develop a workable engineered synapse organizer from the natural one. I considered the working principle of the synapse organizer and then thought to apply it in prob/sharp microelectrode-based electrophysiology for the recording of a specific cell. Also, we need to consider the microelectrode array with a specific medium and voltage for the prob/sharp microelectrode-based electrophysiological specific cell recording. In the future, a specific probe/sharp microelectrode with orthogonal tested protein will be developed for the desired development of the prob/sharp microelectrode-based electrophysiological technique to initiate specific cell recording. I succeeded in the development of engineered synapse organizers. In conclusion, it can be said that my engineered synaptic organizers responded positively.
Keywords: Synapse organizer, neurexin, neuroligin, synapse, electrophysiology.

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General Introduction

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- **Concept of Prob/sharp microelectrode preparation for Prob/sharp microelectrode-based electrophysiological technique.**

General Introduction:

The ongoing research into the physiology of the animal body system advances with other basic parts of the electronic components. Then, a new era has been started as electrophysiology. Physiology cannot be understood broadly as the basic function of the cell in the different organs without the help of electrophysiology. Electrophysiology helps to know the molecular level of functions of the different physiological systems of the animal body systems. Electrophysiology is the section of physiology, where biological cells' and tissue's electrical properties are studied comparatively. It concerns the voltage measurements or electrical current in a whole cell or a culture medium of the neuron or other kind of cell. It measures the action potentials of the neuron and can define neuroscience also. Electrophysiology is very necessary for electrodiagnosis. For electrodiagnosis electrophysiological recording is mandatory.

Through electrophysiological techniques, a big range of neuronal activity can be known by the spiking of neurons with the slower connections of oscillations in a narrow population [1-2]. Exploring the cell and neurons is important for better understanding the physiology of the body system. The complexity of the brain with the drawbacks of technical or ethical constraints led the researchers to depend on brain slices or in vitro cultures of neurons for recording and analyzing the dynamics of neurons [3]. Knowing how the nervous system pursues external environments, keeping memories or origin to cognition, and controlling the body system has become familiar from the various scientific area working together [4]. With exposure to biological science and nanomaterials technology, it can be possible to determine or investigate the measurement at the nanoscale and their properties with their characteristics. This investigation is elaborated to convince the recording of biological work in non-invasive techniques with various micro- or nano-electrodes. In these ways, electrophysiology has become one of the best biological methods in ongoing research.

1.1 Electrophysiological tools: There are a lot of electrophysiological tools in the modern era. Using those electrophysiological tools electrophysiological works are ongoing to achieve the basic recordings of different in vitro or in vivo systems. Those recordings help to understand the common functions of different types of cells or neuron networks. Among those tools, microelectrode arrays (MEAs), Patch clamps settings, prob, calcium indicators, voltage sensor

domain (VSD), genetically encoded voltage indicators (GEVI), VSD-based GEVIs, genetically encoded calcium indicator (GECI), fluorescence dye are famous for various recordings. All these electrophysiological tools can record voltage, electrical activity, or membrane potentials. All those electrophysiological tools also have a lot of varieties to work in a designed and specific cell medium. From the beginning of electrophysiological tools, researchers have been updating the tools and their design in many ways like using various patterns and sizes of electrodes in MEAs and VSD. Researchers also tested various types of proteins in those devices and methods of recording.

1.2 Electrophysiological techniques: There are a lot of electrophysiological techniques for recording, in the following only some famous techniques are named in a brief:

Classical intracellular electrode: Under this type of electrophysiological technique many techniques are available. From those patch clamps for the whole cell, sharp glass electrodes, Planar microelectrode array (Planner MEA), Gold mushroom microelectrodes (GMµEs), and Vertical nanowire electrode arrays (VNEA) are famous for current research. These devices work as alterations in ionic content on the cytoplasm. Followed by dilution of diffusion, which causes necrosis and hampers the normal physiology of the cell [5].

Gold mushroom microelectrodes (GMµEs): This is the latest and most famous 3D vertical model for recording neuron signals [5]. To increase the coupling coefficient Spira et al. fabricated the GMµEs which mimic the neuron's culture with their shape and specific dimensions [6]. This microelectrode has a specific process that results from better seal resistance value and decreased cleft within the neuron [5]. This microelectrode can measure subthreshold activity created through individual neurons [7]. It also has a better capability to record extended periods with more effective stimulation without causing harmful effects on the cell for several days [8]. Which allows a better signal-to-noise ratio in electrophysiology techniques [5,8].

Whole-cell patch-clamp: The whole-cell patch clamping is the most famous way for membrane potential measuring [9-10]. The whole-cell patch settings can measure electrical potential directly, although this technique is invasive and only worked in isolated membranes [11]. It is

also unable to measure membrane potential dynamics under the functional study of various physiological conditions [12].

Genetically encoded voltage indicators (GEVI): GEVI with chemo genetic sensors can work with voltage indicators in the specific neuron culture and plasma membrane of the cell [13]. Recently GEVI presents new logic that using engineered protein can remove some of the drawbacks of the voltage-sensitive dyes (organic). Development of GECI, VSD separated from phosphatase are made a subfamily of GEVI by conjugating to fluorescence proteins [14]. An updated category of GEVI is a genetic voltage sensor with intact organic or protein particles, which help to furnish chemical as well as genetic patterns [15].

Genetically encoded calcium indicator (GECI): In the neurons calcium ion makes intracellular signals which hold the main functions. Calcium ions express specific functions in neurons, so it is important to imaging calcium [16]. Chemical fluorescence and protein-based genetically encoded calcium indicators achieved high praise from the researcher due to their ability to image specific inputs in cortical neurons [16]. Calcium ions can generate adaptable intracellular signals and can measure a lot of functions in almost all cell types [17], especially in cells related to heart muscle contraction [18], and can regulate the major properties of cell necrosis [19]. In the presynaptic end, calcium influences the exocytosis of neurotransmitters along with synaptic vesicles [20]. On the other hand, in the postsynaptic end increase of calcium in the dendritic section for induction of synaptic plasticity [21].

Voltage-gated calcium channels are highly selective for calcium and have a broad range of animating or deactivating features. Based on their threshold of voltage-dependent facilitation, they are classified as high and low-voltage-activated channels [22]. Somatic calcium can be recorded for showing the activity of action potential [23]. Somatic calcium can trigger gene transcription [24]. N-methyl-D-aspartate is called an ion-based glutamate receptor and can mediate postsynaptic calcium ions in the cortex or pyramidal neurons [25,26]. Calcium imaging is generally operated for the monitoring of interconnected neurons such as analyzing the circuitry in the cortex [27]. This idea is also applied to recognize synaptically bridged neurons [28,29].

The somatic calcium signals in neurons are liaised by the entry of calcium ions with the help of voltage-gated calcium ion channels because of the action potential. Neural circuits are analyzed in vitro or in vivo by imaging of calcium when useful voltage imaging is not approaching in vivo [23, 30,31]. One part role of dendritic signals is the amplification of calcium ion signals that are induced by synaptic activity [32,33]. A few years ago, Transgenic mice exhibiting troponin-C mediated calcium indicator called CerTN-L15 can record glutamate-influenced calcium signals in vivo systems, especially from dendrites [34]. ‘‘Fiberoptic periscope’ is the way to record dendritic signals, especially calcium signals action in vivo [35-36].

From the above introductory discussion, we can say that there are mainly two types of electrophysiological techniques. Those are probe/sharp microelectrode-based electrophysiological techniques and optical approach-based electrophysiological techniques. Previously, it was the major problem that cell-type specific recording was not possible but with time optical-approach-type electrophysiological techniques now can do this job. This was done by the genetically encoded protein which only detects the specific cell which is matched with their cell type-specific promoter. But it is still a problem for the probe/sharp microelectrode-based electrophysiological techniques to determine cell-type-specific recording. So, using the properties of a synapse organizer we are trying to develop a methodology by which we can soon record cell-type-specific recordings. Figure 1 described the probable technology which I desired for the near future.

For the development of probe/sharp microelectrode-based electrophysiology, especially for specific cell recording, I need to develop engineered synapse organizers. It will be helpful to attach specific proteins attached with a probe/sharp microelectrode and then we can create a bridge for the specific cell recordings. Synapse organizer properties are helpful to make those types of recordings, but for that, I must use a genetically engineered organizer. For making genetically engineered synapse organizers I used their extracellular part for genetic engineering. Both post and pre-synaptic organizers were used to make genetically engineered synapse organizers. Used various types of protein which are orthogonally attached with the specific protein. Pre-synaptic Neurexin (Nrx) showed better results than post-synaptic Neuroligin (NL). So, we give more emphasis to presynaptic Nrx for making genetically engineered synapse

organizers. Later I discussed details about those but to understand the synapse organizers principle we must know their mode of action and their nature. So, in the following, I have discussed the nervous system and its functions with synaptic organizers.

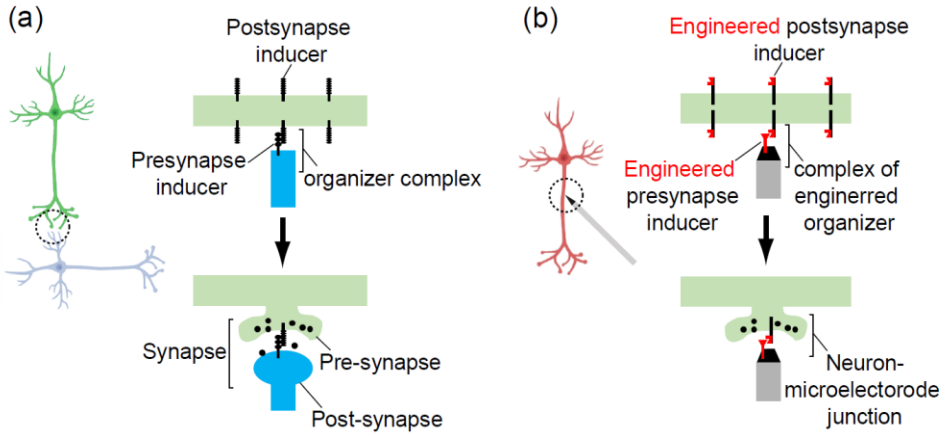

Figure 1: Desired probe/sharp microelectrode technology for cell-specific recording in probe/sharp microelectrode-based electrophysiology.

The animal body is made of cells. A bundle of cells makes tissue, organ, and other parts of the body. Cells are the fundamental unit of the animal body. There are also different types of cells in the animal body like hepatic cells, pancreatic cells, heart muscle cells epithelial tissue cells, blood cells, kidney cells, brain cells (Neuron), etc. Every cell has specific works to keep the organ fit and maintain the body's basic functions. Animal body functions are maintained by the brain and help to recognize the problem in the body and their solutions. In the brain, neurons are the fundamental unit. The brain produces impulses and transmits the impulses through neurons. Through impulse transmission and reception, the brain controls the body's functions and essential needs. For impulse transmission and reception, a single neuron cannot complete the work. So, neurons can make junctions with other neurons and can build long transmission and reception channels. When one neuron pairs with other neurons then the pairing junction is called the synapse. For making synapses there are a lot of synapse organizers. Synapse organizers are associated with synapse-making and destruction.

1.3 Neurons: Neurons are the functional and structural units of an animal's nervous system. Neurons can be electrically excitable [37] with other cells through synapses. Synapses are particularized connections between two or more neurons. For this reason, neurons are the main part of the nervous system. Excepting sponges and placozoans, all animals have neurons but in the case of plants, it's absent. In a basic normal neuron, there are three parts. They are cell bodies named soma, dendrites, and an axon. Dendrites are branched but the cell body/soma is compact. Generally, dendrites extend from the cell body/soma and have branches. Dendrites are usually a few hundred micrometers in length. The axons are about 1 meter in humans and longer in other animals. Axon's heads form the cell body/soma and in the endpoints, they branch but maintain a perpetual diameter. The axon branches are named axon terminals. Through these terminals, neurons can transmit electrical signals via synapses. Most of the neurons get their signals via cell body/soma and the dendrites then pass those signals with the help of axons. In most natural synapses, signals proceed from axons to dendrites between two neurons or more.

1.4 Synapse: Synapse is a bridge between two or more neurons. In the brain, they are the exclusive junction between the neurons. Neurons are connected by synapses and transmit millions of information overlapping neural circuits that are interdigitated [38]. One neuron's axon part bonds with another neuron's dendrite and then makes a synapse bond. Neuron passes the electrical signals via a synapse. In this way, neurons are connected and then send electrical signals and receive electrical signals from the body parts of animals. The central nervous system depends on synapses because if there is any disruption of synapses the central nervous cannot send signals and cannot receive signals as well. So, the synapse has a vital role in the central nervous system. Figure 2 shows the neuron's part and their synapse-making. On the right side of that picture in the box is the synapse region where pre- and post-synaptic organizers are available. Here I showed only a synapse between two neurons but in nature, it can be more than two in number. Organizers are not connected directly but they make the environment form a synapse and relay information as impulses.

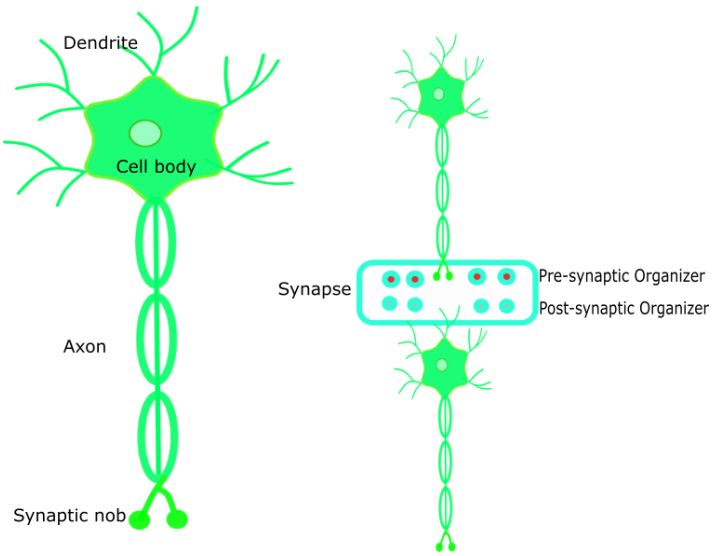

Figure 2: Neuron and synapse formation with the association of synaptic organizers.

An idea named neuron doctrine suggests that neurons are not continual in the whole body but can rely on signals [39]. Some researchers also gave some ideas that synapses may involve the transmission of signals from the neuron to any cell of the animal body [40]. Neurons are not directly associated with synapse formation. For synapse formation, the presynaptic molecule creates differentiation, and the postsynaptic molecule also showed its receptive activity [41]. All those reactions are maintained by synapse-organizing proteins [41]. The presynaptic neuron's plasma membrane ends position with the postsynaptic neuron's plasma membrane for desired signal processing. When the molecular machinery process is confirmed, then they are ready for signal processing. Generally, the presynaptic site takes a position in the axonic end of the neuron, and the postsynaptic site takes a position in the dendritic end of the neuron. Astrocytes are also involved in neurotransmission by exchanging stimuli with synaptic neurons [42].

Most of the synapses are chemical synapses and they are stabilized by synaptic adhesion molecules (SAMs). SAMs are expected from presynaptic and postsynaptic organizers and conjugate together where synapses are overlapped [43]. The neurotransmitter is a chemical released by calcium channels. Calcium channels release the neurotransmitter when they are activated through electrical signals released by presynaptic neurons in a chemical synapse. The neurotransmitter connects to the receptors of postsynaptic neurons and may start electrical reactions or partial message carrier ways which are either inhibitory or excitatory. There are four

types of chemical synapses based on their neurotransmitter-releasing nature. GABAergic chemical synapse sometimes shows inhibitory actions on the other hand glutamatergic chemical synapse shows excitatory actions.

The neuromuscular junction of vertebrates acts as a cholinergic synapse and an adrenergic synapse releases norepinephrine. Chemical synapses can produce various complex effects for the postsynaptic cell and then enhance the complexity of receptor signal transduction. In an electrical synapse, there is a gap junction called a synaptic cleft where postsynaptic and presynaptic cells can make a bridge through limited channels [44]. Those channels can pass electrical signals through voltage differentiation between presynaptic cells and postsynaptic cells. Rapid transfer of electrical current from one cell to another cell is the principal matter of the electrical synapse [44]. Based on cellular compositions in the presynaptic and postsynaptic elements, synapses can be divided into several numbers. Most familiar synapses called axodendritic are found in mammalian nervous systems. However, other varieties are also present, like axo-axonic, axo-secretory, somato-dendritic, dendro-dendritic, dendro-somatic, and somato-somatic synapses.

1.5 Introduction of Synapse Organizer:

Synapse organizer has the leading role in cell adhesion and protein organizing for interacting networks. The interaction of synapse organizers with their collaborators can either be strengthened or weakened by alternative splicing [45]. In vivo, these organizers may be used to control synaptic communications [45]. Synapse organizers are tethered to pre- or post-synaptic by transmembrane and elaborate their extracellular domains within the synaptic cleft [45]. Synapse organizers can perform the heterophilic with the different adhesion molecule families [45]. Through similar synaptic functions, synapse organizers can bind with proteins and form trans-complexes or cis-complexes. The trans-complex relays the adhesive work monitored for synapse organizer into the cell-dependent assays. However, synapse organizers are encouraged for crucial roles surpassing adhesion to the presynaptic membrane and the postsynaptic membrane in conjunction [45]. For neuron-neurons conceding and generating scaffolds, the synapse organizer has a distinguished role in binding additional proteins [45]. Some of the synapse organizers can bind partners chained to a homogenous membrane side by side and form a "*cis*-complex." That *cis* protein congregates are regulatory in nature, binding, forming, or

altering a crucial pre-complex onto which another partner can harbor to bring in the *trans*-synaptic connection [45].

1.6 Introduction of the pre-synaptic organizer:

There are several numbers of pre-synaptic organizing proteins. Neurexin (Nrx), Receptor type protein tyrosine phosphatases (RPTPs), Neuronal Pentraxins, Teneurins, Netrin-G1, and Netrin-G2 are the well-known presynaptic organizers [38]. Nrx and RPTPs survive in multiple isoforms originated by alternative splicing and then interact in a splice-selective code including diverse postsynaptic partners [41]. In vitro, authentication suggests that Nrx differentiation in the early stage of the axonic part into a presynaptic terminal [58,62]. Nrx is exhibited in thousands of substitute spliced isoforms. Nrx maintains synaptic characters through differentiation and bonding to numerous postsynaptic organizers like neuroligin (NL), and netrin G ligand 1, thereby continuing their neural circuit's ingoing or outgoing connections [38].

Nrx works with presynaptic organizers and are well-known directors of synapse effects thus playing a vital role in gathering and rebuilding structure through performing with many presynaptic and postsynaptic molecules or ligands [38]. In the co-culture system of the neuron and the cells other than the neuron, Nrx shows the postsynaptic activity on the neuron culture from the cells other than the neuron [64]. Nrxs, other molecules, and ligands in the next turn deal with different intra and extracellular signaling proteins through a controlled way of building an active molecular network [38]. Affinity chromatography technique was used for the identification of Nrx 1α 1992 [46,50] from the extract of rat brain through an α-latrotoxin column. Former studies by Sudhof and collaborators have shown Nrx and tethering partners of them like NLs [47]. Nrxs are naturally monomeric and remain unbound state [58-61].

There are two types of Nrxs. α-Nrx and β-Nrx are encoded by three and two genes respectively. In the neuron, all of those can show expression. In the adhesion molecule, α-Nrx contains six LNS domains. For the settlement of the Ca^{2+} channel part and NMDA (*N*-methyl-D-aspartate) receptors, α-Nrx plays a crucial role in the synapse environment [53,54]. On the other hand, β-Nrx trims α-Nrx and has a single LNS domain. However, Nrxs are only one type of molecule in a trans-synaptic system. When Nrxs are recognized, they show surface identification in the specific

synapse through alternative splicing [38]. This hypothesis is expanding and validated. The characterization of Nrxs roles and interactions is only starting. The logical means from the valid knowledge says that the Nrxs do not work in a single but also, but they are involved in various connections and conduct some different performances in distinct synapses [38].

1.7 Introduction of the post-synaptic organizer:

There are several numbers of post-synaptic organizing proteins. Neuroligin (NL), dystroglycan, GABA-A receptors, Calsyntenins, Latrophilins, netrin G ligand 1, netrin G ligand 2, netrin G ligand 3, and GluA1-4 are the famous postsynaptic organizers [38]. In vitro, authentication suggests that NL accumulates in the early step differentiation of the dendrite portion into a postsynaptic terminal [58,62]. NLs showed their performances on the non-neuronal cell's surface by influencing synaptic vesicle accumulation. In the glutamatergic synapse connection, they developed functional release spots [47]. In the co-culture system of the neuron and the cells other than the neuron, NL shows the presynaptic differentiation on the neuron culture from the cells other than the neuron [63]. In the co-culture system of the neuron and the cells other than the neuron, Nrx shows the postsynaptic differentiation on the neuron culture from the cells other than the neuron [64].

NLs are transmembrane proteins, which are detected as ligands/receptors for presynaptic Nrxs. NL can form homophilic adhesions with the same molecules, semi homophilic adhesions with associated members. They can form heterophilic connections with another interacting molecular family. A huge number of synapse organizers are present in the brain, especially for mammals, which are involved in development and control [45]. Inducible dimerization experiments proposed that dimerized NL induces presynaptic molecules as the main part of the correlated convention of a chemical synapse [62]. Naturally, NL remains in a dimer form [58-61]. NLs are the most especially known Nrx partners. Five NL genes are present in human beings, and they are called NL1, NL2, NL3, NL4, and NL5 [51]. Among them, NL1 and NL2 are famous among scientists, because they showed good results in lab research. NL1 showed excellent presynaptic differentiation on cultured neurons which are placed in a cell to cells interconnected with HEK293 cells [48]. So, as a postsynaptic organizer, NL1 has been well recognized.

1.8 Mode of action of synapse organizer and engineered synapse organizer:

Nrx 1β with laminin, Nrx, and sex hormone binding globulin forms the shape of a crystal and is named Nrx1βLNS. In the alternative splice sites, they showed remarkable maintenance with the agrin LNS domain [49]. The second LNS domain called Nrx 1α disclosed that the splice plot makes an extremely variable exterior rounding of a synchronized Ca^{2+} ion [50]. Through the depletions in split Ca^{2+} concentrations accompanying the synaptic task, some Ca^{2+}-dependent exchange of Nrxs might be triggered. Nrxs have three well-noticed outer cellular binding participants in the brain, especially in mammals: NLs, neurexophilins, and dystroglycan [50]. Naturally, NL is a dimer-forming molecule and Nrxs are naturally monomeric and remain unbound state monomeric [59]. An NL dimer binds with two Nrxs in an asymmetric tetramer [58-61].

Through alternative splicing, NL and Nrx make their bonding, and both are controlled by their gene. For example, un-spliced NL can make chain β-Nrx not but with α-Nrx. Splicing initiates isoforms that are the tie between β-Nrx and α-Nrx by detaching an eight-amino-acid sequence located from NLs. That bonding could be participated in balancing synapse properties [55]. NL1 was recognized because of its potential to attach definite isoforms, particularly from three β-Nrx [56]. Nrx and NL furnish trans-synaptic affinity through Ca^{2+}-dependent interlinkages of their substitute spliced outer cellular domains [48]. NLs show Ca^{2+}-dependent binding to α-Nrx and β-Nrx, neurexophilin binding is Ca^{2+}-independent with α-Nrx only, dystroglycan expresses Ca^{2+}-dependent bonding with α-Nrx preferentially [50]. In Figure 3, I showed the working module for natural and engineered synapse organizers.

Through affinity chromatography, Nrx 1β, and NLs were first identified [50]. The principal outer cellular domain of NLs expresses analogous with acetylcholinesterase (AChE) but misses cholinesterase interest and they mediate linking to Nrx. Over exhibition of AChE, losses amount of β-Nrx in vivo and cell culture then lessen the evolution of glutamatergic synapses in cell culture, which indicates that may be crosstalk within the two or more proteins [52]. Thus, Nrxs and NLs have numerous isoforms that navigate from alternative splicing and numerous genes. Both Nrx and NL have relatively small inner cellular domains that are close to PDZ-domain

attaching sites, which are probably important for associating with different synaptic proteins [50].

In general, the natural synaptic organizer can associate with several opposite synaptic organizers at the same time. Nrx can bind with NL, dystroglycan, calsyntenins, and latrophilins at the same time. Latrophilins also can bind with Nrx and teneurins at the same time. The outer membrane of the natural synaptic organizers has a complex structure. It is still difficult to understand how they work with several partners. The most significant mode of action of an engineered synapse organizer is that it only binds with one opposite synapse organizer. There are some specific proteins in nature that have an attraction to some specific protein of their group. In the engineered synapse organizer, the outer part of the natural synaptic organizer was removed and replaced by a specific protein. So, only a specific protein containing two engineered synaptic organizers can associate for making the synapse.

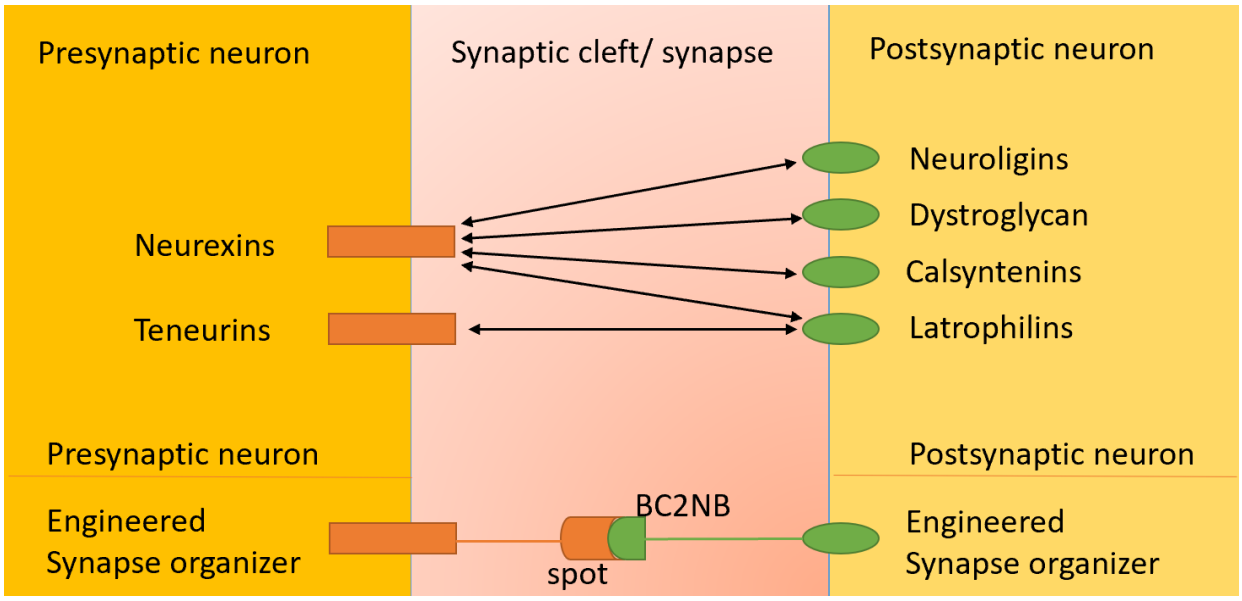

Figure 3: Working module of natural and engineered synapse organizers.

1.9 Relation to various diseases:

Synaptic adhesion/organizing molecules (SAMs) such as Nrx, NLs, contactins, and cadherins are used in neurodevelopmental or neuropsychiatric diseases. For the development of therapeutics

for those diseases, SAMs are studying for knowing molecular mechanisms[45]. Immense proteomics surveys and various literature reviews judge that about 1,900 to 2,700 proteins are contained at synapses [77-79]. Those synaptic proteins can be identified through participation in exocytosis and recovery of synaptic vesicles, various receptors for distinct neurotransmitters, extracellular matrix proteins, ion channels, cell adhesion molecules, scaffolding proteins, cytoskeletal proteins, membrane transporters, phosphatases, GTPases, and involved molecules in protein mortification [45]. The exact area of a synapse is not identified, so researchers have generally relied on protein, confinement with synaptosomal membrane scrapings, then microscopy to appoint a synaptic identity [45]. Another note is that those proteins are not confirmed in their exact location at a definite synapse and how they distribute and express in different types of synapses.

Synapse defects are responsible for neuropsychiatric and neuron-related diseases [80]. A lot of genes are exposed to diseases like autism spectrum disorder, and other behavioral, schizophrenia, and cognitive disorders, thus many of those code synaptic proteins [81-84]. Hence, “synaptopathies” is popularly used to mention neurodegenerative, neuropsychiatric disorders, and neurodevelopmental diseases, that suggest the disordering damaging of synaptic proteins [85-86]. Synapses can be explored as vast protein interaction networks through the host of proteins based on synapses. They are plastic or change in feedback to synaptic activity; moreover, damaging these synaptic channels supports the pathology of behavioral and neurological disorders.

Mutated genes associated with Nrxs and ligands of Nrxs are responsible for schizophrenia, autism, and neuropsychiatric disorders. Nrxs dominate the general trans-synaptic network, which maintains synapse materials, thus creating the synaptic responses to specific point patterns in the neuron and circuit which endangered defacement in neuropsychiatric abnormalities [38,41]. In a broad sense, there are various challenges in understanding synapse formation and its functioning. Thomas C. Sudhof proposed that synapses are mediated through various molecular machines and those operate side by side along with or without other interactions such as lateral interactions [38]. Nrx complexes are the main component of these machines and others are also important. The mode of action of these machines is necessary to understand their interactions with their

compositions then which will give priority to look insight into how synapses are building with neural circuits. In this way, the compromise of synapses in neuropsychiatric disorders may be shown [38].

Receptor-type protein tyrosine phosphatases (RPTPs) encoded genes with their postsynaptic collaborator are related to neuropsychiatric diseases like schizophrenia, Tourette syndrome, and autism [38,41,66-70]. Those genes that have a deleterious mutation in Nrx and their related partners [41,65,71] may support the possibility to make aberrant synaptic coordination would be the basic pathophysiology of neuropsychiatric diseases. RPTP hubs can cause the adaptation of genetic threats [41]. The densely interconnected and partly reduced character of the design, especially Nrx and RPTP hubs maintains through functions along with losses of single synaptic proteins. However, this allows moving in enhancing or inhibiting equilibrium and plasticity or stability on account of detrimental mutation of independent genes [41]. Shifts may be corrected by enhancing or inhibiting the role of other remaining proteins. This matter is clinically appropriate given how these mutated or disordered genes are used for neuropsychiatric diseases or neuropsychiatric disorders [41].

