The Effects of Nicotine on NMDA Receptor Expression in the Brain and Peripheral Blood Lymphocytes of Mice as a Possible Treatment for Schizophrenia.

Celia Hutchins

Nyack High School

Acknowledgement

I would like to thank my mentor, Dr. Henry Sershen at Nathan Kline Institute for Psychiatric Research, for his mentorship and my teachers Ms. Foisy, Ms. Kleinman, and Ms. OHagan for their support towards the completion of this project.

Abstract

This study looked at the effect of nicotine on NMDA receptor function in the brain and peripheral blood lymphocytes of mice as a possible treatment for schizophrenia. The RNA was isolated from the prefrontal cortex, left hippocampus, right striatum, and peripheral blood lymphocytes, of an experimental group of mice that were injected with nicotine, and a control group of mice injected with a saline solution. The RNA converted to cDNA, which was analyzed using a comparative polymerase chain reaction (PCR) quantification, to determine the cycle threshold (Ct) values of the NMDA receptor and control gene, Beta (β) - Actin. The values were then analyzed using the $2^{-\Delta\Delta Ct}$ method to show the fold change (relative up-regulation or down-regulation of the NMDA receptor) of the experimental group compared to the control group, and a t-test to show the statistical significance of the Ct values. The results of the $2^{-\Delta\Delta Ct}$ method showed that the NMDA receptor mRNA in the prefrontal cortex of the experimental mice was up-regulated by a factor of 1.259, and the Ct values were statistically significant based on data converted to the logarithmic scale of $2^{-\Delta\Delta Ct}$. The left hippocampus showed that the NMDA receptor mRNA of the experimental mice was up-regulated by a factor of 1.162, but the Ct values were not statistically significant. The right striatum showed that the NMDA receptor mRNA of the experimental mice was up-regulated by a factor of 1.523, with statistical significance both using the given values, and in the $2^{-\Delta Ct}$ form.

Table of Contents

List of Figures and Tables

Introduction

Schizophrenia is a severe and inheritable neuropsychiatric disease that affects one percent of the general population (Noetzel, et al., 2012). The disease is characterized by positive symptoms, which are extra behaviors, or feelings that are usually not present in people; and negative symptoms, which are a lack of behavior or feelings usually present. Positive symptoms include hallucinations and delusions, while negative symptoms include loss of interest, and lack of emotion. Aside from these symptoms, the disease is marked by cognitive deficits that are more related to the negative symptoms. These include memory loss, trouble paying attention, decreased judgment skill and poor problem solving ability. Antipsychotic medications are the primary pharmacological treatment of schizophrenia, and effectively treat positive symptoms. However, they have limited efficacy for negative symptoms and cognitive impairments (Buchanan, et al., 2007). This indicates an imperative necessity for more effective treatments which can target both sets of symptoms.

A current mechanistic model for schizophrenia is suggested to involve glutamate receptor subtype N-methyl–D- aspartate (NMDAR) hypofunction. Decreased functioning of the NMDAR has been shown to produce symptoms similar to the negative symptoms and cognitive deficits in schizophrenia (Siegal, et al., 2009). Symptoms that mirror those found in schizophrenia can be induced by the NMDAR inhibitors ketamine and phencyclidine (PCP) in healthy patients with no history of psychiatric illness (Mathalon, et al., 2014). The glutamatergic system of receptors plays a critical role in learning, memory and regulation. The NMDAR is one glutamate receptor thought to be critical in synaptic plasticity, a cellular mechanism for learning and memory.

Several current studies have focused on using the different NMDAR agonists, chemicals that bind to a receptor and activate it, such as D-cycloserine, d-serine, and glutamate to increase the functioning of the NMDAR as a possible way to treat the cognitive deficits and negative symptoms of schizophrenia. Studies by Citrome, et al., (2014), and Falkenberg, et al., (2014), found that glutamate increased NMDAR function. The Citrome study showed that D-cycloserine, an agonist or stimulator of the NMDA receptor, affected NMDAR function. Buchanan, et al., (2007) also found that D-cycloserine improved cognitive deficits and negative symptoms in schizophrenia.

