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ARTICLE

Antipsychotic drug-associated gene–miRNA interaction in T-lymphocytes

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Abstract

Antipsychotic drugs (APDs) can have a profound effect on the human body that extends well beyond our understanding of their neuropsychopharmacology. Some of these effects manifest themselves in peripheral blood lymphocytes, and in some cases, particularly in clozapine treatment, result in serious complications. To better understand the molecular biology of APD action in lymphocytes, we investigated the influence of chlorpromazine, haloperidol and clozapine *in vitro*, by microarray-based gene and microRNA (miRNA) expression analysis. JM-Jurkat T-lymphocytes were cultured in the presence of the APDs or vehicle alone over 2 wk to model the early effects of APDs on expression. Interestingly both haloperidol and clozapine appear to regulate the expression of a large number of genes. Functional analysis of APD-associated differential expression revealed changes in genes related to oxidative stress, metabolic disease and surprisingly also implicated pathways and biological processes associated with neurological disease consistent with current understanding of the activity of APDs. We also identified miRNA–mRNA interaction associated with metabolic pathways and cell death/survival, all which could have relevance to known side effects of APDs. These results indicate that APDs have a significant effect on expression in peripheral tissue that relate to both known mechanisms as well as poorly characterized side effects.

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Introduction

The molecular mechanisms underlying the therapeutic activity and side effects of antipsychotic drugs (APD) are not well understood. It is generally accepted that they are mediated through target receptors in the brain, which induce intracellular signaling cascades necessary for regulating biological pathways that are dysfunctional in schizophrenia (Sedvall et al., 1986). Owing to the strong affinity of APDs for the dopamine D_2 receptors, these are thought to be a principle therapeutic target (Seeman, 2010), although many other neurotransmitter systems are also implicated and it is unlikely that schizophrenia is simply the result of imbalance in one or even many different signaling systems (Miyamoto et al., 2005; Miller, 2012).

There are two major classes of APD: first generation (typicals) such as chlorpromazine and haloperidol, which generally show strong antagonistic activity at the D_2 dopamine receptors and second generation (atypicals) such as clozapine, which have a broader range of affinity for other neurotransmitter systems including serotonergic signaling (Schotte et al., 1996; Miyamoto et al., 2005; Carpenter and Koenig, 2008). APDs can produce a wide array of side effects, most likely due to excessive or off-target effects at many different receptors. Some side effects impact on the central nervous system (CNS) such as extra-pyramidal symptoms (EPS). It has been argued that weaker, transient binding of atypicals at D_2 dopamine receptors reduces the risk of EPS that are associated with stronger binding by typical APDs. Other side effects manifest in peripheral tissues and whether they originate from APD action in the CNS or peripheral tissue is unknown. The broader receptor binding profiles of atypicals is thought to underlie their greater propensity for metabolic side effects (Meltzer and Huang, 2008; Miller, 2012). Moreover, the rare but potentially life-threatening reduction in granule-containing white blood cells, known as agranulocytosis, is a well-documented side effect associated with clozapine (clozapine-induced agranulocytosis; CIA). Indeed, despite it having the greatest effect size of all APDs in reducing schizophrenia symptoms (Davis et al., 2003) and being the most effective APD for non-refractory schizophrenia

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(Woerner et al., 2003; Essali et al., 2009; Leucht et al., 2009), clozapine is generally not the first line choice given the risk of CIA (Taylor et al., 2003; Flanagan and Dunk, 2008). However, not all patients using APDs will develop all these side effects, suggesting underlying genetic susceptibility in certain individuals and variable biological mechanisms through which they occur.

Whole genome expression analysis in rodent brain after APD exposure revealed altered expression of genes involved in synaptic plasticity and pre-synaptic function potentially related to their therapeutic mechanism of action (MacDonald et al., 2005; Le-Niculescu et al., 2007; Duncan et al., 2008; Fatemi et al., 2012; Rizig et al., 2012). Additionally, biological pathways unrelated to neurotransmission were altered in rodent studies (Thomas et al., 2003; Mehler-Wex et al., 2006; Sondhi et al., 2006) and in human CNS cell lines (Ferno et al., 2005) such as lipid metabolism, which could be involved in metabolic side effects of APDs. Similarly, microRNA (miRNA), critical post-transcriptional regulators of gene expression, may be novel targets for APDs, because they may be involved in processes in the brain that are relevant to APD activity (Dinan, 2010). In addition, miRNA expression is altered in the brain (Perkins et al., 2007; Beveridge et al., 2008, 2010; Santarelli et al., 2011), olfactory neuroepithelium (Mor et al., 2013) and peripheral blood mononuclear cells (PBMCs) (Gardiner et al., 2011; Lai et al., 2011) of patients with schizophrenia and miRNA may regulate the expression of schizophrenia-associated genes and pathways (Beveridge and Cairns, 2012; Wright et al., 2013). Recently we observed differential miRNA expression in mouse brain upon APD exposure and that these miRNA target genes involved in metabolic pathways (Santarelli et al., 2013).

In view of the possibility that genetic and environmental risk factors for schizophrenia also cause changes in peripheral tissue, we investigated gene and miRNA expression in PBMCs in a large cohort of participants with schizophrenia and non-psychiatric controls (Gardiner et al., 2011, 2013). These participants self-reported the use of APDs and as such, we were unable to definitively attribute the differential expression patterns solely to the disorder and exclude the possibility that APDs contributed to the molecular profiles. Therefore to increase our understanding of the complex activity of APDs, the evolution of their side effects and differentiate them from the schizophrenia-associated changes, we investigated the influence of the typical APDs chlorpromazine and haloperidol and the atypical clozapine on mRNA and miRNA expression in a T-lymphocyte cell line. Since the acute effects of APDs generally stabilize within 1 wk and therapeutic benefit is achieved within 2 wk compared to chronic treatment (Kapur et al., 2005; Agid et al., 2006; Li et al., 2007; Raedler et al., 2007; Kinon et al., 2010), we examined expression changes over 15 d of APD exposure.