Various neuropsychiatric diseases like attention deficit hyperactivity (ADHD), schizophrenia, OCD, and autism spectrum disorders (ASD) are highly heritable, thinking vast genetic research for the above disorders [41]. Whole exome sequencing, copy number variant (CNV), and genome-wide single nucleotide polymorphism (SNP) have exposed various gene mutations in alliance with the above disorders. A group of a subset of those mutations is directly engaged in the functional activity and the development of synapses. Additionally, mutated genes encode cerebellin-GluRδ, LRRTMs, NLs, and their common partner Nrx. Hideto Takahashi also reviewed PTPδ, PTPα [41,65,71-74], and IL1RAPL1, slitrk-associated family, and TrKC are showed an independent alliance with neuropsychiatric disorders. Ishizuka et al. revealed that Nrx1 has an LNS4 domain that is responsible for maintaining the membrane localization and delocalization which is directly linked with the etiology of autism spectrum disorder and schizophrenia.

Mutations in one gene on a specific synaptic organizer are partnered with a vast number of

Neuropsychiatric abnormalities such as exonic removal of Nrx1 are linked with an intellectual disorder, ASD, language delay, and schizophrenia [41,75,76]. On the other hand, a single neuropsychiatric disorder is associated with the mutation of various genes encoding synaptic molecules. In a nutshell, Nrx complexes, RPTP complexes, and dysfunction of any synaptic organizer complex through changing the structure and function in the synapse may indicate basic pathogenesis for a lot of neuropsychiatric abnormalities [41].

Many SAMs, like as NLs, LRRTMs, Nrxs, leucine-containing proteins, CNTNAPs, contactins, and cadherins are involved in neurodevelopmental diseases, and neuropsychiatric problems, such as bipolar disorder, epilepsy, autism spectrum disorder, mental retardation, and schizophrenia, [81, 87-91]. It was hypothesized that SAMs played vital roles in the formation and their lesions may be responsible for the inhibition of synapse formation or disruption. Hence it was confusing why a shortage in those molecules, if so crucial for synapse development, was particularly connected to cognitive or behavioral disorders or leaving other brain issues like the synchronization of mobility and the operation of auditory as well as visual information seemingly undisturbed. Recently it is accepted that a lot of SAMs belong to the mammalian brain. Deletion of a single SAM is not enough to prevent synapse development on a wide scale to their partially useless and overlapping activities.

SAMs are nuanced and have complex localization to various groups of synaptic contacts and impart their role in a synapse selectively [45]. Recent work is giving attention to unraveling the exact subsidization of various SAM members at exact synaptic contacts to know how they moderate selected neural circuits [92]. In addition, high attention is being given to SAMs that selectively concentrate on inhibitory or excitatory synapses[45]. The imbalance between excitatory and inhibitory synaptic transmission is hypothesized to play a vital role in the pathogenesis of various neuropsychiatric diseases [93]. Although it is not known whether this is the major cause or an outcome of other molecular processes is not clear [94]. Recently some reviewed that altering the selection of SAMs in animals may alter inhibitory or excitatory transmission which can lead to social and cognitive deficits in parallel [87, 94,95].

In conclusion, it is unknown about the molecular mechanisms of different SAMs for contributing to neurodevelopmental diseases and neuropsychiatric diseases with their pathogenesis [45].

Some points are also not understandable by the researchers like as, like which exact synapse SAM is present, what are their role in the mature brain as well as their developing process, and how they control dynamic information to accelerate the consequence of that SAM to behavioral or cognitive disorders [45]. SAMs play a vital role in forming and maintaining synapses. SAMs are engaged in synapse stabilization, maturation, and elimination. With their vital roles in synapses, they can impact the process of information along the brain and beyond [45]. Exciting research is being conducted to investigate the expanded SAMs that respond to synaptic work to modify their protein relationship and function. Because SAMs are involved in neuropsychiatric diseases and neurodevelopmental disorders, researching their specific molecular appliance and interaction modes along with their partners kept the pledge that this instruction can finally be manipulated to design completely narrative therapeutic techniques that maintain aberrant synaptic communication.

1.10 Concept of Prob/sharp microelectrode preparation for Prob/sharp microelectrode-based electrophysiological technique:

I already made effective engineered synapse organizers for the initiation of prob/sharp microelectrode-based electrophysiological technique. My engineered synapse organizers will attach the specific protein. That specific protein will be attached to the prob/sharp microelectrode. If I think about a sharp microelectrode, then I must attach the specific protein to that sharp microelectrode. As I know it is not possible directly attached protein with the prob/sharp microelectrode. So, in that case, there should be a linker protein, which can attach to the prob/sharp microelectrode. Then that protein will provide a splice site for the desired protein conjugation. The connecting factor that can be used as a linker here is protein A. After binding a specific protein to ProteinA, a cross-link will form between the Specific protein and ProteinA using DSS (disuccinimidyl suberate) and an organic solvent DMSO(Dimethyl sulfoxide). Then, specific proteins will be identified on the electrode by our engineered synaptic organizer's protein such as venus or GFP protein. Finally, neurons culture on the surface of the electrode. After the cross-link formation, it is necessary to check whether the cross-link is formed or not. It can be confirmed by DIV（Immunostaining）whether a synapse formed between the nerve cell and the electrode.

Cross-link is a technology that induces the formation of strong crosslinking such as covalent or ionic bonds, between polymers by changing polymers using physical and chemical properties. Cross-link formation is possible by a variety of chemicals. DSS is an amine-specific protein crosslinker with NHS-ester reactive groups for selective conjugation of primary amines. Therefore, DSS reacts readily with primary amines. Since DSS is not water-soluble, it can be dissolved using an organic solvent such as DMSO. DSS has no toxic by-products, so it is widely used in protein cross-linking experiments. Some proteins (ProteinA or G) having an antibody binding site generally have a lysine (K) residue and several primary amines at the amino terminus of the polypeptide. Therefore, DSS reacts with the primary amine of ProteinA to form an amide bond. The formation of synapses can be confirmed through light microscopy also. Before experiments using nerve cells, it requires high safety to the cross-link formation process.

1.11 Purpose and outlines:

At glutamate synapse, a transsynaptic link forms through β-Nrxs and NLs [64]. Nrxs alone can induce glutamate postsynaptic differentiation and induces GABA postsynaptic differentiation [64]. On the other hand, NLs can trigger presynaptic differentiation in GABA and glutamate axons [64]. Nrx-NL linkage is a basic constituent mediating glutamatergic and GABAergic synaptogenesis, thus differences in isoform restraining [64]. So, the main purpose was to develop a presynaptic-engineered organizer or postsynaptic-engineered organizer for the improvement of a prob/sharp microelectrode-based electrophysiological technique for cell-specific recording. Nrx was chosen for the presynaptic-engineered organizer and NL was chosen for the postsynaptic-engineered organizer.

The purposes are:

1. The first objective is to explore the possibility of engineered synaptic organizers as a molecular genetic approach to manipulate patterns in neural circuits. This will allow us to observe the effects of changes in nerve fiber contact on neural function with unprecedented precision.
2. The second objective is to develop the next generation of electrophysiological techniques.

Conventional electrophysiological techniques, such as patch-clamp and extracellular recording methods, can record electrical activity with a good signal-to-noise ratio but have the limitation of not being able to discriminate the genetic types of the target cell. There is potential to generate new electrophysiological techniques that overcome this critical limitation.

3. Control of autism diseases related to synaptic organizers.

There are a lot of autistic humans around the world. Several autism-related diseases are directly related to the dysfunction of synaptic organizers like schizophrenia, bipolar disorders, and neuropsychiatric disorders. So, soon engineered synapse organizers can be replaced with dysfunctional synaptic organizers then maybe those kinds of diseases can be reduced through treatment. For future research transgenic animals can be developed through the input of engineered synapse organizers into their brains. Then neural circuit chip may insert into the brain to connect the neural activity directly with the association of engineered synapse organizers which were previously inserted. Through the transgenic animal, we can study the specific synaptic organizer's role in synapse making and loss in different situations. There are a lot of electrophysiological methods like extracellular recording, intracellular recording, patch clamp recording, and multi-electrode array. But now the advanced world demands a new electrophysiological method to analyze circuits specifically and perfectly. So, in a nutshell, an engineered synapse organizer can help to gain all purposes shortly.

For the generation of the test synapse organizers, conventional molecular biology techniques (e.g., combinations of polymerase-chain-reaction, restriction enzyme digestions, DNA ligation, infusion cloning, agarose gel electrophoresis, bacterial transformation and cultures, protein purification, plasmid purifications, DNA sequencing, BAP treatment, etc.) were used to generate a variety of test synapse organizer constructs. NLs, Nrxs, and various proteins, e.g., Venus, GFP, GFP-nanobody, mCherry-nanobody, mCherry, GFP null, Spot, Spot-nanobody, BC2, BC2-nanobody, T2A, EGFP, Rab3, dClover2, NL1Δ1, NL1Δ2, NrxΔ1, NrxΔ2, etc. were used to generate engineered synapse organizers. Then precise evaluations were done. Figures 4 and 5 represent the methods of making engineered pre- and post-synaptic organizers.

Figure 4: Construction map of engineered pre-synaptic organizers.

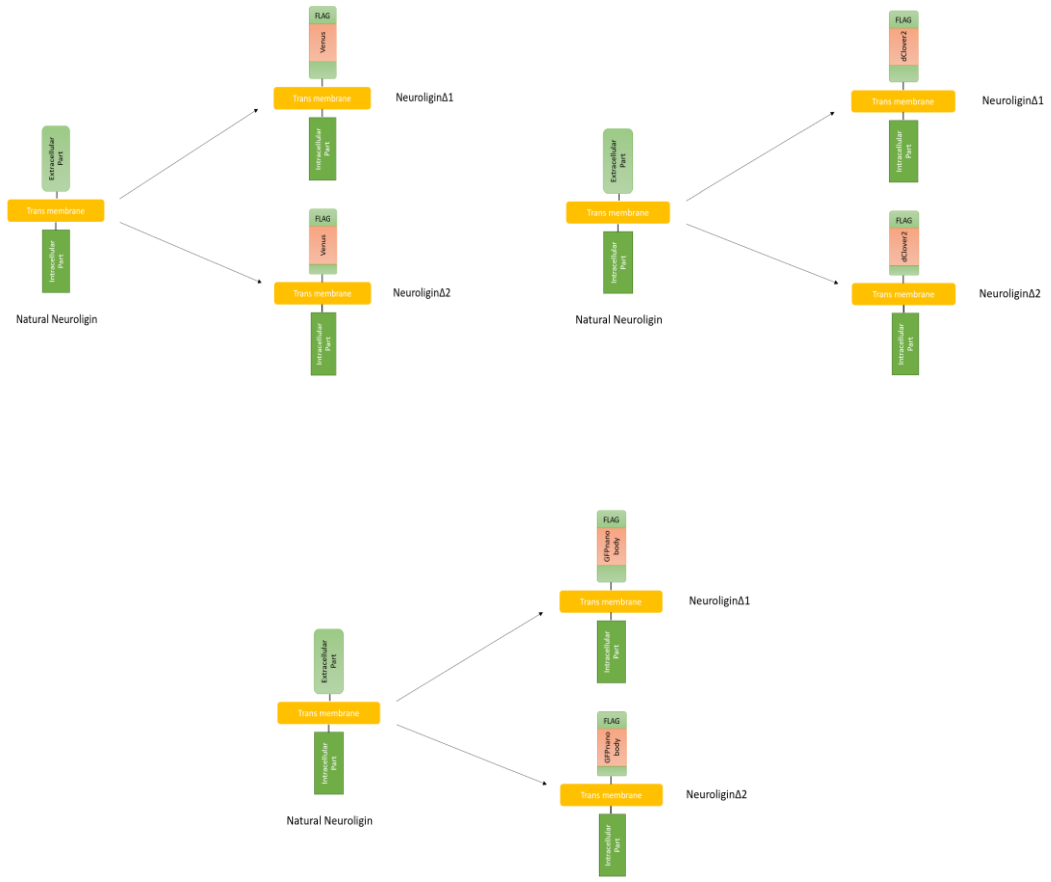

Figure 5: Construction map of engineered post-synaptic organizers.

The orthogonal test showed that GFP-nanobody contains construct bounds with GFP and venus protein. mCherry-nanobody contains constructs bound with mCherry protein. mCherry contains constructs bounds with mCherry-nanobody protein. Spot and BC2 contain constructs bound with BC2-nanobody and spot-nanobody proteins respectively. GFP-containing constructs showed their existence extracellularly in the dark fluorescence view and can bind with GFP-nanobody protein. After neuron expression, we considered those as engineered synapse organizers. Cell-microbead interaction examination, in which a spot containing NrxΔ1 showed the best results among all the constructs.

In the cell-microbeads interaction experiment, pCAG-GS-FLAG-Spot-Nrx1bΔ1-P2A-EGFP-Rab3 was on the presynaptic position, and BC2-nanobody microbead in the postsynaptic position. Then we confirmed with a presynaptic marker called an anti-synaptophysin antibody. GFPnull/YFP containing engineered NrxΔ1 bound with GFP-nanobody microbead and

confirmed by postsynaptic Anti-FLAG and Rab-3 markers. Thus, those presynaptic-engineered organizers are considered for the next level experiment such as interaction with postsynaptic organizers or postsynaptic-engineered organizers. In conclusion, it can be said that our engineered synaptic organizers responded positively. Soon, we can easily manipulate the natural neurons to make artificial synapses.

For the construction of a presynaptic-engineered organizer, monomeric proteins were used for ligating the deleted outer part of natural Nrx. Venus, GFP, GFP-nanobody, mCherry-nanobody, mCherry, GFP null, Spot, Spot-nanobody, BC2, BC2-nanobody, T2A, EGFP, Rab3 all of these are monomeric protein and some of them are fluorescent in nature. NrxΔ1 and NrxΔ2 were used to construct the presynaptic-engineered organizer. Where NrxΔ2 was shorter than NrxΔ1 extracellularly. On the other hand, both monomeric and dimeric proteins were used for the deleted outer part of natural NL. Venus and GFP-nanobody were monomeric but dClover2 was dimeric. Where GFP-nanobody was no fluorescence in nature. NLΔ1 and NLΔ2 were used to construct the postsynaptic-engineered organizer. Where NLΔ2 was shorter than NLΔ1 extracellularly. Soon, we can easily manipulate natural neurons to make artificial synapses.

In a nutshell, I developed engineered synapse organizers for the prob/sharp microelectrode-based electrophysiological specific cell recording. Although it was not easy to develop a workable engineered synapse organizer from the natural one. I considered the working principle of the synapse organizer and then thought to apply it in prob/sharp microelectrode-based electrophysiology for the recording of a specific cell. Also, we need to consider the microelectrode array with a specific medium and voltage for the prob/sharp microelectrode-based electrophysiological specific cell recording. Moreover, we must prove whether the conventional microelectrodes array will be workable with the desired technique, or whether we just need to design a new one. In the future, a specific prob/sharp microelectrode with orthogonal tested protein will be developed for the desired development of the prob/sharp microelectrode-based electrophysiological technique to initiate specific cell recording. In a sentence, I have fulfilled the first step in the development of an engineered synapse organizer, and I succeeded and proved it. Then I can say it can work for the new technique development.

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Chapter 2

Characterization of Engineered Synapse Organizers Toward the Development of Electrophysiological Technique with Target Recognition Capability

Key Points of Chapter 2

- **Confirmed a reliable engineered Neurexin frame for making an orthogonal engineered synapse organizer.**
- **Confirmed that without the LNS domain Neurexin can induce differentiation. LNS domain is only needed for NL association.**
- **Confirmed that engineered organizers can be used in the development of electrophysiological techniques.**

2.1 Introduction

A neuron is an electrically excitable cell [1] that transmissions with other cells via particularized connections called synapses. Synapses transmit information and connect neurons with millions of overlapping neural circuits that are interdigitated [2]. Some authors generalize the idea of the synapse to involve the communication from a neuron to any other cell type [3]. Synapse development needs differentiation of presynaptic secretion and postsynaptic receptive molecules maintained by synapse organizing proteins [4]. Synaptic adhesion molecules (SAMs) may also serve in the generation and working of synapses [5]. The interaction of synapse organizers with their collaborators can either be strengthened or weakened by alternative splicing [6]. In vivo, these organizers may be used to control synaptic communications [6]. Synapse organizers are tethered to pre-or post-synaptic by transmembrane and elaborate their extracellular domains within the synaptic cleft [6]. In the co-culture system of the neuron and nonneuronal cells neuroligin (NL) shows in nonneuronal cells can induce the formation of functional presynaptic terminals onto those cells from cocultured neurons [7]. On the other hand, Neurexins (Nrxs) show in nonneuronal cells and support the formation of postsynaptic activity at the connection of those nonneuronal cells to cocultured neurons [8]. NL exists naturally as a dimer and Nrx remains in an unbound state like a monomeric [9]. An asymmetric tetramer contains an NL dimer and two Nrxs [9–11].

In vitro, authentication suggests that Nrx differentiation in the early stage of an axon part into a presynaptic terminal and NL clustering may be an early step in the differentiation of a dendrite segment into a postsynaptic terminal [11,12]. In the location of alternative splice sites, the crystal shape of the Nrx1β LNS (laminin, Nrx, sex-hormone-binding globulin) domain showed remarkable maintenance with the agrin LNS domain [13]. Using an affinity column of Nrx1β, NLs were first identified [14]. Overexpression of AChE losses levels of β-Nrxs in vivo and culture then impairs the genesis of glutamatergic synapses in culture, which indicates that there is crosstalk within the two proteins [15,16]. α-Nrxs are crucial for the localization and activity of Ca^{2+} channels and NMDA (*N*-methyl-D-aspartate) receptors [17,18]. Splicing initiates isoforms that bind both α-Nrxs and β-Nrxs by detaching an eight-amino-acid sequence from NLs, and this binding could be participated in modulating synapse properties [19]. NL1 was recognized because of its potential to bind definite isoforms of all three β-Nrxs [20].

Exploring the cell and neurons is important for better understanding the physiology of the body system. This investigation is elaborated to convince the recording of biological work in non-invasive techniques with various micro- or nano-electrodes. In these ways, electrophysiology has become one of the best biological methods in ongoing research. Voltage-gated calcium channels are highly selective for calcium and have a broad range of animating or deactivating features. Based on their threshold of voltage-dependent facilitation, they are classified as high and low-voltage-activated channels [21]. Somatic calcium can be recorded to show the activity of action potential [22]. Somatic calcium can trigger gene transcription [23]. N-methyl-D-aspartate is called an ion-based glutamate receptor and can mediate postsynaptic calcium ions in the cortex or pyramidal neurons [24,25]. Calcium imaging is generally operated for the monitoring of interconnected neurons such as analyzing the circuitry in the cortex [26]. This idea is also applied to recognize synaptically bridged neurons [27,28]. In the case of a fluorescence probe technique, it is often used in experiments that require high selectivity because it has very high selectivity. The fluorescence probe technology has lower temporal resolution than the conventional electrophysiology technology and is easily contaminated.

Previously, it was the major problem that cell-type specific recording was not possible but with time optical-approach-type electrophysiological techniques now can do this job. This was done by the genetically encoded protein which only detects the specific cell which is matched with their cell type-specific promoter. But it is still a problem for the prob/sharp microelectrode-based electrophysiological techniques to determine cell-type-specific recording. So, using the properties of a synapse organizer, I am trying to develop a methodology by which I can soon record cell-type-specific recordings (Fig. 1). Synapse organizer properties are helpful to make those types of recordings, but for that, I must use a genetically engineered organizer (Fig. 1). For the development of prob/sharp microelectrode-based electrophysiology, especially for specific cell recording, I need to develop engineered synapse organizers. It will be helpful to attach specific proteins attached with prob/sharp microelectrode and then I can create a bridge for the specific cell recordings.

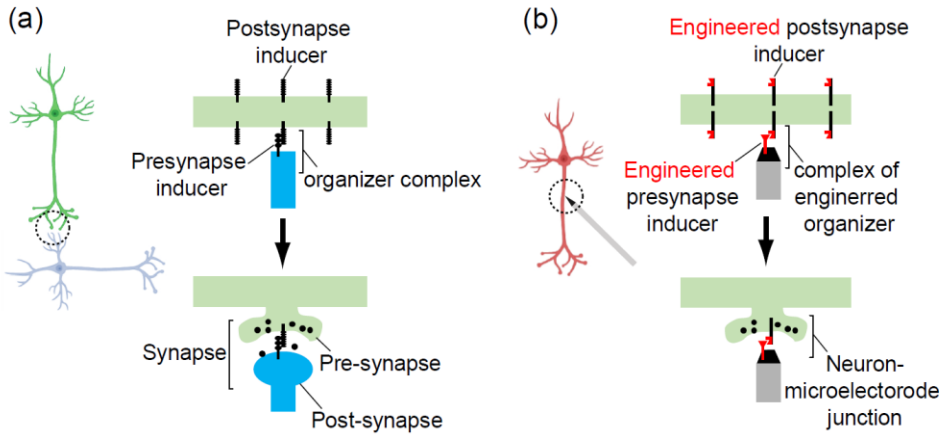

Figure 1: Concept of an engineered synapse organizer and cell-specific formation of the neuron-microelectrode junction.

For making genetically engineered synapse organizers I used their outer part for genetic engineering because the extracellular part clusters with other opposite organizers extracellular part (Fig. 2a). Pre-synaptic organizers were used to make genetically engineered synapse organizers. Then again, I engineered the Nrx as the Nrxn1bΔECD version where the extracellular part is more flexible to take any epitope protein for specific contraction (Fig. 2b). Finally, I did cell microbead reactions with those various types of engineered Nrx like as FLAG-Venus-Nrxn1b, FLAG-Venus-Nrxn1b-ΔECD, FLAG-Nrxn1b-ΔECD (Fig. 2c). Various types of protein which are orthogonally attached with the specific protein. In the orthogonal test, I found GFP-nanobody containing Nrx bounds with GFP and venus protein. mCherry-nanobody contains Nrx bounds with mCherry protein. mCherry contains Nrx bounds with mCherry-nanobody protein. Spot and BC2 contain Nrx bound with BC2-nanobody and spot-nanobody proteins respectively. GFP-containing Nrx showed their existence extracellularly in the dark fluorescence view and can bind with GFP-nanobody.

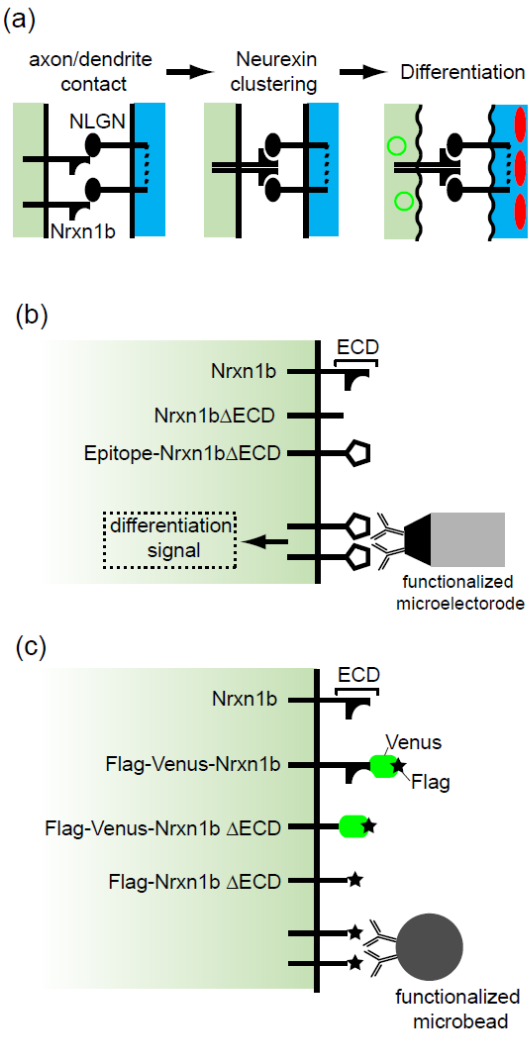

Figure 2: Mechanism of neurexin signaling and strategy for molecular engineering.

In the cell-microbeads interaction experiment, I confirmed with a presynaptic marker called an anti-synaptophysin antibody. Flag-YFP-Nrxn1b-ΔECD1-P2A-Rab3-EGFP bound with GFP-nanobody microbead and confirmed by postsynaptic Anti-FLAG and Rab-3 markers. It was not easy to develop a workable engineered synapse organizer from the natural one. I considered the working principle of the synapse organizer and then thought to apply it in prob/sharp microelectrode-based electrophysiology for the recording of a specific cell. Also, I need to consider the microelectrode with a specific medium and voltage for the prob/sharp microelectrode-based electrophysiological specific cell recording. In the future, a specific

prob/sharp microelectrode with orthogonal tested protein will be developed for the desired development of the prob/sharp microelectrode-based electrophysiological technique to initiate specific cell recording. I succeeded in the development of engineered synapse organizers. In conclusion, it can be said that our engineered synaptic organizers responded positively.

2.2 Materials and Methods

2.2.1 Molecular biology

Genes constructions were performed with the standard PCR/ligation and in-Fusion based cloning techniques. I mainly used Prime STAR Max DNA polymerase (Takara Bio, Shiga, Japan) and In-Fusion HD cloning kit (Clontech, CA, USA). Synaptophysin-EGFP construct used. Initially, I generated four types of engineered Nrxn1b for analyzing presynaptic reaction between HEK cells and neurons (Fig. 3a). In FLAG-Nrxn1b I introduced FLAG into the extracellular domain (ECD) of Nrxn1b immediately after the laminin/neurexin/sex hormone binding globulin (LNS) domain. In FLAG-Venus-Nrxn1b I introduced venus protein into the ECD of FLAG-Nrxn1b immediately upstream of amino acid 48. In FLAG-Venus-Nrxn1b-ΔECD1 I removed the LNS domain extracellularly from downstream of amino acids 80 to 233. For FLAG-Venus-Nrxn1b-ΔECD2 I removed the LNS domain extracellularly from downstream of amino acids 80 to 314. Finally, I generated two more types of engineered Nrxn1b for analyzing the presynaptic reaction between neuron and anti-FLAG beads. Where P2A-EGFP-Rab3 is inserted into the intracellular domain of FLAG-Nrxn1b-ΔECD1 and FLAG-Nrxn1b-ΔECD2 immediately downstream of amino acid 438 (Fig. 4a).

2.2.2 Cell Culture

HEK293T cells are cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS, Gibco) in a standard CO_2 incubator. Transfection was performed using Lipofectamine 2000 reagent (Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. Primary cortical neurons are prepared from mice on embryonic day 18 as described previously. Neurons are cultured on ϕ 13 mm coverslips coated with poly-L-lysin

and mouse laminin. The culture medium was Neurobasal-A supplemented with 0.5 mM L-glutamine, 2% B-27 supplement (Thermo Fisher Scientific, MA, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, and in the initial culture of ~24 hours after preparation, 5% FBS was also added. Transfection was performed on 6 days in vitro (DIV6) cultures using calcium phosphate methods as previously described or by using Lipofectamine 3000 reagent (Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol.

2.2.3 Evaluation of engineered synapse organizer

The synaptic inducing activity of the protein of interest was evaluated by analysis of neurons in contact with protein-conjugated microbeads or HEK cells expressing the protein.

For the microbead-based assay, the cDNAs of the protein of interest were sub-cloned into an Fc fusion protein expression vector and transfected into HEK293T cells. Proteins were secreted into the medium in the form of Fc fusion proteins. During protein production, the concentration of FBS in the culture medium of HEK cells was reduced to 2 %. The medium was harvested, passed through a 0.22 μm pore syringe filter (Advantech, Tokyo, Japan), and incubated with protein-A magnetic microbeads (ϕ ~ 4.7 mm, PAMS-40-S, Spherotech, IL, USA) in a low protein binding microcentrifuge tube at 4^{o} C for overnight. Typically, 15 μl beads solution was used with ~10 μg Fc-fusion protein in 500 μl medium. The protein-conjugated microbeads were deeply washed with neuron culture medium and transferred onto the neuron culture, typically at the density of 10^{5} beads per a ϕ 13 mm coverslip of neuron culture. After overnight culture, the neurons were subject to immunocytochemistry and/or confocal microscopy analysis.

2.3 Results

After a lot of biological work, I found some pairs of engineered synapse organizers, and those are working as presynaptic Nrxs. All those were endotoxin-free. I have chosen the best quality synapse organizers based on their expression on HEK 293 cells and the orthogonal molecular test. Most of those synapse organizers' making protocols are the same. I used different kinds of restriction enzymes for various inserts and vector plasmids. Temperature and steps are different

in different inserts also. At first, I digested the NrxΔECD1 and NrxΔECD2 with respective restriction enzymes and then checked them in agarose gel. I also checked every insert after PCR in agarose gel. For a definite construct, I used the same restriction enzymes both in vector plasmid and insert. For as Not1 restriction enzyme was used in the NrxΔECD1/NrxΔECD2 and GFP-nanobody. So, after making the construct I digested the construct and found both NrxΔECD1/NrxΔECD2 and GFP-nanobody because of digestion of the same place with Not1, where I ligated.

In the orthogonal test results of different engineered synapse organizers, I have tested every possible protein for engineered neurexin like as GFP containing engineered neurexin was tested with GFP-nanobody, mCherry, mCherry-nanobody, BC2-nanobody, GFP, Spot-nanobody, neuroligin in neuron culture, and HEK cell culture. After a long experimental period, I found the desired results. When a specific protein is bound with the specific protein-containing engineered neurexin then it shows the expression in the HEK cell border as Ill as inside the HEK cells.