People with schizophrenia are three times more likely to smoke cigarettes than the rest of the world population (75%-90% vs. 25%-30%), indicating that smoking may be a way of self-medicating (Smith, et al., 2002). Some scientists have therefore considered nicotine as a possible way of treating schizophrenia. Smith, et al., found that acute smoking of a cigarette had the effect of reducing negative symptoms in patients with schizophrenia, with a stronger effect from the cigarette that had higher nicotine content (2002). Different ways of administering nicotine into the body were found to have different effects on symptoms of schizophrenia. One study looked at the interaction between nicotinic treatments and antipsychotic drugs; nicotine was shown to improve cognitive functioning in the presence of the antipsychotic drug clozapine. The study's authors concluded that it was important to understand how the drugs interact in individual patients and how they affect their personal behaviors and cognitive functioning (Levin and Rezvani, 2007).

Nicotine is an agonist of the NMDAR that can activate the receptor so that it will function. Release-modulating nicotine receptors (nAChRs) and NMDARs coexist in the brain. Acetylcholine is the neurotransmitter that usually binds to the AChRS, but nicotine can also acts at this site. Nicotine permits NMDAR activation in the presence of magnesium ions, possibly because the nicotine-induced influx of sodium ions depolarizes the nerve ending membrane sufficiently to remove the magnesium ion block (Risso, et al., 2004). Other studies have used methods such as real-time polymerase chain reaction (RT-PCR) to study the effect of smoking on the genes expressed in schizophrenic and control smokers. One study, by Leonard, et al., found that patients with schizophrenia that smoke have nicotinic receptors that fail to increase activity in response to stimulus (2005). They also found that the immune response and NMDA transcripts between smokers and nonsmokers, regardless of mental health, were different. Specifically in patients with schizophrenia, the smokers and nonsmokers had altered subsets of the NMDA genes expressed. Leonard's team also found 277 different genes that differed between nonsmoking patients with, and without schizophrenia. These results imply that there is a genetic link to schizophrenia and that it is related to the NMDAR. Most current research has looked at the effects of nicotine administration on the symptoms of schizophrenia. Little research has been done on the effect of nicotine on a cellular level. To address this, this study researched the effects of nicotine on a cellular level, and the mechanisms behind schizophrenia by looking to see if it altered the expression of the NMDAR.

This study used RT-PCR to evaluate the effects of nicotine on NMDA receptor expression in post mortem brain tissue of mice that had been injected with nicotine. The prefrontal cortex was studied because its proper function is thought to be integral to a person's personality by playing a role in functions such as complex cognitive behavior, decision making, moderating social behaviors, and personality expression. The hippocampus was included because it is important to memory, especially long-term, and spatial navigation, which is a person's ability to navigate their environment and remember events that occurred. The striatum was also studied because it plays an important role in voluntary movement, controlling different cognitive functions and behaviors, and mediating reward. Analysis was also applied to peripheral blood to determine if it mimicked changes in the brain.

It was hypothesized that mice injected with nicotine would show altered levels of the NMDAR expressed in their tissue and peripheral blood lymphocytes. If the levels of NMDAR mRNA were higher, it could explain the relationship between nicotine and schizophrenia, and aid in the understanding of the mechanisms behind the negative symptoms and cognitive deficits associated with schizophrenia. This study is important because it will help to research nicotine, nicotine analogs, or drugs that target the nicotinic receptor, as a possible treatment for the negative symptoms and cognitive deficits of schizophrenia that are not presently improved by the current generation of antipsychotic medications. Since nicotine is associated with cigarettes, it is rightly viewed as harmful. Therefore, the hope is that a nicotine analog can be developed that targets a specific subset of the nicotine receptors. A second part of the study aimed to see if there are changes in the brain that are then replicated in the blood, which would be a very noninvasive way to screen patients, compared to physically analyzing part of their brain.