Methods

Cell culture and APD treatment

JM-Jurkat T-lymphocyte cells (Schneider et al., 1977) were cultured in a humidified, 5% CO_2 environment in RPMI 1640 (Hyclone, Thermoscientific) supplemented with 10% fetal calf serum and 2 mM L-glutamine. The APDs chlorpromazine, haloperidol and clozapine (Sigma-Aldrich, Australia) were dissolved in ethanol (or nuclease-free water in the case of chlorpromazine), filtered with a 0.2 μM syringe filter (Millex GP, Merck Millipore, Australia) and added to culture media to final concentrations reflective of therapeutic/clinical concentrations during typical treatment regimes with the lowest toxicity: clozapine (400 ng/ml or 1.2 μmol/l), haloperidol (10 ng/ml or 26.6 nmol/l), chlorpromazine (500 ng/ml or 1.6 μmol/l) (Heiser et al., 2007; Mauri et al., 2007; Weidenhofer et al., 2009; Jain et al., 2011; Chen et al., 2012). Cells were seeded at 5×10^5 cells/ml in T75 flasks and cultured to 70% confluence prior to treatment, in triplicate, with drug-supplemented media (or an equivalent volume of ethanol as a baseline control). Every 3 d, cells were re-suspended to 5×10^5 cells/ml in fresh drug-supplemented media while excess cells were washed in 5 ml warm PBS and harvested for RNA extraction.

RNA extraction and purification

RNA extraction using Trizol (Sigma-Aldrich, Australia) and assessment of total RNA quality using an Agilent 2100 bioanalyzer and the RNA 6000 Nano kit (Agilent, Australia) was conducted as previously described (Beveridge et al., 2013). The mean RNA integrity number (RIN) was 9.8 and samples with RIN >6.9 were utilized for microarray and quantitative real-time polymerase chain reaction (Q-PCR) analysis.

Gene expression analysis

Total RNA was purified using the RNeasy minikit (Australia) according to the manufacturer's instructions. Each sample was prepared and hybridized to Illumina HT_12_v4 beadchips as previously described (Gardiner et al., 2013).

Quality control, background subtraction and quantile normalization were performed in GenomeStudio V3 (USA) according to the manufacturer's guidelines. Expressed genes were determined with respect to negative control probes to provide a mean detection p value, calculated across technical replicates. Only genes expressed above this threshold ($p<0.01$) were included for differential expression analysis. Unsupervised hierarchical clustering (Cluster 3.0, Stanford University, USA (Eisen et al., 1998), visualized in Java Treeview V.1.1.1 (Saldanha, 2004)) identified two clusters composed mainly of samples collected throughout the first week (<7 d) termed 'acute' and second week (>7 but

<15 d) termed 'subacute', respectively). Gene expression data were loaded into GeneSpring v11 (Agilent, Australia), median centered to the median of all genes and baseline transformed. A 2-class differential expression analysis (grouped by APD type and duration of exposure, i.e. 'acute' and 'subacute' averaged over replicates, relative to respective controls) was conducted: an unpaired *t* test with unequal variances (Welch's *t* test) with correction for multiple testing (Benjamini–Hochberg false discovery rate (FDR)). Following filtering of genes with poorly annotated/discontinued Entrez gene records, lists of differentially expressed genes (DEGs) were compiled (1.5 fold up or down-regulation, corrected $p<0.001$) (Supplementary Tables S1–6).

miRNA expression analysis

1μg of total RNA was amplified, labeled and hybridized to miRNA microarrays (Illumina miRNA sentrix array) as described previously (Santarelli et al., 2011). Quality control, background subtraction and normalization were conducted on the miRNA expression data as described above. Differential expression analysis was performed on expressed miRNA (mean detection $p<0.05$) using the significance analysis of microarrays statistical analysis program (SAM; full academic version 2.23, http://www-stat.stanford.edu/~tibs/SAM/) (Tusher et al., 2001). An unpaired 2-class comparison (as described above for the genes) of un-logged expression values was performed (Wilcoxon T-statistic) with 5000 permutations of the data (FDR=0) as described (Beveridge et al., 2010; Santarelli et al., 2011).

Quantitative real-time PCR (Q-PCR)

Q-PCR was used to validate differential mRNA and miRNA expression observed on the microarrays with efficiency correction and the relative quantitation method as previously described (Santarelli et al., 2011, 2013). Primers for gene expression validation of five genes chosen for their consistent change in expression in the same direction after acute and subacute exposure to both haloperidol and clozapine, as well as another two genes altered after acute treatment with both haloperidol and clozapine, were designed in Oligo Explorer V1.5 (USA) such that the forward primer amplified across an exon-exon junction and the reverse primer resided within the same exon detected by the microarray probe. Similarly, primers for miRNA were designed based on their miRBase mature miRNA sequence (www.mirbase.org) with a non-specific sequence added to the reverse primer and, where required, locked nucleic acids added to the forward primer for increased stability as described previously (Santarelli et al., 2011). Primer sequences are listed in Supplementary Table S7. After efficiency correction, relative expression was determined with respect to the most stably expressed reference genes, *GUSB* and *HMBS* (genes) and small nucleolar RNAs U44 and U49 (miRNA), as determined by geNorm analysis (Vandesompele et al., 2002). A ratio of relative expression (APD/control) was calculated and the significance of differential expression determined using an unpaired student's one tailed *t*-test (outliers >3 standard deviations from the mean were excluded).