In Figure 3, FLAG-Venus-Nrxn, FLAG-Venus-Nrxn-ΔECD1, and FLAG-Nrxn-ΔECD2 show activity in HEK and neuron cell adhesions. The first two columns were controlled where only HEK cell neurons were present with EGFP. When the anti-FLAG marker was used with those HEK cells then, there was no expression or no differentiation (Fig. 3b). After those two columns, the next two columns show the differentiation in HEK cells and Neurons (Fig. 3c). In those HEK cells, I added FLAG-Venus-Nrxn and neuron with NL. The red color indicates the differentiation because of the FLAG marker. The next two columns show no differentiation where HEK cells contained FLAG-Venus-Nrxn-ΔECD1 without extracellular part and neuron with NL (Fig. 3d). Then the last two columns show no differentiation where HEK cells contained FLAG-Venus-Nrxn-ΔECD2 without extracellular part and neuron with NL (Fig. 3e).

In principle HEK cells cannot attach with neurons or cannot make synapse-like connections, so I preferred to express our engineered Nrx in HEK cells first then added neurons with natural NL (Fig. 3f). Then I added a Shank2 marker for the detection of extracellular interactions in the borderline of neuron and HEK cells (Fig. 3g). FLAG-Venus-Nrxn-ΔECD1, and FLAG-Venus-

Nrxn-ΔECD2 were not shown any interactions because those have no active extracellular domain (Fig 3g, 3i). It means without the extracellular part of Nrx or the extracellular part of engineered Nrx cannot work for inducing differentiation. In our engineered Nrx design I deleted a small part from the extracellular portion of natural Nrx and added desired protein. However, my designed engineered Nrx can work efficiently as I wished. Moreover, I can say that it responds accurately, and soon it will be proven to make many electrophysiological policies as Ill as techniques.

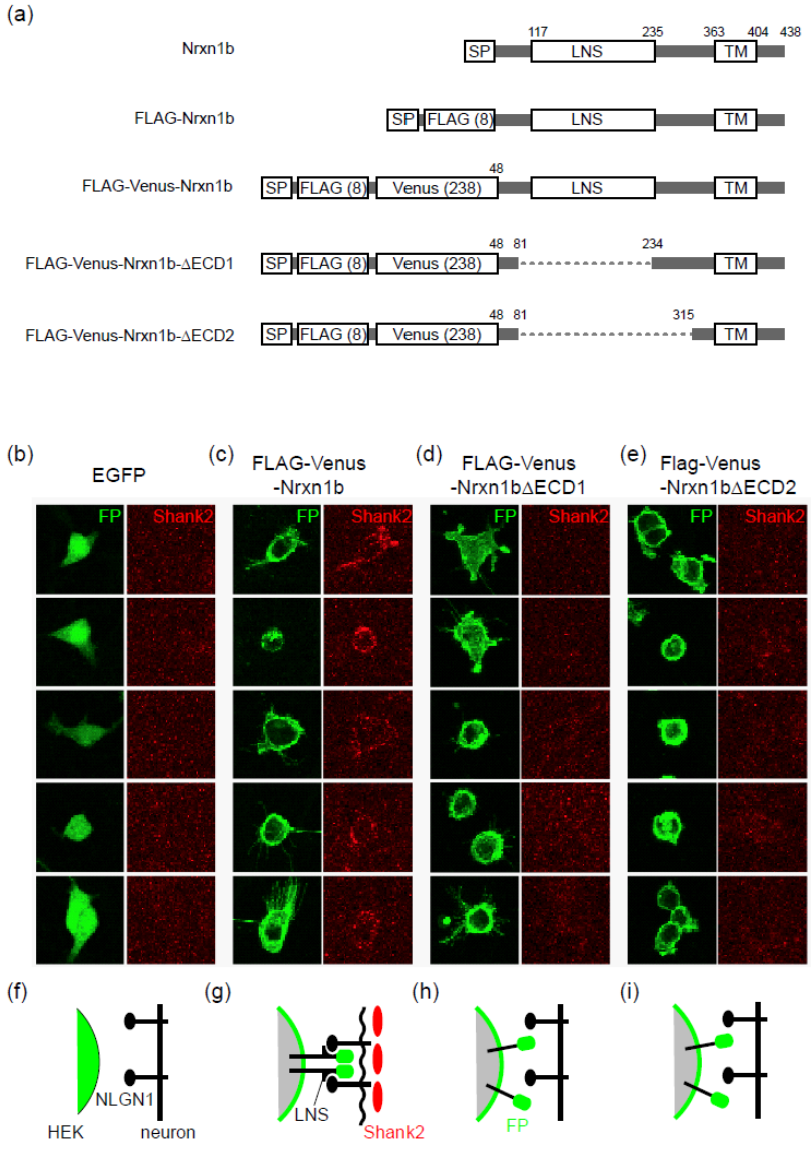

Figure 3: ΔECD version goes to membrane Ill, but null interaction with neuroligin.

After the Hek cell and neuron cell experiment, I decided to make our engineered Nrx a little longer by adding P2A-Rab3-EGFP. I made Flag-Nrxn-ΔECD1-P2A-Rab3-EGFP and Flag-Nrxn-ΔECD2-P2A-Rab3-EGFP versions and made run our experiment in neuron and bead reaction (Fig. 4a). P2A has a flexible nature between the junction of the attached part of engineered neurexin. It will help to move the bond freely when it attaches to the nearby ligand or anything for differentiation. On the other hand, Rab3 and EGFP will be helpful for the initiation of synaptic reaction and for distinguishing from the other cell activity (Fig. 4b).

For the neuron and bead reaction, I did it in three forms where Flag-Nrxn-ΔECD1-P2A-Rab3-EGFP, and Flag-Nrxn-ΔECD2-P2A-Rab3-EGFP with anti-FLAG beads in the separation medium (Fig. 4c and 4d). The last one was Flag-Nrxn-ΔECD2-P2A-Rab3-EGFP with control bead only where anti-FLAG antibody was not used (Fig. 4e). In the neuron bead experiment, anti-FLAG antibody reacted with the Flag part of the Flag-Nrxn-ΔECD1-P2A-Rab3-EGFP, and Flag-Nrxn-ΔECD2-P2A-Rab3-EGFP and made a presynaptic reaction (Fig. 4c and 4d). Then, it can be shown in green fluorescence accumulation on the borderline of the beads but in control, I did not find that (Fig. 4e). So, from this experiment I can say that Flag-Nrxn-ΔECD1-P2A-Rab3-EGFP and Flag-Nrxn-ΔECD2-P2A-Rab3-EGFP can response in the attachment of other influenced protein or their relatives.

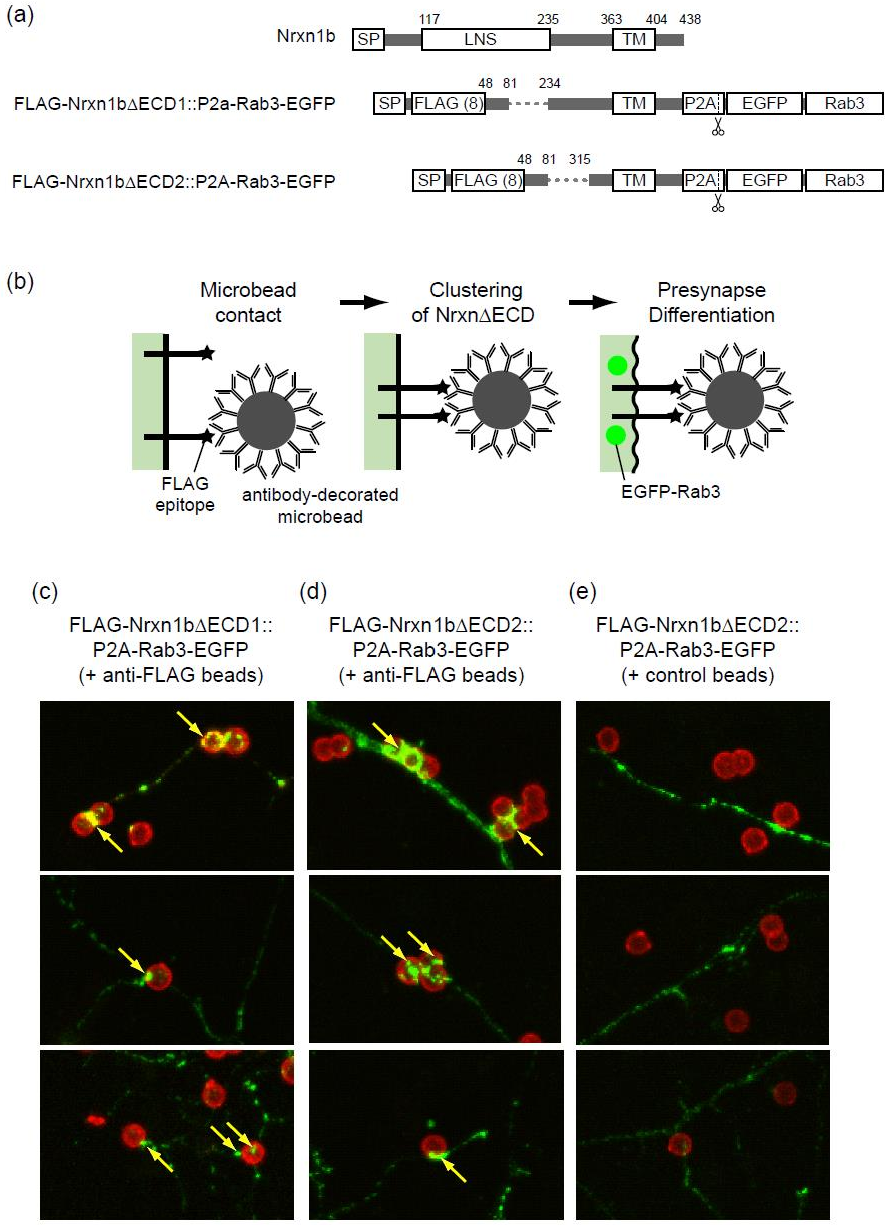

Figure 4: Presynaptic induction triggered by FLAG epitope and anti-FLAG antibody interaction.

In the case of neuron culture when a specific protein is bound with the specific protein containing engineered Nrx then it showed the presynaptic reaction in nearby neurons. Presynaptic reactions were marked and identified through various markers like Shank2, Rab3, anti-FLAG, etc. I used micro beads for the carrier of specific proteins to attach with the engineered Nrx. Anti-GFP-nanobody serves as the receptor for the yellow fluorescence protein

(YFP) and exerts differentiation into pre-synapse. For the polycistronic expression of Rab3-EGFP as a presynaptic marker, its cDNA was added downstream of Flag-YFP-Nrxn1b-ΔECD via the self-cleaving P2A peptide and was named Flag-YFP-Nrxn1b-ΔECD1-P2A-Rab3-EGFP. Fig 5 shows representative images of the primary neurons transfected with Flag-YFP-Nrxn1b-ΔECD1-P2A-Rab3-EGFP and contacted with the microbeads bound with anti-GFP nanobody. As indicated by arrows, accumulations of Rab3-EGFP are evident, showing that anti-GFP nanobody successfully exerted presynaptic differentiation via the interaction with GFP upstream of the modified Nrx1b. Flag-YFP-Nrxn1b-ΔECD1-P2A-Rab3-EGFP bound with GFP-nanobody microbead and induced presynaptic reaction (Fig. 5). Anti-FLAG marker showed in red color and Rab-3 marker showed in green color. The Grey color indicates the microbead existence and the right-side picture is a merged view of Anti-FLAG and Rab-3 markers.

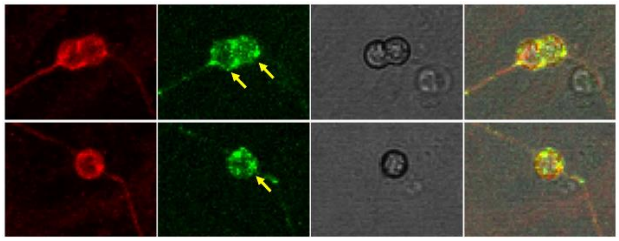

Figure 5: Flag-YFP-Nrxn1b-ΔECD1-P2A-Rab3-EGFP bound with GFP-nanobody microbead and induced presynaptic reaction. Anti-FLAG marker showed in red color and Rab-3 marker showed in green color. The Grey color indicates the microbead existence and the right-side picture is a merged view of Anti-FLAG and Rab-3 markers.

After cell-microbead interaction, I can say that they showed positive results. In our experiment, I got success to generate those constructs and they all bound their specific protein extracellularly in HEK cells. I tested our engineered synapse organizers through the orthogonal technique. In many cases, one engineered organizer can bind two types of proteins but there was a difference in binding nature and efficiency. Through that orthogonal testing, I compared and found the specific one.

2.4 Discussion:

For making engineered synapse organizers, I worked on a presynaptic organizer named neurexin. In the natural synapse, neurexin can induce postsynaptic activation for a synapse to make by interacting with several postsynaptic organizers at a time [2]. Neurexin can work with neuroligin, dystroglycan, calsyntenins, and latrophilins at a time when forming synapses [2]. But engineered neurexin may only induce neuroligins and/or engineered neuroligins. In engineered neurexin, I discarded the outer transmembrane part of natural neurexin and then ligated our desired protein. I think that the outer transmembrane part of natural neurexin is responsible for the manipulation of several postsynaptic organizers at a time. So, I removed the outer transmembrane part of natural neurexin and set a specific protein that can bind with another specific protein. However, when the specific protein binds with the engineered neurexin's protein, then it will induce the presynaptic reaction. In this way, the neuron can be manipulated artificially through the engineered synapse organizers. However, I can use that engineered Nrx for the development of prob/sharp microelectrode-based electrophysiological techniques for specifically selected cell recordings in a handled way.

For the Nrx/NL complex in synapse formation, either independent manipulation of Nrx/NL can cause moderation of presynaptic and postsynaptic assembly, which suggests an instructive role [11]. In our experiment, I synthesized engineered synapse organizers. I synthesized presynaptic organizers like Nrxs. I simply manipulated the natural Nrxs and firstly made FLAG-Nrxn-ΔECD1, and FLAG-Nrxn-ΔECD2 by digesting the extracellular part of the Nrx with restriction enzymes. Then I generated various proteins like GFPnull/YFP, GFP-nanobody, and venus through PCR and then ligated with those manipulated Nrxs. Seth L. Shipman et al. also suggested that dimerized NL induces the gathering of presynaptic organizers which is a part of the functional chemical synapse [11]. Dean C et al. In vitro, authentication suggests that Nrx differentiation in the early stage on an axon part into a presynaptic terminal and NL clustering may be an early step in the differentiation of a dendrite segment into a postsynaptic terminal [12].

Demet Arac et al. suggested that NL1 showed in nonneuronal HEK293 cells influenced presynaptic differentiation in co-cultured neurons at the places of cell-to-cell contact [9]. Similarly, Nrxs showed in nonneuronal cells influence postsynaptic differentiation in cultured neurons at associate sites. These bits of knowledge led to the assumption that NL and Nrx act as

trans-neuronal signals for the enrollment of synaptic molecules and might be associated with initial synapse evolution in vivo [9]. Nrxs can bind with many postsynaptic molecules, for this it was difficult to generate an engineered synapse organizer, but our engineered Nrxs can only bind with the specific protein or ligands. So, soon, I expect these engineered Nrxs will bind with engineered NL containing the specific protein extracellularly. Moreover, I expect these engineered Nrxs will bind with a specific protein attached to prob/sharp microelectrode and can induce a presynaptic reaction in the specific cell for the development of a prob/sharp microelectrode-based electrophysiological technique.

After doing orthogonal testing, I found the specific protein for each construct. Flag-YFP-Nrxn1b-ΔECD1-P2A-Rab3-EGFP can bind with GFP-nanobody protein. GFP-containing constructs showed their existence extracellularly in the dark fluorescence view and can bind with GFP-nanobody protein. In cell-microbead reaction, I found positive results for presynaptic reactions marked by pre and postsynaptic markers. GFPnull/YFP containing engineered Flag-YFP-Nrxn1b-ΔECD1-P2A-Rab3-EGFP bound with GFP-nanobody microbead and confirmed by presynaptic Anti-FLAG and Rab-3 markers. In conclusion, it can be said that our engineered synaptic organizers responded well.

From the above research and the outcome of our expectations, it can be said that in the future I will be able to make a new electrophysiological method or technique. In that technique, specific cell recording can be possible with the help of prob/sharp microelectrode bound with a specific protein. That specific protein will be reacted only with the specific engineered Nrx and then I can record that cell activity only. In other words, a specific protein bound with prob/sharp microelectrode will initiate the presynaptic reaction in specific cells/neurons that have a relation with the engineered Nrx. Then cell specificity will be confirmed because that engineered Nrx will be associated only with that specific protein bound with prob/sharp microelectrode. Then there will be a new era for the prob/sharp microelectrode-based electrophysiological technique for specific cell recordings.

2.5 Conclusions

I have confirmed from the above experiments and discussions that my designed engineered Nrx can be induced by outer protein/particle and showed their presynaptic reaction. Flag-YFP-Nrxn1b-ΔECD1-P2A-Rab3-EGFP can attach with GFP-nanobody connected microbead and confirmed by presynaptic Anti-FLAG and Rab-3 markers. So, if a specific protein deposits on the top of a prob/sharp microelectrode, then that specific protein can attach with designed engineered Nrx. Then will initiate a presynaptic reaction in that specific cell who have an attachment with the designed engineered Nrx. In this way, my planned prob/sharp microelectrode-based electrophysiological technique will fruitfully come out for the specific cell recordings. Thus, I concluded that my engineered organizer can be able to work for the development of prob/sharp microelectrode-based electrophysiological technique.

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Chapter 3

Generation of Engineered Presynaptic Organizers

Key Points of Chapter 3

- **Made various types of orthogonal engineered Neurexin.**
- **Confirmed exitance in HEK cell culture as structural integrity.**
- **Confirmed initiation of presynapse in Neurons with non-biological materials such as silica beads. Such activity leads to initiating next generation electrophysiology for selective recordings.**
- **Confirmed that adding other proteins with the organizers can bind with their orthogonal protein and can induce presynapse reaction.**

3.1 Introduction:

A neuron is an electrically excitable cell [1] that transmissions with other cells via particularized connections called synapses. Synapses transmit information and connect neurons with millions of overlapping neural circuits that are interdigitated [2]. Some authors generalize the idea of the synapse to involve the communication from a neuron to any other cell type [3], like to a motor cell, while such non-neuronal contacts could be mentioned as junctions. Synapse development needs differentiation of presynaptic secretion and postsynaptic receptive molecules maintained by synapse organizing proteins [4]. Synapses especially chemical synapses are stabilized in location by synaptic adhesion molecules (SAMs) expecting from both the pre-and post-synaptic neuron and conjugating together where they overlap; SAMs may also serve in the generation and working of synapses [5]. Here, we called SAMs synapse organizers.

The interaction of synapse organizers with their collaborators can either be strengthened or weakened by alternative splicing [6]. In vivo, these organizers may be used to control synaptic communications [6]. Synapse organizers are tethered to pre-or post-synaptic by transmembrane and elaborate their extracellular domains within the synaptic cleft [6]. In the co-culture system of the neuron and nonneuronal cells, NL shows in nonneuronal cells can induce the formation of functional presynaptic terminals onto those cells from cocultured neurons [7]. On the other hand, Nrxs show in nonneuronal cells and support the formation of postsynaptic activity at the connection of those nonneuronal cells to cocultured neurons [8]. They can form strict homophilic interactions with the same molecules, semi homophilic interactions with associated family members. They can form heterophilic interactions with other adhesion molecule families. NL exists naturally as a dimer and Nrx remains in an unbound state like a monomeric [9]. An asymmetric tetramer contains an NL dimer and two Nrxs [9,10,11].

Synapse organizers can bind with proteins and form trans-complexes or cis-complexes in the same synaptic manner [6]. These *trans*-complexes carry the adhesive function observed for synapse organizers in cell-based assays. However, synapse organizers are increasingly being supported for crucial roles beyond adhering to the presynaptic membrane and postsynaptic membrane in conjunction [6]. For neuron-neuron recognition and generating scaffolds, the synapse organizer has a great role in binding additional proteins. Some synapse organizers can

bind partners chained to a similar membrane in a side-by-side mode forming a "*cis*-complex." Such cis protein congregates are often regulatory in nature, binding, altering, or forming a crucial pre-complex onto which a third partner can harbor to yield the final *trans*-synaptic bridge [6].

In vitro, authentication suggests that Nrx differentiation in the early stage on an axon part into a presynaptic terminal and NL clustering may be an early step in the differentiation of a dendrite segment into a postsynaptic terminal [11,12]. Nrxs are presynaptic cell-adhesion molecules that are currently the best-understood directors of synapse properties and thus perform a vital role in neural circuit gathering and restructuring via dealing with multifarious presynaptic and postsynaptic ligands [2]. These Nrxs, ligands, in turn, deal with other extracellular and intracellular signaling proteins in a controlled manner, making a dynamic molecular network [2].

In the location of alternative splice sites, the crystal shape of the Nrx1β LNS (laminin, Nrx, sex-hormone-binding globulin) domain showed remarkable maintenance with the agrin LNS domain [13]. The crystal shape of the second LNS domain of Nrx1α disclosed that this part containing the splice plot forms an extremely variable surface rounding a coordinated Ca^{2+} ion [14]. Through the depletions in cleft Ca^{2+} concentrations accompanying synaptic activity, some Ca^{2+}-dependent interactions of Nrxs might be influenced. Nrxs have three well-noticed extracellular binding participants in the mammalian brain: NLs, neurexophilins, and dystroglycan [14]. NLs show Ca^{2+}-dependent binding to α-Nrxs and β-Nrxs, neurexophilin binding is Ca^{2+}-independent with α-Nrx only, dystroglycan shows Ca^{2+}-dependent binding with α-Nrxs preferentially. Using an affinity column of Nrx1β, NLs were first identified[14]. NLs are the most exclusively studied Nrx binding partners. Overexpression of AChE losses levels of β-Nrxs in vivo and culture then impairs the genesis of glutamatergic synapses in culture, which indicates that there is crosstalk within the two proteins [15,16].

α-Nrxs are crucial for the localization and activity of Ca^{2+} channels and NMDA (*N*-methyl-D-aspartate) receptors [17,17]. Splicing initiates isoforms that bind both α-Nrxs and β-Nrxs by detaching an eight-amino-acid sequence from NLs, and this binding could participate in modulating synapse properties [19]. NL1 was recognized because of its potential to bind definite isoforms of all three β-Nrxs [20]. However, Nrxs are just one component of a varied trans-

synaptic molecular machine. When Nrxs were recognized, their extensive alternative splicing suggested a role as surface-identifying molecules that specify synapses [2]. This hypothesis is expanding and validated.

In a chemical synapse, electrical action in the presynaptic neuron is transformed via the activation of voltage-gated calcium channels then releases a chemical named a neurotransmitter that ties to receptors settled in the plasma membrane of the postsynaptic neuron. The neurotransmitter may start an electrical reaction or a secondary message carrier pathway which may either excite or inhibit the postsynaptic neuron. Chemical synapses can be divided according to the neurotransmitter released: GABAergic which is often an inhibitor, glutamatergic which is often excitatory, cholinergic e.g. vertebrate neuromuscular junction, and adrenergic which releases norepinephrine.

Chemical synapses can have complex effects on the postsynaptic cell for the complexity of receptor signal transduction. The presynaptic and postsynaptic cell membranes are connected by special channels in an electrical synapse, which is called gap junctions or synaptic cleft that is able of passing an electric current, doing voltage changes in the presynaptic cell to inspire voltage changes in the postsynaptic cell. The rapid transfer of signals from one cell to the next is the main advantage of an electrical synapse [21]. Synapses can be divided by the type of cellular compositions serving as pre-and and post-synaptic elements. Axo-dendritic synapses are the highest number of synapses in the mammalian nervous system; however, other varieties are also present. These include but are not restricted to axo-axonic, axo-secretory, somato-dendritic, dendro-dendritic, somato-somatic, and dendro-somatic synapses.

The characterization of Nrxs roles and interactions is only starting, but it is logical from the available knowledge that Nrxs are not molecularly or work as monogamous; they involve in numerous interactions and conduct at least some different roles in distinct synapses [2]. Nrxs and NLs furnish trans-synaptic affinity by the Ca^{2+}-dependent interaction of their substitute spliced extracellular domains. NLs specify synapses that have an activity-dependent manner, apparently by binding to Nrxs [9]. NLs are transmembrane proteins on the postsynaptic cell surface. which

were detected as ligands (or receptors) for Nrxs, they are synaptic cell adhesion proteins on the presynaptic cell surface.

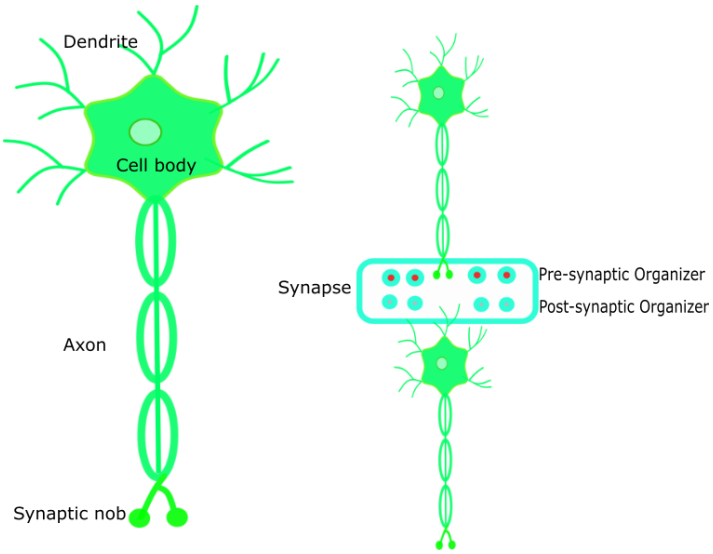

Figure 1: The left side is the motor neuron, and the right side is the sensory neuron showing the transmission of impulses from the central nervous system (CNS) and to the CNS respectively. Shows the synapse connections.

NL1 showed in nonneuronal HEK293 cells influence presynaptic differentiation in co-cultured neurons at the places of cell-to-cell contact [9]. Similarly, Nrxs showed in nonneuronal cells influence postsynaptic differentiation in cultured neurons at associate sites. These bits of knowledge led to the assumption that NL and Nrx act as transneuronal signals for the enrollment of synaptic molecules and might be associated with initial synapse evolution in vivo [9]. At glutamate synapse, a transsynaptic link forms through β-Nrxs and NLs [8]. Nrx alone can induce glutamate postsynaptic differentiation and induces GABA postsynaptic differentiation[8]. On the other hand, NLs can induce presynaptic differentiation in both GABA and glutamate axons [8]. Nrx-NL linkage is a basic constituent mediating both glutamatergic and GABAergic synaptogenesis, and differences in isoform restraining [8].

Exploring the cell and neurons is important for better understanding the physiology of the body system. This investigation is elaborated to convince the recording of biological work in non-invasive techniques with various micro- or nano-electrodes. In these ways, electrophysiology has

become one of the best biological methods in ongoing research. Voltage-gated calcium channels are highly selective for calcium and have a broad range of animating or deactivating features. Based on their threshold of voltage-dependent facilitation, they are classified as high and low-voltage-activated channels. Somatic calcium can be recorded to show the activity of action potential. Somatic calcium can trigger gene transcription. Calcium imaging is generally operated for the monitoring of interconnected neurons such as analyzing the circuitry in the cortex. This idea is also applied to recognize synaptically bridged neurons. In the case of a fluorescence probe technique, it is often used in experiments that require high selectivity because it has very high selectivity. The fluorescence probe technology has lower temporal resolution than the conventional electrophysiology technology and is easily contaminated.

Previously, it was the major problem that cell-type specific recording was not possible but with time optical-approach-type electrophysiological techniques now can do this job. This was done by the genetically encoded protein which only detects the specific cell which is matched with their cell type-specific promoter. But it is still a problem for the prob/sharp microelectrode-based electrophysiological techniques to determine cell-type-specific recording. So, using the properties of the pre-synaptic organizer, I am trying to develop a methodology by which I can soon record cell-type-specific recordings. Pre-synaptic organizer properties are helpful to make those types of recordings, but for that, I must use a genetically engineered pre-synaptic organizer. For the development of prob/sharp microelectrode-based electrophysiology, especially for specific cell recording, I need to develop engineered pre-synaptic organizers. It will be helpful to attach specific proteins attached with prob/sharp microelectrode and then I can create a bridge for the specific cell recordings.

For the generation of the test synapse organizer, we used conventional molecular biology techniques (e.g., combinations of polymerase-chain-reaction, restriction enzyme digestions, DNA ligation, infusion cloning, agarose gel electrophoresis, bacterial transformation and cultures, protein purification, plasmid purifications, DNA sequencing, BAP treatment, etc.) to generate a variety of test synapse organizer constructs. We used various proteins and Nrxs (e.g., Venus, GFP, GFP-nanobody, mCherry-nanobody, mCherry, GFPnull/YFP, Spot, Spot-nanobody, BC2, BC2-nanobody, T2A, EGFP, Rab3, NrxΔ1, NrxΔ2, etc.) to generate an engineered synapse

organizer. Then we started precise evaluations. In the orthogonal test, we found GFP-nanobody containing construct bounds with GFP and venus protein. mCherry-nanobody contains constructs bound with mCherry protein. mCherry contains constructs bounds with mCherry-nanobody protein. Spot and BC2 proteins contain constructs bound with BC2-nanobody and Spot-nanobody proteins respectively. GFP-containing constructs showed their existence extracellularly in the dark fluorescence view and can bind with GFP-nanobody and protein. After neuron expression, we considered those as engineered synapse organizers. We did a cell-microbead interaction examination, in which a spot containing NrxΔ1 showed the best results among all the constructs. In the cell-microbeads interaction experiment, we used pCAG-GS-FLAG-Spot-Nrx1bΔ1-P2A-EGFP-Rab3 in the presynaptic position and BC2-nanobody microbead in the postsynaptic position. Then we confirmed with a presynaptic marker called an anti-synaptophysin antibody. Soon, we can easily manipulate natural neurons to make Prob/sharp microelectrode-based specific cell recordings.

Figure 2: Formation of Engineered Pre-synaptic Organizers. The above diagram shows how to convert natural Nrxs to engineered Nrxs.

3.2 Materials and Methods:

Many synapse organizers were prepared for my experiment. Of those only six pairs were used for the final work. I have chosen the best quality synapse organizers based on their expression on HEK 293 cells and neurons. Most of those synapse organizers' making protocols were the same. So, I discussed materials and methods as a review for all synapse organizers in this part.