Materials and Methods

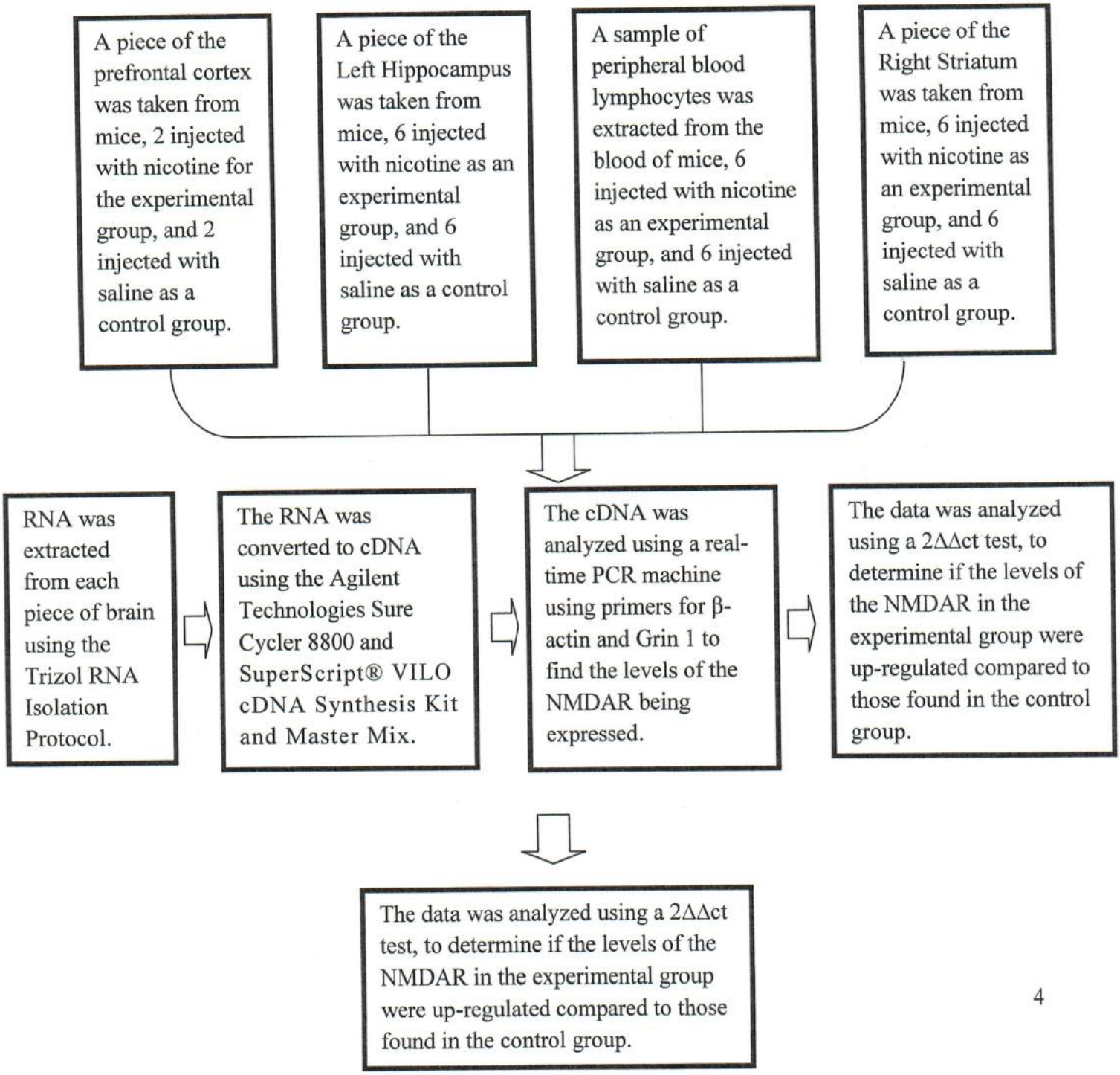
The brain tissue and blood plasma of twelve mice, of the genetically modified strain Mus musculus (C57BL/6), were obtained from a previous study. The mice numbered 7-12 had been

 ılly injected with nicotine, with 0 .4 mg/mL (nicotine-di-tartrate salt), for that study,

while the mice numbered 1-6 were a control group injected with a 0.9% saline solution. Both groups received a daily injection for 14 days. The tissues were frozen in a -80 degree Celsius

freezer to be saved for this experiment. NMDAR expression was examined in samples from the prefrontal cortex, left hippocampus, right striatum, and blood plasma. Lymphocyte separation was performed on the mouse blood.