Functional annotation of differentially expressed genes

DEGs and corresponding fold change, were uploaded to Ingenuity Pathway Analysis (IPA) knowledge base v6.3 (Ingenuity Systems, USA, www.ingenuity.com) for functional annotation. IPA summarizes functional annotation terms into top Biological Functions (divided into 3 major categories: Disease and Disorders, Molecular and Cellular Functions and Physiological System Development and Function) and Canonical Pathways with which DEGs are associated. A Fisher's exact *p* value is calculated representing the probability that these genes are associated with a particular function or pathway by chance alone, with correction for multiple testing (Benjamini-Hochberg). For the Canonical Pathways this is expressed as -log(*p*-value) (>1.3 is equivalent to $p<0.05$). A ratio of the number of DEGs involved in the pathway divided by the total number of genes in that pathway is also calculated as an indicator of enrichment. In some instances, an activation *z*-score is calculated, providing a prediction of the overall effect of dysregulated expression of groups of genes in a given pathway/annotation term, i.e. activation ($\geqslant 2$) or inhibition ($\leqslant -2$), and is based upon sources in the Ingenuity Knowledge Base and the direction of change in the expression of genes within the pathway.

mRNA–miRNA interactions

Potential regulatory relationships between mRNA and miRNA expression levels were investigated in IPA by cross-referencing mRNA and miRNA that are differentially expressed after the same treatment, highlighting predicted mRNA:miRNA pairs (where a miRNA contains a seed region that is predicted to bind the 3′-UTR of its target mRNA).

Results

Genome-wide gene and miRNA expression was investigated in JM-Jurkat T-lymphocytes after a 7–15 d exposure to the APDs chlorpromazine, haloperidol and clozapine.

Differential gene expression

JM-Jurkat T-lymphocytes expressed 11 520 genes (24.4% of the total number of transcripts on the array). A 2-class differential expression analysis was performed, identifying genes that were considered significantly up or down-regulated ($1.5 \geqslant$ fold change $\leqslant -1.5$) following acute and subacute APD exposure compared to

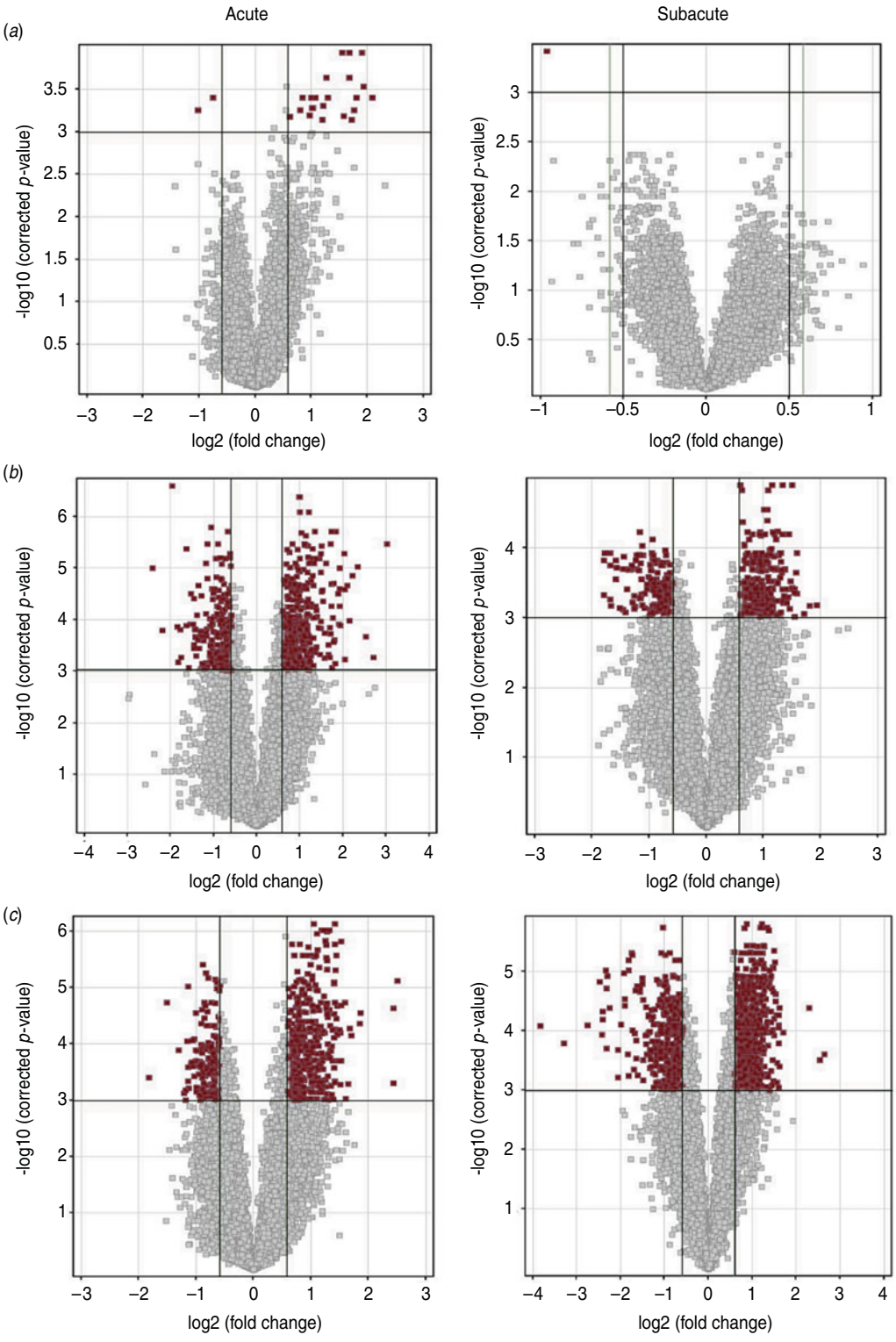

Fig. 1. Volcano plots of differentially expressed genes in Jurkat T-lymphocytes after antipsychotic exposure. Log_2 fold change in expression are plotted against $-log_{10}$ corrected p values of significance. (*a*) acute and subacute chlorpromazine, (*b*) acute and subacute clozapine and (*c*) acute and subacute haloperidol. Each point represents a gene, with genes shown in red in the upper left and right quadrants differentially expressed (fold change >1.5 up or down-regulation and corrected $p<0.001$).

respective controls (Benjamini-Hochberg corrected $p<0.001$) (Fig. 1 and Table 1). DEGs in each experimental group are listed in Supplementary Tables S1–6.