3.2.1. Experimental design:

This study was designed according to the following schematic experimental diagram:

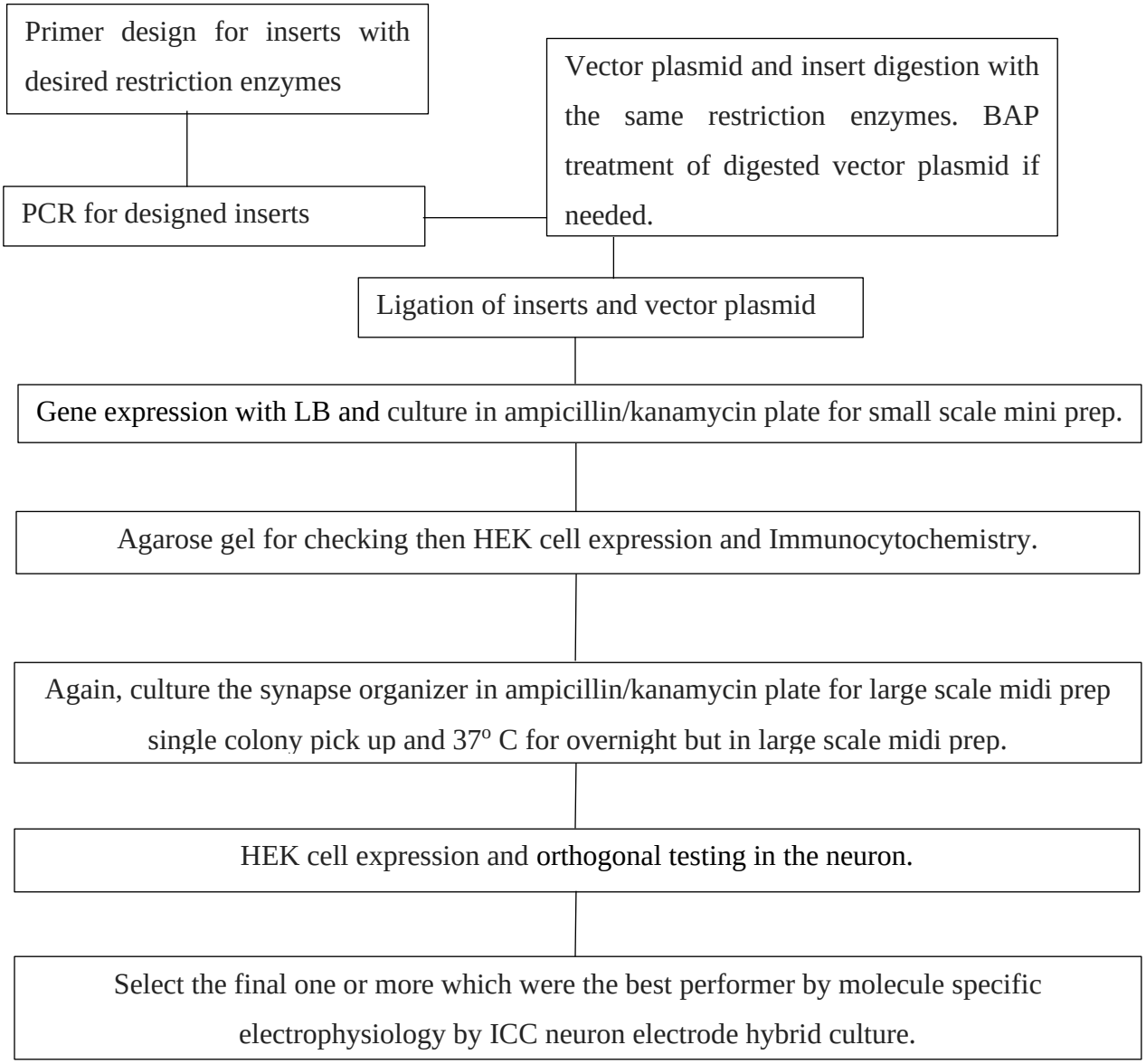

Figure 3: Flow chart for experimental design.

3.2.2. Primer design:

Primer design is the most important factor in insert-making for the desired vector plasmid. This primer design is crucial for successful synapse organizer making. Primers designed for the PCR were about 500 to 900 base pairs (bp) in total. Inserts were the same lengths with restriction enzymes as in the plasmid vector digestion sites. In this research, firstly I input the targeted bp genomic DNA sequences into the online NCBI primer BLAST software; then about 18-22 bp nucleotide sequences were selected as a primer to amplify 500-900 bp for inserts. After that I made reverse primer then I added the restriction enzymes in both forward and reverse primer. After completion, I ordered the primers online. Some factors like the percentage of GC content, melting temperature, and self-complementary were considered.

During the design of the primer, I followed some techniques for proper and better primer design. As for the GFP nanobody primer, in forwarding the GFP nanobody primer I added an extra G after the Not1 restriction enzyme design. So that GCGGCCGC (Not1) gained an extra G, then made 9 bonds. We know that 3 bonds make an amino acid, so 9 bonds will make proper 3 amino acids. This reduced the risk during digestion of the Not1 restriction enzyme. In reverse primer, I was not added extra G or other bonds because Not1 was the outer side. Another extra thing I did was added some extra light bonds before restriction enzyme-like AAAA. In the case of all primers, I added those kinds of bonds before the restriction enzyme. I skipped G bonds in case of adding extra bonds because G makes strong bonds called GC bonds. EcoR1 and BanH1generally cut with six bonds, but Not1 cuts eight bonds.

3.2.3. PCR amplification:

For most of the inserts, the PCR protocol was the same. I did PCR amplification after making the following dilution:

Prime star max enzyme:	10 µl
Pure water:	10 µl
Primer mix (Forward and Reverse):	1.0 µl
Template diluted (1:50):	0.5 µl
Total:	21.5 µl

For the primer mix, I took 1 µl forward primer and 1 µl reverse primer from 100 µM forward primer and 100 µM reverse primer respectively. Then I took 3 µl pure water in the same tube, then got a 5 µl primer mix. From that primer mix, I used only 1 µl primer mix for PCR. The following temperature cycle was maintained:

Segment	Repeat	Temperature ^{O}C	Time
1	1	95	2 minutes
2	30	98	10 seconds
		55	15 seconds
		72	10 seconds
3	1	4	99.99 minutes

I maintained the specific protocol for the Prime star max enzyme for PCR amplification.

3.2.4. Agarose gel electrophoresis:

For gel preparation, I added 0.5 gm of agarose into a 50 ml 1 x TAE buffer. Sometimes one agarose table is used instead of 0.5 gm agarose into 50 ml 1 x TAE buffer. Then heat in an oven for about 1 and a half minutes. When the solution got a clear appearance, it was ready for use. Then prepared the cassettes with the desired comb and poured them into the cassette. Then keep that cassette for hardening. After half an hour gel generally gets a hard structure. Then I removed the comb carefully and placed the gel with the upper cassette into an agarose gel electrophoresis box chamber. Then I set the wire with the Volta miter.

Before running the agarose gel electrophoresis, I loaded the marker and sample into the gel's wells. At first, I put the 4 µl GL Fast1marker then I added the samples with loading buffer into the gel's wells. Then I connected the Volta miter with electricity and 100 V was applied. Agarose gel electrophoresis needs about 30 minutes for the proper running of the sample and marker. So, after 30 minutes I stopped the electrophoresis and put the gel into the Ethidium bromide solution. I made the Ethidium bromide solution previously. For the making of Ethidium bromide solution, I used 200 ml of 1 x TAE buffer and 5 µl of Ethidium bromide solution and

mixed. After dissolving into the Ethidium bromide solution, I kept the gel on the UV display board. Then I checked the band pattern of those desired inserts.

3.2.5. PCR gels clean up:

The following protocol was followed for PCR clean-up as well as DNA concentration and removal of salts, enzymes, etc. from enzymatic reaction (SDS < 0.1%). There are several steps I maintained.

Adjust DNA binding condition:

For very small sample volumes < 30 µl adjust the volume of the reaction mixture to 50-100 µl with water. It is not necessary to remove mineral oil. Mix 1 volume of sample with 2 volumes of Buffer NT1 (e.g., mix 100 µl PCR reaction and 200 µl Buffer NT1). For melting gel, I kept the tube at 42^{o} C for 5 minutes.

Bind DNA:

Place a NucleoSpin® Gel and PCR Clean-up Column into a Collection Tube (2 ml) and load up to 700 µl sample. Centrifuge for 30 seconds at 11000 x g. Discard the flow-through and place the column back into the collection tube. Load the remaining sample if necessary and repeat the centrifugation step.

Wash silica membrane:

Add 700 µl Buffer NT3 to the NucleoSpin® Gel and PCR Clean-up Column. Centrifuge for 30 seconds at 11000 x g. Discard the flow-through and place the column back into the collection tube. Repeat the previous step to minimize chaotropic salt.

Dry silica membrane:

Centrifuge for 1 minute 11000 x g to remove Buffer NT3 completely. Make sure the spin column does not encounter the flow through while removing it from the centrifuge and the collection tube.

Elute DNA:

Place the NucleoSpin® Gel and PCR Clean-up Column into a new 1.5 ml microcentrifuge tube. Add 15-30 µl Buffer NE and incubate at room temperature (18-25 ^{o}C) for 1 minute. Centrifuge for 1 minute at 11000 x g.

3.2.6. Digestion of vector plasmid and inserts:

In the case of digestion, I followed various methods and techniques for better digestion. Almost all digestion procedures were the same. There were some differences in using the restriction enzymes because some inserts and vector plasmids were digested with only one restriction enzyme and some inserts and vector plasmids needed two restriction enzymes. Some restriction enzymes work better in the H buffer, some were the in K buffer. Some restriction enzymes needed BSA (Bovine Serum Albumin) and triton, on the other hand, some need only BSA, some restriction enzymes do not need of those both, etc. In some cases, two restriction enzymes like different buffers, in those cases sometimes I used the only one which is works in both restriction enzymes. I used a technique like at first, I used the buffer with two restriction enzymes then after 1-2 hours I used another buffer with that restriction enzyme which was not well suited in the previous buffer. In general, I used the following digestion method-

For GFP nanobody:

Eluted PCR (GFP nanobody)	: 30 µl
10 x H buffer	: 4 µl
BSA	: 4 µl
Triton	: 4 µl
Not1	:0.4 µl
Total	: 42.5 µl

Then digested for 3 hours at 37^{o} C. Then purified with PCR clean-up method and found about 15 µl digested GFP nanobody. I kept the inserts for ligation in the -5^{o} C refrigerator.

For, pCAG-GS-FLAG-Nrxn1bΔ1-P2A-EGFP-Rab3 (Plasmid vector):

Water	: 21.5 µl
10 x H buffer	: 3 µl
BSA	: 3 µl
Not1	: 0.5 µl

Plasmid vector	: 2	µl
Total	: 30	µl

Then digested for 3 hours at 37° C.

BAP treatment:

Water	: 10	µl
BAP buffer	: 4.5	µl
BAP C75	: 0.5	µl
Digested Plasmid vector	: 30	µl
Total	: 45	µl

Keep that mixture for 30-40 minutes at 55° C. Then purified with PCR clean-up method and found about 15 µl digested plasmid vector. I kept the inserts for ligation in the -5° C refrigerator.

3.2.7. Ligation and Transformation:

Plasmid vector	0.5	µl
PCR inserts	1	µl
Ligation mix enzyme	1.5	µl
Total	3	µl

For ligation, I kept that for 30-60 minutes at 16° C. Then I did transmission in 25 µl *E. coli* (DH5α). For transmission, I kept the mixture on ice for 10 minutes then at 42° C for 45 seconds then again immediately on ice for 10 minutes. After that, I spread the DH5α to the ampicillin or kanamycin plate. Then I kept the plate overnight at 37° C.

3.2.8. Miniprep procedure:

I did colony pick up with a sterile toothpick stick and placed it in a glass tube containing LB with a respective antibiotic which I used in the plate culture. Then I kept the tube overnight at 37° C. The next morning, I decant the fluids into a 2 ml bacterial tube and then centrifuge them for 3 minutes at 5000 rpm. Discarded the LB medium because cells were clotted. Then added Cell

Resuspension (CRA) about 200 µl then vortex and added Cell Lysis (CLA) about 200 µl but this time no vortex was done. I just went up and down then waited for 3 minutes and kept it open.

After 3 minutes I added Alkaline Protease 5 µl, then up and down, and kept it open for 3 minutes. Then added Neutralization Solution (NSB)of about 300 µl then mixed properly up and down. After that centrifugation was done at 14000 rpm for 10 minutes. Then I prepared the column and poured the solution into the column and centrifugation at 5000 rpm for 1 minute. The lower solution was discarded and the 600 µl column was into the column and centrifuged at 5000 rpm for 1 minute. Then again discarded the lower solution and added more 200 µthan l column wash into the column and centrifuged at 5000 rpm for 1 minute. Then again discarded the lower solution.

For drying, I centrifuged the column again at 14000 rpm for one minute. Then changed the lower column and replaced it with a 1.5 ml tube. Then put 100 µl water for elution into the middle of the dried column and wait for 1 minute. For elution, I centrifuged at 10000 rpm for one minute. After centrifugation discarded the column and preserved in a 1.5 ml tube containing plasmids. I preserved the plasmids in a -5^{o} C refrigerator for future agarose gel checking and various work like midi prep, HEK 293 cell checking, etc. I kept those plasmids that show positive results in agarose gel electrophoresis only. Then I went to proceed with other checking and midi prep.

3.2.9. Midi prep (Neuron type; Endotoxin Free):

After picking up the colony from the plate culture, pre-cultured in 2 ml LB with ampicillin or Kanamycin for several hours. Then cultured in 100 ml LB with ampicillin or Kanamycin overnight. Harvested the culture in a 50 ml tube by using twice, for harvesting centrifuged at 7000 rpm for five minutes. After every centrifugation, I discarded the fluids. Sometimes I stored the pellet in the n -80^{o} fridge when there were difficulties in time.

At first, I added 8 ml buffer RES into the 50 ml tube for resuspending the pellet. I used the vortex machine, then added 8 ml buffer LYS EF and mixed that well, thus turning blue color. After waiting 2-3 minutes, I added 8 ml buffer NEU EF which neutralized the blue color and made the solution whitish. Then I centrifuged at 10000 rpm for 5 minutes. I set the column with a filter to the black stand using a ring holder. I poured 15 ml buffer EQU EF into the edge of the

filter. Then I poured the supernatant into the filter column. After the passage of the solution from the filter, I added 5 ml FIL EF to the edge of the filter. After passing the buffer FIL EF, I discarded the column filter. Then I added 35 ml ENDO EF into the column. After that, I added 15 ml buffer WASH EF and set a new 50 ml collection tube under the column. Then I poured a 5 ml elution buffer into the column. After elution, I removed the column and added 5 ml of Isopropanol (IPA) into the collection tube. After a gentle and brief vortex, I centrifuged the collection tube at 15000 rpm for 30 minutes. Then I found a clear crystal pellet and carefully discarded the solution. Then I washed the crystal pellet with a cool 70% EtOH. After a gentle and brief vortex, I centrifuged the collection tube at 15000 rpm for 10 minutes. After that carefully I discarded the fluid and dried it for a few minutes. Finally, I melted the crystal pellet with 100 µl TE EF and measured the concentration.

3.2.10. Cell culture:

HEK 293 cells were carefully cultured in Dulbecco's Modified Eagle's Medium (DMEM, Nacalai Tesque, Inc., Japan) and added with 10% fetal bovine serum (Gibco, Life Technology, Canada). Cell plate density was at 2 x 10^6 per well in sterile 35 x10 mm cell culture dishes (Falcon, USA) and cultured in an additional incubator at 37ºC, 5% CO_2, and 95% humidity for 48-96 hours, then constructs were placed in those media.

3.2.11. Transfection of engineered synapse organizer in HEK 293 cells:

At first, I took the sample in laminar airflow and started the air flowing, and waited for 1 minute. For transfection, previously I passaged HEK 293 cells in some 60 mm sterile cell culture dishes. Then I took DMEM from the 5º C refrigerator and PEI from the -20º C refrigerator. Then I checked the HEK 293 cells' condition in the dishes through a microscope. I kept the PEI at 47º C for 5 minutes. After that, I took 100 µl DMEM containing 10% FBS and 1/100 Ampicillin. Then added 10 µl miniprep engineered synapse organizer and 4 µl PEI then pipetted several times and waited for 10 minutes. In the case of a midiprep-engineered synapse organizer, I used only 0.5 µl only. After that, I fixed the pipette to 140 µl for better pipetting. After pipetting several times, I added the mixture to the HEK 293cells dish drop by drop and spread it to the whole dish. After a little jarring the dish, I kept it at 37at º C incubator overnight.

The next morning, I added PBS 10 µl with GFP (arbitrary 3-4 µl) to the HEK 293 cell's dish and waited for 20 minutes. At various times I used various proteins for various engineered synapse organizers. If the engineered synapse organizer contained a GFP nanobody then I added GFP and if the engineered synapse organizer contained d mCherry nanobody then I added mCherry protein. After 20 minutes I sucked the DMEM medium from the HEK 293 cell's dish and washed it slowly with PBS (-) for the cell following through the dish's wall. Then sucked the PBS and again added 200 µl PBS (-) to the cell. After that, I checked under the fluorescence microscope and found the adhesion between two or three HEK 293 cells through the bonding between GFP with GFP nanobody or mCherry protein with mCherry.

3.2.12: Orthogonal testing:

Orthogonal testing is a technique that is a systematic, statistical way of testing. It is used when the total number of inputs is comparatively small but too large to permit comprehensive testing of every possible input. It is particularly effective in finding errors associated with faulty causes. The orthogonal test can be appealed in user interface testing, regression testing, positioning testing, and production testing. Each treatment gives a unique piece of information because the permutations of factor levels incorporating a single treatment are so selected that their feedbacks are uncorrelated. The net effect of organizing the experiment in such treatments is that the same piece of information is gathered in the minimum number of experiments.

I tested my engineered synapse organizers through this technique. I tested the specifically engineered synapse organizer with various specific proteins. In many cases, one engineered organizer can bind two types of proteins but there was a difference in binding nature and efficiency. Through this orthogonal testing, I compared and found the specific one.

3.2.13. Immunocytochemistry:

For immunocytochemistry, I needed 4% PFA and 4% sucrose but making them was very hard so, I took 1.2 ml of 20% PFA and 4.8 ml of sucrose in PBS. I took a little bit of NaOH to clear the solution but after checking PH by PH paper. PH 7-8 was right for working. In some cases, when I found high PH then I added 6 HCL to reduce the PH. Then I took off the HEK 293 cells which were previously transfected then sucked the DMEM fluids. After that, I added the PFA and sucrose mixture of about 500 µl to each well and waited for 20 minutes for fixation.

After 20 minutes I washed three times every well with PBS 500 µl and sucked every time. Last time I kept some PBS because of not too dry. Then I took 120 µl Donkey serum in 1200 µl PBS. For antibody preparation I have taken 1.2 ml PBS, 24 µl Donkey serum, and 1.2 µl rabbit antihuman Fc, which were made of 2% Donkey serum and 1/1000 rabbit antihuman Fc. After the application of the antibody, I waited about 2 hours, then washed again with 1 ml PBS three times. For anti-antibody, I took 1.2 ml PBS, 2 µl Donkey serum, and 2 µl antirabbit Fc, then again sucked the PBS and applied 100 µl mixture in each well and kept those in a dark place for one hour. At last, I checked under the fluorescence microscope.

3.2.14. Sequencing:

We sent our synapse organizer's sample of about 500-1000 ng for each sample to a company named Eurofin Genomics for Sanger sequencing. Within one week we got the sequencing results and then compared the results with the help of SnapGene Viewer and ApE software.

3.3 Results:

After doing many biological works I found six pairs of engineered synapse organizers that work as presynaptic Nrxs. After making them measured the concentration and all of them were endotoxin-free. I have chosen those best quality synapse organizers based on their expression on HEK 293 cells and the ICC test. Most of those synapse organizers' making protocols were the same. I used different kinds of restriction enzymes for various inserts and vector plasmids. The temperature and steps of PCR were different in different inserts also. Different constructs manipulation was also done in different ways. Those constructs are as follows-

pCAG-GS-FLAG-GFP-nanobody-Nrxn1bΔ1-P2A-mCherry-Rab3 (Endo toxin free 1.9 µg/µl)

pCAG-GS-FLAG-GFP-nanobody-Nrxn1bΔ2-P2A-mCherry-Rab3 (Endo toxin free 2.2 µg/µl)

pCAG-GS-FLAG-GFPNull-Nrxn1bΔ1-P2A-EGFP-Rab3 (Endo toxin free 5.04 µg/µl)

pCAG-GS-FLAG-GFPNull-Nrxn1bΔ2-P2A-EGFP-Rab3 (Endo toxin free 3.5 µg/µl)

pCAG-GS-FLAG-mCherry-Nrxn1bΔ1-P2A-EGFP-Rab3 (Endo toxin free 5.0 µg/µl)

pCAG-GS-FLAG- mCherry -Nrxn1bΔ2-P2A-EGFP-Rab3 (Endo toxin free 5.7 µg/µl)

pCAG-GS-FLAG-mCherry-nanobody-Nrxn1bΔ1-P2A-EGFP-Rab3 (Endo toxin free 4.2 µg /µl)

pCAG-GS-FLAG-mCherry-nanobody-Nrxn1bΔ2-P2A-EGFP-Rab3 (Endo toxin free 4.9 µg/µl)

pCAG-GS-FLAG-BC2-Nrxn1bΔ1-P2A-EGFP-Rab3 (Endo toxin free 3.4 µg/µl)

pCAG-GS-FLAG-BC2-Nrxn1bΔ2-P2A-EGFP-Rab3 (Endo toxin free 1.8 µg/µl)

pCAG-GS-FLAG-Spot-Nrxn1bΔ1-P2A-EGFP-Rab3 (Endo toxin free 2.16 µg/µl)

pCAG-GS-FLAG-Spot-Nrxn1bΔ2-P2A-EGFP-Rab3 (Endo toxin free 4.8 µg/µl)

At first, I digested the Nrx1Δ1 and Nrx1Δ2 with respective restriction enzymes and then checked them in agarose gel. I also checked every insert after PCR in agarose gel. I digested those inserts with different kinds of restriction enzymes. For a definite construct, I used the same restriction enzymes both in vector plasmid and insert. For as Not1 restriction enzyme was used in the Nrx1Δ1/ Nrx1Δ2 and GFP-nanobody. So, after making the construct I digested the construct and

found both NrxΔ1/NrxΔ2 and GFP-nanobody because of digestion of the same place with Not1, where I ligated.

In Fig. 4, the left side is for pCAG-GS-FLAG-GFP-nanobody-Nrxn1bΔ1-P2A-mCherry-Rab3, and the right side is for pCAG-GS-FLAG-GFP-nanobody-Nrxn1bΔ2-P2A-mCherry-Rab3 were expressed in HEK 293 cells in red views. In both pictures, it can easily see the expression of internal GPF of those constructs but the extracellular GFP-nanobody cannot be expressed. For the evidence of GFP-nanobody extracellularly, I added Venus protein.

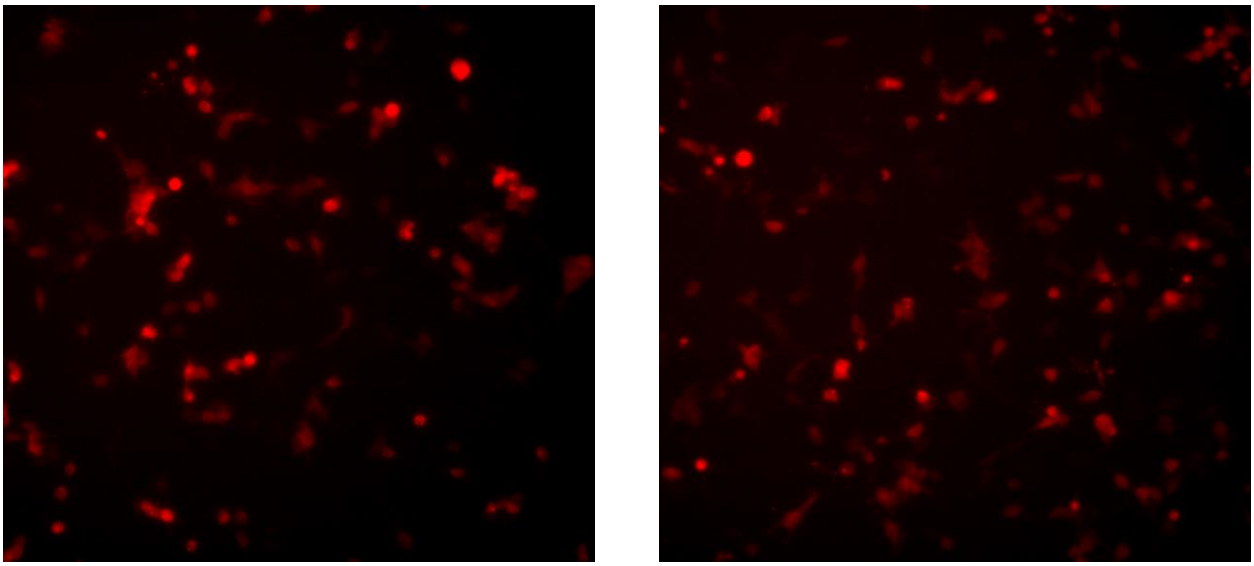

Figure 4: The left side is for pCAG-GS-FLAG-GFP-nanobody-Nrxn1bΔ1-P2A-mCherry-Rab3 before binding with Venus protein in the red view and the right side is for pCAG-GS-FLAG-GFP-nanobody-Nrxn1bΔ2-P2A-mCherry-Rab3 before binding Venus protein in red view.

In Fig. 5, the pCAG-GS-FLAG-GFP-nanobody-Nrxn1bΔ1-P2A-mCherry-Rab3 construct is expressed after adding Venus protein. Venus protein can attach to the GFP nanobody so that it is expressed extracellularly in HEK 293 cells. In the red view, we can just see the internal fluorescence part of the constructs but in the yellow view, we can detect the outer part which was bound with Venus protein. Venus protein has a fluorescent nature, so it is expressed. From the extracellular expression of the Venus protein, we can easily say that GFP-nanobody was bound

with the Venus protein. Figure 5 shows from left, red view then in middle yellow view, and the right-side merged view of red and yellow views.

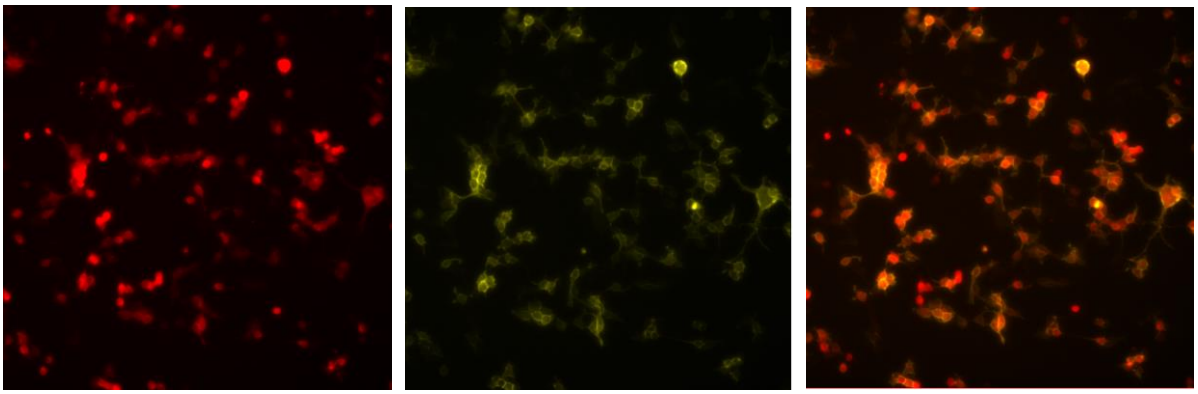

Figure 5: pCAG-GS-FLAG-GFP-nanobody-Nrxn1bΔ1-P2A-mCherry-Rab3 after binding Venus protein in red view (Left), yellow view (middle), and merged view (Right).

In Fig. 6, pCAG-GS-FLAG-GFP-nanobody-Nrxn1bΔ2-P2A-mCherry-Rab3 construct is expressed after adding Venus protein. Venus protein can attach to the GFP nanobody so that it is expressed extracellularly in HEK 293 cells. In the red view, we can just see the internal fluorescence part of the constructs but in the yellow view, we can detect the outer part which was bound with Venus protein. Venus protein has a fluorescent nature, so it is expressed. From the extracellular expression of the Venus protein, we can easily say that GFP-nanobody was bound with the Venus protein. Figure 6 shows from left, red view then the middle yellow view and the right-side merged view of red and yellow views.

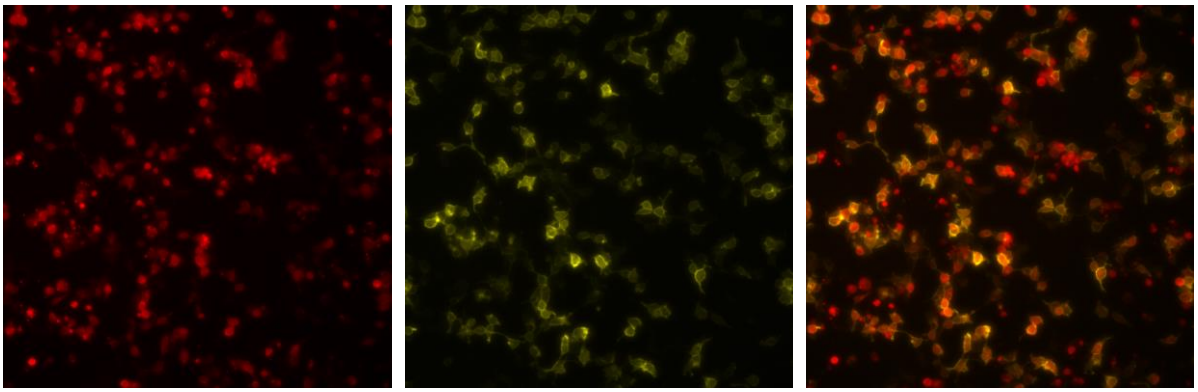

Figure 6: pCAG-GS-FLAG-GFP-nanobody-Nrxn1bΔ2-P2A-mCherry-Rab3 after binding Venus protein in red view (Left), yellow view (middle), and merged view (Right).

