

Elevated *DISC1* transcript levels in PBMCs during acute psychosis in patients with schizophrenia

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Abstract

Background: Severe mental disorders like schizophrenia are a leading cause of disability in people in the prime years of their lives (aged 15 to 44 years). Relapse is a primary contributor to schizophrenia disease burden and is frequently attributed to medication noncompliance and inadequate doses. Currently, a patient's neuroleptic dose is titrated to clinical response within recommended dose ranges. Use of unbiased biomarkers of effective neuroleptic treatment-response would greatly facilitate the identification of a person's lowest effective dose to minimize unsafe side effects and improve compliance. Biomarkers may allow precisely tailored adjustments of neuroleptic dose to reduce relapse due to variable disease course.

Methods and Findings: Biomarkers of active psychosis were sought among persons with schizophrenia hospitalized with acute psychosis. The transcriptional response of peripheral blood mononuclear cells (PBMCs) to treatment of psychosis was measured using RNA expression profiling in 12-paired samples from patients with schizophrenia. The paired samples were collected early after treatment initiation and again just before patients were released from the hospital. Patients showed significant improvement in positive symptoms of psychosis assessed at each sample collection using a brief psychiatric rating scale (BPRS) ($P < 0.05$). Preliminary evidence is presented indicating that decreased transcript levels of isoforms of disrupted in schizophrenia 1 (*DISC1*) measured in PBMCs were associated with treatment in 91% of samples ($P = 0.037$).

Conclusion: Further studies are warranted to identify neuroleptic-response biomarkers and to replicate this initial finding of association of *DISC1* transcript levels with treatment of psychosis.

Introduction

Neuroleptic medication is clearly efficacious in treating schizophrenia, however, 61% of patients with schizophrenia have a relapsing and remitting course [1]. Relapse is a primary contributor to schizophrenia disease burden [2] that results from non-compliance and inadequate dosing. Neuroleptics frequently do not alleviate all psychotic symptoms making identification of an optimal dose by subjective means a challenge and ap-

proximately 46% of patients in community mental health services receive suboptimally prescribed neuroleptics [3]. In addition, 27-50% of patients on injectable neuroleptic medication relapse each year into acute psychosis consistent with a need to adjust medications in accordance with a variable disease course [4]. An objective measure of neuroleptic-response is required that would allow identification of the lowest efficacious dose and reduce unsafe side effects associated with high doses of neuroleptic medication [5].

Two of 3 multicenter clinical antipsychotic trials showed that neuroleptics have similar clinical efficacy [6-8] and the therapeutic window is achieved by 50-80% dopamine receptor D2 (DRD2) occupancy [9, 10]. Increased DRD2 mRNA levels in peripheral blood lymphocytes served as a diagnostic biomarker for 13 medication-naïve male and female patients with schizophrenia [11], but not in 30 patients with variable treatment histories [12] suggesting DRD2 receptor levels on lymphocytes may be altered by neuroleptic treatment. This evidence together suggests that some neuroleptic-response biomarkers may be related to the DRD2 pathway. Plasma haloperidol levels are correlated with D2 occupancy in the brain [13] showing the utility of using peripheral blood as a surrogate for relevant neuroleptic effects in the brain.

Identification of a large Scottish family with a balanced translocation breakpoint in the disrupted in schizophrenia 1 (DISC1) gene that cosegregates with all 7 family members with schizophrenia and 10 other members with major depression, is the best genetic evidence that DISC1 is involved in schizophrenia (LOD score 7.1) [14]. When identifying causative genes in Mendelian diseases, the focus is on the identification of mutations that clearly disrupt the gene in more than one family and co-segregate with disease. DISC1 is promising in this regard. There appears to be clinical heterogeneity and reduced clinical penetrance in the two families described with 2 different rare mutations (the second family has a 4 base pair deletion in the coding region of DISC1) [15, 16]. Several common single nucleotide polymorphisms (SNPs) in DISC1 [17] and DRD2 [17] have been associated with schizophrenia and they are among the genes most strongly associated with schizophrenia through meta-analysis [18]. Multiple proteins have been identified that interact with DISC1 during neurodevelopment [19-25]. The N terminal region of DISC1 physically interacts with glycogen synthase kinase 3 beta (GSK3 β) [24]. GSK3 β is also regulated through both the dopamine D2 receptor (DRD2) and serotonin pathway as shown by pharmacological studies and analyses of knockout mice [26]. AKT1 also directly inhibits GSK3 β and haplotypes of AKT1 have been associated with both reduced levels of AKT1 protein and with schizophrenia [27]. In addition, neuroleptic treatment was shown to change levels of AKT1 and GSK3 β phosphorylation in mouse brain [27] and in vitro [28]. Together this evidence is consistent with the DRD2/DISC1 pathway being involved in schizophrenia and the treatment of schizophrenia.

