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DRD2 Polymorphisms Imparting Risk for Schizophrenia

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Abstract

Schizophrenia is a disorder that affects 1% of the population and causes enormous deficits in functioning. The development of this disorder is through unclear mechanisms, yet studies suggest that genetics and dopamine processes play a major role in the manifestation of schizophrenia. This study considers the gene that encodes dopamine receptor D2 (*DRD2)* and how single nucleotide polymorphisms (SNPs) affect alternative splicing of the gene and the balance between the two different protein isoforms, Long (D2L) and Short (D2S). Four mutations (rs12363125 and rs2511521 from intron 5 and rs6275 and rs6277 from exon 7) were studied. Constructs were derived from extracted DNA from *postmortem* brain tissue of Brodmann's Area 10 of deceased patients with schizophrenia and non-psychiatric comparison subjects. Enzyme digests and ligations of patient DNA were used to create constructs that have a spectrum of mutation combinations. Quantitative polymerase chain reaction (qPCR) analysis was used to measure the proportional expression of the different isoforms. A linear regression model with an r^2 of 0.255 showed that more than 25% of the variance in D2L to D2S expression ratios (L/S Ratios) were attributable to SNPs, and rs6275 and rs6277 were found to independently and significantly affect L/S Ratios expressed by cells, with $p<0.001$ and 0.048, respectively.

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Acknowledgements

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Advice to Future Honors Students

Don't choose a project on the basis that it is easy to do, nor a laboratory because of its convenience. Find out what makes you excited, something you actually want to discover, and then find a lab to suit you, not the other way around. Anticipation of results can be just as exciting as the ending of a novel if you choose well.

I. Introduction

Schizophrenia is a severely debilitating disorder that interferes with the ability to think logically, experience emotion, succeed socially, and recognize reality. Schizophrenia (SZ) affects the emotional and social functioning of 1% of the world's population (Tsuang et al. 1997). There are many different types of schizophrenia. Some people experience psychosis, which is the manifestation of delusions or auditory or visual hallucinations. Another category of symptoms is extremely disorganized thinking and situationally inappropriate behavior. Alternatively, some individuals with schizophrenia may become extremely inactive with rigid movements and show inappropriate or total lack of emotion (National Institute of Mental Health 2009). SZ is identified as a public health concern due to its economic impact and prevalence (Rice 1999 and Goldner et al. 2002). Because this disorder typically affects individuals from early adulthood until their death, it is incredibly important to identify its underlying mechanisms so it can be more effectively treated and, eventually, prevented. The aim of this study was to identify one aspect of a potential mechanism of development by focusing on how genetic elements of dopamine receptors may contribute to schizophrenia.

Genes have been shown to have a great contribution to the development of schizophrenia. The likelihood of developing schizophrenia increases dramatically if one is related to someone with the disorder; twin studies suggest a strong

genetic influence; the heritability of schizophrenia is estimated to be from 73% to 90% (Sullivan et al. 2003).Other studies show that monozygotic twins of an affected individual have a 50% risk of developing SZ, dizygotic twins have 15% risk, and other first-degree relatives are at 10% risk (Faraone et al. 1999), which are all much higher than the 1% lifetime risk seen in the general population. Schizophrenia is thought to be derived from interactions among many genes (Risch 1990) and the environment (Gottesman and Shields 1982). This study focuses on the genetic elements that are related to schizophrenia, specifically the ones involved in coordinating environmental interactions.

The Dopamine Hypothesis is the belief that schizophrenic symptoms are a result of excess dopamine in the brain, illustrated in the experiments of Schmidt and Beninger (2006). Dopamine (DA) is a neurotransmitter that functions in motivation and reward, mood regulation, attention, working memory, and voluntary movement, all related to schizophrenia symptoms. DA has been implicated in many other neurological and psychiatric disorders including Parkinson's disease, attention-deficit hyperactivity disorder (ADHD), and drug abuse. Schizophrenic patients have been found to have elevated presynaptic dopamine synthesis and availability (Howes and Kapur 2009). Medications that target the dopamine receptor D2 (DRD2) are the most effective for treating schizophrenia symptoms (Seeman 2006). Kestler has clarified this phenomenon by finding that dysregulation and dysfunction of D2 receptors occur in the brains of schizophrenic patients (Kestler et al. 2001), further justifying our research in dopaminergic pathways.

Several mutations in the *DRD2* gene have been previously associated with schizophrenia. When a mutation causes an amino-acid substitution in the protein, the disruption is obvious. However, many SZ-associated mutations are located on non-coding regions (introns) or are silent mutations (do not cause amino acid substitution). It is important to not dismiss these "silent" mutations since these are the elements that may bind DNA-binding proteins and RNA-binding proteins and thus coordinate transcription, RNA splicing, and translation. RNA must undergo several processes before it can be translated into a protein. One of these steps is splicing; pre-mRNA molecules contain exons, which are segments of genetic sequence used directly for protein synthesis, and introns, whose functions are currently not well understood. During splicing, introns and some exons are removed from the molecule; the differential removal of exons causes different variants or isoforms of the RNA, which, subsequently, encodes different proteins. This process is modulated by alternative splicing regulator proteins (ASRs) that bind to regions surrounding segments that are to be included or removed from the pre-mRNA. Mutations may alter binding motifs of different ASRs that might cause a shift in the quantities of different protein isoforms.