Seven differentially expressed mRNA were selected for validation by Q-PCR (Fig. 2 and Table 2). With one exception, the direction of change in expression detected

Table 1. Summary of differentially expressed genes after antipsychotic drugs (APD) exposure (microarray)

Experimental group		Differentially expressed genes (>1.5 fold difference treated/control, FDR (false discovery rate) $p<0.001$)		
Duration of exposure	Treatment			
Acute		Up-regulated	Down-regulated	Total
	Chlorpromazine	16	2	18
	Clozapine	339	196	535
	Haloperidol	361	126	487
Subacute	Chlorpromazine	0	1	1
	Clozapine	237	134	371
	Haloperidol	667	311	978

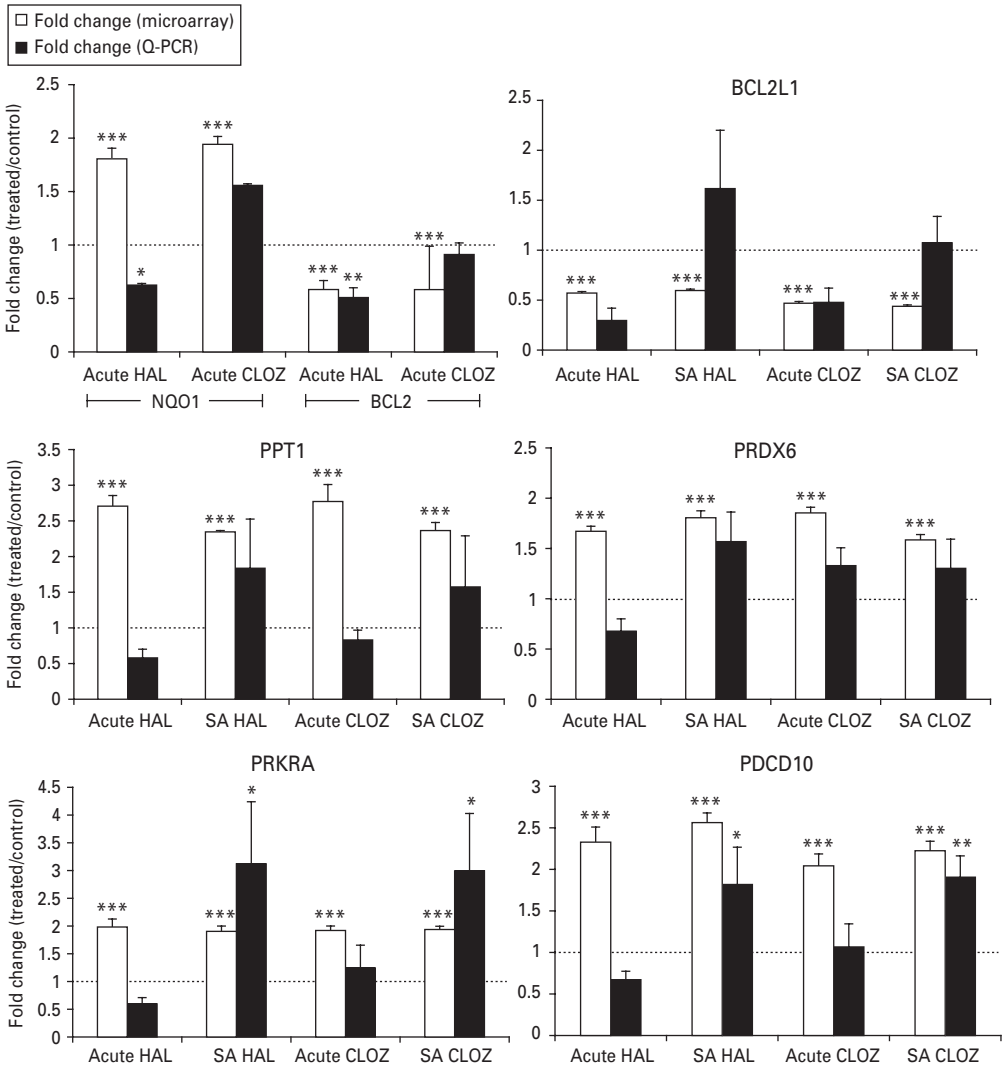

Fig. 2. Quantitative real-time polymerase chain reaction (Q-PCR) validation of differential gene expression after antipsychotic exposure. *NQO1, BCL2, BCL2L1, PPT1, PRDX6, PRKRA* and *PDCD10*. Bars represent mean fold change in expression (antipsychotic-treated relative to controls; +S.E.M.) by microarray (***corrected $p<0.001$) and Q-PCR (*$p<0.05$ one tailed student's *t*-test). HAL, haloperidol; CLOZ, clozapine; SA, subacute.

by Q-PCR was consistent with the microarray. B-cell CLL/lymphoma 2 (*BCL2*) was down-regulated after acute haloperidol exposure. Protein kinase interferon-inducible double-stranded RNA dependent activator (*PRKRA*) and programmed cell death 10 (*PDCD10*) were up-regulated after subacute haloperidol and clozapine. NAD(P)H

Table 2. Quantitative real-time polymerase chain reaction (Q-PCR) gene expression summary of fold changes and *p* values