In Fig. 7, pCAG-GS-FLAG-GFPNull-Nrxn1bΔ1-P2A-EGFP-Rab3 construct was expressed. The left side is the green view which expresses the intracellular fluorescence part of the construct, and the middle is the red view which expresses the extracellular part of the construct after binding with a fusion protein of anti-GFPnanobody and mCherry. The right side marge view indicates the GFPNull existence after binding with fusion pa protein of anti-GFPnanobody and mCherry.

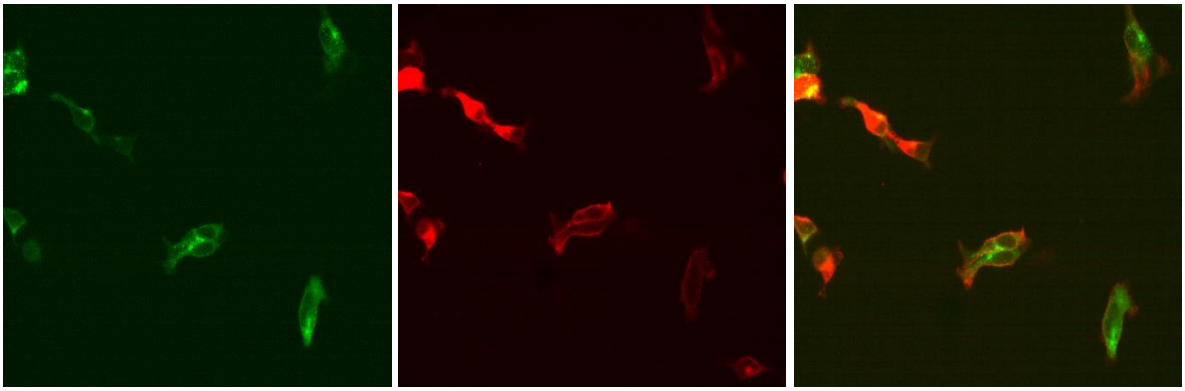

Figure 7: pCAG-GS-FLAG-GFPNull-Nrxn1bΔ1-P2A-EGFP-Rab3 green view (Left), red view (Middle), and marge view (Right).

In Fig. 8, pCAG-GS-FLAG-GFPNull-Nrxn1bΔ2-P2A-EGFP-Rab3 construct was expressed. The left side is the green view which expresses the intracellular fluorescence part of the construct, and the middle is the red view which expresses the extracellular part of the construct after binding with a fusion protein of anti-GFPnanobody and mCherry. The right side marge view indicates the GFPNull existence after binding with a fusion protein of anti-GFPnanobody and mCherry.

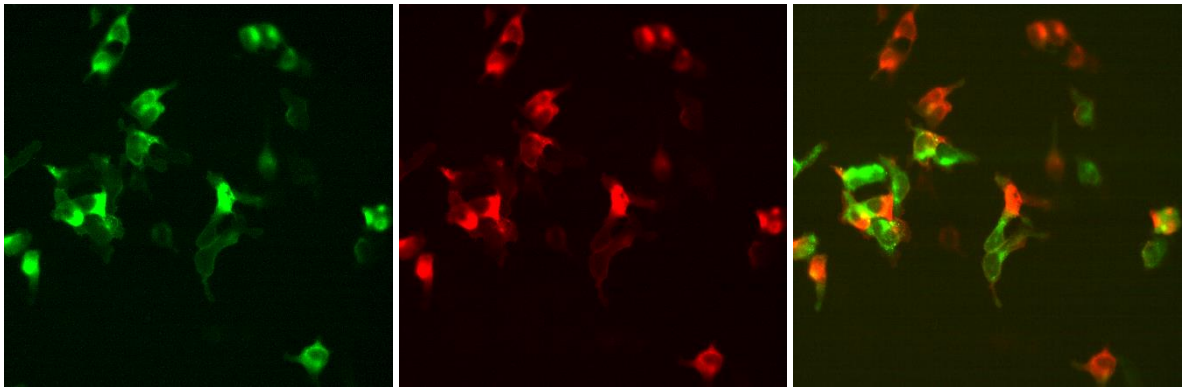

Figure 8: pCAG-GS-FLAG-GFPNull-Nrxn1bΔ2-P2A-EGFP-Rab3 green view (Left), red view (Middle), and marge view (Right).

In Fig. 9, the pCAG-GS-FLAG-mCherry-Nrxn1bΔ1-P2A-EGFP-Rab3 construct was expressed. The left side is the green view which expresses the intracellular fluorescence part of the construct, and the right side is the dark view which expresses the extracellular fluorescence part of the construct. The extracellular part contains mCherry which is fluorescence in nature, so I have not added any extra kind of fluorescence protein. In a dark view, the outer lining indicates the extracellular part of the construct containing mCherry.

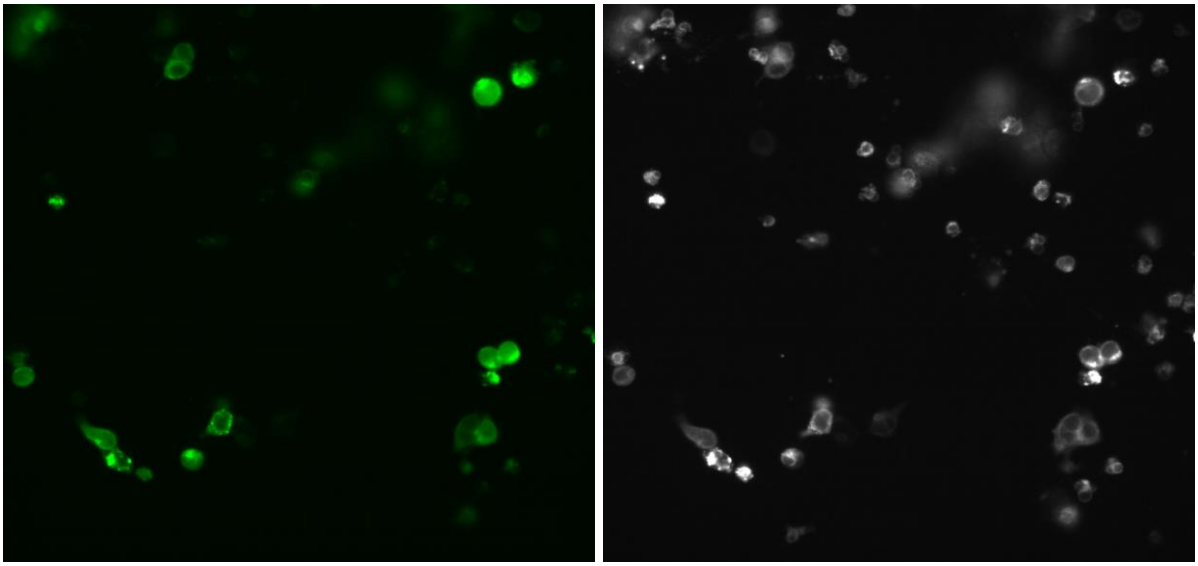

Figure 9: pCAG-GS-FLAG-mCherry-Nrxn1bΔ1-P2A-EGFP-Rab3 green view (Left) and dark view (Right).

In Fig. 10, the pCAG-GS-FLAG-mCherry-Nrxn1bΔ2-P2A-EGFP-Rab3 construct was expressed. The left side is the green view which expresses the intracellular fluorescence part of the construct, and the right side is the dark view which expresses the extracellular fluorescence part of the construct. The extracellular part contains mCherry which is fluorescence in nature, so I have not added any extra kind of fluorescence protein. In a dark view, the outer lining indicates the extracellular part of the construct containing mCherry.

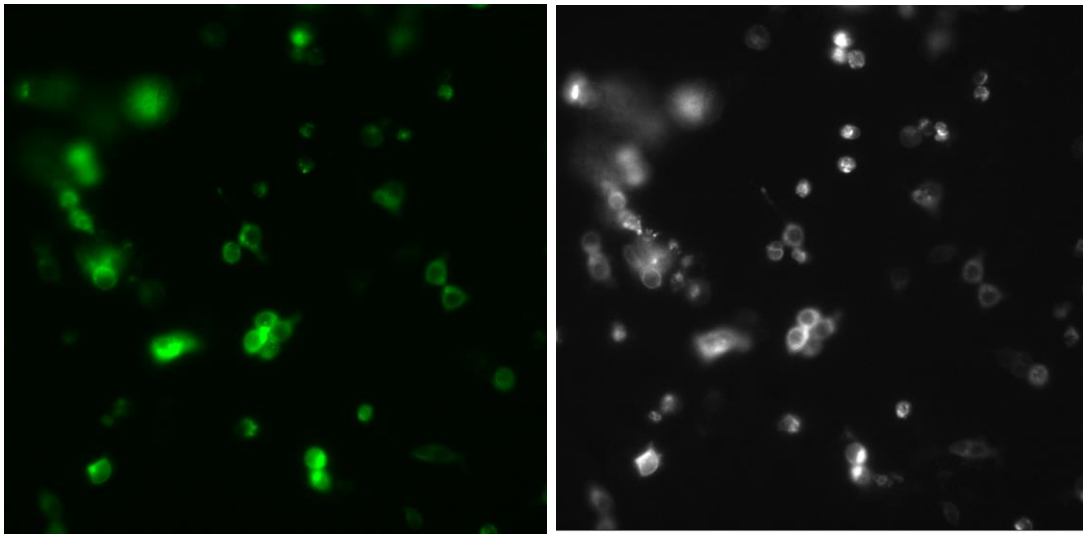

Figure 10: pCAG-GS-FLAG-mCherry-Nrxn1bΔ2-P2A-EGFP-Rab3 green view (Left) and dark view (Right).

In Fig. 11, the left side is the green view, and the right side is a red view for the pCAG-GS-FLAG-mCherry-nanobody-Nrxn1bΔ1-P2A-EGFP-Rab3 construct. In the green view, we can see the intracellular part of the construct which is expressed in HEK 293 cells. The extracellular mCherry-nanobody part of the construct was not detectable in both green and red views.

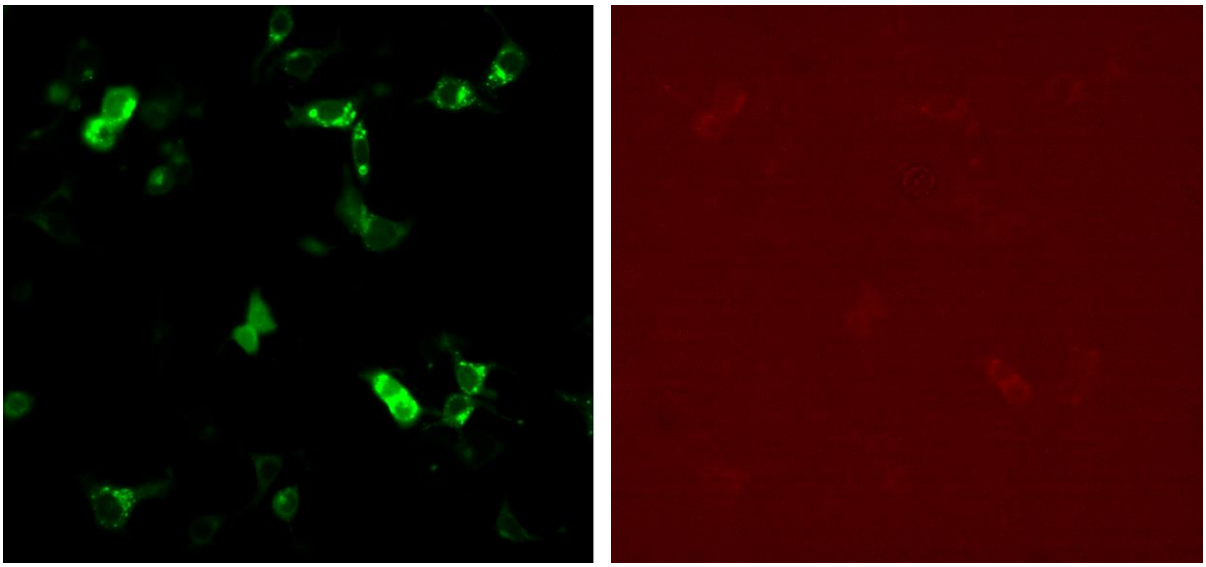

Figure 11: pCAG-GS-FLAG-mCherry-nanobody-Nrxn1bΔ1-P2A-EGFP-Rab3 before binding with mCherry protein in green view (Left), red view (Right).

In Fig. 12, the pCAG-GS-FLAG-mCherry-nanobody-Nrxn1bΔ1-P2A-EGFP-Rab3 construct is expressed after adding mCherry protein. mCherry protein can ligate with the mCherry-nanobody so that it is expressed extracellularly in HEK 293 cells. In the green view, we can just see the internal fluorescence part of the constructs but in the red view, we can detect the outer part which was bound with the mCherry protein. mCherry protein has a fluorescence nature, so it was expressed. From the extracellular expression of the mCherry protein, we can easily say that mCherry-nanobody was bound with mCherry protein. The figure shows from left, green view then in middle red view and on right side merged view of green and red views. In the merged view both intracellular and extracellular expressions are shown. The outer red lining is for the binding of mCherry protein with the extracellular part mCherry-nanobody of the construct.

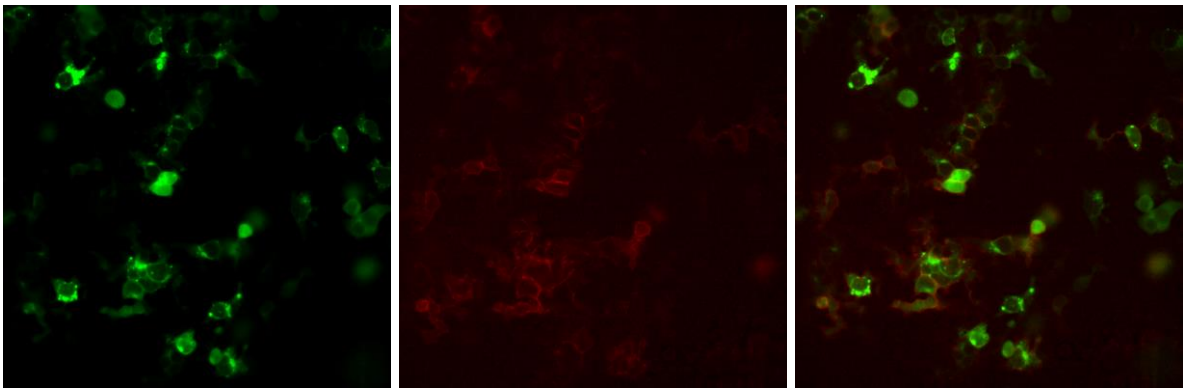

Figure 12: pCAG-GS-FLAG-mCherry-nanobody-Nrxn1bΔ1-P2A-EGFP-Rab3 after binding Cherry protein in green view (Left), red view (middle), and merged view (Right).

In Fig. 13, the left side is the green view, the middle is the red view, and the right side is the merged view of green and red views for pCAG-GS-FLAG-mCherry-nanobody-Nrxn1bΔ2-P2A-EGFP-Rab3 construct. In the green view, we can see the intracellular part of the construct which is expressed in HEK 293 cells. The extracellular mCherry-nanobody part of the construct was not detectable in both green and red views. In the merged view, there is nothing like the extracellular part because mCherry-nanobody is not fluorescent in nature. If mCherry-nanobody binds with any fluorescence protein, then we can see an extracellular lining in HEK 293 cells.

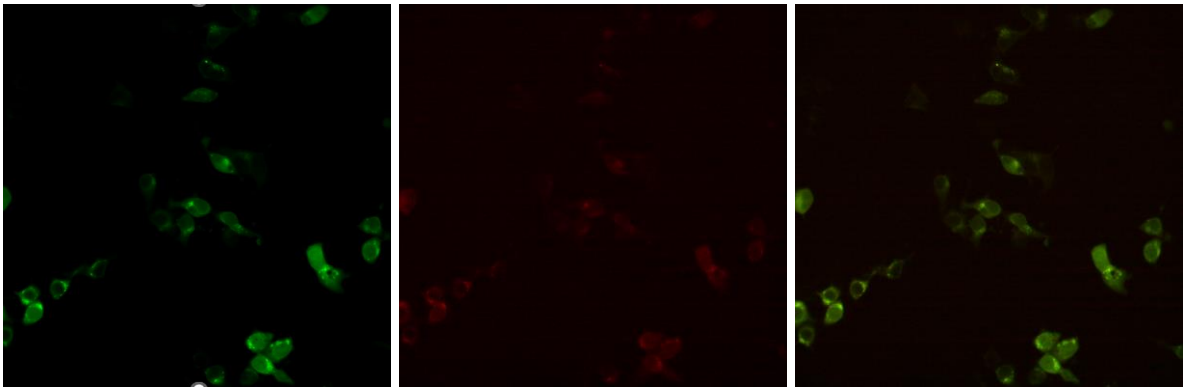

Figure 13: pCAG-GS-FLAG-mCherry-nanobody-Nrxn1bΔ2-P2A-EGFP-Rab3 before binding with Cherry protein in green view (Left), red view (middle), and merged view (Right).

In Fig. 14, the pCAG-GS-FLAG-mCherry-nanobody-Nrxn1bΔ2-P2A-EGFP-Rab3 construct is expressed after adding mCherry protein. mCherry protein can ligate with the mCherry-nanobody so that it is expressed extracellularly in HEK 293 cells. In the green view, we can just see the internal fluorescence part of the constructs but in the red view, we can detect the outer part which was bound with the mCherry protein. mCherry protein has a fluorescence nature, so it was expressed. From the extracellular expression of the mCherry protein, we can easily say that mCherry-nanobody was bound with mCherry protein. The figure shows from left, green view then in middle red view and on right side merged view of green and red views. In the merged view, both intracellular and extracellular expressions are shown. The outer red lining is for the binding of mCherry protein with the extracellular part mCherry-nanobody of the construct.

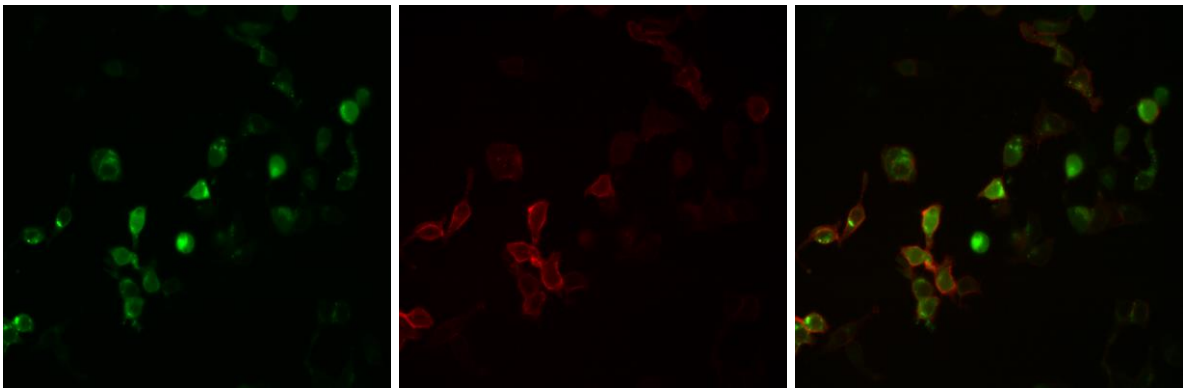

Figure 14: pCAG-GS-FLAG-mCherry-nanobody-Nrxn1bΔ2-P2A-EGFP-Rab3 after binding with Cherry protein in green view (Left), red view (middle), and merged view (Right).

In Fig. 15, the left side is the green view, the middle is the red view, and the right side is the merged view of green and red views for the pCAG-GS-FLAG-BC2-Nrxn1bΔ1-P2A-EGFP-Rab3 construct. In the green view, we can see the intracellular part of the construct which is expressed in HEK 293 cells. The extracellular BC2 part of the construct was not detectable in both the green or red views. In the merged view, there is nothing like the extracellular part because BC2 is not fluorescence in nature. If BC2 binds with any fluorescence protein, then we can see an extracellular lining in HEK 293 cells.

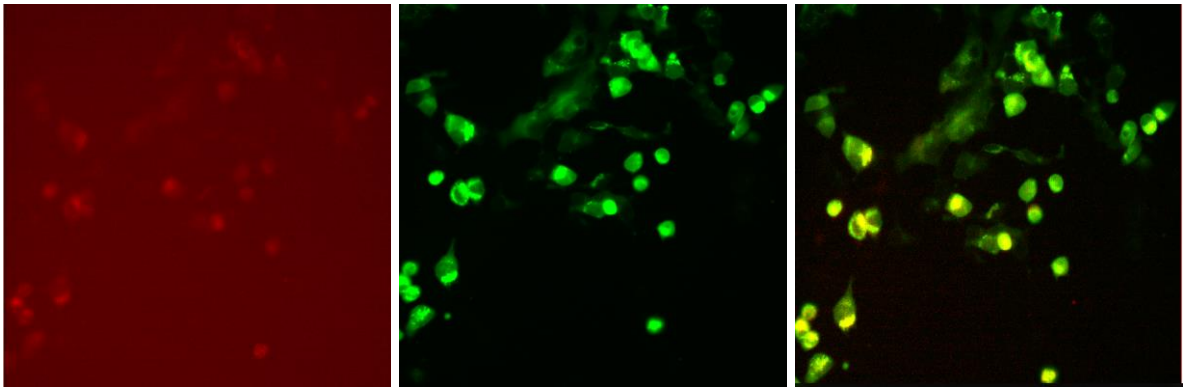

Figure 15: pCAG-GS-FLAG-BC2-Nrxn1bΔ1-P2A-EGFP-Rab3 before binding with BC2-nanobodymCherry protein in red view (Left), green view (middle), and merged view (Right).

In Fig. 16, the pCAG-GS-FLAG-BC2-Nrxn1bΔ1-P2A-EGFP-Rab3 construct is expressed after adding BC2-nanobodymCherry protein. BC2-nanobodymCherry protein can ligate with the BC2 so that it is expressed extracellularly in HEK 293 cells. In the green view, we can just see the internal fluorescence part of the constructs but in the red view, we can detect the outer part which was bound with BC2-nanobodymCherry protein. BC2-nanobodymCherry protein has fluorescence in nature, so it was expressed. From the extracellular expression of the BC2-nanobodymCherry protein, we can easily say that BC2 was bound with the BC2-nanobodymCherry protein. The figure shows from left, green view then in middle red view and on right side merged view of green and red views. In the merged view, both intracellular and extracellular expressions are shown. The outer red lining is for the binding of BC2-nanobodymCherry protein with the extracellular part BC2 of the construct.

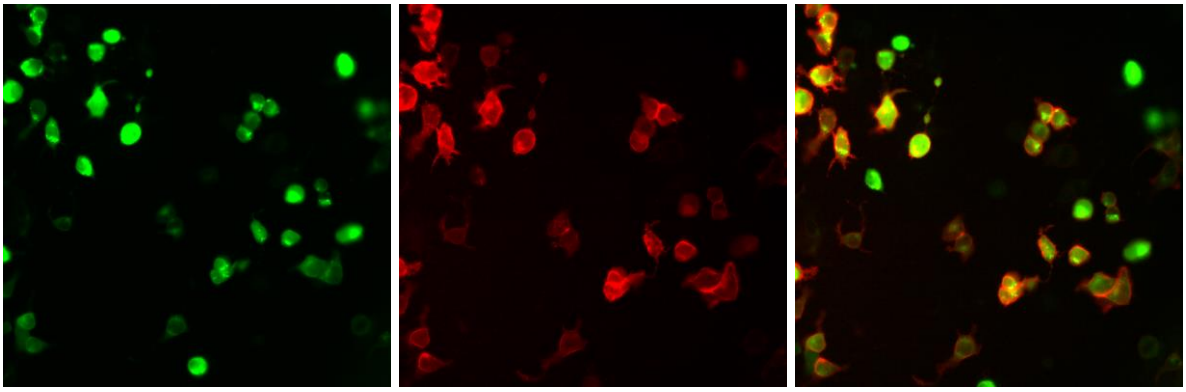

Figure 16: pCAG-GS-FLAG-BC2-Nrxn1bΔ1-P2A-EGFP-Rab3 after binding with BC2-nanobodymCherry protein in green view (Left), red view (middle), and merged view (Right).

In Fig. 17, the left side is the green view, the middle is the red view, and the right side is the merged view of green and red views for the pCAG-GS-FLAG-BC2-Nrxn1bΔ2-P2A-EGFP-Rab3 construct. In the green view, we can see the intracellular part of the construct which is expressed in HEK 293 cells. The extracellular BC2 part of the construct was not detectable in both the green or red views. In the merged view , there is nothing like the extracellular part because BC2 is not fluorescence in nature. If BC2 binds with any fluorescence protein, then we can see an extracellular lining in HEK 293 cells.

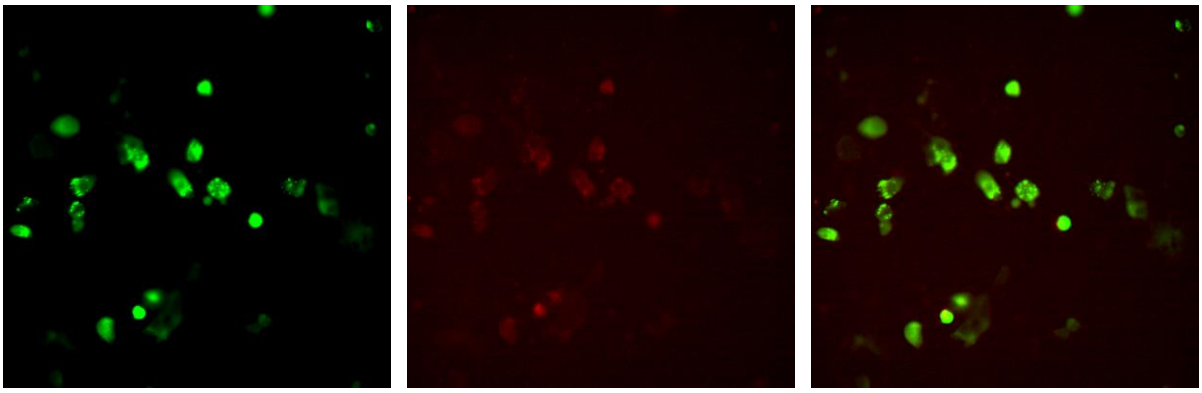

Figure 17: pCAG-GS-FLAG-BC2-Nrxn1bΔ2-P2A-EGFP-Rab3 before binding with BC2-nanobodymCherry protein in red view (Left), green view (middle), and merged view (Right).

In Fig. 18, the pCAG-GS-FLAG-BC2-Nrxn1bΔ2-P2A-EGFP-Rab3 construct is expressed after adding BC2-nanobodymCherry protein. BC2-nanobodymCherry protein can ligate with the BC2

so that it is expressed extracellularly in HEK 293 cells. In the green view, we can just see the internal fluorescence part of the constructs but in the red view, we can detect the outer part which was bound with BC2-nanobodymCherry protein. BC2-nanobodymCherry protein has fluorescence in nature, so it was expressed. From the extracellular expression of the BC2-nanobodymCherry protein, we can easily say that BC2 was bound with the BC2-nanobodymCherry protein. The figure shows from left, green view then in middle red view and on right side merged view of green and red views. In the merged view, both intracellular and extracellular expressions are shown. The outer red lining is for the binding of BC2-nanobodymCherry protein with the extracellular part BC2 of the construct.

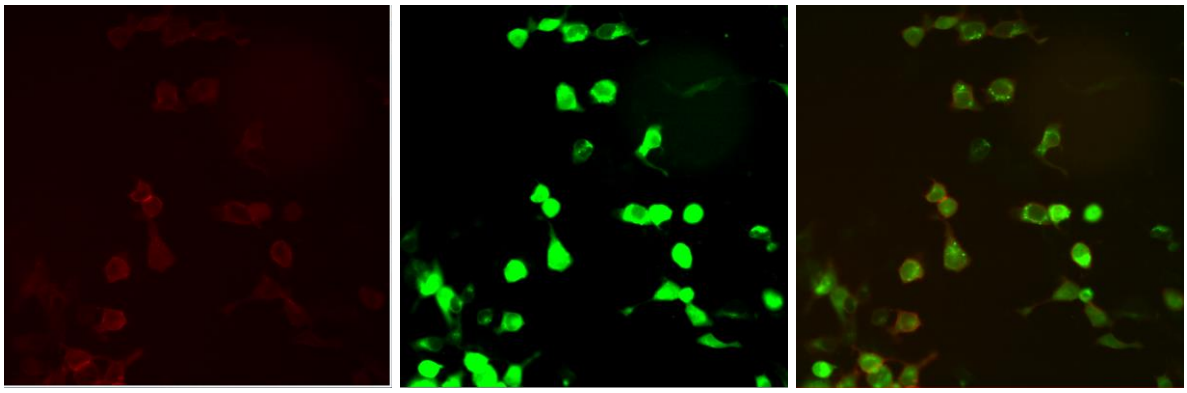

Figure 18: pCAG-GS-FLAG-BC2-Nrxn1bΔ2-P2A-EGFP-Rab3 after binding with BC2-nanobodymCherry protein in red view (Left), green view (middle), and merged view (Right).

In Fig. 19, the left side is the green view, the middle is the red view, and the right side is the merged view of green and red views for the pCAG-GS-FLAG-Spot-Nrxn1bΔ1-P2A-EGFP-Rab3 construct. In the green view, we can see the intracellular part of the construct which is expressed in HEK 293 cells. The extracellular Spot part of the construct was not detectable in both green and red views. In the merged view, there is nothing like the extracellular part because Spot is not fluorescent in nature. If Spot binds with any fluorescence protein, then we can see an extracellular lining in HEK 293 cells.