In *DRD2*, a long isoform (D2L) of the protein is made which includes exon 6, whereas a short isoform (D2S) has exon 6 excluded from the molecule via alternative splicing (Bertolino et al. 2009). The D2S is a presynaptic autoreceptor associated with biosynthesis of dopamine and dopamine reuptake (Lindgren et al. 2003) and thus attenuates the DA signal. D2L is a postsynaptic receptor (Khan et al. 1998) and propagates the DA signal. DRD2 density has shown significant

effects ~~t~~on mouse working memory (Kellendonk et al. 2006). Furthermore, functional magnetic ~~-~~resonance imaging (fMRI) studies in humans have shown that in patients that had decreased D2S expression, working memory performance declined (Zhang et al. 2007); these data are interesting for the implications of this experiment because schizophrenic patients have abnormal working memory.

Studies in Dr. Stephen Glatt's laboratory indicated that a single nucleotide polymorphism(SNP) in intron 6 of *DRD2* is associated with schizophrenia and splicing (Glatt et al 2003). The rs1076560 allele shows highly significant evidence for association with schizophrenia under two models of inheritance (Glatt, S. submitted). There are alternative splicing regulators that are affected by rs1076560 because it alters their binding sites (Li et al. 2007). This allele has been found to influence the production of different forms of mRNA via alternative splicing by causing a shift in the expression ratios of D2S and D2L splice variants, where more D2L mRNA is expressed in the presence of the schizophrenia-risk allele. This is consistent with the dopamine hypothesis; if there are fewer D2S receptors regulating the presence of DA in the synapse, one would expect there to be extra dopaminergic activity. There is also an association of greater fMRI activity of the striatum and prefrontal cortex during working memory for those with the risk-conferring T allele of rs1076560 (Zhang et al. 2007).

Genetic association results from a family-based association study of *DRD2* polymorphisms in Han Chinese families from Taiwan implicated mutations in the intron 5 to exon 7 region as influencing risk for schizophrenia (Glatt, SJ et al. 2009). Others have found these same mutations to contribute to changes in the ratio of long to short DRD2 isoforms (Zhang 2007).This study was designed to pinpoint the precise mutations that have been found to be associated with schizophrenia in the intron 5 to exon 7 region of *DRD2* that result in a high ratio of D2L to D2S expression in order to clarify the factors that alter splicing through different interactions with ASRs at these loci. My hypothesis is that these SNPs increase susceptibility to schizophrenia by causing splicing imbalances.

II. Material and Methods

DNA Sample Preparations. *Postmortem* brain tissue (PMB) of patients with schizophrenia and controls was provided by Harvard Brain Tissue Resource Center (Belmont, MA) with consent of the patients' families. QIAgen (Valencia, CA) Allprep procedures and kit were used to extract DNA. 30mg of Broadman's Area 10 tissue from the PMB was lysed with 600µl of Buffer RLT Plus. The lysate was homogenized via QIAshredder. 700µl of 70% ethanol, cell contents and 500µl of Buffer AW1 were added to spin columns to separate out DNA from other contaminants. Buffer AW2 was added in 500µl volumes to wash columns. DNA was eluted with 100µl Buffer EB.

DNA was amplified via polymerase chain reaction with DRD2 e5-e7 forward and reverse primers (Figure 1).This procedure utilized Phusion High-Fidelity PCR kits from New England Biolabs (Ipswich, MA). To 10µl of template DNA, 10µl of 5x Phusion Master Mix, 1µl 10mM dNTPs, 1µl DMSO, 0.5µl of DNA Polymerase, 1µl of Mg^{2+} at 50mM, and 100µM of each primer were added to PCR. Using a MJ Research Peltier Thermocycler 225 from Harlow Scientific (Arlington, MA) the following incubation steps were completed: denaturing step for 30s at 98^{o}C, 30 cycles of denaturing at 98^{o}C for 10s, annealing at 60^{o}C for 20s and extension steps at 72^{o}C for 90s, and a final step at 72^{o}C for 10 minutes. The reaction was then run on a 0.75% agarose gel to confirm successful amplification.

The amplified DNA region was then inserted into a pcDNA 3.1 vector from Invitrogen (Grand Island, NY). First, both the DNA and vector were digested using 1µleach of HindIII and XhoI in 5µl 10x Buffer 2, and 0.5µlof BSA. (All reagents were from New England Biolabs). The reaction was incubated at 37^{o}C for 1 hour and ran on a 0.75% agarose gel.