		Acute haloperidol	Acute clozapine	Subacute haloperidol	Subacute clozapine
NQO1	Fold change	0.62	1.56		
	p-value	**0.023**	0.068		
BCL2	Fold change	0.52	0.91		
	p-value	**0.006**	0.303		
BCL2L1	Fold change	0.30	0.47	1.61	1.07
	p-value	0.051	0.088	0.138	0.414
PPT1	Fold change	0.59	0.84	1.85	1.59
	p-value	0.068	0.259	0.095	0.178
PRDX6	Fold change	0.68	1.33	1.57	1.30
	p-value	0.182	0.173	0.063	0.198
PRKRA	Fold change	0.60	1.24	3.13	2.99
	p-value	0.057	0.276	**0.026**	**0.027**
PDCD10	Fold change	0.68	1.07	1.83	1.91
	p-value	0.093	0.404	**0.036**	**0.006**

P values in bold are significant (one-tailed student's *t*-test $p<0.05$).

Table 3. Differentially expressed microRNA (miRNA) after antipsychotic drugs (APD) exposure (microarray)

Experimental group		miRNA	Fold change	miRNA family	Chromosome
Duration of exposure	Treatment				
Acute	Chlorpromazine	hsa-miR-942	1.25	miR-942	1p13.1
		hsa-miR-362-5p	1.29	miR-362	Xp11.23
		hsa-miR-421	1.41	miR-95	Xq13.2
	Clozapine	hsa-miR-17-3p	−1.27	miR-17	13q31.3
	Haloperidol	hsa-miR-200c-3p	−1.61	miR-8	12p13.31
		hsa-miR-28-5p	−1.3	miR-28	3q28
		hsa-miR-624-5p	−1.39	miR-624	14q12
Subacute	Chlorpromazine	None			
	Clozapine	hsa-miR-21	1.29	miR-21	17q23.1
	Haloperidol	None			

All miRNA listed were differentially expressed compared to controls at false discovery rate (FDR)=0. There were no miRNA differentially expressed between acute and subacute controls.

dehydrogenase, quinone 1 (*NQO1*) was significantly down-regulated after acute haloperidol in contrast to the up-regulation shown by the microarray. Down-regulation of BCL2-like 1 (*BCL2L1*) after acute haloperidol was borderline non-significant by Q-PCR ($p=0.05$).

Differential miRNA expression and mRNA:miRNA integration

A total of 247 mature miRNA were expressed, which is 29% of all annotated/validated miRNA transcripts present on the array. Differential expression analysis revealed the significantly altered expression of 8 miRNA after APD exposure compared to controls (Table 3). After acute APD exposure the following was observed: up-regulation of miR-942, miR-362-5p and miR-421 (chlorpromazine); down-regulation of miR-17-3p (clozapine); down-regulation of miR-200c-3p, miR-28-5p and miR-624-5p (haloperidol). After subacute APD exposure, miR-21-5p was up-regulated (clozapine).

The expression of a selection of these miRNA was also analyzed by Q-PCR (Fig. 3 and Table 4). miR-200c-3p and miR-28-5p were confirmed to be significantly down-regulated in haloperidol-treated JM-Jurkat cells compared to controls (one tailed *t*-test: −2.46 fold, $p=0.026$; −3.73 fold, $p=0.014$ respectively). miR-421 and miR-17-3p showed non-significant trends in the same direction as the microarray (1.31 fold up-regulation, $p=0.114$ and −1.21 fold down-regulation, $p=0.214$) while miR-21-5p showed no change compared to controls.

Differentially expressed mRNA and miRNA were cross referenced for the following experimental groups: chlorpromazine acute, clozapine acute and subacute, haloperidol acute. Considering the current model in

Table 4. Differential microRNA (miRNA) expression (microarray and quantitative real-time polymerase chain reaction (Q-PCR)

		miR-421 (acute – chlorpromazine)	miR-17-3p (acute – clozapine)	miR-200c-3p (acute – haloperidol)	miR-28-5p (acute – haloperidol)	miR-21-5p (subacute – clozapine)
Microarray[a]	Fold change	**1.41**	**−1.27**	**−1.61**	**−1.30**	**1.29**
Q-PCR	Fold change	1.31	−1.21	**−2.46**	**−3.73**	−1.06
	P value	0.114	0.214	**0.026**	**0.014**	0.434

Values in bold are significant: [a]False discovery rate (FDR)=0 in all instances; Q-PCR student's one-tailed *t*-test, $p<0.05$.

