Validation of Two CRISPR sgRNA's Directed at the α-5-Nicotinic acetylcholine Receptor Subunit.

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ABSTRACT: Nicotinic acetylcholine receptors, or nAChRs, form the basis of nicotine addiction. The α4β2α5 nAChR(Nicotinic acetycholine receptor) is the focus of this study. When an individual uses either a cigarette or an e-cigarette, nicotine is released, rapidly moving from the bloodstream to the brain. From there, it binds to the nAChRs and results in the opening of the receptor which causes sodium and calcium to enter into the receptor and allows potassium to exit. The effect of calcium entering a neuron releases the neurotransmitter dopamine. Once this transmitter is released the user experiences a pleasurable feeling, which reinforces continued use of the substance to achieve more pleasure, thereby strengthening the addiction. This process is known as the "Dopamine Reward Pathway." Thus, defining the nicotinic receptor subunit composition in vivo remains a critical issue to be addressed in characterizing the mechanisms underlying addiction. Western Blot tests were conducted to help both characterize the α5 receptors. The new tool that was used to help with the knockdown of α4β2α5 nAChR is the gene editing tool called CRISPR-Cas9. After finding the correct antibody, I was able to, with the help of the pX330 DNA plasmid, knock-down the α4β2α5 nAChR. To help visualize the knockdown, western blots were performed, allowing me to identify the presence of specific proteins.

KEYWORDS: Gene editing; CRISPR-Cas9; Nicotine Addiction; Molecular Biology; Alpha5.

Introduction

Addiction is a dependence on an item or substance. One of the most common addictions in society today is smoking/vaping. Vaping has grown in popularity with the rise of e-cigarettes and is becoming a massive issue. Additionally, smoking cigarettes increases the risk of COPD (Chronic Obstructive Pulmonary Disease) or Lung cancer (Figure 1). The American Cancer Society shows that so far in 2019 alone there were 228,150 new cases of lung cancer, and of those 142,670 died, which means that 63% people died from smoking. Someone who smokes only five cigarettes a day (which is considered low) has a 15% chance of lung cancer after smoking 45 years. However, a person who smokes 40 cigarettes a day has a 55% chance of getting lung cancer.[1] The current rate of lung cancer is staggering; hence it is crucial to develop new methods to stop the addiction to cigarettes.

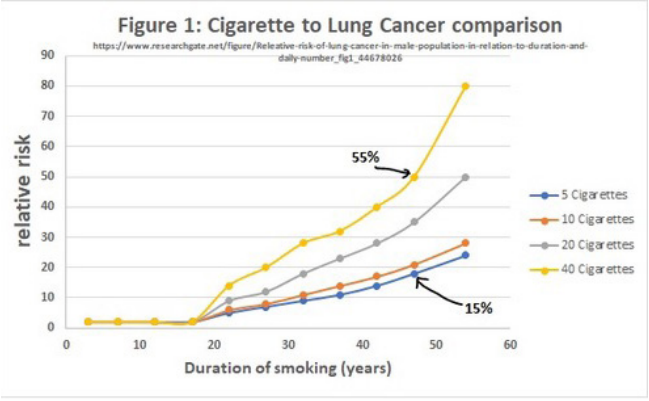

Figure 1. The relative lung cancer risk increases as the number of cigarettes and the duration of smoking are increased.

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels which allow Na^+ and Ca^{2+} ions to enter a cell and K^+ ions exit when selective ligands bind to the receptor.[2] The α4β2α5 nAChR is the focus of this study. These receptors are made up of two α4, two β2 and one α5 subunits as seen in Figure 2, a single nucleotide polymorphism (SNP) in the α5 subunit causes "heavy smoking" which is the receptor that I have targeted in this research. Inhalation of smoke from a cigarette releases nicotine (NIC) from the tobacco in the cigarette which carries the NIC into the lungs, where it is rapidly absorbed into the pulmonary venous circulation. The NIC then enters the arterial circulation and moves quickly from the lungs to the brain, where it binds to nAChRs (ligand-gated ion channels that usually bind and are activated by acetylcholine). The binding of nicotine at the interface between two subunits of the receptor opens the channel, therefore allowing the entry of sodium and calcium into the cells. Following the open channel state, the channel remains desensitized for a period of time before returning to the closed channel state (Figure 3). One of the effects of the entry of calcium into a neuron is the release of neurotransmitters.[3]