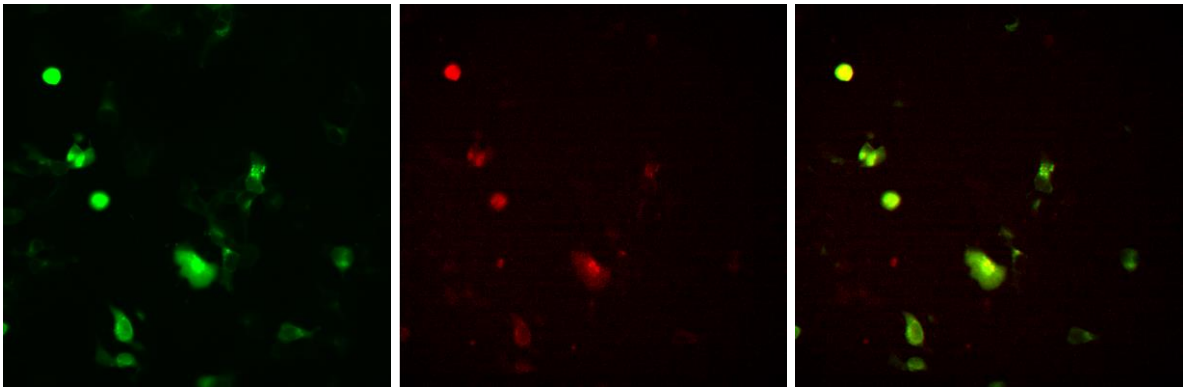

Figure 19: pCAG-GS-FLAG-Spot-Nrxn1bΔ1-P2A-EGFP-Rab3 Rab3 before binding with BC2-nanobodymCherry protein in green view (Left), red view (middle), and merged view (Right).

In Fig. 20, the pCAG-GS-FLAG-Spot-Nrxn1bΔ1-P2A-EGFP-Rab3 construct is expressed after adding BC2-nanobodymCherry protein. In Fig. 33, HEK cells are expressed due to the presence of an engineered Nrx construct. In green view, it shows the presence of the construct because of the EGFP part which is one kind of green fluorescence that remains an intracellular part of the construct. In the red view, it shows the outer red line which indicates that the extracellular part Spot binds with the specific protein BC2-nanobody mCherry. In the figure, the right-side picture is a merged view of green and red views after adding BC2-nanobobymCherry. So, from the figure, it can be easily confirmed the presence of constructs and the efficiency of the constructs. It indicates they can be bound like a lock and key with their respective proteins. So, it can be assumed that these constructs may bind with the postsynaptic-engineered red organizer containing a specific protein in the extracellular part.

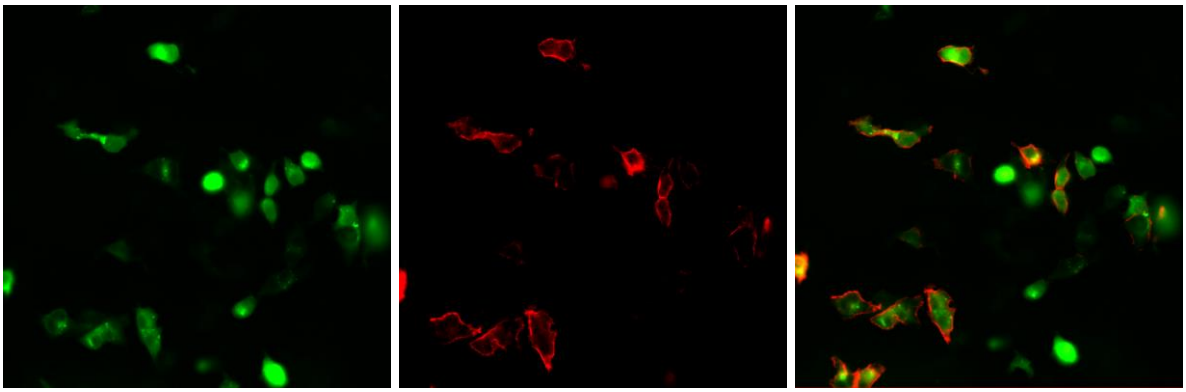

Figure 20: pCAG-GS-FLAG-Spot-Nrxn1bΔ1-P2A-EGFP-Rab3 after binding with BC2-nanobodymCherry protein in green view (Left), red view (middle), and merged view (Right).

In Fig. 21, the left side is the green view, the middle is the red view, and the right side is the merged view of green and red views for the pCAG-GS-FLAG-Spot-Nrxn1bΔ2-P2A-EGFP-Rab3 construct. In the green view, we can see the intracellular part of the construct which is expressed in HEK 293 cells. The extracellular Spot part of the construct was not detectable in both green and red views. In the merged view, there is nothing like the extracellular part because Spot is not fluorescent in nature. If Spot binds with any fluorescence protein, then we can see an extracellular lining in HEK 293 cells.

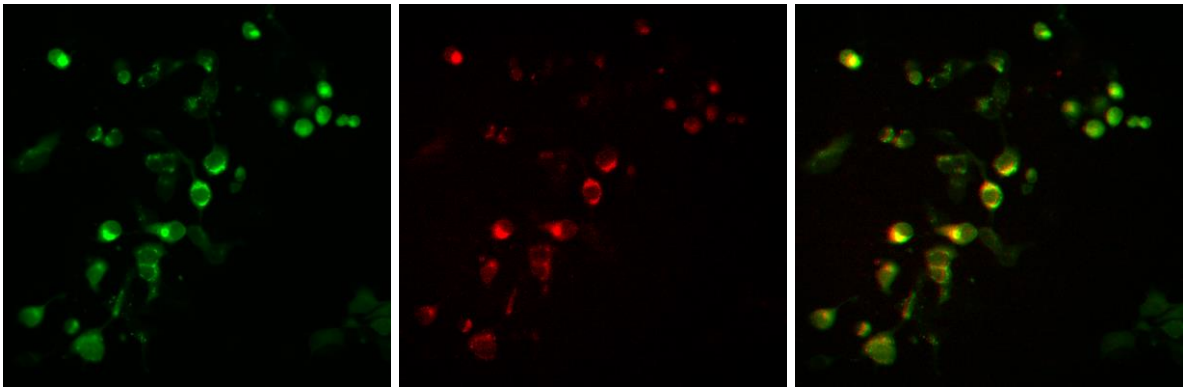

Figure 21: pCAG-GS-FLAG-Spot-Nrxn1bΔ2-P2A-EGFP-Rab3 before binding BC2-nanobodymCherry protein in green view (Left), red view (middle), and merged view (Right).

In Fig. 22, the pCAG-GS-FLAG-Spot-Nrxn1bΔ2-P2A-EGFP-Rab3 construct is expressed after adding BC2-nanobodymCherry protein. In Fig. 35, HEK cells are expressed due to the presence of an engineered Nrx construct. In green view, it shows the presence of the construct because of the EGFP part which is one kind of green fluorescence that remains an intracellular part of the construct. In the red view, it shows the outer red line which indicates that the extracellular part Spot binds with the specific protein BC2-nanobody mCherry. In the figure, the right-side picture is a merged view of green and red views after adding BC2-nanobobymCherry. So, from the figure, it can be easily confirmed the presence of constructs and the efficiency of the constructs. It indicates they can be bound like a lock and key with their respective proteins. So, it can be assumed that these constructs may bind with another postsynaptic-engineered organizer containing a specific protein in the extracellular part.

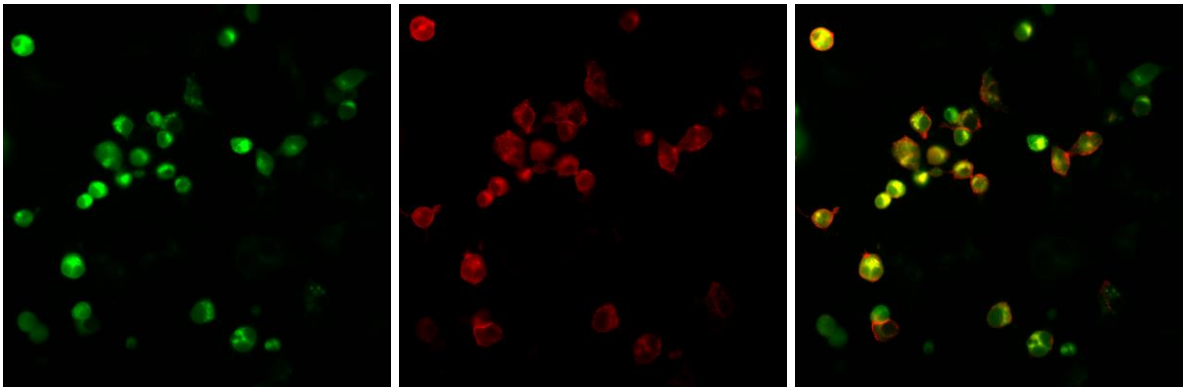

Figure 22: pCAG-GS-FLAG-Spot-Nrxn1bΔ2-P2A-EGFP-Rab3 before binding with BC2-nanobodymCherry protein in green view (Left), red view (middle), and merged view (Right).

Some of them also showed positive results in the ICC test. In my experiment, I got success to generate those constructs and they all were bound to their specific protein extracellularly in HEK cells. I tested my engineered synapse organizers through the orthogonal technique. I tested the specifically engineered synapse organizer with various specific proteins. In many cases, one engineered organizer can bind two types of proteins but there was a difference in binding nature and efficiency. Through this orthogonal testing, I compared and found the specific one. GFP-nanobody contains constructs bound with Venus protein and GFP. GFP-containing constructs showed their existence extracellularly in the dark fluorescence view and can bind with GFP-nanobody protein. mCherry-nanobody containing constructs bound with mCherry protein. mCherry contains constructs bound with mCherry-nanobody protein. Spot and BC2-containing constructs bound with BC2-nanobodymCherry protein. Through orthogonal testing I found, a spot containing construct can bind with BC2-nanobody protein and a BC2-containing construct can bind with spot-nanobody protein. In conclusion, it can be said that my engineered synaptic organizers responded positively.

3.4 Discussion:

For the Nrx/NL complex in synapse formation, either independent manipulation of Nrx/NL can cause moderation of presynaptic and postsynaptic assembly, which suggests an instructive role [11]. In our experiment, we synthesized engineered synapse organizers. We synthesized presynaptic organizers like Nrxs. We simply manipulated the natural Nrxs and first made NrxΔ1 and NrxΔ2 by digesting the extracellular part of the Nrx with restriction enzymes. Then we generated various proteins like GFP, GFP-nanobody, BC2, Spot, mCherry, and mCherry-nanobody through PCR and then ligated them with those manipulated Nrxs. Seth L. Shipman et al. also suggested that dimerized NL induces the gathering of presynaptic organizers which is a part of the functional chemical synapse [11]. Dean C et al. In vitro, authentication suggests that Nrx differentiation in the early stage on an axon part into a presynaptic terminal and NL clustering may be an early step in the differentiation of a dendrite segment into a postsynaptic terminal [12].

Demet Arac et al. suggested that NL1 shown in nonneuronal HEK293 cells influences presynaptic differentiation in co-cultured neurons at the places of cell-to-cell contact [9]. Similarly, Nrxs showed in nonneuronal cells influence postsynaptic differentiation in cultured neurons at associate sites. These bits of knowledge led to the assumption that NL and Nrx act as trans-neuronal signals for the enrollment of synaptic molecules and might be associated with initial synapse evolution in vivo [9]. Nrxs can bind with many postsynaptic-specific proteins. It was difficult to generate an engineered synapse organizer, but our engineered Nrxs can only bind with the specific protein or ligands. So, soon, we expect these engineered Nrxs will bind with engineered NL containing the specific protein extracellularly.

After doing orthogonal testing, we found the specific protein for each construct. GFP-nanobody contains constructs bound with GFP protein. GFP-containing constructs showed their existence extracellularly in the dark fluorescence view and can bind with GFP-nanobody protein. mCherry-nanobody contains constructs bound with mCherry protein. mCherry contains constructs bounds with mCherry-nanobody protein. Spot and BC2 contain constructs bound with spot-nanobody and BC2-nanobody proteins, respectively. In cell-microbead reaction, we found positive results for pre and postsynaptic reactions marked by pre and postsynaptic markers. Spot containing engineered NrxΔ1 bound with BC2-nanobody microbead and confirmed by

presynaptic Anti-synaptophysin antibody. In conclusion, it can be said that our engineered synaptic organizers responded well.

From the above research and the outcome of our expectations, it can be said that in the future I will be able to make a new electrophysiological method or technique. In that technique, specific cell recording can be possible with the help of prob/sharp microelectrode bound with a specific protein. That specific protein will be reacted only with the specific engineered Nrx and then I can record that cell activity only. In other words, a specific protein bound with prob/sharp microelectrode will initiate the presynaptic reaction in specific cells/neurons that have a relation with the engineered Nrx. Then cell specificity will be confirmed because that engineered Nrx will be associated only with that specific protein bound with prob/sharp microelectrode. Then there will be a new era for the prob/sharp microelectrode-based electrophysiological technique for specific cell recordings.

3.5 Conclusions

I have confirmed from the above experiments and discussions that my designed engineered Nrx can be induced by outer protein/particle and showed their presynaptic reaction. Spot protein containing engineered NrxΔ1 bound with BC2-nanobody microbead and confirmed by presynaptic Anti-synaptophysin antibody. So, if a specific protein deposits on the top of a prob/sharp microelectrode, then that specific protein can attach with designed engineered Nrx. Then will initiate a presynaptic reaction in that specific cell who have an attachment with the designed engineered Nrx. In this way, my planned prob/sharp microelectrode-based electrophysiological technique will fruitfully come out for the specific cell recordings. Thus, I concluded that my engineered pre-synaptic organizer can be able to work for the development of a prob/sharp microelectrode-based electrophysiological technique.

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Chapter 4

Development of Engineered Post-Synaptic Organizer

Key Points of Chapter 4

- **Confirmed orthogonal engineered post-synaptic organizers (NL).**
- **Confirmed existence in HEK cell culture as structural integrity.**
- **So, this confirmed that these can be used in target binding for the development of electrophysiological techniques.**

4.1 Introduction:

The animal brain is composed of neurons and those are the fundamental units of the nervous system. Neurons can be excited electrically [1] and related to the transmission by specialized connections named synapses. Based on their function neurons are of three types. In a neural circuit, a bunch of neurons work significantly. Sensory neurons initiate the cells related to the sensory organs and then transmit signals to the spinal cord. Motor neurons gather signals from the brain or spinal cord to regulate all. An ordinary neuron includes a cell body, dendrites, and an axon. The cell body is generally compact. Dendrites are subdivided and widen from the cell body. Dendrites are normally few and far between a hundred micrometers in range. A signal can be transferred from one neuron to another neuron over the synapse in axon terminals. Signals are gathered through the dendrites and cell body then advanced signals through the axon. However, synapses can be made between an axon to another axon or between a dendrite to another dendrite.

In the brain, synapses work as exclusive junctions in the middle of neurons. They connect neurons and then transmit information with millions of interdigitated crisscrossing neural circuits [2]. Neurons are not constant in the whole body, yet they still, communicate with each other, an idea named the neuron doctrine[4]. Synapse-organizing proteins maintain the presynaptic secretion and postsynaptic receptive molecules to make differentiation for the development of synapses [6]. The plasma membrane of the signal-passing presynaptic neuron comes into close collocation with the membrane of the targeted postsynaptic neuron. To carry, out the signaling process, both the presynaptic and postsynaptic sites hold extensive arrangements of the molecular machinery that link the two membranes together. The presynaptic part is placed on an axon and the postsynaptic part is placed on a dendrite or soma is shown in many synapses. Astrocytes also interchange information with the synaptic neurons, greeting synaptic activity and, in turn, controlling neurotransmission [7].

Chemical synapses are balanced in location through synaptic adhesion molecules (SAMs) that look forward from both the pre-synaptic and post-synaptic neurons and assemble where they imbricate; SAMs can provide for the initiation and running of synapses [8]. The presynaptic and postsynaptic cell membranes are connected by special channels in an electrical synapse, which is called gap junctions or synaptic cleft that is able of passing an electric current, doing voltage

changes in the presynaptic cell to inspire voltage changes in the postsynaptic cell. The rapid transfer of signals from one cell to the next is the main advantage of an electrical synapse [9].

For protein assembling and cell adhesion, the synapse organizer plays the main role to form an interacting network. Through alternative splicing, the interaction of the synapse organizers can be moved as weakened or reinforced with their collaborators [10]. To guide synaptic communications, these synaptic organizers can be used in the living system [10]. In the synaptic cleft, synapse organizers are tied up with pre-synaptic or post-synaptic through the transmembrane along with their extracellular domain elaboration [10]. NL and Nrx showed support for pre-synaptic and post-synaptic activity on the connection of cultured neurons with non-neuronal cells respectively [29,30]. They can form solid homophilic interactions with identical molecules and semi-homophilic with partner family members. They also form heterophilic interactions with different adhesion molecule backgrounds. A huge number of synapse organizers are present in the mammalian brain, which is involved in the development and control [10]. Inducible dimerization experiments proposed that dimerized NL induces presynaptic molecules as the main part of the correlated convention of a chemical synapse [24]. NL binds with two Nrx in an asymmetric tetramer [24-27]. In nature, NL is a dimer and Nrx remains in a monomeric form [25].

In the same synaptic manner, Synapse organizers can initiate trans-compound or cis compounds through binding with proteins [10]. Additional protein binding plays a great role in synapse organizers for making scaffolds and neuron-neuron recognition [10]. Synapse organizers are highly supported for important roles in the bonding of postsynaptic and presynaptic membranes in synapse conjunctions[10]. Nrxs, Receptor type protein tyrosine phosphatases (RPTPs), neuronal pentraxins, teneurins, netrin-G1, and netrin-G2 are the well-known presynaptic organizers [2]. NLs, dystroglycan, GABA-A receptors, Calsyntenins, Latrophilins, netrin G ligand 1, netrin G ligand 2, netrin G ligand 3, and GluA1-4 are the famous postsynaptic organizers [2]. In vitro, authentication suggests that Nrx differentiation in the early stage on an axon part into a presynaptic terminal and NL clustering may be an early step in the differentiation of a dendrite segment into a postsynaptic terminal [24,28]. Nrxs and RPTPs survive in multiple isoforms originated by alternative splicing and then interact in a splice-selective code including diverse postsynaptic partners [6]. Presynaptic Nrxs regulate synapse

properties through differential binding to numerous postsynaptic ligands, like NLs and latrophilins, thereby configuring the input/output connections of their resident neural circuits [2]. The affinity chromatography technique was used for the identification of Nrx1α 1992 [11] from rat brain extract through a column of α-latrotoxin. Pioneering studies by Sudhof and collaborators have characterized Nrxs and their tethering partners the NLs. NLs showed on the surface of non-neuronal cells influence synaptic vesicle clustering and the development of functional release sites in connecting glutamatergic axons [12].

NLs are Ca^{2+}-dependent for pairing to α- and β-Nrx, neurexophilin is Ca^{2+}-independent for pairing α-Nrx only, and dystroglycan is Ca^{2+}-dependent with α-Nrx especially. NLs were first identified through the affinity column of Nrx1β [15]. In humans, there are five NL genes: *NL1, NL2, NL3, NL4,* and *NL4Y* [16]. The principal extracellular domain of NLs is analogous to acetylcholinesterase (AChE) but misses cholinesterase activity and they mediate binding to Nrxs. Overexpression of AChE losses levels of β-Nrx in vivo and culture then impairs the genesis of glutamatergic synapses in culture, which indicates that there is crosstalk within the two proteins [17,18]. Thus, both Nrx and NL exist in numerous isoforms that navigate from alternative splicing and multiple genes. Nrx and NL both have relatively small intracellular domains that are close to PDZ-domain-binding sites, which are probably important for linking with other synaptic proteins [15].

Splicing initiates isoforms that bind both α- Nrx and β- Nrx by detaching an eight-amino-acid sequence from NLs, and this binding could participate in modulating synapse properties [21]. NL1 was recognized because of its potential to bind definite isoforms of all three β- Nrxs [22]. However, Nrxs are just one component of a varied trans-synaptic molecular machine. When Nrxs were recognized, their extensive alternative splicing suggested a role as surface-identifying molecules that specify synapses [2]. This hypothesis is expanding and validated. The characterization of Nrxs roles and interactions is only starting, but it is logical from the available knowledge that Nrxs are not molecularly or work as monogamous; they involve in numerous interactions and conduct at least some different roles in distinct synapses [2]. Nrxs and NLs furnish trans-synaptic affinity by the Ca^{2+}-dependent interaction of their substitute spliced

extracellular domains. NLs specify synapses that have an activity-dependent manner, apparently by binding to Nrxs [13].

Exploring the cell and neurons is important for better understanding the physiology of the body system. This investigation is elaborated to convince the recording of biological work in non-invasive techniques with various micro- or nano-electrodes. In these ways, electrophysiology has become one of the best biological methods in ongoing research. Voltage-gated calcium channels are highly selective for calcium and have a broad range of animating or deactivating features. Based on their threshold of voltage-dependent facilitation, they are classified as high and low-voltage-activated channels. Somatic calcium can be recorded to show the activity of action potential. Somatic calcium can trigger gene transcription. Calcium imaging is generally operated for the monitoring of interconnected neurons such as analyzing the circuitry in the cortex. This idea is also applied to recognize synaptically bridged neurons. In the case of a fluorescence probe technique, it is often used in experiments that require high selectivity because it has very high selectivity. The fluorescence probe technology has lower temporal resolution than the conventional electrophysiology technology and is easily contaminated.

Previously, it was the major problem that cell-type specific recording was not possible but with time optical-approach-type electrophysiological techniques now can do this job. This was done by the genetically encoded protein which only detects the specific cell which is matched with their cell type-specific promoter. But it is still a problem for the prob/sharp microelectrode-based electrophysiological techniques to determine cell-type-specific recording. So, using the properties of a post-synaptic organizer, I am trying to develop a methodology by which I can soon record cell-type-specific recordings. Post-synaptic organizer's properties are helpful to make those types of recordings, but for that, I must use a genetically engineered post-synaptic organizer. For the development of prob/sharp microelectrode-based electrophysiology, especially for specific cell recording, I need to develop engineered post-synaptic organizers. It will be helpful to attach specific proteins attached with prob/sharp microelectrode and then I can create a bridge for the specific cell recordings.

NL1 and Nrxs showed their influence in HEK293 cells and other nonneuronal cells for presynaptic differentiation and postsynaptic differentiation respectively [13]. This study led to the presumption that NLs and Nrx might relate to early synapse evolution in living organisms and may work as transneuronal signals for inducting synaptic molecules [13]. Nrxs can induce glutamate and GABA postsynaptic differentiation. On the other hand, NLs can induce GABA and glutamate axon's presynaptic differentiation [30]. In glutamatergic and GABAergic synapse formation, Nrx-NL linkage is a principal constituent media and variance in isoform restraining [30]. For the development of post-synaptic organizers, we used various molecular biological techniques (e.g., combinations of PCR, restriction enzyme digestion, DNA ligation, BAP treatment, agarose gel electrophoresis, transformation, plasmid purification, DNA sequencing, etc.) to generate a different type of post-synaptic organizer. We used NL1 and various proteins (e.g., Venus, GFP-nanobody, and dClover2) to produce engineered post-synaptic organizers.

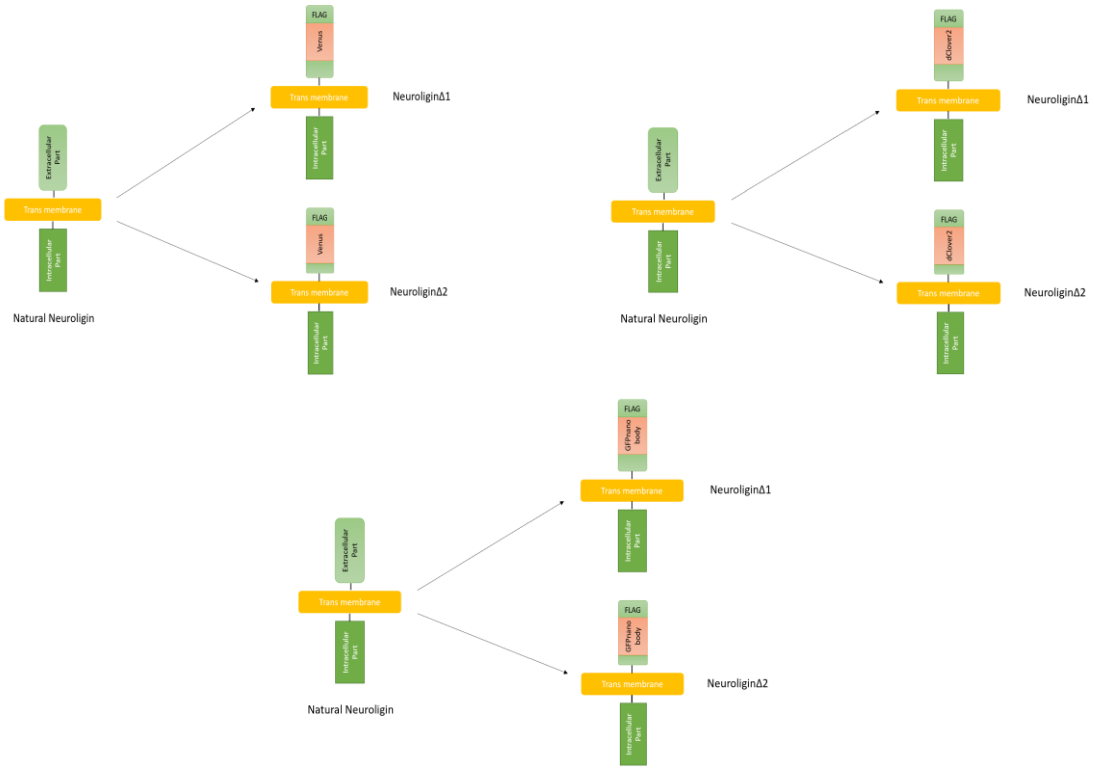

Figure 1: Construction map of engineered post-synaptic organizers.

4.2 Materials and Methods:

Some post-synaptic synapse organizers were prepared for my experiment. We have chosen the best quality, post-synaptic synapse organizers, based on their expression on HEK 293 cells and neurons. Most of those synapse organizers' making protocols were the same. So, we discussed materials and methods as a review for all synapse organizers in this part.

In NL1Δ1, the gray region was deleted:

(Signal peptide and FLAG from vector)

MSALLILALVGAAVADYKDDDDKLAAASQKLDDVDPLVTTNFGKIRGIKKELNNEILGPVIQFL
GVPYAAPPTGEHRFQPPEPPSPWSDIRNATQFAPVCPQNIIDGRLPEVMLPVWFTNNLDVVSSY
VQDQSEDCLYLNIYVPTEDDIRDSGGPKPVMVYIHGGSYMEGTGNLYDGSVLASYGNVIVITVN
YRLGVLGFLSTGDQAAKGNYGLLDLIQALRWTSENIGFFGGDPLRITVFGSGAGGSCVNLLTLS
HYSEGLFQRAIAQSGTALSSWAVSFQPAKYARILATKVGCNVSDTVELVECLQKKPYKELVDQD
VQPARYHIAFGPVIDGDVIPDDPQILMEQGEFLNYDIMLGVNQGEGLKFVENIVDSDDGVSASD
FDFAVSNFVDNLYGYPEGKDVLRETIKFMYTDWADRHNPETRRKTLLALFTDHQWVAPAVATAD
LHSNFGSPTYFYAFYHHCQTDQVPAWADAAHGDEVPYVLGIPMIGPTELFPCNFSKNDVMLSAV
VMTYWTNFAKTGDPNQPVPQDTKFIHTKPNRFEEVAWTRYSQKDQLYLHIGLKPRVKEHYRANK
VNLWLELVPHLHNLNDISQYTSTTTKVPSTDITLRPTRKNSTPVTSAFPTAKQDDPKQQPSPFS
VDQRDYSTELSVTIAVGASLLFLNILAFAALYYKKDKRRHDVHRRCSPQRTTTNDLTHAPEEEI
MSLQMKHTDLDHECESIHPHEVVLRTACPPDYTLAMRRSPDDIPLMTPNTITMIPNTIPGIQPL
HTFNTFTGGQNNTLPHPHPHPHSHSTTRV*

In NL1Δ2, the gray region was deleted:

MSALLILALVGAAVADYKDDDDKLAAASQKLDDVDPLVTTNFGKIRGIKKELNNEILGPVIQFL
GVPYAAPPTGEHRFQPPEPPSPWSDIRNATQFAPVCPQNIIDGRLPEVMLPVWFTNNLDVVSSY
VQDQSEDCLYLNIYVPTEDDIRDSGGPKPVMVYIHGGSYMEGTGNLYDGSVLASYGNVIVITVN
YRLGVLGFLSTGDQAAKGNYGLLDLIQALRWTSENIGFFGGDPLRITVFGSGAGGSCVNLLTLS
HYSEGLFQRAIAQSGTALSSWAVSFQPAKYARILATKVGCNVSDTVELVECLQKKPYKELVDQD
VQPARYHIAFGPVIDGDVIPDDPQILMEQGEFLNYDIMLGVNQGEGLKFVENIVDSDDGVSASD
FDFAVSNFVDNLYGYPEGKDVLRETIKFMYTDWADRHNPETRRKTLLALFTDHQWVAPAVATAD
LHSNFGSPTYFYAFYHHCQTDQVPAWADAAHGDEVPYVLGIPMIGPTELFPCNFSKNDVMLSAV
VMTYWTNFAKTGDPNQPVPQDTKFIHTKPNRFEEVAWTRYSQKDQLYLHIGLKPRVKEHYRANK
VNLWLELVPHLHNLNDISQYTSTTTKVPSTDITLRPTRKNSTPVTSAFPTAKQDDPKQQPSPFS
VDQRDYSTELSVTIAVGASLLFLNILAFAALYYKKDKRRHDVHRRCSPQRTTTNDLTHAPEEEI
MSLQMKHTDLDHECESIHPHEVVLRTACPPDYTLAMRRSPDDIPLMTPNTITMIPNTIPGIQPL
HTFNTFTGGQNNTLPHPHPHPHSHSTTRV*

4.2.1 Experimental design:

This study was designed according to the following schematic experimental diagram:

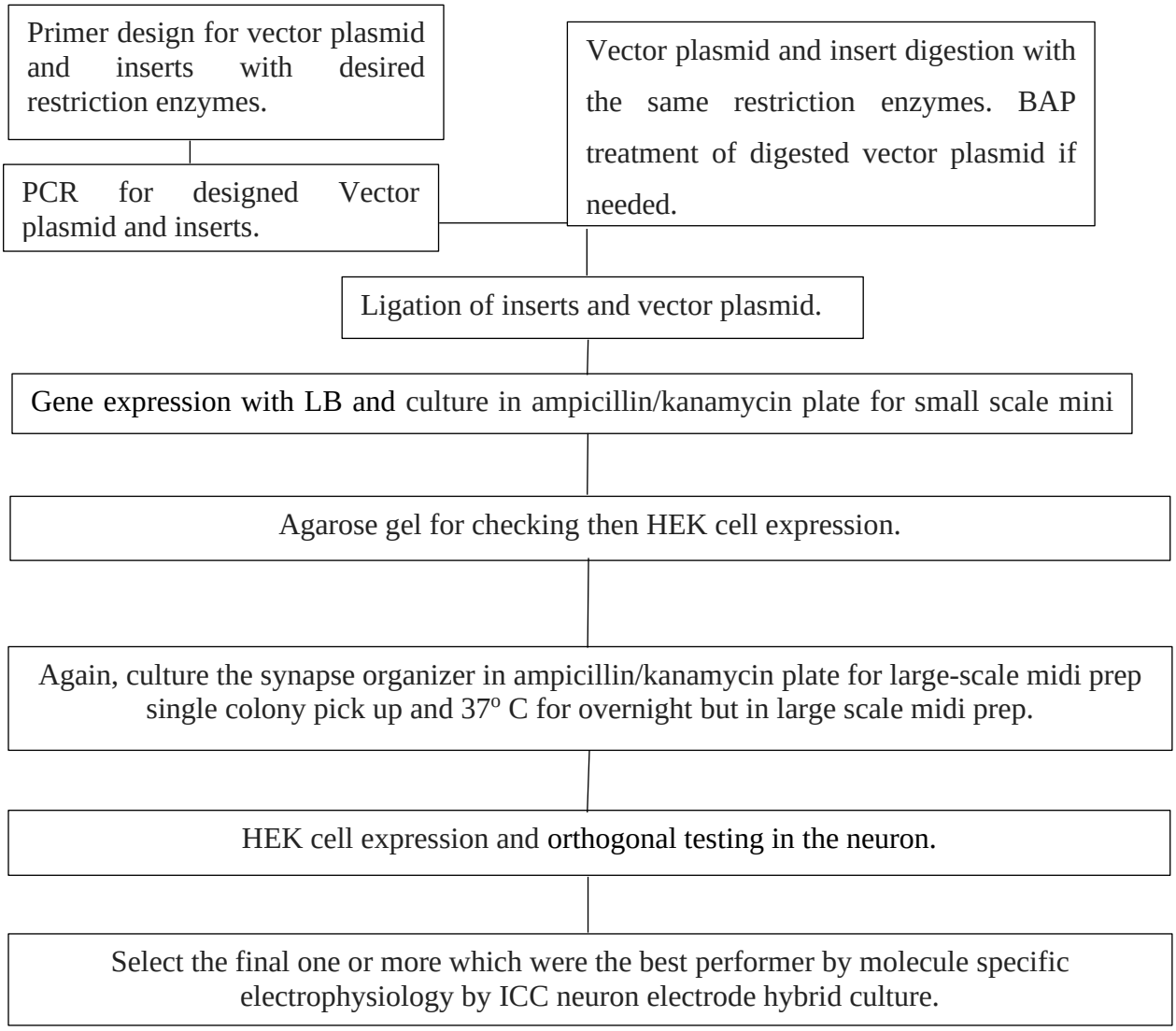

Figure 2: Flow chart for experimental design.