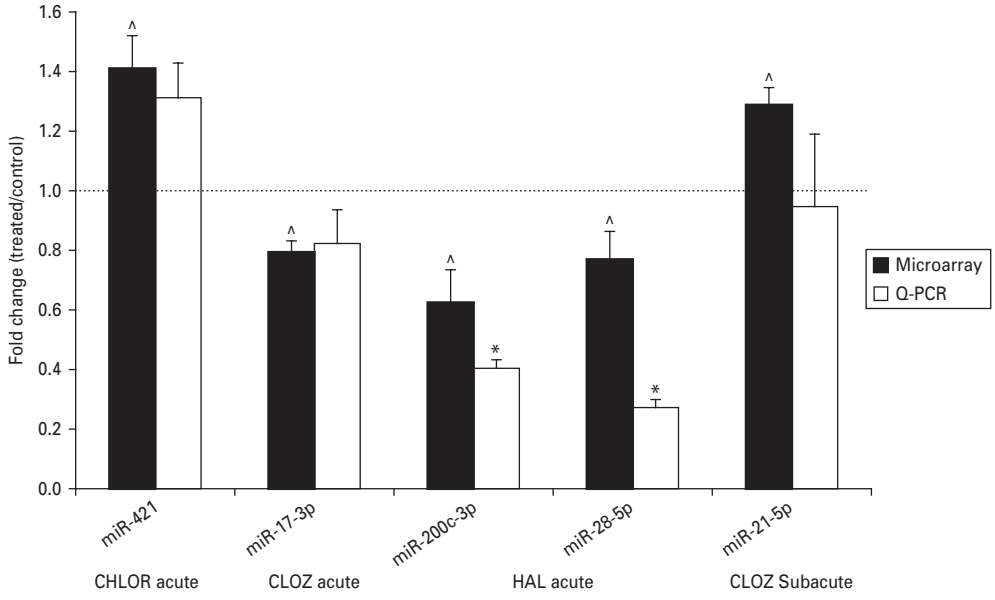

Fig. 3. Quantitative real-time polymerase chain reaction (Q-PCR) validation of differential miRNA expression after antipsychotic exposure. Bars represent mean fold change in expression (antipsychotic-treated relative to controls; +S.E.M. All values for the microarray were statistically significant (^FDR=0). Q-PCR *$p<0.05$ one tailed student's *t*-test. miR-421 showed a trend for up-regulation after acute chlorpromazine treatment, miR-17-3p showed a trend for down-regulation after acute clozapine treatment and there was no change in miR-21-5p after subacute clozapine treatment. Both miR-200c-3p and miR-28-5p were significantly down-regulated after acute haloperidol treatment by both the microarray and Q-PCR. CHLOR, chlorpromazine; CLOZ, clozapine; HAL, haloperidol; FDR, false discovery rate.

which miRNA typically act as inhibitors/destabilizers of mRNA expression (post-transcriptional gene silencing), up-regulation of a miRNA would be expected to lead to silencing of their target mRNA (and *vice versa*), thus we focused on inversely expressed pairs. We identified 73 unique mRNA:miRNA pairs after acute haloperidol exposure, 58 of which were inversely expressed, i.e. miR-200c-3p and miR-28-5p were predicted to target 40 and 18 genes respectively, that showed reciprocal up-regulation. Similarly there were eight pairings between miR-21-5p, which were up-regulated after subacute treatment with clozapine, and mRNA differentially expressed in the same experimental group. No mRNA: miRNA pairs were identified after acute exposure to chlorpromazine or clozapine. The lists of mRNA: miRNA pairs for acute haloperidol-exposed cells and subacute clozapine-exposed cells are listed in Supplementary Table S13. Functional annotation of all 73 mRNA:miRNA pairs and the 58 inversely expressed pairs for acute haloperidol-exposed cells revealed top Molecular and Cellular Functions such as 'Carbohydrate metabolism', 'Lipid metabolism' and 'Small molecule biochemistry' as well as 'Cell death and survival' and many processes related to development (Supplementary Table S14).

Functional annotation of differentially expressed genes

A stringent inter-treatment comparison and functional annotation was performed on genes differentially expressed in response to multiple APDs and/or in response to both acute and subacute APD exposure, since

Table 5. Top ranked functional categories of genes commonly differentially expressed in Jurkat T-lymphocytes after antipsychotic drugs (APD) exposure

Functional category		*Corrected p value*	*Genes*
Haloperidol (acute & subacute) (n=242)			
Disease & Disorders	Developmental Disorder	7.61E-04 – 4.47E-02	23
	Hereditary Disorder	7.61E-04 – 4.47E-02	32
	Metabolic Disease	7.61E-04 – 4.47E-02	11
	Renal & Urological Disease	7.61E-04 – 2.26E-02	4
	Neurological Disease	1.87E-03 – 4.01E-02	34
Molecular & Cellular Functions	RNA Post-transcriptional Modification	2.33E-05 – 3.37E-02	14
	Cell Cycle	3.81E-04 – 4.88E-02	18
	Carbohydrate Metabolism	1.87E-03 – 3.37E-02	8
	Lipid Metabolism	1.87E-03 – 4.79E-02	13
	Small Molecule Biochemistry	1.87E-03 – 4.79E-02	18
Physiological System Development & Function	Organismal Development	2.38E-02 – 4.83E-02	24
	Tumor Morphology	5.45E-03 – 4.47E-02	8
	Tissue Morphology	6.62E-03 – 4.55E-02	13
	Embryonic Development	7.88E-03 – 4.83E-02	26
	Nervous System Development & Function	7.88E-03 – 4.83E-02	7
Clozapine (acute & subacute) (n=116)			
Disease & Disorders	Cancer	8.66E-04 – 4.41E-02	18
	Hematological Disease	8.66E-04 – 3.35E-02	4
	Neurological Disease	1.68E-03 – 4.44E-02	12
	Organismal Injury & Abnormalities	1.68E-03 – 3.35E-02	3
	Cardiovascular Disease	5.66E-03 – 5.66E-03	1
Molecular & Cellular Functions	Carbohydrate Metabolism	3.13E-04 – 3.35E-02	5
	Nucleic Acid Metabolism	3.13E-04 – 4.44E-02	9
	Small Molecule Biochemistry	3.13E-04 – 4.98E-02	13
	RNA Post-transcriptional Modification	4.14E-04 – 1.69E-02	4
	Post-translational Modification	4.14E-04 – 3.89E-02	13
Physiological System Development & Function	Organ Morphology	1.363–03 – 4.26E-02	7
	Nervous System Development & Function	2.01E-03 – 4.44E-02	7
	Emrbyonic Development	5.66E-03 – 4.99E-02	8
	Hematopoiesis	5.66E-03 – 4.44E-02	2
	Humoral Immune Response	5.66E-03 – 3.89E-02	1
Haloperidol & clozapine (acute & subacute) (n=68)			
Disease & Disorders	Neurological Disease	6.52E-04 – 4.81E-02	9
	Organismal Injury & Abnormalities	6.52E-04 – 3.46E-02	3
	Cancer	3.51E-03 – 4.47E-02	4
	Cardiovascular Disease	3.51E-03 – 3.51E-03	1
	Connective Tissue Disorders	3.51E-03 – 3.36E-02	5
Molecular & Cellular Functions	RNA Post-transcriptional Modification	4.29E-04 – 1.05E-02	3
	DNA Replication, Recombination & Repair	5.78E-04 – 3.29E-02	9
	Cellular Development	7.81E-04 – 3.8E-02	4
	Post-Translational Modification	1.34E-03 – 1.88E-02	6
	Free Radical Scavenging	2.92E-03 – 8.22E-03	3
Physiological System Development & Function	Nervous System Development & Function	7.81E-04 – 4.81E-02	7
	Organ Morphology	3.3E-03 – 5E-02	8
	Embryonic Development	3.51E-03 – 5E-02	7
	Hematopoiesis	3.51E-03 – 3.46E-02	1
	Humoral Immune Response	3.51E-03 – 2.43E-02	1
Haloperidol & clozapine (acute and/or subacute) (n=377)			
Disease & Disorders	Cancer	8.9E-04 – 3.83E-02	64
	Connective Tissue Disorders	1.76E-03 – 3.44E-02	4
	Developmental Disorder	1.76E-03 – 3.83E-02	21
	Infectious Disease	4.21E-03 – 2.9E-02	29
	Gastrointestinal Disease	5.61E-03 – 3.44E-02	45