The neurotransmitter we are focused on in this research is dopamine. This neurotransmitter works in the system called the Dopamine Reward Pathway. Once this transmitter is released the user receives a pleasurable feeling. This feeling reinforces the behavior of the person to continue using the substance to achieve more pleasure and strengthens the addiction. Thus, defining nicotinic receptor subunit composition in vivo remains a critical issue to be addressed in characterizing the mechanisms underlying addiction.[4]

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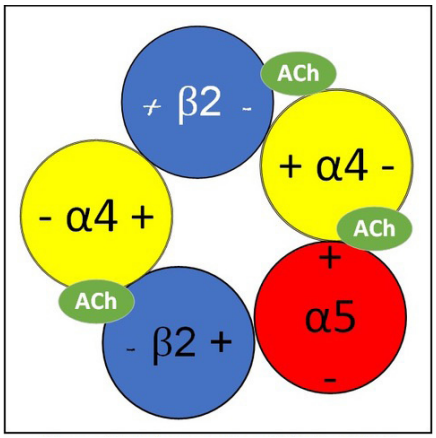

Figure 2. Heteromic α4β2α5 nAChR www.lookfordiagnosis.com/mesh_info.php?term=Nicotini Agonist&lang=1

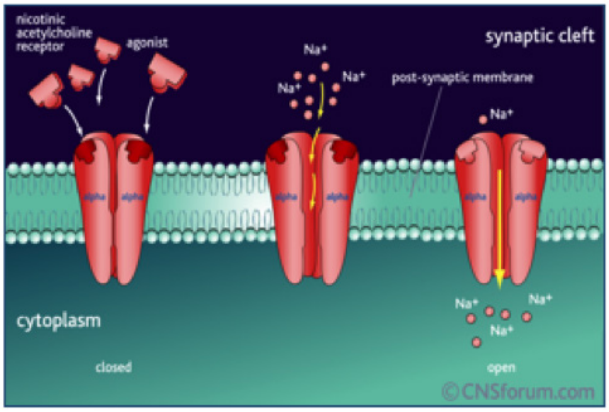

Figure 3. Membrane view of nAChRs www.cnsforum.com/upload/imagebank/download/rcpt_sys_nic_ag1.png.

Current Issues:

More need to be understood about the role of the α5 nAChR SNP in smoking addiction.

Goals of the study:

1. To create the CRISPR tools to knock down α5 nAChR in specific brain regions.

2. Prepare and characterize CRISPR plasmids to be used the study the effect of α5 nAChR subunit knockdowns of mice that constituently express either the α5 nAChR D- or N-variant subunit.

This study provides a crucial first step in order to understand the role of the α5 nAChR subunit in smoking addiction and perhaps to develop drugs that target these receptors. The experiments presented here demonstrate the creation of new CRISPR tools and show that they will be useful in future "in vivo" studies.

The tool that was used to help with the knockdown of the α4β2α5 nAChR is the gene editing tool called CRISPR-Cas9. The CRISPR-Cas9 system consists of two key molecules that induce a change in DNA: (1) Cas9 acts as molecular scissors," cutting both strands of DNA at a specific location in the genome. This allows a bit of the DNA to be inserted or deleted, which is called an indel; (2) Guide RNA (gRNA) consists of a small piece of pre-designed RNA sequence (around ~20 bases) located within a more extended RNA scaffold. The scaffold

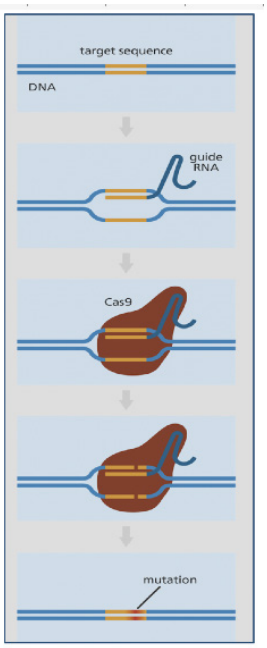

Figure 4. Heteromic α4β2α5 nAChR www.lookfordiagnosis.com/mesh_info.php?term=Nicotini Agonist&lang=1.