In the present study, treatment-response biomarkers were sought in PBMCs by identifying transcripts that were similarly up or down regulated by microarray analysis of 6 paired samples collected early and late during treatment of psychosis in a heterogeneous group of patients hospitalized with schizophrenia. Further quantitative analysis was performed on one promising treatment-response biomarker, DISC1, that was selected based on known genetic involvement in schizophrenia [14] and involvement in the dopamine DRD2 pathway [24].

Methods

Participants

Patients experiencing psychosis were enrolled with consent within 3 days of being admitted to the psychiatric ward at Denver Health Medical Center (Colorado Multiple Institutional Review Board protocol #04-0853). Upon collection of paired blood samples, patient's symptoms were assessed with a BPRS performed by the same interviewer. Therapeutic interventions had been initiated before the time of the 1st blood draw. Electronic records of neuroleptic doses administered up to 24 hrs before each blood draw were employed to calculate the haloperidol equivalents using dose equivalencies determined by Andreasen et al. 2010[29]. Blood samples (30 ml) were collected in ice cold sodium heparin 1 ml tubes BD Vacutainer® (BD, Franklin Lakes, NJ) around 2 pm. Lymphocytes were separated from 16 ml of peripheral blood as directed using Unisep™ (Axis-Shield, Oslo, Norway). RNA was isolated from lymphocytes as directed using TRIZOL® Reagent (Invitrogen, Carlsbad, CA). Diagnoses were made based on a Structured Clinical Interview for Diagnostic Interview for Genetic Studies (DIGS) and review of medical records[30].

Microarray transcript analysis

Six paired RNA samples from patients with schizophrenia showing the most improvement between paired samples were further purified using QIAGEN RNeasy mini kit (Qiagen, Valencia, CA) for gene chip analysis. RNA quality was assessed using the Agilent Bioanalyzer 2100 (Quantum Analytics, Inc. Foster City, CA). RNA (50 ngs) was amplified and labeled as directed by Ovation™ System (NuGEN Technologies Inc., San Carlos, CA). The labeled cRNA was hybridized to Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA). Affymetrix data was normalized with the MAS5 filter. Pair wise comparison analysis was done using Affymetrix GCOS v1.4 data analysis software to identify genes similarly up or down regulated across the 6 paired samples. Paired T-test analysis was performed using Partek Genomic Solutions (Partek, St. Charles, MO) to determine p-values for the differentially expressed genes.

Reverse-transcribed Quantitative PCR (RT-qPCR)

Bioanalyzed RNA of good quality was reverse transcribed in triplicate using 0.29 ugs of RNA per tube in a 20 ul reaction. For each sample one reaction without reverse transcription was performed in a 16.8 ul reaction. RNA incubated at 65°C for 5', then 4°C. Reverse transcription mix was added and incubated at 25°C for 10' then at 42°C for 1 hour, then 99°C for 5'. Reaction mix was Superscript™ II (2.5 Units per ul in 1x First-Strand Buffer, 0.01M dithiothreitol (DTT), 8 mM random hexamers (GE Healthcare, Uppsala, Sweden), and 0.5 U/ml placental RNase inhibitor (Roche Applied Science, Indianapolis, IN).

Quantitative PCR was performed using the DISC1 primer set VI (Table 1) designed using PerlPrimer v1.1.14 [31] and normalized with an 18S amplicon primer set VII (Table 1). The DISC1 and 18S amplicons had correlation coefficients of 0.979 and 0.985 with PCR efficiencies of 89.5% and 97.6% based on analysis of 5 and 9 point standard curves respectively. The cDNA template (2 ul) was qPCR'd with the primers at 140 nM final concentrations in 1X SYBR green I dye and fluorescein mix with Thermo-Start DNA polymerase in a total reaction volume of 27 ul(Thermo Fisher Scientific Inc. Waltham, MA). Both assays were performed on a BioRad iCycler® (BioRad, Hercules, CA) using the following program: 95°C for 15'; (95°C for 15"; 60°C for 1') for 40 cycles followed by a melt curve. Relative gene expression was determined using both the Livak $2^{-\Delta\Delta C(t)}$ and Pfaffl methods with iQ5™ version 2.0 (BioRad, Hercules, CA). Significance was tested with a paired T-test. The DISC1-biomarker assay was tested using various control templates including DNA only, DNase treated RNA, poly A isolated RNA, water, RNA without reverse transcriptase. DISC1 isoforms were sought in RNA from PBMCs (Table 1).