4.2.2 PCR for NL1:

We have made two types of NL1 PCR. There were two forward but one reverse primer. Apa1_NLGN1_V601_Fwd with the pFLAG-CMV-Signal_Not1_Apa1 Rev primer named NL1Δ1 and Apa1_NLGN1_V585_Fwd with the pFLAG-CMV-Signal_Not1_Apa1 Rev primer named NL1Δ2.

4.2.2.1 Primers:

Apa1_NLGN1_V585_Fwd: 5'-AAA GGG CCC GTT AAA GAG CAT TAC AGA GCC AAT AAG GTA-3'

Apa1_NLGN1_V601_Fwd: 5'-AAA GGG CCC GTA CCT CAT CTG CAT AAT CTC AAT GAC ATT-3'

pFLAG-CMV-Signal_Not1_Apa1 Rev: 5' TTT GGG CCC CGC GGC CGC AAG CTT GTC GTC ATC GTC TTT-3'

We added TE buffer with those primers to make 100 µM.

4.2.2.2 PCR solution preparation:

Water	10 µl
Template	01 µl (100-time dilution if mini prep and 1000 times if midi prep)
Prime Star Max	10 µl
Forward Primer	0.2 µl from 100 µM
Reverse Primer	0.2 µl from 100 µM

4.2.2.3 Temperature:

95^{o} C	2 minutes	
98^{o} C	10 seconds	
62^{o} C	15 seconds	30 cycles
72^{o} C	50 seconds	(because above 6 Kbp)
72^{o} C	5 minutes	
4 ^{o} C	99.99 minutes	

4.2.3 DPN treatment:

Then added 10 X T buffer 1 µl, Dpn1 0.5 µl, and water 10 µl with the PCR product and incubated at 37^{o} C for 20 minutes.

Then agarose gel was run and found about 6 Kbp after dissolving in ethidium bromide for 20 minutes. We used the λ Hind III marker. Then gel clean up.

4.2.4 Agarose gel electrophoresis:

For gel preparation, we added 0.5 gm of agarose into a 50 ml 1 x TAE buffer. Sometimes one agarose table is used instead of 0.5 gm agarose into 50 ml 1 x TAE buffer. Then heat in an oven for about 1 and a half minutes. When the solution got a clear appearance, it was ready for use. Then prepared the cassettes with the desired comb and poured them into the cassette. Then keep that cassette for hardening. After half an hour gel generally gets a hard structure. Then we removed the comb carefully and placed the gel with the upper cassette into an agarose gel electrophoresis box chamber. Then we set the wire with the Volta miter.

Before running the agarose gel electrophoresis, we loaded the marker and sample into the gel's wells. At first, we put the 3 µl λ Hind III marker and then added the samples with loading buffer into the gel's wells. After connecting the Volta miter with electricity, 100 V was applied. Agarose gel electrophoresis needs about 30 minutes for the proper running of the sample and marker. So, after 30 minutes just stopped the electrophoresis and took the gel into the Ethidium bromide solution. We made the Ethidium bromide solution previously. For the making of Ethidium bromide solution, we used 200 ml of 1 x TAE buffer and 5 µl of Ethidium bromide solution and mixed. After dissolving into the Ethidium bromide solution, we kept the gel on the UV display board. Then we checked the band pattern of those desired inserts.

4.2.5 PCR gels clean up:

The following protocol was followed for PCR clean-up as well as DNA concentration and removal of salts, enzymes, etc. from enzymatic reaction (SDS < 0.1%). There are several steps we maintained.

Adjust DNA binding condition:

For very small sample volumes < 30 µl adjust the volume of the reaction mixture to 50-100 µl with water. It is not necessary to remove mineral oil. Mix 1 volume of sample with 2 volumes of Buffer NT1 (e.g., mix 100 µl PCR reaction and 200 µl Buffer NT1). For melting gel, I kept the tube at 42^{o} C for 5 minutes.

Bind DNA:

Place a NucleoSpin® Gel and PCR Clean-up Column into a Collection Tube (2 ml) and load up to 700 µl sample. Centrifuge for 30 seconds at 11000 x g. Discard the flow-through and place the column back into the collection tube. Load the remaining sample if necessary and repeat the centrifugation step.

Wash silica membrane:

Add 700 µl Buffer NT3 to the NucleoSpin® Gel and PCR Clean-up Column. Centrifuge for 30 seconds at 11000 x g. Discard the flow-through and place the column back into the collection tube. Repeat the previous step to minimize chaotropic salt.

Dry silica membrane:

Centrifuge for 1 minute 11000 x g to remove Buffer NT3 completely. Make sure the spin column does not encounter the flow-through while removing it from the centrifuge and the collection tube.

Elute DNA:

Place the NucleoSpin® Gel and PCR Clean-up Column into a new 1.5 ml microcentrifuge tube. Add 15-30 µl Buffer NE and incubate at room temperature (18-25 ^{o}C) for 1 minute. Centrifuge for 1 minute at 11000 x g.

***4.2.6 Digestion of NL1*:** (NL1Δ1/NL1Δ2 in Separate tube)

NL1Δ1/NL1Δ2:	30 µl
10 X L buffer:	4 µl
Apa1:	0.5 µl

Then incubated at 25^{o} C for 3 hours.

Then agarose gel runs and is found to be about 6 Kbp after dissolving in ethidium bromide for 20 minutes. We used the λ Hind III marker. Then gel clean up.

4.2.7 Ligation and transformation:

NL1Δ1/NL1Δ2 in separate tube: 1 µl in both 1.5 ml tube

Ligation high enzyme: 1 µl in both 1.5 ml tube

Incubated at 16^{o} C for 2 hours.

In control, we used only NL1Δ1/NL1Δ2, with no ligation enzyme.

For transformation, 25 µl $DH_{5\alpha}$ *E. coli* cells took in each tube.

We kept those tubes for 10 minutes on ice, 45 seconds in a 42^{o} C water bath, and again for 10 minutes on ice. Then spread those on different ampicillin plates. We kept those overnight in an incubator at 37^{o} C.

4.2.8 Miniprep procedure:

We did colony pick up with a sterile toothpick stick and placed it in a glass tube containing LB with a respective antibiotic which we used in the plate culture. Then we kept the tube overnight at 37^{o} C. The next morning, we decant the fluids into a 2 ml bacterial tube and then centrifuge for 3 minutes at 5000 rpm. Discarded the LB medium because cells were clotted. Then added Cell Resuspension (CRA) about 200 µl then vortex and added Cell Lysis (CLA) about 200 µl but this time no vortex was done. We were just done up and down then waited for 3 minutes and kept it open.

After 3 minutes we added Alkaline Protease 5 µl, then up and down and kept it open for 3 minutes. Then added Neutralization Solution (NSB) of about 300 µl then mixed properly up and down. After that centrifugation was done at 14000 rpm for 10 minutes. Then we prepared the column and poured the solution into the column and centrifugated it at 5000 rpm for 1 minute. The lower solution was discarded and the 600 µl column wash into the column and centrifuged at 5000 rpm for 1 minute. Then again discarded the lower solution and added more than 200 µl of column wash into the column and centrifuged at 5000 rpm for 1 minute. Then again discarded the lower solution.

For drying, centrifuged the column again at 14000 rpm for one minute. Then changed the lower column and replaced it with a 1.5 ml tube. Then put 100 µl of water for elution into the middle of the dried column and wait for 1 minute. For elution, centrifuged at 10000 rpm for one minute.

After centrifugation discarded the column and preserved in a 1.5 ml tube containing plasmids. We preserved the plasmids in a -5^{o} C refrigerator for future agarose gel checking and various work like midi prep, HEK 293 cell checking, etc. We kept those plasmids showing positive results in agarose gel electrophoresis only. Then we went to proceed with other checking and midi prep.

4.2.9 Checking the NL1Δ1/NL1Δ2:

We had two PCR products named NL1Δ1 and NL1Δ2. But detected those PCR products through Xho1 and Apa1 digestion and then showed the band pattern in the agarose gel. For that, we have checked the restriction enzyme of the NL1 sequence and the pFLAG-CMV-1 map sequence. In our PCR samples, there were Apa1 and Xho1 because we added Apa1. In the NL1 sequence, there were no Apa1 and Xho1. In the pFLAG-CMV-1 map, there was Xho1 but no Apa1.

So, we digested NL1Δ1 and NL1Δ2 with Xho1 and Apa1 along with our template NL1. We found NL1 as a linear band one band because there was Xho1 in the 2000 bps position but no Apa1. But NL1Δ1 and NL1Δ2 showed two bands because within the NL1 primer, we added Apa1 and in the promoter there was Xho1. So, we found one extra 2000-band pattern with a 4000-band pattern which indicated the value of my NL1Δ1 and NL1Δ2.

4.2.10 Digestion of NL1Δ1 and NL1Δ2 for checking:

Water	25 µl
10 X L buffer	03 µl
Apa1	0.5 µl

Kept at 25^{o} C for 2 hours.

Then added the followings-

Xho1	0.5 µl
10 X H buffer	04 µl

Kept at 37^{o} C for 2 hours.

Then agarose gel was run and found about 4Kbps and 2Kbps after dissolving in ethidium bromide for 20 minutes. We used the λ Hind III marker.

4.2.11 Dclover2:

Incent Dclover2 is fluorescence and dimer from the protein. For primer, we added the Apa1 restriction enzyme on both sides. In reverse primer, we discard the stop codon and added Apa1 then did the reverse sequence for reverse primer.

4.2.11.1 Primers:

Apa1_XFP_Fwd: 5'-AAA AGG GCC CAT GGT GAG CAA GGG CGA GGA-3'

XFP!*_Apa1_Rev: 5'- TTT TGG GCC CCT TGT ACA GCT CGT CCA TGC -3'

We added TE buffer with those primers to make 100 µM.

4.2.11.2 PCR for Dclover2:

Water	10 µl
Prime Star Max	10 µl
Template	01 µl (100-time dilution if mini prep and 1000 times if midi prep)
Primer mix	01 µl (reverse primer 1 µl and forward primer 1 µl then added 3 µl water. Then from that 5 µl primer mix I took 1 µl for the PCR)

3.2.11.2 Temperature:

95° C	2 minutes	
98° C	10 seconds	
62° C	15 seconds	30 cycles
72° C	10 seconds	
72° C	5 minutes	
4 ° C	99.99 minutes. Then agarose gel and PCR clean up.	

4.2.12 NL1Δ1 and NL1Δ2 digestion with Apa1:

Water	: 20 µl
Cut smart buffer	: 03 µl
Apa1	: 0.5 µl
NL1Δ1 or NL1Δ2	: 03 µl
Total	: 26.5 µl then incubate at 25^{o} C for 2.5 hours.

4.2.13 BAP treatment:

We just took those products which were digested with Apa1 only.

Water : 27 µl with the digested product.

BAP buffer: 0.3 µl

BAP enzyme: 0.5 µl

Then incubated at 55^{o} C for 30 minutes.

4.2.14 dClover2 digestion with Apa1:

dClover2 after PCR	: 30 µl eluted after cleaning up.
Apa1	: 0.5 µl
Cut smart buffer	: 04 µl

Then incubated at 25^{o} C for 3 hours. After that, PCR clean-up only with no gel checking.

4.2.15 Ligation:

NL1Δ1 / NL1Δ2	: 0.5 µl
dClover2	: 01 µl
Ligation mix	: 1.5 µl
Total	: 2.5 µl

For control,

NL1Δ1 / NL1Δ2	: 0.5 µl
Ligation mix	: 01 µl
Water	: 01 µl
Total	: 2.5 µl

Then incubate for 1.5 hours at 16^{o} C.

4.2.16 Transformation:

$DH_{5\alpha}$ 25 µl into the transformation tubes. Then 10 minutes in ice kept in a 42^{o} C water bath for 45 seconds then again 10 minutes in ice. Then spread on an ampicillin plate.

Colony picks up and cultures with LB ampicillin 2 ml at 37^{o} C for 1 day with 180 rpm.

4.2.17 Miniprep:

The next morning, we decant the fluids into a 2 ml bacterial tube and then centrifuge for 3 minutes at 5000 rpm. Discarded the LB medium because cells were clotted. Then added Cell Resuspension (CRA) about 200 µl then vortex and added Cell Lysis (CLA) about 200 µl but this time no vortex was done. We were just done up and down then waited for 3 minutes and kept it open.

After 3 minutes added Alkaline Protease 5 µl, then up and down and kept open for 3 minutes. Then added Neutralization Solution (NSB) of about 300 µl then mixed properly up and down. After that centrifugation was done at 14000 rpm for 10 minutes. Then we prepared the column and poured the solution into the column and centrifugated it at 5000 rpm for 1 minute. The lower solution was discarded and the 600 µl column was into the column and centrifuged at 5000 rpm for 1 minute. Then again discarded the lower solution and added more than 200 µl column wash was into the column and centrifuged at 5000 rpm for 1 minute. Then again discarded the lower solution.

For drying, centrifuged the column again at 14000 rpm for one minute. Then changed the lower column and replaced it with a 1.5 ml tube. Then put 100 µl of water for elution into the middle of the dried column and wait for 1 minute. For elution, centrifuged at 10000 rpm for one minute.

After centrifugation discarded the column and preserved in a 1.5 ml tube containing plasmids. We preserved the plasmids in a -5^{o} C refrigerator for future agarose gel checking and various work like midi prep, HEK 293 cell checking, etc. We kept those plasmids showing positive results in agarose gel electrophoresis only.

4.2.18 Checking the construct:

Water	: 11 µl
Buffer Cut smart	: 03 µl
Apa1	: 0.5 µl
NL1Δ1 / NL1Δ2	: 06 µl
In total	: 19.5 µl

Then incubate it for 3 hours at 25^{o} C.

We found the desired two-band pattern in the agarose gel.

4.2.19 HEK cell culture:

HEK 293 cells were carefully cultured in Dulbecco's Modified Eagle's Medium (DMEM, Nacalai Tesque, Inc., Japan) and added with 10% fetal bovine serum (Gibco, Life Technology, Canada). Cell plate density was at 2 x 10^{6} per well in sterile 35 x10 mm cell culture dishes (Falcon, USA) and cultured in an additional incubator at 37^{o}C, 5% CO_2, and 95% humidity for 48-96 hours, then constructs were placed in those media.

4.2.20 HEK cell passage for confluent culture in 60 mm dish:

At first, we removed the medium. Then add 0.5 ml PBS (-) twice and wash gently by tilting down and up; do not discard the tip (take care not to touch the dish with the tip during pipetting; in that case discard the tip after use). Removed PBS then 0.5 ml Trypsin/EDTA and kept for 3 minutes in the CO_2 incubator at 37^{o} C. We prepared a new dish with 5 ml DMEM (using the NEW tip and did not discard). 1 ml was taken from the new dish and gently suspended in the cell with a pipette using the same tip. Transfer the appropriate amount of the suspension (0.2-0.5) to the new dish using the same tip. Then placed the new dish into the CO_2 incubator at 37^{o} C.

4.2.21 Transfection of engineered synapse organizer NL1Δ1 / NL1Δ2 in HEK 293 cells:

At first, the sample was taken in laminar airflow and started the air flowing, and waited for 1 minute. For transfection, previously passaged HEK 293 cells were in some 60 mm sterile cell culture dishes. Then took DMEM (-) from the 5^{o} C refrigerator and PEI from the -20^{o} C refrigerator. Then checked the HEK 293 cells were conditioned in the dishes through a microscope. The PEI was at 47^{o} C for 5 minutes. After that, 100 µl DMEM (-) containing 10% FBS was taken, and 1/100 Ampicillin. Then added 10 µl miniprep engineered synapse organizer and 4 µl PEI then pipetted several times and waited for 10 minutes. In the case of a midi prep-engineered synapse organizer, we used only 0.5 µl only. After that, we fixed the pipette to 140 µl for better pipetting. After pipetting several times, we added the mixture to the HEK 293 cell dish drop by drop and spread it to the whole dish. After a little jarring the dish was kept in the CO_2 incubator at 37^{o} C overnight. For the NL1 check, we did not add any protein, we just washed it with PBS (-) and added 200 µl PBS (-) then showed it under a fluorescence microscope. We found NL1Δ1 / NL1Δ2 may be ok because there is a very low expression. So, we did PCR for checking the right direction.

4.2.22 Checking dClover2 direction:

Primer sequence: pFLAG-CMV-1-5'S_F
5'- GGG AGT TTG TTT TGG CAC C-3'

Primer sequence: pFLAG-CMV-Signal_Not1_F
5'-AAA GAC GAT GAC GAC AAG CTT GCG GCC GC -3'

We used the reverse primer from the previous dClover2 reverse primer. We ordered two primers, but we chose the forward primer which was similar temperature to the dClover2 reverse primer both were about 67 ^{o} C. We added TE buffer with those primers to make 100 µM.

4.2.22.1 Gotaq PCR enzyme: It contains a loading buffer, so we did not add a loading buffer during loading into the agarose gel.

4.2.22.2 PCR solutions:

Water : 10 µl

GOtaq : 10 µl

Primer Fwd : 0.05 µl

Primer Rev : 0.05 µl

Template : 0.2 µl

Then equally distributed in four tubes for four constructs.

For making 0.05 µl primer Fwd and Rev, we took two tubes and added 10 µl TE to each tube. Then added 0.5 µl Fwd and Rev, separately to that tube respectfully. Finally, from that tube, we took 0.5 µl to the main tube.

4.2.22.3 PCR temperature:

95° C	2 minutes	
95° C	10 seconds	
55° C	1minute	30 cycles
72° C	1 minute	
72° C	7 minutes	
4 ° C	99.99 minutes	

Then the agarose gel ran and found NL1Δ1 / NL1Δ2 constructs were ok. We have NL1Δ1 and NL1Δ2 miniprep again because it was the low amount and finally for NL1Δ1 / NL1Δ2 preserver 3 tubes for each construct.

4.2.23 For GFP-nanobody and Venus:

4.2.23.1 GFP-nanobody and Venus primers:

Not(AAA)_GFP-nanobody_Fwd: 5'-AAA AAG CGG CCG CGG CGC AGG TTC AGC-3'

GFP-nanobody_Apa1_Rev: 5'-TTT TGG GCC CTT TGC TGC TAA CGG TAA-3'

We previously had venus primers, so we did not order those again. PCR protocol and temperature were the same as Dclover2 because GFP-nanobody and Venus proteins are almost the same lengths as fluorescence proteins.

4.2.23.2 Digestion of GFP-nanobody and Venus:

GFP-nanobody / Venus : 25 µl eluted after cleaning up.

Cut smart buffer : 04 µl

Apa1 : 0.5 µl

Not1 : 0.5 µl

Total : 30 µl

Then incubated at 25^{o} C for 3 hours. After that, PCR clean-up only with no gel checking.

4.2.23.3 NL1Δ1 / NL1Δ2 digestion with Not1 and Apa1:

NL1Δ1 / NL1Δ2 (miniprep) : 10 µl

Cut smart buffer : 03 µl

Apa1 : 0.5 µl

Not1 : 0.5 µl

Total : 14 µl then incubate at 25^{o} C for 2.5 hours.

4.2.23.4 BAP treatment:

We just took those products which are digested with Apa1 and Not1.

Water : 27 µl with the digested product.

BAP buffer: 0.3 µl

BAP enzyme: 0.5 µl

Then incubated at 55^{o} C for 30 minutes.

4.2.23.5 Ligation:

NL1Δ1 / NL1Δ2 : 0.5 µl

GFP-nanobody / Venus : 01 µl

Ligation mix : 1.5 µl

Total	: 2.5 µl

For control,

NL1Δ1 / NL1Δ2	: 0.5 µl
Ligation mix	: 01 µl
Water	: 01 µl
Total	: 2.5 µl

Then incubate for 1.5 hours in 16^{o} C.

Transformation and miniprep were the same procedure as NL1Δ1/NL1Δ2-Dclover2.

4.2.23.6 Checking the construct:

Water	: 11 µl
Buffer Cutsmart	: 03 µl
Apa1	: 0.5 µl
Not1	: 0.5 µl
NL1Δ1 / NL1Δ2	: 05 µl
In total	: 20 µl

Then incubate it for 3 hours at 25^{o} C.

We found the desired two-band pattern in the agarose gel.

4.2.24 **Transfection of engineered synapse organizer NL1Δ1 / NL1Δ2 containing GFP-nanobody / Venus in HEK 293 cells:**

At first, the sample was taken in laminar airflow and started the air flowing, and waited for 1 minute. For transfection, previously passaged HEK 293 cells were in some 60 mm sterile cell culture dishes. Then took DMEM (-) from the 5^{o} C refrigerator and PEI from the -20^{o} C refrigerator. Then checked the HEK 293 cells were conditioned in the dishes through a microscope. The PEI was at 47^{o} C for 5 minutes. After that, 100 µl DMEM (-) containing 10%

FBS was taken, and 1/100 Ampicillin. Then added 10 µl miniprep engineered synapse organizer and 4 µl PEI then pipetted several times and waited for 10 minutes. In the case of a midi prep-engineered synapse organizer, we used only 0.5 µl only. After that, we fixed the pipette to 140 µl for better pipetting. After pipetting several times, we added the mixture to the HEK 293 cell dish drop by drop and spread it to the whole dish. After a little jarring the dish was kept in the CO_2 incubator at 37^{o} C overnight. For the NL1Δ1 / NL1Δ2 containing venus check, we did not add any protein, we just washed it with PBS (-) and added 200 µl PBS (-) then showed it under a fluorescence microscope. We found NL1Δ1 / NL1Δ2 containing venus were expressed. For the NL1Δ1 / NL1Δ2 containing GFP-nanobody check, we added GFP protein, because GFP-nanobody has no fluorescence nature. We just washed with PBS (-) and added 200 µl PBS (-) then added 2-3 µl GFP protein and jerked the dish then kept it for 30 minutes. After washing with PBS (-), added 200 µl PBS (-) into the dish and showed it under a fluorescence microscope. We found NL1Δ1 / NL1Δ2 containing GFP-nanobody were expressed due to binding with GFP protein extracellularly.

4.2.25 Midi prep (Neuron type; Endotoxin Free):

For all the engineered post-synaptic organizers, we did midi prep (neuron type) after the successful checking in HEK cells. After picking up the colony from the plate culture, pre-cultured in 2 ml LB with ampicillin for several hours. Then cultured in 100 ml LB with ampicillin or Kanamycin overnight. Harvested the culture in a 50 ml tube by using it twice, for harvesting centrifuged at 7000 rpm for five minutes. After every centrifugation, we discarded the fluids. Sometimes we stored the pellet in the -80^{o} fridge when there were difficulties in time.

At first, we added 8 ml buffer RES into the 50 ml tube for resuspending the pellet. we used the vortex machine, then added 8 ml buffer LYS EF and mixed that well, thus turning blue color. After waiting 2-3 minutes, we added 8 ml buffer NEU EF which neutralized the blue color and made the solution whitish. Then we centrifuged at 10000 rpm for 5 minutes. We set the column with a filter to the black stand using a ring holder. We poured 15 ml buffer EQU EF into the edge of the filter. Then we poured the supernatant into the filter column. After the passage of the solution from the filter, we added 5 ml FIL EF to the edge of the filter. After passing through the buffer FIL EF, we discarded the column filter. Then we added 35 ml ENDO EF into the column.

After that, we added a 15 ml buffer WASH EF and set a new 50 ml collection tube under the column. Then we poured 5 ml elution buffer into the column. After elution, we removed the column and added 5 ml of Isopropanol (IPA) into the collection tube. After a gentle and brief vortex, we centrifuged the collection tube at 15000 rpm for 30 minutes. Then we found a clear crystal pellet and carefully discarded the solution. Then we washed the crystal pellet with a cool 70% EtOH. After gently and briefly vortex, we centrifuged the collection tube at 15000 rpm for 10 minutes. After that, I carefully discarded the fluid and dried it for a few minutes. Finally, we melted the crystal pellet with 100 µl TE EF and measured the concentration.

4.2.26 Orthogonal testing:

Orthogonal testing is a technique that is a systematic, statistical way of testing. It is used when the total number of inputs is comparatively small but too large to permit comprehensive testing of every possible input. It is particularly effective in finding errors associated with faulty reasons. The orthogonal test can be appealed in user interface testing, regression testing, positioning testing, and production testing. Each treatment gives a unique piece of information because the permutations of factor levels incorporating a single treatment are so selected that their feedbacks are uncorrelated. The net effect of organizing the experiment in such treatments is that the same piece of information is gathered in the minimum number of experiments. We tested our engineered post-synaptic organizers through this technique. We tested the specifically post-synaptic synapse organizer with various specific proteins. In many cases, one engineered organizer can bind two types of proteins but there was a difference in binding nature and efficiency. Through this orthogonal testing, we compared and found the specific one.

4.2.27 Immunocytochemistry:

For immunocytochemistry, we needed 4% PFA and 4% sucrose but making them was very hard so, we took 1.2 ml of 20% PFA and 4.8 ml of sucrose in PBS. We took a little bit of NaOH to clear the solution but after checking PH by PH paper. PH 7-8 was right for working. In some cases, when we found high PH then we added 6 HCL to reduce the PH. Then we put off the HEK 293 cells which were previously transfected then sucked the DMEM fluids. After that, we added the PFA and sucrose mixture of about 500 µl to each well and waited for 20 minutes for fixation.

After 20 minutes we washed three times every well with PBS 500 µl and sucked every time. Last time we kept some PBS because of not too dry. Then we took 120 µl Donkey serum in 1200 µl PBS. For antibody preparation we have taken 1.2 ml PBS, 24 µl Donkey serum, and 1.2 µl rabbit antihuman Fc, which were made of 2% Donkey serum and 1/1000 rabbit antihuman Fc. After the application of the antibody, we waited about 2 hours, then washed again with 1 ml PBS three times. For anti-antibody, we took 1.2 ml PBS, 2 µl Donkey serum, and 2 µl antirabbit Fc, then again sucked the PBS and applied 100 µl mixture in each well and kept those in a dark place for one hour. At last, I checked under the fluorescence microscope.

4.3 Results:

At first, After PCR of NL1Δ1 and NL1Δ2, I checked in agarose gel and found desired band pattern. Then, I did mini prep of several colonies after ligation. Then, all of them were checked for proper lengths. For checking again, I used Apa1 and Xho1 restriction enzymes for digestion because if it cut the desired location then I can show the two desired bands. The constructs were shown-band band patterns like 4000 and 2000 base pairs were considered as correct plasmids because Apa1 and Xho1 cut the desired points of the plasmids, and the position difference of those restriction enzymes is about 2000 base pairs. For the insert, I used dClover2 fluorescence protein. I used dClover2 because it is a dimer-forming fluorescence protein and NLs were working as dimer form. For the generation of the dClover2 protein, I did PCR and checked the band pattern in agarose gel. When I used primer making, I added the Apa1 enzyme on both sides of the primer. So dClover2 protein with Apa1 restriction enzyme was made by successful PCR. Then, I ligated the NL1Δ1/ NL1Δ2 and dClover2 after digestion with the Apa1 restriction enzyme. The next morning, I found lots of colonies from them separated colonies were taken for miniprep and I made some engineered NL for our next experiment. The NL constructs are as follows-

pFLAG-CMV1-NL1Δ1-dClover2, pFLAG-CMV1-NL1Δ2-dClover2

pFLAG-CMV1-NL1Δ1-GFP-nanobody, pFLAG-CMV1-NL1Δ2-GFP-nanobody

pFLAG-CMV1-NL1Δ1-Venus, pFLAG-CMV1-NL1Δ2-Venus

After successfully making an engineered NL containing dClover2, I checked again to see the proper band pattern for plasmid and inserts. In the engineered NL, I used the Apa1 restriction enzyme on both sides of the plasmid and inserted it. So, for checking, I used Apa1 digestion and checked in the agarose gel. For plasmid, it's about 6000 kbs and for dclover2 it's about 700 kbs. After that, I did a sequence to check the exact mapping of our engineered NL. The sequence map also showed the correct sequence. Then, finally, I checked the direction of the dClover2 and for that, I did PCR again with the newly designed primer based on our DNA map, where the forward primer was started before the Apa1 restriction enzyme with desired methionine position. The reverse primer was the same as the dClover2 reverse primer and the template was engineered NL which I made. Then, finally, engineered NLs were ready for the next experiment.