Using T4 DNA Ligase Kit from Invitrogen, cut DNA inserts and vectorswere ligated. Reactions were conducted with 4µl of 5x ligase reaction buffer, 0.1µl of T4 DNA ligase, and brought to a total volume of 20µl via addition of water. All water used in procedures is autoclaved, distilled, RNAse, DNAse, and Protease free water from Acros Organics of Thermo Fisher Scientific (New Jersey). The reaction was held at room temperature for 10 minutes.

Minipreps of the ligation reaction were made to amplify the quantity of DNA. First, 2µl of the reaction was added to 50µl of Top 10 Competent cells (e. coli) from Invitrogen. To complete this transformation, reactions were incubated on ice for 30 minutes, heat shocked at 42^{o}C for 30s, returned to ice for 2 minutes, and then added to SOC (Super Optimal Broth with Catabolite repression) medium. Cells were incubated at 37^{o}C at 250rpm for 1 hour, and plated on agar with Ampicillin antibiotic (at 100µg per ml) to grow overnight. Using QIAgen Plasmid DNA Miniprep kit, the DNA constructs were sequestered from the transformed bacterial colonies.Cells were suspended in 250µl of Buffer P1 and lysed by 250µl of Buffer P2. 350µl of Buffer N3 and cell contents were added to QIAprep spin column and washed with 500µl of Buffer PB and 750µl of Buffer PE. DNA was eluted twice with 40µl of water.

Sequencing of these products, and all others, wasdone by Genewiz (South Plainfield, NJ).Samples were sent with 10µl of 80ng/µl of DNA sample and 5µl of 4µM of primer. Results were analyzed using Geneious Pro software (New Zealand).

Creation of Constructs. Patient constructs were digested with BamHI and XhoI restriction enzymes from New England Bio Labs (NEB) in Buffer 4 supplemented with bovine serum albumin. Reaction volumes were brought to 50µl through the addition of water. Reactions were incubated at 37^{o}C for 1 hour, and terminated by running on a 1.5% agarose gel for 90 minutes at 120V.

The subsequent digest was performed with BsaI restriction enzyme from NEB in the same conditions as the first digestion procedure, except a 1% agarose gel was used to terminate the reaction and separate fragments.

A spectrophotometer, NanoDrop 2000 from Thermoscientific was used to measure the DNA concentration of each fragment of DNA. Ligations were done using the Invitrogen T4 DNA Ligase Kit with the same protocol as described above. Reactions were performed with a 3:3:1 molar ratio of the two larger DNA fragments to the smallest DNA fragment. Reactions were conducted with 4µl of 5x ligase reaction buffer, 1µl of T4 DNA ligase, and brought to a total volume of 20µl via addition of water. Reactions were incubated at 37^{o}C for 1 hour and transformed using the same procedure previously described.

Ligation reactions were plated on agar with ampicillin (100µg/1ml) via glass beads, and left to grow overnight while incubated at37^{o}C and shaking at 250rpm. Colonies were picked and grown in 4ml of LB broth with 40µl of ampicillin and grown overnight in the same conditions for QIAgen Minipreps.

Constructs were digested with Bsa I restriction enzyme using previous digestion protocols to identify potential successful ligation reactions. Those that matched the same gel profile of original patient constructs were selected to send out for sequencing.

Cell Culture and Transfection. Human embryonic kidney cells were grown in 24 well plate in bovine serum free media and grown to 80% confluency. Cells were transfected using 1.6µl of FuGene transfection factor from Promega (Madison, WI) per reaction with 0.5 ug of DNA and media to bring the total reaction volume to 40µl. Each sample was transfected into twelve wells and kept separate throughout the experiment. Cells were left to grow for 48 hours at 37^{o}C.

Harvesting RNA. Using QIAgen RNeasy kit, cells were lysed with 250µl Buffer RLT. 350µl of 70% ethanol was added, and all was added to an RNeasy spin column. 350µl RW1 buffer was added to wash RNA. 80µl of DNase Incubation Mix was added to the column to degrade possible DNA contaminants. The samples were washed again with 350µl Buffer RW1. 500µl Buffer RPE was added twice, and RNA was eluted with 40µl of water twice. RNA concentrations were found with a Synergy 2 plate reader from Biotek (Winooski, VT).

RNA was then transformed to cDNA using a High Capacity cDNA Reverse Transcription kit from Applied Biosystems (Carlsbad, CA). 2µl 10x RT Buffer, 2µl Primers, 0.8µl 25x dNTPs, 1µl of Reverse Transcriptase and 4.2µl of water were added to 10µl of 100ng/µl RNA. Using the same Thermocycler, samples were incubated at 25 ^{o}C for 10 minutes, 37 ^{o}C for 120 minutes, and 85 ^{o}C for 5 minutes.