Table 5 (*cont.*)

Functional category		*Corrected p value*	*Genes*
Molecular & Cellular Functions	Post-translational Modification	9.56E-05 – 4.9E-02	37
	Cell Morphology	3E-04 – 3.83E-02	20
	Cellular Function & Maintenance	3E-04 – 3.83E-02	12
	DNA Replication, Recombination & Repair	3.41E-04 – 3.83E-02	28
	Cell-to-cell Signaling & Interaction	8.9E-04 – 3.44E-02	5
Physiological System Development & Function	Tumor Morphology	8.9E-04 – 3.44E-02	2
	Nervous System Development & Function	1.76E-03 – 3.44E-02	6
	Cardiovascular System Development & Function	2.9E-03 – 3.83E-02	13
	Hematological System Development & Function	2.9E-03 – 4.23E-02	9
	Organismal Development	2.9E-03 – 3.44E-02	17

they are more robustly altered and less likely to be false positives, i.e. true targets of the APDs (compared to those only altered by a single treatment/time-point which tend to generate a higher false negative rate) (Supplementary Fig. S1, Supplementary Tables S8 and S9). Of the 18 genes altered after acute chlorpromazine, 10 were also altered (up-regulated in all instances) by acute clozapine and haloperidol. Subacute treatment with chlorpromazine or acute clozapine resulted in down-regulation of fibulin 2 (FBLN2). A greater overlap was observed between clozapine and haloperidol with 68 genes altered in response to acute and subacute exposure. To identify processes and pathways unique to clozapine and haloperidol exposure and commonly dysregulated by both haloperidol and clozapine, the following four experimental groups (treatment-timepoints) were submitted to IPA for functional annotation (note that in all cases where a gene was altered by both drugs, they were altered in the same direction by a similar magnitude): (1) Clozapine acute and subacute (n=116); (2) Haloperidol acute and subacute (n=242); (3) Clozapine and haloperidol acute AND subacute (n=68) and (4) Clozapine and haloperidol acute AND/OR subacute (n=377).

The top five biological functions under the categories 'diseases and disorders', 'molecular and cellular functions' and 'physiological system development and function' are summarized for the four experimental groups in Table 5. Interestingly, in Jurkat T-lymphocytes (a non-neuronal tissue), genes with canonical functions within the brain were among those altered by APDs: the top Disease/Disorder for genes in experimental group 3 (genes altered by both haloperidol and clozapine after both acute and subacute exposure) was 'Neurological disease', which also features among the top five for the haloperidol and clozapine-specific gene lists (Table 5 and Supplementary Table S10). Moreover, 'Nervous system development and function' featured in the top five terms under the category 'Physiological system development and function' for all four experimental groups (Table 5).

Functional annotation also revealed other processes/pathways with potential relevance to APD-induced side effects. For genes differentially expressed after acute and subacute haloperidol exposure, 'Metabolic disease' was among the top five diseases and disorders. In the top five molecular and cellular functions were 'Carbohydrate metabolism' and 'Lipid metabolism'. For haloperidol, the functional annotation term 'Accumulation of lipid' carried a z-score of 1.715 (trend for an increased lipid accumulation). Moreover, 'Accumulation of lipid', 'Accumulation of fatty acid' and 'Oxidation of fatty acid' were terms represented in the significant biological functions containing altered genes from the four experimental groups (Supplementary Table S11). The canonical pathways analysis also suggested that genes involved in lipid metabolism are altered by APDs: the most significant canonical pathway for experimental group 4 (acute AND/OR subacute haloperidol and clozapine) was 'Fatty acid β-oxidation I', which is in accordance with APD-induced weight gain (Supplementary Table S12). Furthermore, the pathway 'Mitochondrial dysfunction' and other biological functions including 'Free radical scavenging', 'Permeability of mitochondrial membrane', 'Quantity of hydrogen peroxide', 'Quantity of NADPH' and 'Quantity of reactive oxygen species' featured among the four experimental groups suggesting dysregulation of genes related to oxidative/cellular stress (Supplementary Tables S11 and S12).