Figure 1: Methods used to find the effect of nicotine on NMDA receptor expression



RNA was extracted from the tissue of both the control mice and nicotine-injected mice, using the Trizol RNA Isolation Protocol (Chomczynski and Mackey, 1995). Once the RNA was extracted, it was dissolved in 50 uL of RNAase-free water. The samples were then converted to cDNA using the Agilent Technologies Sure Cycler 8800 (see figure 2) and SuperScript® VILO cDNA Synthesis Kit and Master Mix. 2 uL of each sample dissolved in water were tested to determine the amount of RNA. To determine how much sample was used, each sample was adjusted so that a final assay volume contained about 125 ng/uL RNA; the RNA of each sample was determined with the NanoDrop Lite (See Figure 2). Then water was added to each sample in a different test tube to reach a volume of 14 uL. If the volume of sample was greater than or equal to 14 uL, then only 14 uL of the sample were added to the test tube without adding water. Then 4 uL of the 5x Vilo reaction mix and 2 uL of the 10x Superscript Enzyme Mix were added to each sample, for a total volume of 20 uL. The samples were then put in the Agilent Technologies Sure Cycler 8800 that mixed and incubated them at 25 degrees Celsius for 10 minutes, incubated during the reaction at 42 degrees Celsius for 60 minutes, and terminated the reaction at 85 degrees Celsius for 5 minutes, thus turning the samples of RNA into cDNA. These were then tested in the NanoDrop Lite (see figure 3) to determine how much cDNA was in each sample.

Figure 2: Agilent Technologies Sure Cycler 8800

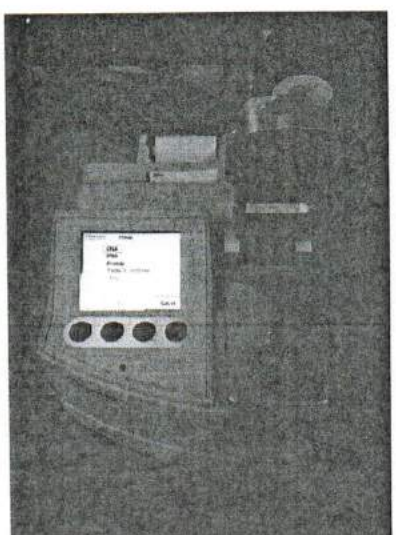


Figure 3: NanoDrop Lite

The samples were then analyzed using the Agilent Technologies Mx3000P qPCR System that operates through the real time polymerase chain reaction (RT-PCR) of selected primers for the NMDAR (see figure 4). 1 uL of each sample and 8 uL of water were added to each well of a polymerase chain reaction (PCR) plate (See figure 5) with either 1 uL of the Taqman probe Beta Actin (β–actin), a housekeeping gene, as a baseline, or 1 uL of the NMDAR primer Grin1, both specific to the genes found in

mice, and 14 uL of the PCR Mastermix. Each sample was tested in triplicate, for 6 wells, three with β–actin, and three with Grin 1. The PCR machine then quantified the cycle threshold (Ct) levels of the NMDAR mRNA that were expressed in the tissue of the mice for both the β–actin and Grin1.

Figure 4: Agilent Technologies Mx3000P qPCR

The data collected was analyzed by using the 2ΔΔCt (2 delta delta ct) test. This analysis was performed on the resulting data using the cycle threshold (Ct) values, obtained through the comparative PCR quantification, of NMDAR expression in each sample and for each primer. The test was used to determine the fold change, the relative change of NMDA expression of the experimental group compared to the control mice. This also showed if the receptors in the experimental group were up-regulated, (showed increased NMDAR expression) or down-regulated (decreased NMDAR expression) as compared to the control group.

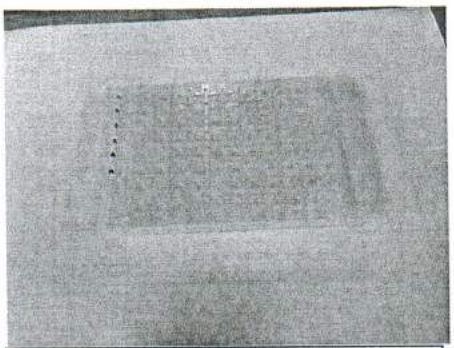


Figure 5: Polymerase Chain Reaction Plate

The standard deviation test of the averages for the control and experimental mice was computed and a two sample t-test assuming equal variance was performed to compare the nicotine–treated and the control data gathered from the comparative PCR quantification. The t-test was used to show the statistical significance of the results based on whether they had a p value less than 0.05, which means that there is a less than 5% chance that the results occurred randomly. These test were done using the raw Ct values collected, and by using the Ct values converted to the form $2^{-\Delta Ct}$, which is the linear form, because the Ct values are based on a logarithmic scale so this data may be a more accurate representation of the results.