Qualitative PolymeraseChain Reaction. Taqman reagents and protocol from Applied Biosciences were used for this experiment. The cDNA used was diluted to 5ng/µl, and 4µl were used with 2µl of primer, 10µl Taqman Master mix, and 4µl of water per reaction, was loaded into a well on a 384-well plate. There were 12 samples per construct, and three primers used, and each reaction was replicated. The primers measured *DRD2* Long, *DRD2* Short, and B-Actin, and are also found in Figure 1. The plate was then placed into a LightCycler 480 from Roche Applied Science (Indianapolis, IN). The following incubation cycles were used for the reaction: 50^{o}C for 2 minutes, 95^{o}C for 10 minutes, and 50 cycles of 95^{o}C for 15s and 60^{o}C for 1 minute. To calculate threshold, a default setting called 2ndDerivative Maximums was used.

Statistical Analysis. Cycle numbers of D2L and D2S were normalized using β-Actin (βA)cycle numbers. Ratios were computed using the following formula:

$$\textbf{L/S Ratio} = 2^{-[(D2L-\beta A)-(D2S-\beta A)]}$$

Data included were found within 1.5x Interquartile Range $\pm$ the upper and lower quartile values One value was excluded from sample from construct ATTTT. Kolmogorov-Smirnov statistics were used to determine the normality of the L/S Ratio results, and a multivariate linear regression model calculated with STATA software from StataCorp (College Station, Texas) was used to analyze the significance and weights of the various SNPs and the coefficient of the data.

III. Results

Postmortem brain tissue samples of identified persons with schizophrenia and controls (non-schizophrenic individuals) were used to find the *DRD2* genetic sequence of these individuals. The brain tissue provided *DRD2* DNA for PCR amplification of the region from intron 5 to exon 7, and segments were inserted into plasmids; thus one patient could yield two constructs with different haplotypes from its two natural alleles. Selected subjects varied at four mutation sites: rs12363125 with a G or A base pair (bp), rs2511521 with a C or T, rs6275 with a C or T, and rs6277 with C or T. However, many of the SNPs were linked; those with rs12363125A would always have a rs2511521T, and rs6275C would always have a rs6277T. Because of this linked heritage, I had to create new constructs that would demonstrate the entire spectrum of SNP combinations.

The exon 7 alleles, rs6275 and rs6277, are only 18 nucleotides apart. Only one restriction enzyme had a binding site between these SNPs that could separate these two mutations. However, because this enzyme had multiple restriction sites on the *DRD2* gene and its plasmid vector, more steps had to be taken. First, a double digest was produced to excise exon 7 and its surrounding region from the patient constructs, while the remainder contained intron 5 which has both rs1236125 and rs2511521 sites. Next, the exon 7 fragment was digested to separate rs6275 and rs6277. See figure 2.

Construct Map:

Enzyme Cut Sites, Fragments, and SNPs

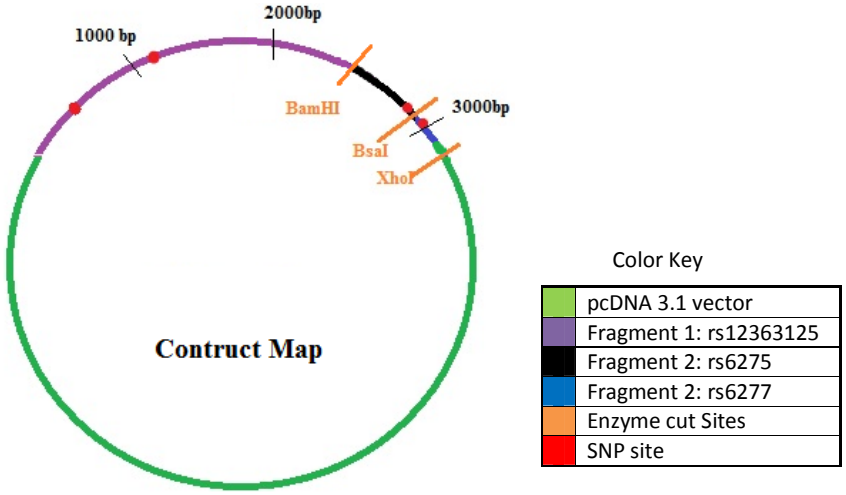

Figure 2. Construct map is provided, with increments of 1000bp shown with the first base pair at the start of *DRD2* region. SNPs are shown, indicated in red: from left to right are allele sites rs12363125, rs2511521, rs6275, and rs6277. The fragments produced through enzyme digests are shown in purple, black and blue, while the pcDNA vector is shown in green. Orange marks the sites of the enzymes used to create constructs.

This entire process was done on two reciprocal patient constructs, which allowed six different *DRD2* profiles to be created through ligation reactions. The first segment of the construct would have rs2511521 and rs1236125, the middle segment would contain rs6275, and the remaining segment would have rs6277. Because the first segment contained both rs2511521 and rs1236125, the alleles became linked in the experiment: a "G" at the rs12363125 site was always paired with a "C" at the rs2511521 site. Further work could be done to separate the two linked loci and ligate them to existing rs6275-rs6277 combinations to provide a full compendium of constructs for future experiments. The ligation reactions were grown overnight, and colonies were selected and sequenced to confirm ligation success.