Results

Sample

A total of 16 paired samples were collected from patients. Four paired samples were removed from analysis due to poor quality RNA (2 paired samples), no improvement between paired samples (1 paired sample), a compromised 2nd BPRS interview (1 paired sample). All 12 subjects whose samples were utilized for transcript quantification met DSM IV criteria for schizophrenia. Patient S33 was diagnosed with schizophrenia and bipolar disorder. There was significant improvement in positive symptoms assessed with the BPRS ($P = 0.01$; Table 2) and improvement in total BPRS scores between samples ($P = 0.08$). The 12 patients were on average 48 years old and paired samples were collected an average of 5.8 days apart. Patient S31 was the only female. Patient S39 was the only black male all others were Caucasian. Neuroleptics administered within 24 hours before sample collection were at a mean dose of 12.5 haloperidol equivalents [29] and did not significantly change from

TABLE 1 Primer sets.

Set	Transcripts	Sequence	Location	bp
I	DISC1q	F1-CAGCCCAGGCGGAGCGGGAGGA	exon 1 [32]	
		F2-GGAGCTGGCAGCGGGCGCATG	exon 1 [32]	1179
		R1-CCCTGGTAGAGATGATCAGAGGAACA	3'UT of exon 3 [32]	
		R2-GGGACATGATGACAAAACAATC	3'UT of exon 3 [32]	
II	AK025293	F-TCACCCAAGATGCCTCCAGAAGAA(AG)	AK025293 exon 1	1271
		R1-CCCTGGTAGAGATGATCAGAGGAACA	3'UT of exon 3 [32]	
		R2-GGGACATGATGACAAAACAATC	3'UT of exon 3 [32]	
III	AK023443	F-GAAGAAGTTCAGTTTTGAAACAG		
		R-AACTATTCCCTGGTAGAGATG	3'UT of exon 3	312
IV	TSNAX-DISC1-7	F1-TTTCCAGGTTCCCTCGGCCTGTA	TSNAX exon 1 [32]	
		F2-TGAGGAACATGGACGAGATGGGAA	TSNAX exon 7 [32]	1207
		R1-CCCTGGTAGAGATGATCAGAGGAACA	3'UT of exon 3 [32]	
V	TSNAX-DISC1-7	R2-TCCCTGGTAGAGATGATCAGAGGA	3'UT of exon 3 [32]	
		F-TCACAGTGCCTTTACCTCAAGCTTTAGCT	exon 2	798
		R-ATGGGCAGACAGGTGGCAAG	3'UT of exon	
VI	DISC1-biomarker	F-GGCAGATGGAG/GTAATATCC	exon 2/3	216
		R-AACTATTCCCTGGTAGAGGTG	3'UT of exon 3	
VII	18S	F1-CGGCTACCACATCCAAGGAA	exon 1	187
		R1-GCTGGAATTACCGCGGCT	exon 1	

TABLE 2 Patient Information

Subject	Age	Days between Samples	BPRS 1st (+/total)	BPRS 2nd (+/total)	Current Smoker	Neuroleptics during hospital stay	Other
S18	44	10	21/53	7/33	yes	risperidone, VPA, olanzapine, haloperidol, benztropine	drugs not detected, noncompliant
S22*	37	3	24/56	15/46	yes	risperidone, aripiprazole	marijuana + homeless
S29*	59	2	16/50	6/36	no	quetiapine aripiprazole	drugs not detected, compliant, homeless
S30*	54	2	17/61	8/22	yes	risperidone	marijuana +, noncompliant
S39*	45	7	9/28	10/28	yes	olanzapine, quetiapine, VPA	cocaine + compliant homeless
S40*	53	6	20/54	6/25	yes	risperidone, VPA aripiprazole quetiapine, amantadine, benztropine	drugs not detected compliant
S23*	39	15	18/60	14/40	yes	olanzapine, aripiprazole, quetiapine	drugs not detected, noncompliant homeless
S26*	38	3	18/60	13/50	yes	fluphenazine olanzapine, Li+, benztropine	reported marijuana/ cocaine use noncompliant homeless
S31*	59	2	6/34	10/37	no	haloperidol, quetiapine	compliant female
S32*	45	14	14/23	12/53	yes	risperidone, VPA, Li+	drugs not detected, compliant, homeless
S33*	49	2	15/39	16/34	yes	quetiapine, risperidone, VPA,	drugs not detected, noncompliant homeless
S37*	58	3	6/40	6/36	pipe	olanzapine quetiapine,	drugs not detected compliance not known