Results

Prefrontal Cortex

Table 1 shows the cycle threshold (C_t) values for the control and experimental samples corrected against the house-keeping gene β–actin in the Prefrontal cortex. The results showed that the NMDARs in the experimental mice injected with nicotine were up-regulated by a factor

of 1.259, compared to the NMDARs in the control mice. This result is consistent with the hypothesis because it shows increased expression of the NMDA messenger RNA and therefore may be increasing NMDAR functioning. Using a t-test, the results of this experiment were shown to be statistically significant when converted to the $2^{-\Delta ct}$ form, with a p value of 0.046. The results are approaching statistical significance using the given values with a p value of 0.051.

Left Hippocampus

The cycle threshold (C_t) values for the control and experimental samples corrected against the house keeping gene β-actin collected from the left hippocampus are shown in Table 1. The results of analysis show that the NMDARs in the experimental mice that were injected with nicotine were up-regulated by a factor of 1.162, compared to the NMDARs in the control mice. Since this shows that the mRNA expression for the NMDAR increased, the result is consistent with the hypothesis because it may then be increasing the functioning of the NMDAR. However, these results did not reach statistical significance with a p value of 0.21, and 0.17 when converted to the $2^{-\Delta ct}$ form.

Right Striatum

The cycle threshold (C_t) values for the control and experimental samples corrected against the house keeping gene β-actin collected from right striatum are represented in Table 1. The results, after being analyzed, show that the NMDARs of the experimental group that were injected with nicotine were up-regulated by a factor of 1.523, compared to the NMDARs of the control group. The result confirms the hypothesis, because it showed that the expression of the NMDAR mRNA was increased, and as a result could cause increased function of the NMDAR. The results are statistically significant both using the given numbers, and when converted to the $2^{-\Delta ct}$ form with p values of 0.015 and 0.0101, respectively.

Peripheral blood lymphocytes

No data was collected for the peripheral blood lymphocytes, because the values for the levels of the NMDARs being expressed were below threshold level. These results were inconclusive, and therefore could not confirm the hypothesis that the level of NMDAR

expression in the peripheral blood lymphocytes would model changes seen in the brain of the mice, for example after exposure to nicotine. Possibly a larger sample size would be needed.

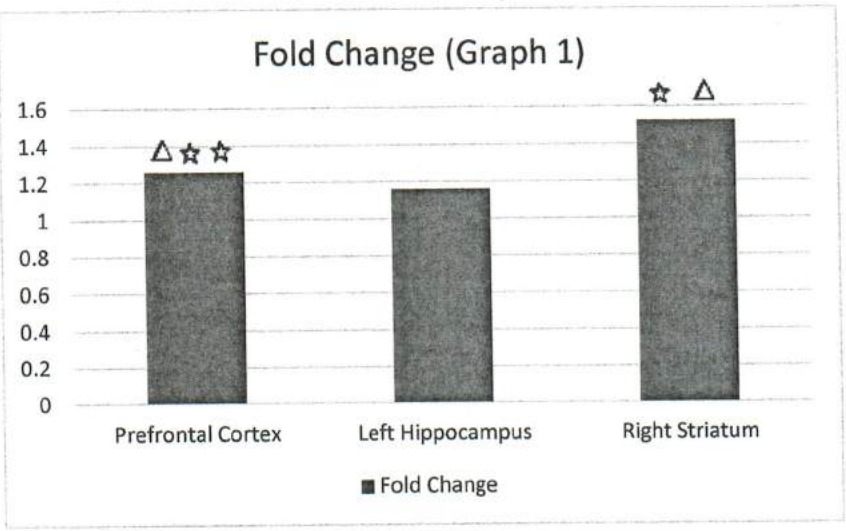


Figure 6: Factor by which the NMDA receptor expression levels of the experimental mice were up-regulated compared to the controls