Overall, five constructs were created through strategic matching of segments from patients with GCTC and ATCT profiles. Because one of the potential *DRD2* profiles that could have been created through this process was actually present in a patient, the patient DNA was used for the ATTC profile instead. The original patient DNA used to create the constructs was also included, making a total of eight constructs for the experiment (Figure 3).

The constructs were transfected into cells that were then grown for 48 hours. The RNA was then harvested from the cells; it was assumed that the amount of RNA representing one isoform directly correlated with the quantity of the protein isoform that was synthesized or expressed. Reverse transcriptions and quantitative polymerase chain reactions were performed.

***DRD2* Experimental Constructs:**

Alleles at the Four Mutation Sites

SNPs	GCTC*	ATTC*	ATCT*	GCTT	GCCC	GCCT	ATCC	ATTT
rs12363125	G	A	A	G	G	G	A	A
rs2511521	C	T	T	C	C	C	T	T
rs6275	T	T	C	T	C	C	C	T
rs6277	C	C	T	T	C	T	C	T

Figure 3. Displayed are the eight constructs (*natural constructs found in patient PMB tissue) used in this experiment. The constructs had identical sequences besides the alleles at the four SNP sites associated with schizophrenia found in the *DRD2* gene.

For the qPCR, primers were used to amplify Short (D2S) and Long isoforms (D2L) of the *DRD2* region by using exon-exon boundary specific oligonucleotides (D2L primer binding site was at the boundary of exon 6 and 7, while the D2S primer binding site was where exon 5 met exon 7). β- Actin (βA) primers acted as the housekeeping gene to control for varying levels of expression by HEK cells. It was assumed that the number of cycles it took to reach threshold represented the original quantity of RNA present in the cell; thus those that reached threshold earlier had more RNA expressed. These data contributed to calculating the Long to Short ratios (L/S Ratios) of the *DRD2* isoforms.

The L/S Ratio values were averaged over 12 trials, and the results are shown in Figure 4. One data point was excluded as an outlier after performing interquartile range calculations; it was a L/S Ratio of 4.155 from construct ATTT. The construct with the highest average L/S ratio was GCTC, and the smallest average was with construct GCCT. The L/S range of the 95 data samples were from 1.564 to 2.420 with a mean of 1.962 and a median of 1.959. The data follows a normal distribution with an insignificant Kolmogorov-Smirnov statistic ($p=.076$). See figure 5.

Experimental Results:

Averaged Long to Short Isoform Ratios

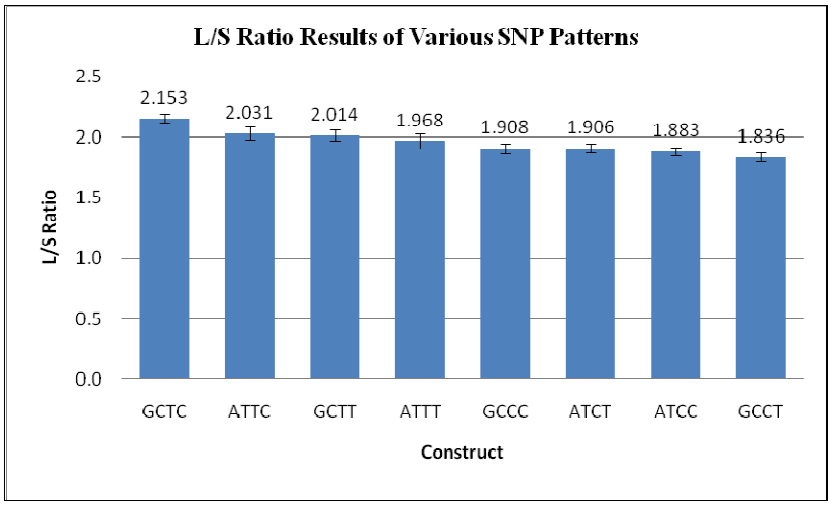

Figure 4.L/S Ratio averages from qPCR for each construct ordered highest to lowest. N=12, except for ATTT: N=11. Error bars are constructed by using the mean ± standard error.