Bold = Gene Chip Samples;

* qRT-PCR samples,

Underline, the sample with higher biomarker levels after treatment. Valproic acid=VPA, lithium= Li+

the 1st to the 2nd sample collection (Table 2). Eight patients were treated for comorbid disease during hospitalization. The patients were not treated for hepatic failure or other illnesses known to interfere with metabolism of neuroleptics.

Differential gene expression analysis

Six paired samples were used for gene chip analysis and 11 paired samples were used for RT-qPCR analysis (Table 2). Pair wise analysis using Gene Chip Operating Software (GCOS) of lymphocyte RNA from 6 paired samples from male patients with schizophrenia suggested there were 470 genes that were similarly differentially expressed during psychosis in at least 3 out of 6 paired samples (318 increased and 153 decreased). Of the 2 DISC1 probe sets that measured corresponding isoforms found in all 12 samples, one probe set detected significant

changes in transcript levels. DISC1 probe set (206090_s_at) measured levels of the L and Lv isoforms, but they were not differentially expressed. DISC1 probe set (207759_s_at) was found up regulated during acute psychosis 1.4 to 1.7 fold in samples S18, S22 and S40 but not significantly changed in samples S30, S39 and S29 on microarray analysis ($p = 0.14$) (Figure 1a). Probe set 207759_s_at measured 5 known unique cDNA sequences together, the TSNAX-DISC1 variant 7 and 54 (GI:257153373 and 238066764 respectively), DISC1 variant q and isoform 46 (GI:257153300 and 238066748 respectively), AK025293 (GI:10437780), AK023443 (GI:10435379) and a novel TSNAX-DISC1 isoform kaje discovered during this investigation (Figure 2). Together these 5 transcripts were detected at intermediate levels in PBMCs and were found increased during psychosis (decreased by 40% after effective treatment) in 10 of the 11 paired samples ($p = 0.037$) using RT-qPCR (Figure 1b).