△ = Statistically significant at p value < 0.05 based on $2^{-\Delta ct}$ form

☆ = Statistically significant at p value < 0.05 using the numbers given

☆ ☆ = Approaching statistical significance using the numbers given

Table 1: (C_t) values for the samples corrected, and analyzed, n = sample number

	Prefrontal Cortex	Left Hippocampus	Right Striatum
Average (Δct) (ct Grin 1 - ct β-Actin)			
Sample 1 (Control)	1.34	2.243	2.687
Sample 2 (Control)	1.55	2.173	2.153
Sample 3 (Control)		2.357	2.83
Sample 4 (Control)		1.703	2.177
Sample 5 (Control)		2.47	2.097
Sample 6 (Control)		2.137	3.453
Sample 7 (Experimental)		2.47	2.023
Sample 8 (Experimental)		1.09	1.916
Sample 9 (Experimental)		2.37	1.963
Sample 10 (Experimental)		1.32	2.09
Sample 11 (Experimental)	1.16	2.22	2.19
Sample 12 (Experimental)	1.065	2.317	1.573
Average Δct Control	1.445	2.1805	2.566166667
Average Δct Experimental	1.1125	1.9645	1.959166667
Standard Deviation (Between the control and experimental groups)	0.235113005	0.152735065	0.429213816
Standard Deviation based on $2^{-\Delta ct}$	0.067316382	0.0394202	0.05393184
ΔΔct (Δct Experiment – Δct Control)	-0.3325	-0.216	-0.607
Fold Change ($2^{-\Delta\Delta ct}$)	1.259193501	1.161508732	1.52308874
P-Value from t-test	**0.05103423	0.20988847	*0.014691552
P-Value from t-test based on $2^{-\Delta ct}$	*0.045898997	0.172184758	*0.010090385

* Indicates data that is Statistically Significant ($p < 0.05$)

**Indicates data that is approaching statistical significance

Discussion/Conclusion

In this experiment the levels of the mRNA for NMDARs expressed in the mice injected with nicotine were found to be higher from those in the control mice. The results of this experiment support the hypothesis that nicotine increases the level of the NMDARs being expressed in certain parts of the brain. It suggests that nicotine could be used as a possible treatment to improve NMDARs' functioning. Therefore, one could treat the negative symptoms associated with schizophrenia that are currently left unaddressed, but are the subject of some recent studies (Mathalon, Daniel H., 2014).

The prefrontal cortex and right striatum showed up-regulation of the mRNA for the NMDARs in the experimental group compared to the NMDARs in the control group, when using the Grin 1 NMDA primer. These results means that repeated nicotine treatment had an effect on the expression of the mRNA for the NMDAR in the two brain regions tested, supporting that they each play a role in the mechanisms behind different symptoms associated with schizophrenia. The effect was region specific and the change in the hippocampus was not significantly different. This promotes the theory that the mouse brains were more sensitive to the neurotransmitters that interact with the NMDARs, which could indicate that the nicotine caused the functioning of the NMDARs to improve. In turn, the findings are consistent with those reported in previous studies on the effect of smoking, which showed a decrease in the severity of negative symptoms (Smith, et al., 2002).

The results of the two sample t-test assuming equal variance showed statistical significance for a majority of the areas of the brain studied. The prefrontal cortex data was significant when the data was converted to the $2^{-\Delta ct}$ and it was approaching significance in its raw form. The data for this area was most likely significant because a major symptom of schizophrenia is abnormal social behavior. This is also a part of the negative symptoms that are associated with the NMDAR and the prefrontal cortex moderates this social behavior. The data for the striatum was significant when the data was in both the given form, and the $2^{-\Delta ct}$ form. The significance is most likely due to the fact that people with schizophrenia also experience symptoms such as abnormal motor behavior, which could take the form of abnormal posture, and unnecessary movements. The results for the left hippocampus were not statistically significant

although there was a trend for an increase. Whether a larger sample size or longer nicotine exposure would induce effects on NMDAR expression in this region needs to be tested.

The results of this study also provide evidence that patients who smoke are using it as a way to self-medicate, since the nicotine is shown to increase the level of the NMDAR mRNA expressed, which could improve its functioning. However, the results may be unclear because they do not directly show the effect this increase has on the negative symptoms of schizophrenia. Additionally the study was done using mice that do not have schizophrenia, so it may not be an accurate model or representation.

In order to increase the validity of the results, more tests could be run, such as using more samples, or examining different parts of the mouse brains such as the frontal lobe and limbic system and by subjecting the mice to nicotine for a longer period of time. Another avenue for improvement could be to try to detect if a signal could be received from the peripheral blood lymphocytes since that was not detected in this round of data collection. This study could have been improved upon by using a different method of isolating the RNA for a greater yield. Since the cDNA used to analyze the peripheral blood lymphocytes were below the threshold, optional testing should be done to determine if this was the result of an error of the researchers, or if there is too little RNA to test in the blood.