Frequency of L/S Ratio Data Points:

A Histogram to Determine Normality of Curve

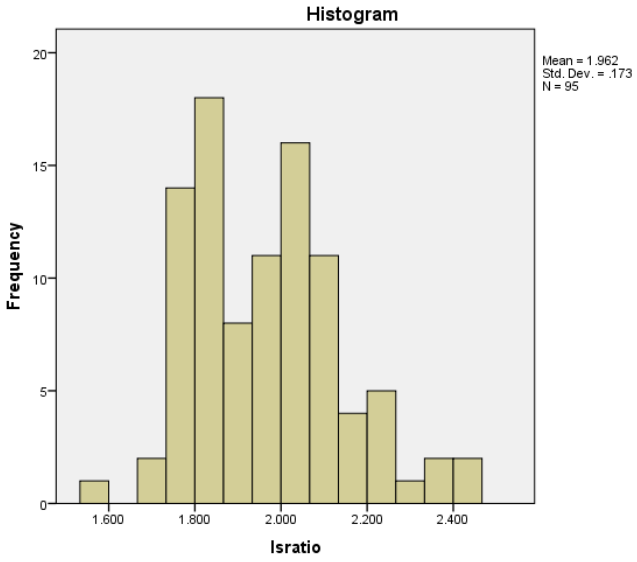

Figure 5.Historgram recording frequency of L/S Ratios from the study with 95 samples. Computed via Stata 9 sofware.

A multiple regression analysis was calculated using rs2511521, rs6275, and rs6277 mutation variables (Rs2511521 and rs12363125 are co-linear because of the links previously described, thus no individual contribution from one or the other mutation could have been identified.) The SNPs in combination were shown to significantly affect expressed L/S ratio with $F(3, 91)$, $df=94$, $p \leq 0.0001$. The coefficient of determination of the model (r^2) was calculated to be 0.255, which indicates that the SNPs account for more than 25% of the variance in the observed D2L:D2S expression ratios.

The combinations of rs2511521 and rs12363125 mutations (GC or AT) showed no significant influence on L/S ratio with a coefficient of -0.0149 and standard error of 0.156, t=-0.96, and p=0.341.

Rs6275 and rs6277 showed significant results. The allele at rs6275 had a 0.793 coefficient, a 0.156 standard error, a 5.09 t value, and a p value of <0.001. The allele at rs6277 had a β-coefficient of -0.0312, standard error of 0.156, a t value of -2.00, and p=0.048.See figure 6.

Multivariate Linear Regression Analysis Results:

The Relative Contribution of Four Alleles

SNP	Coefficient	Std. Err.	t	P
rs12363125	-0.0149	0.156	-0.96	0.341
rs2511521	-0.0149	0.156	-0.96	0.341
rs6275	0.0793	0.156	5.09	0.000
rs6277	-0.0312	0.156	-2.00	0.048

Figure 6.Multivariate linear regression model results. Coefficient represents the direction and amount of contribution of each SNP, t is the value statistic, while p determines the possibility that the data was produced at random. A rs6275T significantly increases L/S Ratio while an rs6277T decreases L/S Ratio. There were no significant contributions to L/S Ratio from rs12363125 and rs2511521 in this study.

IV. Discussion

The results suggest that the rs6275 allelotype contributed the most to determining DRD2 variant ratios and that rs6275T allele in particular was associated with an elevated L/S Ratio. In contrast, the rs6277 T allele was associated with a significantly lower L/S Ratio, though its effects are not as strong. The rs2511521 and rs112363125 SNPs were not found to significantly affect L/S Ratio in this experiment.

The rs6275 SNP is a synonymous mutation in the *DRD2* exon 7. Other studies have found it to be associated to schizophrenia (Monahkov et al.2008 and Vijayan et al. 2007). Viajayan et al. measured the behavioral effects of rs6275 and found those homozygous for the T allele responded well to medication (Viajayan et al. 2007).

The rs6277 allele has also been linked to schizophrenia by more studies (Fan et al. 2009, Monakhov et al. 2008, Betcheva et al. 2009, and Lawford et al. 2005). The T allele at this site has a protective effect from schizophrenia, though it causes decreased D2 synthesis, reduced mRNA stability, and a decreased response to upregulation of *DRD2* by dopamine (Duan et al. 2003). It was also found that healthy humans show decreased striatal binding of D2 with those homozygous with the C allele (Hirvonen et al. 2004). Finally, those with the T allele outperformed others in working memory tests (Xu et al. 2007, and Rodriguez-Jimenez et al. 2006).

These results are very interesting in conjunction with previous studies. Perhaps the altered protein folding pattern caused by rs6277enables more expression of the DRD2 short variant. Furthermore, rs6275 has been strongly linked to higher L/S ratio, independent of the contribution from the rs6277C allele. These observations suggest a complex genetic contribution involved in schizophrenia.

The next step of this study would be to identify the mechanisms through which these mutations affect splicing. The alternative splicing regulators that are affected can be determined through comparisons of co-transfections of constructs with ASRs to transfections with constructs alone, protein mobility shift assays that measure the binding ability of ASRs to mRNA with different genetic profiles of *DRD2*, and research splicing databases can identify ASRs whose binding sites are affected by the SNPs. Understanding the mechanisms of *DRD2* splicing can help unravel a small part of the enigma that is schizophrenia, and bring us closer to identifying, preventing, and treating the disorder.