Results and Discussion

In order to observe a knock-out of the α5 nAChR subunit we first needed to characterize our α5 antibodies. We used a monoclonal rat and rabbit polyclonal anti-α5 nAChR subunit antibody and an anti-rat secondary (green) and an anti-rabbit secondary (red). As seen in both Figure 6 and Figure 7, both antibodies specifically recognized the mouse and human α5 nAChR subunit. To help test whether these antibodies work, we examined the mouse α4β2 and the human α4β2. Importantly, lanes that contained cells only expressing the α4β2 nAChR did not reveal an immunoreactive band where the α5 nAChR subunit should have been present highlighting the specificity of both antibodies.

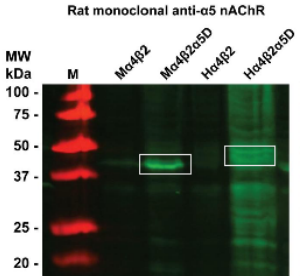

Figure 6. Demonstration of the specificity of the rat anti-α5 nAChR subunit primary antibody. The α5 nAChR subunit was visualized with an anti-rat secondary antibody coupled to a near-infrared green (800 nm) fluorescent dye. The specificity is demonstrated by the presence of an immunoreactive band around 42 kDa only when the α5 nAChR subunit is present.

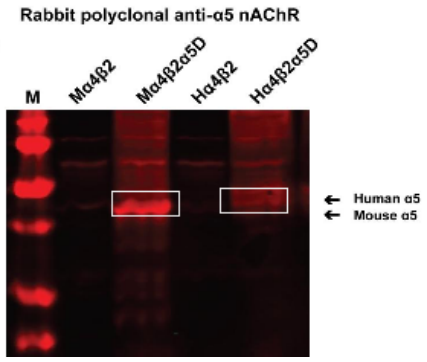

Figure 7. Demonstration of the specificity of the rabbit anti-α5 nAChR subunit primary antibody. The α5 nAChR subunit was visualized with an anti-rat secondary antibody coupled to a near-infrared red (700 nm) fluorescent dye.

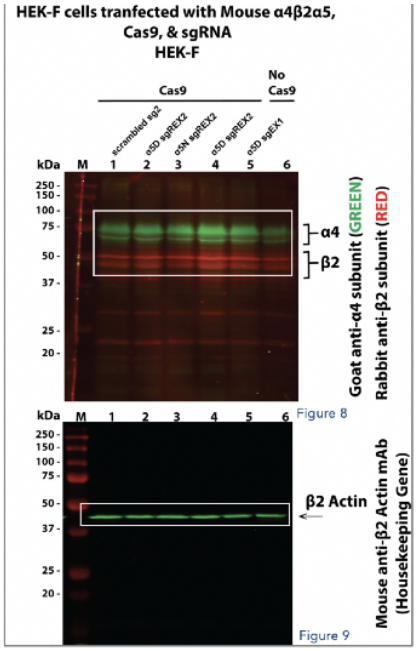

Figure 8. The blot showing only α4 and β2 are the same PX330 samples that used m268. This demonstrates the α4β2α5 D or N nAChR receptor are present in each lane (This is the receptor we are interested in).

Figure 9. Uses β-actin which is a ubiquitous protein in cells to demonstrate that the loading was similar for each sample.

Now that both the rat and rabbit antibody could be used to locate the α5 subunits we could test the CRISPR-Cas9 tools. Before we can achieve a knock-down of the α5 receptor. Figure 8 shows that in the receptor we are interested in has the α4 and β2 which the pX330 contains. In addition, Figure 9 uses β-actin, an ubiquitous protein in cells to demonstrate that the loading was similar for each sample was made. Figure 11 correlates with Figure 10 as this western blot shows that the Cas9 is present in all of the lanes. Figure 10 helps to explain the full potential of the CRISPR-Cas9 tool and its real-life implications in the reduction of nicotine addiction. Lanes 2, 3, 4, and 5 represent the absence of the α5 receptors. These lanes contain both our pX330 plasmid with the Cas9 enzyme and show that a knock-down is possible. Lane 1 has a nonsense sgRNA(pX330) which is shown to make no knock-down. And lane 6 which is our control has no Cas9 and no sgRNA and shows the α5 receptors clearly with the fluorescent dye and acts as a positive control. These studies help us move to the end goal of creating a knock-down, and since the sgRNA in the PX330 is identical to that in 60231, it is highly likely that this exon 2 sgRNA will work in mouse brain as well.