After making dClover2 protein-containing engineered NLs, I focused my mind on making another type of engineered NL. This time I used venus and GFPnanobody proteins to insert the NL plasmid. Venus and GFPnanobody proteins are monomer-forming proteins and venus has a fluorescent nature. Firstly, I digested the dClover2 protein-containing engineered NLs with Apa1 restriction enzyme and did PCR for the venus and GFPnanobody protein after primer design with Apa1 restriction enzyme. Then, I ligated the NL1Δ1/ NL1Δ2 and venus/GFP-nanobody after digestion with the Apa1 restriction enzyme. The next morning, I found lots of colonies from them separated colonies were taken for miniprep and we made some engineered NLs for our experiment. In the engineered NL1Δ1/ NL1Δ2, I used the Apa1 restriction enzyme on both sides of the plasmid and insert. So, for checking, I used Apa1 digestion and checked in the agarose gel. For plasmid, it's about 6000 kbs and for venus and GFP-nanobody it's about 700 and 500 kbs respectively. After that, I did a sequence for checking the exact mapping of our engineered NL. The sequence map also showed the correct sequence.

In Figure 3, the left side is the pFLAG-CMV1-NL1Δ1-dClover2, and the right side is the pFLAG-CMV1-NL1Δ2-dClover2. In the green view, we can see the extracellular part of the construct which is expressed in HEK 293 cells. The extracellular dClover2 part of the construct was detectable in the green view. The pFLAG-CMV1-NL1Δ1-dClover2 was a more prominent expression than the pFLAG-CMV1-NL1Δ2-dClover2. dClover2 is a fluorescence protein so, we

do not need to add extra fluorescence protein after washing with buffer. We cleaned the HEK cells with buffer and saw them under the microscope using a green fluorescence filter. Then we detected the borderline of the HEK cells through extracellular expression.

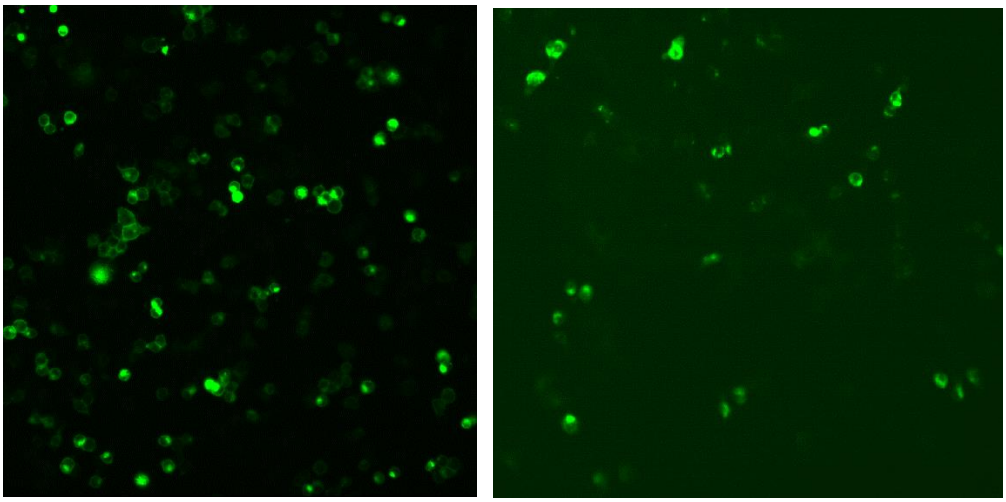

Figure 3: pFLAG-CMV1-NL1Δ1-dClover2 (left), pFLAG-CMV1-NL1Δ2-dClover2 (right)

In Figure 4, the left side is pFLAG-CMV1-NL1Δ1-GFP-nanobody, and the right side is pFLAG-CMV1-NL1Δ2-GFP-nanobody was expressed after adding Venus protein. Venus protein can conjugate with the GFP-nanobody so that it is expressed extracellularly in HEK 293 cells. In the yellow view, we can detect the outer part which was bound with Venus protein. Venus protein has a fluorescent nature, so it is expressed. From the extracellular expression of the Venus protein, we can easily say that GFP-nanobody was bound with the Venus protein. We cleaned the HEK cells with buffer and saw them under the microscope using Venus/Yellow filter. Then we detected the borderline of the HEK cells through extracellular expression.

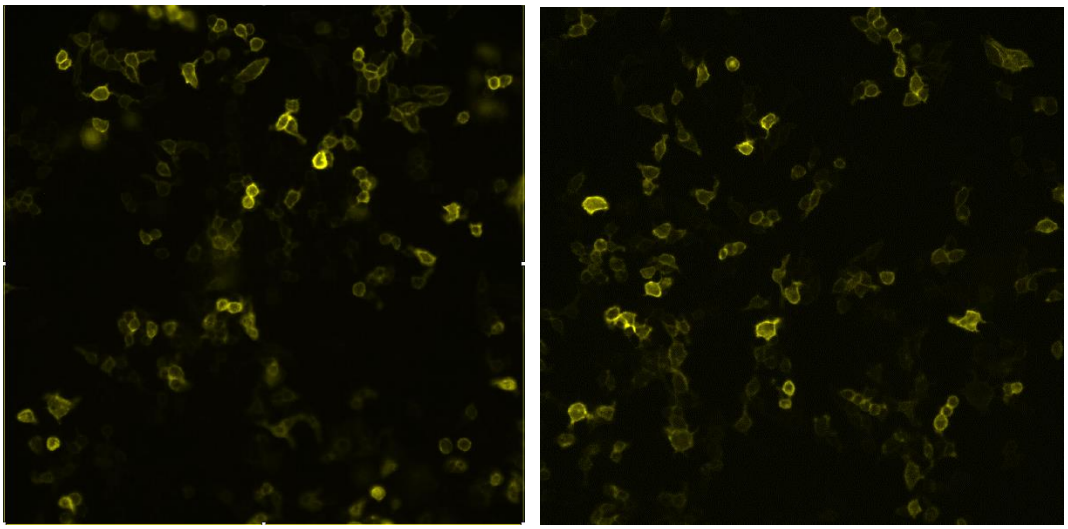

Figure 4: pFLAG-CMV1-NL1Δ1-GFP-nanobody (left) and pFLAG-CMV1-NL1Δ2-GFP-nanobody (right)

In Figure 5, the left side is pFLAG-CMV1-NL1Δ1-Venus, and the right side is the pFLAG-CMV1-NL1Δ2-Venus construct expressed. Venus protein can be expressed alone so it is expressed extracellularly in HEK 293 cells. In the yellow view, we can detect the outer part which was the Venus protein. Venus protein has a fluorescent nature, so it is expressed. From the extracellular expression of the Venus protein, we can easily say that pFLAG-CMV1-NL1Δ1-Venus/pFLAG-CMV1-NL1Δ2-Venus constructs existence, and they can work. We cleaned the HEK cells with buffer and saw them under the microscope using Venus/Yellow filter. Then we detected the borderline of the HEK cells through extracellular expression.

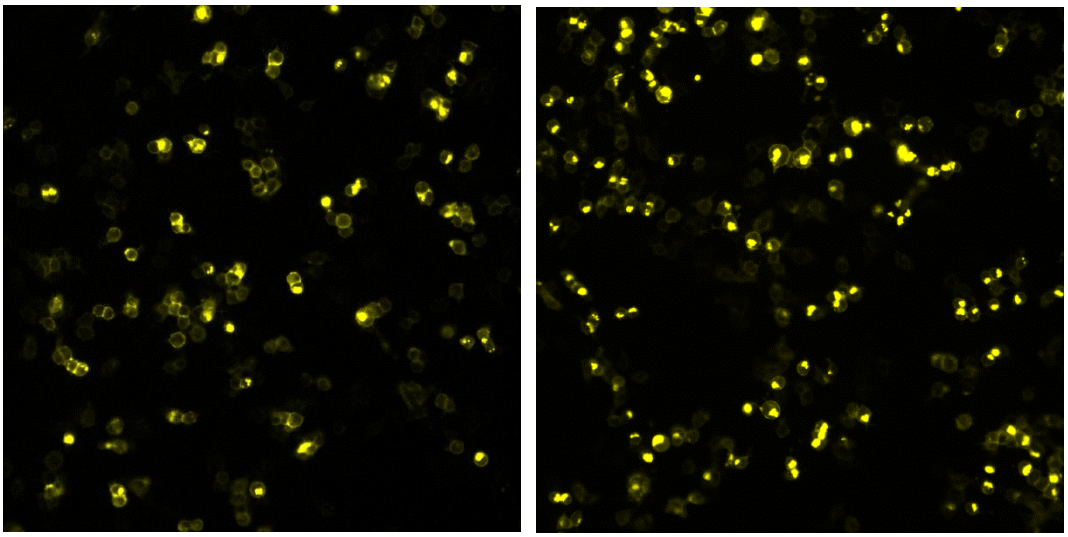

Figure 5: pFLAG-CMV1-NL1Δ1-Venus (left) pFLAG-CMV1-NL1Δ2-Venus (right).

In my experiment, I got success to generate those constructs and they all were bound to their specific protein extracellularly in HEK cells. I tested some of my engineered synapse organizers through the orthogonal technique. I tested the specifically engineered synapse organizer with various specific proteins. In many cases, one engineered organizer can bind two types of proteins but there was a difference in binding nature and efficiency. Through this orthogonal testing, I compared and found the specific one. dClover2-containing constructs can be bound with GFP-nanobody protein. GFP-nanobody containing constructs bound with Venus protein. Venus-containing constructs can be bound with GFPnanobody protein. In conclusion, it can be said that my engineered postsynaptic organizers responded positively.

4.4 Discussion:

NLs can induce presynaptic differentiation in either GABA or glutamate axons. On the other hand, Nrx is sufficient to influence glutamate postsynaptic differentiation by getting in touch with dendrites. Strangely Nrx also influences GABA postsynaptic differentiation [30]. For the Nrx/NL complex in synapse formation, either independent manipulation of Nrx/NL can cause moderation of presynaptic and postsynaptic assembly, which suggests an instructive role [24]. In my experiment, I synthesized engineered synapse organizers. I synthesized engineered postsynaptic organizers like NL. I simply manipulated the natural NL and first made NLΔ1 and NLΔ2 by digesting the extracellular part of the NL with restriction enzymes. Then I generated

various proteins like dClover2, GFP-nanobody, and venus by PCR and then ligated them with those manipulated NL. Seth L. Shipman et al. also suggested that dimerized NL induces the gathering of presynaptic organizers which is a part of functional chemical synapse [24]. Dean C et al. In vitro, authentication suggests that Nrx differentiation in the early stage of an axon part into a presynaptic terminal and NL clustering may be an early step in the differentiation of a dendrite segment into a postsynaptic terminal [28].

Demet Arac et al. suggested that NL1 shown in nonneuronal HEK293 cells influences presynaptic differentiation in co-cultured neurons at the places of cell-to-cell contact [13]. Similarly, Nrxs showed in nonneuronal cells influence postsynaptic differentiation in cultured neurons at associate sites. These bits of knowledge led to the assumption that NL and Nrx act as trans-neuronal signals for the enrollment of synaptic molecules and might be associated with initial synapse evolution in vivo [13]. Thus, the NL-Nrx link may play as a trans-synaptic travers bringing vesicles into Alling to the postsynaptic density. This theory gained assistance when [12] displayed that NL contacting with glutamatergic axons is expressed on congregated synaptic vesicles in nonneuronal cells. Furthermore, antibody-induced assembling of recombinant Nrx directly influenced the co-clustering of many synaptic vesicles [28]. NL can bind with many presynaptic molecules, for this it was difficult to generate an engineered synapse organizer, but my engineered NL can only bind with the specific protein or ligands. So, soon, I expect these engineered NLs will bind with engineered Nrx containing the specific protein extracellularly.

Graf ER et al. suggested that Nrx interfered with the aggregation of NLs imaginably necessary as a signal for persuading receptors and forgathering of scaffolding proteins. In relief, NLs passively may be aggregated with something that actively converts the signal produced from Nrxs to the neurotransmitter receptors and postsynaptic scaffolds. Moreover, the accumulation of the neurotransmitter receptors and scaffolding proteins may need a gathering of NLs with other tracks by local demonstration of Nrxs to dendrites [30]. For differentiation of these possibilities, NLs were aggregated independently of Nrxs. The low amounts of YFP-NL transfected in neurons followed by attaching of beads holding anti-YFP antibodies showed in local accumulating of YFP-NL on transfected dendrites superficially. This accumulation of YFP-NL-1 proceeds in the coaccumulation of PSD-95 but is different in gephyrin [30].

Graf ER et al. showed the molecular relationship of other NLs, and YFP-NL3 and YFP-NL4 were expressed in cultured neurons along with the anti-YFP beads. They also showed the combination of either NL3 or NL4 consequence in co-accumulation of PSD-95 except for gephyrin compared to NL1. This sequel varies from Nrx-Fc attached with beads; Nrx accumulated endogenous NLs containing NL2, thus directing to local accumulation of PSD-95 including gephyrin [30]. These outcomes specify that the accumulation of NLs can influence postsynaptic protein aggregating; no need for other influences in Nrxs to convert the signal. Another specificity is that NL1, 3, and 4 are associated with glutamatergic postsynaptic proteins only, but NL2 can connect with GABAergic and glutamatergic postsynaptic proteins[30]. In my experiment, I got success to generate those constructs and they all were bound to their specific protein extracellularly in HEK cells. After doing orthogonal testing, I found the specific protein for each construct. GFP-nanobody contains constructs bound with Venus or GFP protein. Venus contains constructs bound with GFP-nanobody protein. In conclusion, it can be said that my post-synaptic engineered organizers responded well.

From the above research and the outcome of our expectations, it can be said that in the future I will be able to make a new electrophysiological method or technique. In that technique, specific cell recording can be possible with the help of prob/sharp microelectrode bound with a specific protein. That specific protein will be reacted only with the specific engineered NL and then I can record that cell activity only. In other words, a specific protein bound with prob/sharp microelectrode will initiate the postsynaptic reaction in specific cells/neurons that have a relation with the engineered NL. Then cell specificity will be confirmed because that engineered NL will be associated only with that specific protein bound with prob/sharp microelectrode. Then there will be a new era for the prob/sharp microelectrode-based electrophysiological technique for specific cell recordings.

4.5 Conclusions

I have confirmed from the above experiments and discussions that my designed engineered NL can be induced by outer protein/particle and showed their postsynaptic reaction. So, if a specific protein is deposited on the top of a prob/sharp microelectrode, then that specific protein can attach with designed engineered NL. Then will initiate a postsynaptic reaction in that specific cell who have an attachment with the designed engineered NL. In this way, my planned prob/sharp microelectrode-based electrophysiological technique will fruitfully come out for the specific cell recordings. Thus, I concluded that my engineered post-synaptic organizer can be able to work for the development of a prob/sharp microelectrode-based electrophysiological technique.

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Chapter 5

General Discussion

Key Points of Chapter 5

- **Without the LNS domain Neurexin can induce differentiation. LNS domain is only needed for NL association.**
- **Made various orthogonal engineered pre- and post-synaptic organizers.**
- **Confirmed of existence in HEK cell culture as their structural integrity.**
- **Confirmed that adding other proteins with engineered organizers can bind with their orthogonal protein and can induce a presynapse reaction.**
- **Confirmed initiation of presynapse in neurons with non-biological materials such as silica beads. Such activity leads to initiating next generation electrophysiology for selective recordings.**

Discussion:

In summary, I tried to develop engineered synaptic organizers. Through those engineered synaptic organizers neurons or specific cell types can be manipulated soon. I tried to develop engineered pre-synaptic organizers and then engineered post-synaptic organizers. In the general information chapter, I introduced the neuron, synapse, and synaptic organizers with their association with the animal body system. In the general introduction chapter, I also discussed the structure of the neurons, their working module, and how they are regulated by the synaptic organizers. I noted the synapse formation, their activity, the role of SAMS, and the types of synapses in the animal body. Then I wrote about the synapse organizer and its functions. I introduced the pre-and post-synaptic organizers with their location and functions specifically.

Electrophysiological methods using genetically encoded proteins can record the specific cell. On the other hand, Electrophysiological methods using prob/sharp microelectrode arrays cannot record the specific cell because in the arrays all cells are connected, and prob/sharp microelectrode can detect only from the medium. So, in this case, if we use the engineered synapse organizer with a specific protein in prob then only desired specific cell can relate to the prob. Then maybe it is possible to record specifically the cells through electrophysiological methods. In my research, I want to develop a powerful engineered synapse organizer. Which will be capable of attaching to the specific protein and only a specific cell. I want to use the mode of action of synapse organizer but in one way not in multiple like as in nature. So, I make an engineering change through that the engineered organizer can only bind with one cell and not collaborate with other cells.

In the first chapter, I started with the introduction of electrophysiology and highly used electrophysiology techniques. I made a brief introduction to the tools and some methods of electrophysiology. I was started with electrophysiology because my research work also related to electrophysiology. In that chapter, I briefly cited some advantages and limitations of some electrophysiological techniques. Previously, it was the major problem that cell-type specific recording was not possible but with time optical-approach-type electrophysiological techniques now can do this job. This was done by the genetically encoded protein which only detects the specific cell which is matched with their cell type-specific promoter. But it is still a problem for

the prob/sharp microelectrode-based electrophysiological techniques to determine cell-type-specific recording. Using the properties of a synapse organizer we are trying to develop a methodology by which we can soon record cell-type-specific recordings. Figure 1 described the probable technology which I desired for the near future.

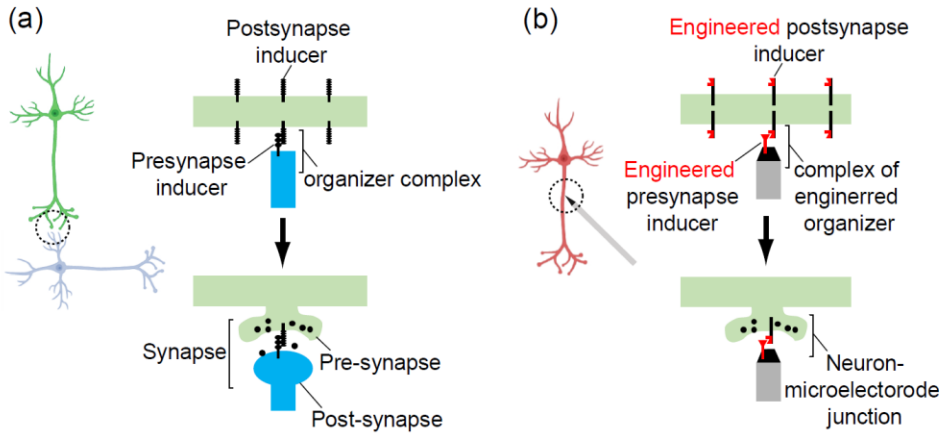

Figure 1: Desired probe/sharp microelectrode technology for cell-specific recording in probe/sharp microelectrode-based electrophysiology.

For the development of prob/sharp microelectrode-based electrophysiology, especially for specific cell recording, we need to develop engineered synapse organizers. It will be helpful to attach specific proteins attached with prob and then we can create a bridge for the specific cell recordings. Synapse organizer properties are helpful to make those types of recordings, but for that, I must use a genetically engineered organizer. For making genetically engineered synapse organizers I used their extracellular part for genetic engineering. Both post and pre-synaptic organizers were used to make genetically engineered synapse organizers. Used various types of protein which are orthogonally attached with the specific protein. Pre-synaptic Nrx showed more desirable results than post-synaptic NL. So, I give more emphasis to presynaptic Nrx for making genetically engineered synapse organizers.

I started to make engineered synaptic organizers because this is very important for the understanding of synapse formation and manipulation of synapses. At present, there is very little scientific information about the artificial synapse organizer and very few laboratories are newly

working on this. Most of the research is disease-oriented and caused by improper synapse formation due to genital causes. So, those scientists are thinking if they replaced the engineered synaptic organizers then may be possible to the treatment of various psychiatric diseases like autism spectrum disorder. In the general introduction chapter, I introduced the relationship between synaptic organizers in various psychiatric diseases. In that section, I discussed various diseases and their associated causes due to defective synaptic organizers.

Then I discussed the purposes of my work. So, the purpose was to develop engineered pre-synaptic organizers and engineered post-synaptic organizers. Nrx was chosen for the engineered pre-synaptic organizer and NL was chosen for the engineered post-synaptic organizer. Other purposes are to explore the possibility of engineered synaptic organizers as a molecular genetic approach to manipulate patterns in neural circuits. This will allow us to observe the effects of changes in nerve fiber contact on neural function with unprecedented precision. Another is to develop the next generation of electrophysiological techniques. Conventional electrophysiological techniques, such as patch-clamp and extracellular recording methods, can record electrical activity with a good signal-to-noise ratio but have the limitation of not being able to discriminate the genetic types of the target cell. There is potential to generate new electrophysiological techniques that overcome this critical limitation. And the last one is the control of autism diseases related to synaptic organizers.

In the second chapter, for making genetically engineered synapse organizers I used their outer part for genetic engineering because the extracellular part clusters with other opposite organizers extracellular part. I engineered the Nrx as the Nrxn1bΔECD version where the extracellular part is more flexible to take any epitope protein for specific contraction. I did cell microbead reactions with those various types of engineered Nrx like FLAG-Venus-Nrxn1b, FLAG-Venus-Nrxn1b-ΔECD, and FLAG-Nrxn1b-ΔECD. Various types of protein are orthogonally attached to the specific protein. I found GFP-nanobody containing Nrx bounds with GFP and venus protein. mCherry-nanobody contains Nrx bounds with mCherry protein. mCherry contains Nrx bounds with mCherry-nanobody protein. Spot and BC2 contain Nrx bound with BC2-nanobody and spot-nanobody proteins respectively. GFP-containing Nrx showed their existence extracellularly in the dark fluorescence view and can bind with GFP-nanobody. In the cell-microbeads

interaction experiment, I confirmed with a presynaptic marker called an anti-synaptophysin antibody. Flag-YFP-Nrxn1b-ΔECD1-P2A-Rab3-EGFP bound with GFP-nanobody microbead and confirmed by postsynaptic Anti-FLAG and Rab-3 markers. So, if a specific protein deposits on the top of a prob/sharp microelectrode, then that specific protein can attach with designed engineered Nrx. Then will initiate a presynaptic reaction in that specific cell who have an attachment with the designed engineered Nrx. In this way, my planned prob/sharp microelectrode-based electrophysiological technique will fruitfully come out for the specific cell recordings.

It was not easy to develop a workable engineered synapse organizer from the natural one. I considered the working principle of the synapse organizer and then thought to apply it in prob/sharp microelectrode-based electrophysiology for the recording of a specific cell. Also, I need to consider the microelectrode with a specific medium and voltage for the prob/sharp microelectrode-based electrophysiological specific cell recording. In the future, a specific prob/sharp microelectrode with orthogonal tested protein will be developed for the desired development of the prob/sharp microelectrode-based electrophysiological technique to initiate specific cell recording. I succeeded in the development of engineered synapse organizers. In conclusion, it can be said that our engineered synaptic organizers responded positively.

In the third chapter, I discussed the generation of the engineered pre-synaptic organizer and the test results for establishing engineered pre-synaptic organizers. For the generation of the test synapse organizer, I used conventional molecular biology techniques (e.g., combinations of polymerase-chain-reaction, restriction enzyme digestions, DNA ligation, infusion cloning, agarose gel electrophoresis, bacterial transformation and cultures, protein purification, plasmid purifications, DNA sequencing, BAP treatment, etc.) to generate a variety of test synapse organizer constructs. I used various proteins and Nrxs (e.g., Venus, GFP, GFP-nanobody, mCherry-nanobody, mCherry, GFP null, Spot, Spot-nanobody, BC2, BC2-nanobody, T2A, EGFP, Rab3, NrxΔ1, NrxΔ2, etc.) to generate an engineered synapse organizer.

In the orthogonal test, I found GFP-nanobody containing construct bounds with GFP and venus protein. mCherry-nanobody contains constructs bound with mCherry protein. mCherry contains constructs bounds with mCherry-nanobody protein. Spot and BC2 contain constructs bound with BC2-nanobody and spot-nanobody proteins respectively. GFP-containing constructs showed their existence extracellularly in the dark fluorescence view and can bind with GFP-nanobody and protein. I did a cell-microbead interaction examination, in which a spot containing NrxΔ1 showed the best results among all the constructs. In the cell-microbeads interaction experiment, we used pCAG-GS-FLAG-Spot-Nrx1bΔ1-P2A-EGFP-Rab3 in the presynaptic position and BC2-nanobody microbead in the postsynaptic position. Then I confirmed with a presynaptic marker called an anti-synaptophysin antibody. GFPnull/YFP containing engineered NrxΔ1 bound with GFP-nanobody microbead and confirmed by postsynaptic Anti-FLAG and Rab-3 markers. So, if a specific protein deposits on the top of a prob/sharp microelectrode, then that specific protein can attach with designed engineered Nrx. Then will initiate a presynaptic reaction in that specific cell who have an attachment with the designed engineered Nrx. In this way, my planned prob/sharp microelectrode-based electrophysiological technique will fruitfully come out for the specific cell recordings. In conclusion, it can be said that our engineered synaptic organizers responded positively.

In the fourth chapter, I discussed the generation of the engineered post-synaptic organizer and the test results for establishing engineered post-synaptic organizers. For the development of post-synaptic organizers, I used various molecular biological techniques (e.g., combinations of PCR, restriction enzyme digestion, DNA ligation, BAP treatment, agarose gel electrophoresis, transformation, plasmid purification, DNA sequencing, etc.) to generate a different type of post-synaptic organizer. I used NL1 and various proteins (e.g., Venus, GFP-nanobody, and dClover2) to produce engineered post-synaptic organizers. In my experiment, I got success to generate those constructs and they all were bound to their specific protein extracellularly in HEK cells. After doing orthogonal testing, I found the specific protein for each construct. GFP-nanobody contains constructs bound with Venus or GFP protein. Venus contains constructs bound with GFP-nanobody protein. In conclusion, it can be said that my post-synaptic engineered organizers responded well.

Previously, it was the major problem that cell-type specific recording was not possible but with time optical-approach-type electrophysiological techniques now can do this job. This was done by the genetically encoded protein which only detects the specific cell which is matched with their cell type-specific promoter. But it is still a problem for the prob/sharp microelectrode-based electrophysiological techniques to determine cell-type-specific recording. So, using the properties of a synapse organizer, I am trying to develop a methodology by which I can soon record cell-type-specific recordings. Synapse organizer properties are helpful to make those types of recordings, but for that, I must use a genetically engineered organizer. For the development of prob/sharp microelectrode-based electrophysiology, especially for specific cell recording, I need to develop engineered synapse organizers. It will be helpful to attach specific proteins attached with prob/sharp microelectrode and then I can create a bridge for the specific cell recordings.

Finally, I am going to talk about the final experiments for the test synapse organizers. I did a lot of experiments on HEK cells as well as on neurons. After several experiments preceded that those synapse organizers can work as engineered synapse organizers for the development of the prob/sharp microelectrode-based electrophysiological technique. Most of the test synapse organizers use the same technique so, I have just focused on a better result showing experiments. Firstly, I did the orthogonal test to select the specific protein for the specifically engineered synapse organizers. Then, I go through the HEK cells and neurons culture with the specific marker, biotechnological beads, and proteins with specific conditions.

Future directions:

1. The main objective is to develop the next generation of electrophysiological techniques. Conventional electrophysiological techniques, such as patch-clamp and extracellular recording methods, can record electrical activity with a good signal-to-noise ratio but have the limitation of not being able to discriminate the genetic types of the target cell. There is potential to generate new electrophysiological techniques that overcome this critical limitation.
2. Control of autism diseases related to synaptic organizers.

Limitations:

For doing those engineering synapse organizers, I took challenges to work with a new thought. It was not easy to work with an almost unknown topic to a furnished one. So, planning and doing experiments takes a lot of time and effort. In many cases, I made some organizers, but they were not responding well in HEK cells, then again, I did those for a recheck. Then I considered those for discard or continuation. In those ways, I discard a lot of pairs of engineered organizers even in the last stage of cell microbeads conjugation.

I took only those engineered synapse organizers which were very good in HEK cells and then passed the cell microbead reaction. Some of the constructs had some problems then I found those problems and corrected or rejected them. I always started two to three pairs of engineered synapse organizers and from start, to finish it took six to eight months. As I remember I made about 15 pairs of engineered synapse organizers, which passed the cell microbead reactions. On the other hand, I discarded more than 10 pairs of engineered synapse organizers because of not suitable for cell microbead reactions and cell expressions. So, it was very challenging because we have limited time and space to work on our project, but my project was too lengthy. But I finished those and finally chose a single pair for future prob generation to initiate a new electrophysiological technique.

There is also another limitation to the continuation of our project for me and that is funding for this project. I am the pioneer of the method development process but the next step I cannot attend the due to a lack of funds and time scale. Maybe another person will handle the project and will finish soon. This is a heart-touching moment that I cannot continue but hope for the best who will continue this soon. Anyhow science will develop with time with someone or without someone. However, I finished my step for the development of the prob-based electrophysiological method for specific cell recording. Soon someone will develop that prob and then will conjugate the engineered synapse organizer’s specific protein with the prob.

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