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VI. Summary

Introduction

Schizophrenia is a severely debilitating disorder that interferes with the ability to think logically, experience emotion, succeed socially, and recognize reality. There are many different manifestations of schizophrenia. Some persons experience psychosis, which is the experience of delusions or auditory or visual hallucinations. Another category of symptoms is extremely disorganized thinking and situationally inappropriate behavior. Alternatively, some individuals with schizophrenia may become extremely inactive with rigid movements and show inappropriate or total lack of emotion(National Institute of Mental Health 2009). Schizophrenia affects about 24 million people across the globe, 1.1% of the United States population (Tsuang et al. 1997). Schizophrenia typically develops in early adulthood and lasts until death. In order to help diagnose, treat, and prevent schizophrenia, it is incredibly important to learn the mechanisms of the disease.

Genes have been shown to greatly contribute to developing schizophrenia. The likelihood of developing schizophrenia increases dramatically if one is related to someone with schizophrenia; it is 50% likely that an individual whose twin has schizophrenia will also develop it(a much higher likelihood than for those in the general population), strengthening the hypothesis that genes contribute to the disorder (Faraone et al. 1999). This study focused on genetic elements concerning a dopamine receptor related to schizophrenia.

The Dopamine Hypothesis is the belief that schizophrenic symptoms are a result of excess dopamine in the brain (Schmidt and Beninger 2006). Dopamine (DA) is a neurotransmitter that functions in motivation and reward, mood regulation, attention, working memory, and voluntary movement- all related to schizophrenia symptoms. It has been implicated in many other neurological and psychiatric disorders including Parkinson's disease, attention-deficit hyperactivity disorder (ADHD), and drug abuse. Research has shown that the medications most effective for treating schizophrenia symptoms target the dopamine receptor D2 (DRD2) (Seeman 2006). Receptors are proteins that dopamine molecules bind to, and subsequently elicit responses in the cell. Another important study found that the regulation and function of D2 receptors was disrupted in schizophrenic patients (Kestler et al. 2001) further justifying our research in dopaminergic pathways.

Several mutations in the gene that encodes the D2 dopamine receptor, *DRD2*, were found to be strongly associated with schizophrenia. The genetic code is the starting block for cellular protein synthesis. DNA in the nucleus of the cell is transcribed into RNA, which is the intermediate substrate used to build proteins. RNA must undergo several processes in the cytosol of the cell before it can be translated into a protein. One of these steps is splicing; RNA molecules contain exons, which are segments of genetic sequence used directly for protein synthesis, and introns, whose functions are currently not well understood. During splicing, introns and some exons are removed from the molecule; the differential removal of exons causes different variants or isoforms of the RNA, which,

subsequently, encodes different proteins. In DRD2, a long isoform (D2L) of the protein is made which includes exon 6, whereas a short isoform (D2S) has exon 6 spliced out of the molecule (Bertolino et al. 2009). The D2S and D2L receptor proteins have different functions and are located on different regions of the neuron (Lindgren et al. 2003 and Khan et al. 1998). Furthermore, functional magnetic resonance imaging, commonly known as fMRI, studies in humans have shown that decreased D2S variant expression (number of protein/RNA present) significantly affects working memory performance, an activity where patients with schizophrenia typically struggle (Zhang et al. 2007).

My project aims were to pinpoint which mutations or SNPs in the *DRD2* gene result in a high ratio of DRD2 Long to DRD2 Short expression, or relatively lower D2S expression, in order to then clarify the splicing factors that alter DRD2 splicing by interacting differently with the alternate alleles at these loci.

Methods

Postmortem brain tissue samples of identified persons with schizophrenia and controls (non-schizophrenics) were used to find the *DRD2* genetic sequence of these individuals. Selected subjects varied at four mutation sites: rs12363125 with a G or A base pair (bp), rs2511521 with a C or T, rs6275 with a C or T, and rs6277 with C or T. However, many of the SNPs were linked; those with rs12363125A would always have rs2511521T, and rs6275C would always have rs6277T. Because of this linked heritage, I had to create new constructs that

would demonstrate the entire spectrum of SNP combinations (Figure 1). These were constructed by taking existing subject DNA and cutting them at strategic sites with enzymes. The newly created segments were ligated, or re-attached to one another in different ways to create the necessary constructs. With this array of constructs, all possible *DRD2* mutation combinations were represented.

SNPs	GCTC*	ATTC*	ATCT*	GCTT	GCCC	GCCT	ATCC	ATTT
rs12363125	G	A	A	G	G	G	A	A
rs2511521	C	T	T	C	C	C	T	T
rs6275	T	T	C	T	C	C	C	T
rs6277	C	C	T	T	C	T	C	T

Figure 1. Constructs used and their varying combinations of SNPs.