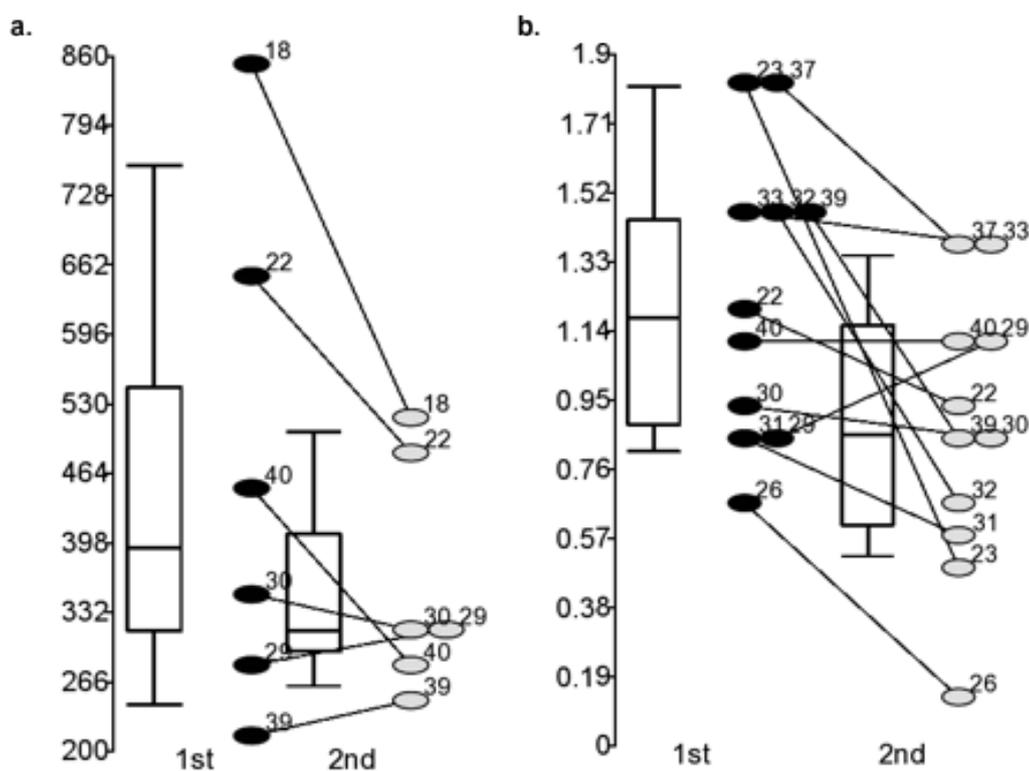


FIGURE 1. Differential DISC1-biomarker expression as shown by **a.** Relative expression levels are shown for microarray analysis of 6 paired samples ($p = 0.14$) and **b.** by RT-qPCR analysis in 11 paired samples ($p = 0.037$). The box represents the interquartile range from the 75TH - 25TH percentile of the data and median. Error bars are the 10TH - 90TH percentile of the data.

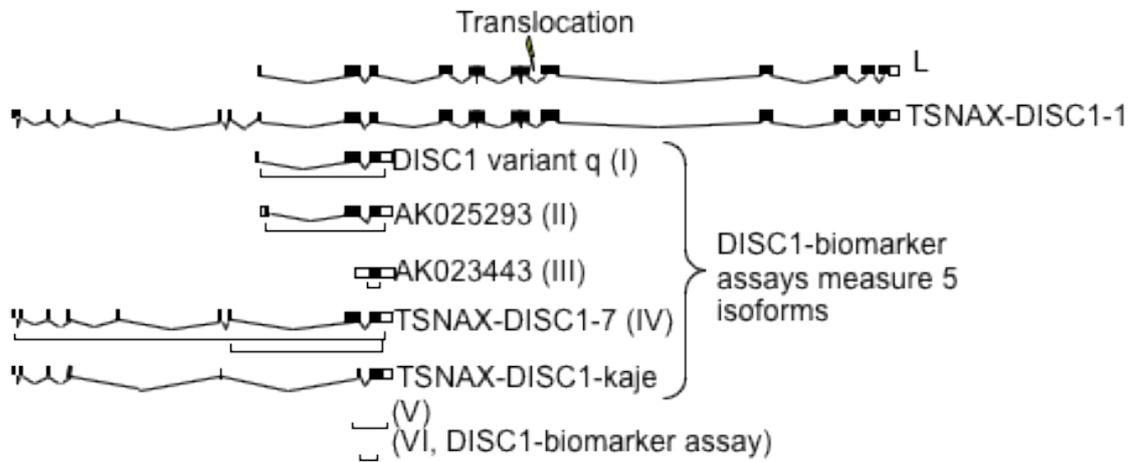


FIGURE 2. Subset of 7 DISC1 transcripts (isoforms). Primer sets I-VI.