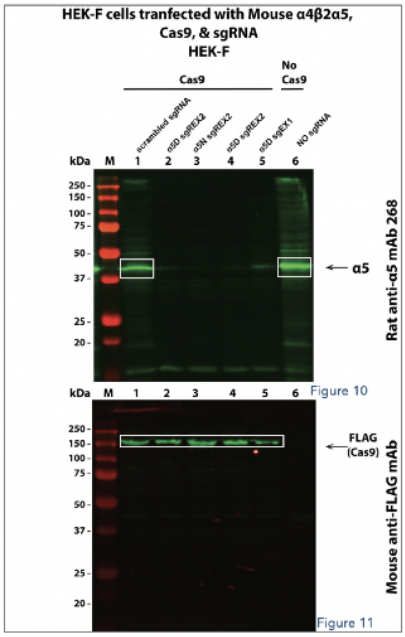

Figure 10. This figure can help to conclude that the exon 2 sgRNA can knock out both the α5D and the α5N subunits (lanes 2,3,4) and that a scrambled sgRNA has no effect that is, α5 is expressed under this condition.

Figure 11. This western blot shows that lanes 1 through 5 contain the cas9 enzyme which is essential for the CRISPR-Cas9 process to knock-down the α5 receptors.

Methods

Western blots (immunoblots) are used to identify the presence of specific proteins in transfected cells and brain regions. In this case, I was detecting the α5 nAChR subunit. Samples were denatured with the sulfhydryl reducing agent, dithiothreitol, and heated to about 90°C. We carried out Gel electrophoresis using a 4% stacking gel and an 11% resolving gel was used to separate proteins by their apparent molecular weight. Proteins were then transferred to PVDF membranes by electrophoresis. The PVDF membranes (immunoblots) were then treated with a propriety protein solution to block the non-specific sites on the membrane. The immunoblots were then incubated overnight with the primary antibodies directed at epitopes present α5 nAChR subunit. The next day the primary antibodies are removed by washing in PBS (including the detergent TWEEN-20). The immunoblots were incubated with species-specific secondary antibodies that are specific for each α5 nAChR subunit primary antibody. These secondaries possess near-infrared (NIR) red (700 nm) and green (800 nm) fluorescent dyes which allow the visualization of the α5 nAChR subunit and other specific and relevant proteins such as Cas9 and Beta-actin. Developing the blot is accomplished after several washes of the secondary antibodies and the utilization of Licor's Odyssey system (Figure 12). The scans are digitally recorded.[6]

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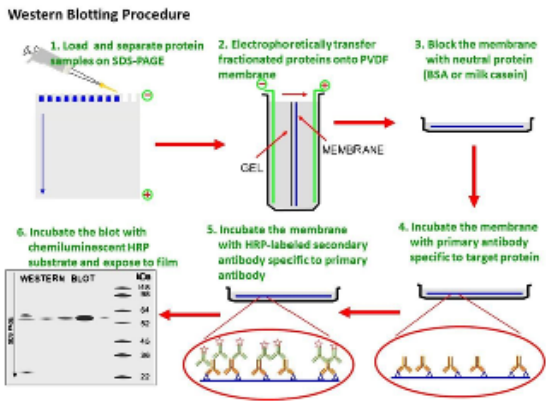

Figure 12. Western Blot Process https://microbeonline.com/western-blot-technique-principle-procedures-advantages-and-disadvantages/

Conclusion

The initial work used molecular biological techniques to create plasmids to knockdown the mouse α5 nAChR subunit. Two antibodies were then characterized by Western blots and demonstrated α5 nAChR subunit specific immunoblots. The PX330 plasmids were expressed with either the mouse α4 and β2 nAChR subunits alone or including mouse α5 nAChR subunit as well. Knockdown the α5 nAChR subunit was demonstrated with no effect on the α4 or β2 nAChR subunits. We are now ready to use these tools to localize and compare the effects of the nAChR subunits in discrete mouse brain regions. Continuation of these studies could lead to a better understanding of smoking and other addictions and hopefully lead to better treatments of addiction.

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Sid Thakker is currently a junior at James Madison High in Vienna, Virginia. He has interests in molecular biology and business and hopes to combine both his passions into a company to help change the world.