To quantify the ratio of D2L and D2S expression, quantitative polymerase chain reaction (qPCR) was conducted. This analysis started by transfecting cells by inserting a circular genetic ring containing a version of the *DRD2* gene into them. As these cells grow, they create RNA which then creates the different DRD2 isoforms. After two days, we extracted the RNA from the cells; the amount of RNA representing one isoform should directly correlate with the amount of that protein isoform synthesized or expressed. The RNA was then transformed back into cDNA(DNA that only includes exons), via reverse transcription reaction. The cDNA was then used for PCR which is a process by which specified gene regions are amplified, where in each cycle of the reaction, the cDNA region is replicated, thus the number exponentially increases. The primers that enable this reaction fluoresce or glow when used, and thus the amount of fluorescence measured by a qPCR machine represents the quantity of that gene region in the reaction. If there

Comment [SG1]: This is an assumption, right? Not demonstrated here…

Comment [SG2]: Re-word for more clarity.

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was more D2L from one cell, it would reach a detectable threshold quantity faster. These data contributed to calculating the Long to Short ratios (L/SRatios).

Results

In my experiment, we ran a qPCR twelve times per sample and found the following results displayed in Figure 2. The L/S range of the 95 data samples were from 1.564 to 2.420 with a mean of 1.962 and a median of 1.959. There was one data point (from construct ATTT) that was a computed outlier, and thus deleted from the study.

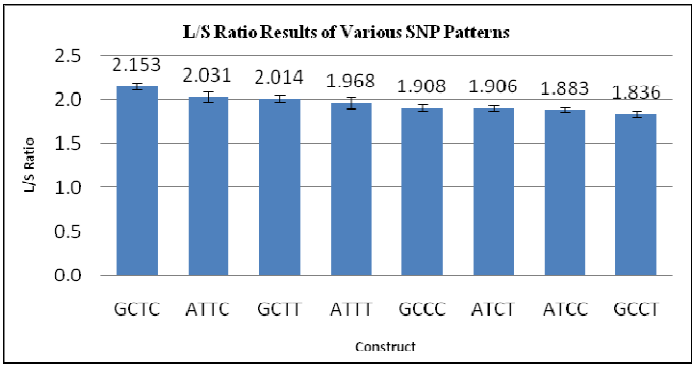

Figure2. Average Long/Short isoform expression measured from QRT-PCR reactions. Data is ordered by highest to lowest L/S ratios.

A multivariate linear regression analysis was calculated to determine the relative weight (represented by coefficients) and significance (represented by p-values) of each mutation to the gene's L/S Ratio by creating a regression line, or formula that would help predict L/S Ratio from assorted SNP combinations. Because rs2511521 and rs12363125 were always paired in the study, rs2511521

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Comment [SG5]: Data are plural, whereas the singular for a data point is "datum"

Comment [SG6]: Mention the use of a housekeeping gene as a means of standardization for mRNA quantity.

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Comment [SG8]: Based on what criteria?

(representing both SNPs), rs6275, and rs6277 were analyzed (see Figure 3). The coefficient of determination of the model (r^2) was calculated to be 0.255, which means that with the regression analysis results, 25.5% of the L/S data can be attributed to the combination of SNPs, which is considered a strong relationship in biology. The alleles at rs6275 and rs6277 were found to be statistically significant in determining L/S Ratios, meaning that the likelihood the data was collected merely by chance was $p<0.001$ and $p=0.048$, respectively.

SNP	Coefficient	Std. Err.	t	P
rs2511521	-0.0149	0.156	-0.96	0.341
rs6275	0.0793	0.156	5.09	0.000
rs6277	-0.0312	0.156	-2.00	0.048

Figure 3. Results from multiple linear regression analysis.

Discussion

The results suggest that rs6275 allelotype contributed the most to determining DRD2 variant ratios and that the rs6275 T allele in particular was associated with an elevated L/S Ratio. In contrast, the rs6277 T allele was associated with a significantly lower L/S Ratio, though its effects are not as strong. Rs2511521 and rs112363125 were not found to significantly affect L/S Ratio in this experiment.

These results are very interesting in conjunction with previous studies. The T allele at the rs6277 site was found to alter protein folding, decrease mRNA stability (which affects its ability to persist as a molecule) and decrease overall expression (Duan et al. 2003). Perhaps the altered protein folding pattern caused

by this mutation enables more expression of the DRD2 short variant. Also, both rs6275 (Monahkov et al.2008 and Vijayan et al. 2007) and rs6277 (Fan et al. 2009, Monakhov et al. 2008, Betcheva et al. 2009,and Lawford et al. 2005) have been associated with schizophrenia; their contribution to DRD2 splicing may lead us to understand how they are involved in the disease.

This study can help launch schizophrenia research down many pathways. I plan to further investigate how these mutations alter DRD2 isoform expression by looking at splicing regulators, proteins that bind to RNA to help direct splicing. Protein mobility shift assay is a technique by which we can determine if a splicing regulator protein binds to an RNA sequence. These results can be compared to the same sequence with mutations to see if the binding site for the splicing regulator is altered. This could help identify what proteins are affected by SNPs, advancing treatment of schizophrenia. Understanding the mechanisms of *DRD2* splicing can help unravel a small part of the enigma that is schizophrenia, and bring us closer to identifying, preventing, and treating the disorder.

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