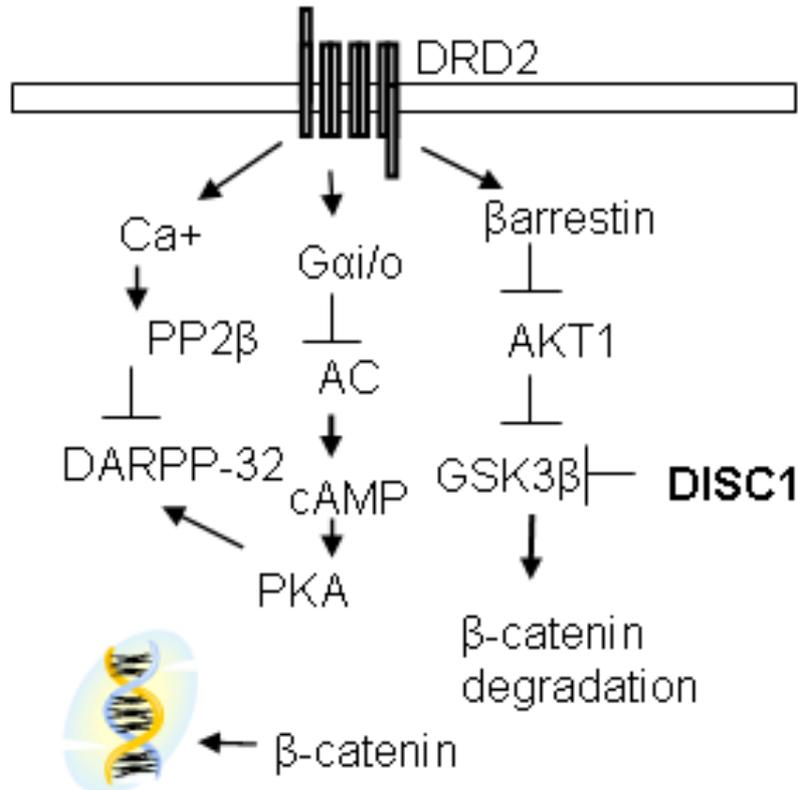


FIGURE 3. DRD2/GSK3β/DISC1 pathway modified from Beaulieu et al. [26] to include gene names ↓ simulation. ⊥ inhibition. Isoforms of DISC1 that include the amino terminal domain responsible for physically interacting with GSK3β may be in a regulatory feedback loop in PBMCs..

Discussion

Paired PBMC samples from patients hospitalized with acute psychosis were analyzed for potential treatment-response biomarkers of psychosis using gene chip analysis and RT-qPCR. Several DISC1 transcripts measured together were the focus of the verification using RT-qPCR because DISC1 is implicated in the genetics of schizophrenia and the DRD2 pathway. We present a novel preliminary finding of decreases in several DISC1 isoforms measured together that is associated with treatment of psychosis during hospitalization.

There are several limitations to this preliminary study. First, the sample size is small and the findings require replication in a larger sample. Control subjects were not included to determine the normal variability of the DISC1-biomarker levels. The patient sample is heterogeneous in regard to street drug use, medication compliance before hospitalization, comorbid disease, age, whether the subjects were homeless and time between samples. While the patients took many different neuroleptics, all the patients were on high doses and their mean haldol-equivalent levels [29] were 12.5 mg/day; haloperidol at 2-5 mg/day corresponds to optimal 60-80% DRD2 occupancy in the brain [33]. In addition to neuroleptics, 6 of the 12 patients in this study were treated with either lithium and/or valproic acid that have been implicated in GSK3 β regulation as well [26] and may contribute to changes in DISC1-biomarker levels. A larger change in DISC1 levels was found in the antipsychotic, VPA and lithium group ($p = 0.12$) when compared to the antipsychotic alone group ($p = 0.22$). Dividing the group up resulted in a loss of statistical significance due to the small sample size.

The mechanism by which levels of DISC1 in PBMCs was associated with neuroleptic treatment may involve the DRD2/GSK3 β pathway (Figure 3). PBMCs may act as a surrogate of the brain through exposure to neuroleptic medications that are antagonists of DRD2 [26, 34] leading to inhibition of GSK3 β in PBMCs. DISC1 protein has been shown to directly bind GSK3 β through the N terminal encoded by exon 2 of the full-length DISC1 protein and inhibit GSK3 β 's activity [24] (Figure 3). Three differentially expressed transcripts, measured as part of the DISC1-biomarker assay, have an ORF through exon 2 and if translated may be able to bind and inhibit GSK3 β . Total dopamine (DA) levels in the plasma are 7.18 nM (0.1-0.33 nM free DA) after fasting, but increase 2-20 fold with sympathetic nervous system activity (exercise, eating, stress and high blood pressure [35]) and are estimated at 100-250 nM at the dendritic cell-T-cell synapse [36]. The affinity (K_D) between DA and DRD2 receptors is 6 nM for D2High affinity and 3600 nM for D2Low affinity [37]. Peripheral dopamine does not cross the blood brain barrier and action at the DRD2 receptor has been implicated in regulating permeability in vascular endothelium, insulin secretion in pancreatic β -cells and blood pressure by modulating sodium balance in the kidneys. Therefore, dopamine levels in blood may be sufficient to stimulate DRD2 receptors on PBMCs if present. Although we (not shown) and others have detected DRD2 transcripts in PBMCs [10-12], detection of PBMC DRD2

protein have been controversial [38-40]. Using flow cytometry, DRD2 receptors were found primarily on B cells (28.3%), NK cells (8.4%) and overall represented less than 5% of PBMCs [41].

In conclusion, changes in transcript levels of DISC1-biomarkers are associated with effective neuroleptic treatment in patients with schizophrenia. Future projects will focus on replicating this finding in a larger patient population including control subjects to estimate the normal variance of DISC1-biomarker levels. Ultimately, the clinical utility of monitoring changes in the DISC1-biomarker to indicate an optimal dose of neuroleptic drug will be determined.

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