For further research, more studies could be done testing the peripheral blood lymphocytes of post-mortem brain samples in patients with schizophrenia versus a control group, or a group of smokers with schizophrenia versus a group of non-smokers with schizophrenia. Research should also look at the side effects of using an addictive substance like nicotine, and whether or not it has the potential to be toxic.

The most important part of this research was to add a base of knowledge for more effective treatments of schizophrenia. It is necessary to run a number of studies to create potential treatments for all of the indicated symptoms to improve the lives of patients. The results of this research add credence to previous studies which show that nicotine could possibly be used as a treatment for schizophrenia. The study also points in a direction for further inquiries that could be helpful in the development of new treatments.

References

1. Buchanan, RW., Javitt, DC., Marder. SR., Schooler., NR., Gold, JM., McMahon, RP., Heresco-Levy, U., Carpenter, WT. "The Cognitive and Negative Symptoms in Schizophrenia Trial,"2007. Am J Psychiatry. 164. 1593–1602.

2. Chana, G., Bousman, CA., Money, TT., Gibbons, A., Gillett, P., Dean, B., Everall, IP., "Biomarker investigations related to pathophysiological pathways in schizophrenia and psychosis." 2013. Front. Cell. Neurosci, 7: 95. 1-18.

3. Chomczynski, P., Mackey, K., "Modification of the TRIZOL reagent procedure for isolation of RNA from Polysaccharide-and proteoglycan-rich sources." 1995. Biotechniques 19(6), 942-945.

4. Citrome, L., "Unmet Needs in the Treatment of Schizophrenia: New Targets to Help different Symptom Domains," 2014. Clinical Psychiatry. 75. 21-26.

5. Falkenberg, L.E., Westerhausen, R., Craven, AR., Johnsen, E., Kroken, RA., L. Berg, EM., Specht, K., Hugdahl, K., ''Impact of glutamate levels on neuronal response and cognitive abilities in schizophrenia." 2014. NeuroImage: Clinica. 4. 576–584.

6. Leonard, S., Mexal, S., Frank, M., Berger, R., Adams, CE., Ross, RG., Freedman, R., "Differential Modulation of Gene Expression in the NMDA Postsynaptic Density of Schizophrenic and Control Smokers." 2005. Brain Res Mol Brain Res. 139. 317-332.

7. Levin, E., Rezvani, A., "Nicotinic Interactions with Antipsychotic Drugs, Models of Schizophrenia and Impacts on Cognitive Function." 2007. Biochem Pharmacol. 74(8). 1182-1191.

8. Mathalon, Daniel H., Ahn, KH., Perry, EB., Cho, HS., Roach, BJ., Blais, RK., Bhakta, S., Ranganathan, M., Ford, JM., D'Souza, DC., "Effects of Nicotine on the Neurophysiological and Behavioral Effects of Ketamine in Humans." 2014. Frontiers in Psychiatry. 5. 1-16.

9. Noetzel, Meredith J., Jones, CK., Conn, PJ., "Emerging Approaches for Treatment of Schizophrenia: Modulation of Glutamatergic Signaling." 2012. Discovery Medical. 14(78), 335-343.

10. Risso, Francesca, Grilli, M., Parodi, M., Bado, M., Raiteri, M., Marchi, M., "Nicotine exerts a permissive role on NMDA receptor function in hippocampal noradrenergic terminals." 2004. Neuropharmacology. 47. 65–71.

11. Siegal, Steven, Ehrlichman, RS., Liang, Y., Christian, EP., Jonak, GJ., Gur, TL., Blendy, JA., Dow, HC., Brodkin, ES., Schneider, F., Gur, RC., Siegel, SJ., "Assessment of NMDA Receptor NR1 Subunit Hypofunction in Mice as a Model for Schizophrenia." 2009. Genes Brain Behav. 8(7). 661-67.

12. Smith, Robert C., Singh, A., Infante, M., Khandat, A., Kloos, A., "Effects of Cigarette Smoking and Nicotine Nasal Spray on Psychiatric Symptoms and Cognition in Schizophrenia." 2002. Neuropsychopharmacology. 27. 479–497.