Functional annotation terms related to T-lymphocyte function and development were among those represented by DEGs that were commonly dysregulated by clozapine and haloperidol. 'Cell cycle progression of T-lymphocytes', 'Arrest in cell cycle progression of T-lymphocytes' and 'Interphase of T-lymphocytes' were identified for experimental group 4 (clozapine and haloperidol acute AND/OR subacute). Similarly, for genes differentially expressed after haloperidol exposure, 'Cell cycle progression of T-lymphocytes', 'Lack of CD8+ T-lymphocyte' and 'Differentiation of CD4+ T-lymphocytes' were observed while 'Quantity of

memory T-lymphocytes' and 'I-kappaB kinase/NF-kappaB cascade' were identified for clozapine-exposed cells (Supplementary Table 11). Functional annotation terms related to infection, in particular with human immunodeficiency virus (HIV, which targets T-helper cells), featured a *z*-score >2, suggesting an overall increase in the activity of this pathway.

Discussion

Although APDs are thought to achieve their therapeutic effects via molecular targets in the brain, they display broad receptor binding profiles and may elicit 'off-target' effects in the brain and periphery (Canfran-Duque et al., 2013). To gain further insight into the molecular effects of APDs at the transcriptional level in peripheral cells, we examined both gene and miRNA expression in Jurkat T-lymphocytes following APD exposure. Functional annotation of the DEGs and miRNA suggests these agents influence pathways associated with oxidative stress and cellular metabolism which could affect T-cell biology, and may also provide insight into the molecular effects of APDs in other cell types, with several neurological diseases relevant to APD-induced EPS also being implicated.

APD-induced differential expression and mRNA: miRNA integration

Genes altered by multiple APDs and/or timepoints are more likely to be true molecular targets of APD treatment. There were 68 genes differentially expressed after acute and subacute exposure to both haloperidol and clozapine (all in the same direction with similar magnitude of fold change). The greater overlap between haloperidol and clozapine (as compared to that between chlorpromazine and haloperidol, both typical APDs) was somewhat surprising since they have distinct neurotransmitter receptor binding affinities (Nielsen et al., 2011). Nevertheless, this suggests possible co-regulatory influences of these APDs on the expression of these genes and similarities in their mechanisms of action. In this study we considered the potential of miRNA to mediate some of the APD-related changes in expression and identified eight miRNA associated with APD exposure, including miR-17-3p. This miRNA, down-regulated after acute clozapine exposure was previously shown to be down-regulated during neural differentiation (Beveridge et al., 2009) and in the serum of patients with schizophrenia (Shi et al., 2012), while up-regulated in post-mortem schizophrenia brain (Santarelli et al., 2011; Wong et al., 2013). Down-regulation of miR-200c-3p and miR-28-5p after acute haloperidol exposure was also consistent with their expression profile in PBMCs from patients with schizophrenia (Gardiner et al., 2011), suggesting these miRNA could be altered in patients through APD treatment.

To garner more information about the interaction between APD–miRNA and their target genes we identified 73 haloperidol-associated mRNA:miRNA pairs for miR-200c-3p and miR-28-5p, 58 of which showed inverse expression. Functional annotation and pathways analysis of the altered mRNA:miRNA pairs suggested involvement in a wide variety of metabolic signaling pathways, including 'Carbohydrate metabolism', 'Lipid metabolism' and 'Small molecule biochemistry' consistent with previous reports associating miR-200c with adipogenesis and obesity (Kennell et al., 2008; Chartoumpekis et al., 2012).

APD impact on T-cell biology

T-lymphocytes express neurotransmitters and their receptors (Cosentino et al., 2007; Chen et al., 2012) and there is evidence that, in addition to their canonical roles in neurotransmission, neurotransmitters affect immune function (Levite, 2008). Moreover, APDs possess immunomodulatory properties (Drzyzga et al., 2006; Himmerich et al., 2011; Roge et al., 2012) which could have implications for treatment of schizophrenia given evidence suggesting an immune component in the disorder (Fillman et al., 2012; Xu et al., 2012; Gardiner et al., 2013; Hwang et al., 2013). Thus we suspect that APDs could alter the expression of miRNA and genes associated with T-lymphocyte function, which may shed light on the molecular mechanism(s) underlying APD-induced immunological side effects. Investigation of the biological processes and pathways featuring genes that were commonly dysregulated by clozapine and haloperidol revealed functional annotation terms related to T-cell development including 'Cell cycle progression of T-lymphocytes'. Moreover, functional annotation terms related to infection were predicted to have increased overall activity so it could be speculated that APDs alter the expression of genes that render T-lymphocytes more vulnerable to viral infection.

Biological terms and pathways including 'Free radical scavenging', 'Permeability of mitochondrial membrane', 'Quantity of hydrogen peroxide', 'Quantity of reactive oxygen species' and 'Mitochondrial dysfunction' suggest that T-lymphocyte function may be affected by APD-induced alterations in oxidative stress/antioxidant defense, mitochondrial function and energy metabolism. APD exposure altered the expression of glutaredoxin family members *GLRX, GLRX2* and *GLRX3*, which are involved in the regulation of antioxidant defense and maintenance of mitochondrial redox homeostasis (Felberbaum-Corti et al., 2007; Sabens Liedhegner et al., 2012; Stroher and Millar, 2012). *GLRX* was among the inversely expressed mRNA targets of both miR-200c-3p and miR-28-5p. The unique antioxidant peroxiredoxin 6 (*PRDX6*) was also up-regulated after APD exposure in contrast to down-regulation in APD-treated rat frontal cortex (Fatemi et al., 2012) but consistent with increased

PRDX6 protein in post-mortem brain from medicated schizophrenia patients (Martins-de-Souza et al., 2010). Anti-apoptotic *BCL2* and *BCL2L1*, associated with cell cycle regulation, survival and mitochondrial membrane permeability (Ogilvy et al., 1999; Akgul et al., 2001; Rolland and Conradt, 2010) were both down-regulated after APD exposure, consistent with down-regulation of *BCL2L1* in rat frontal cortex in response to haloperidol and clozapine (Fatemi et al., 2012). It is plausible that APD-induced down-regulation of these genes confers increased vulnerability of T-lymphocytes to oxidative stress and pro-apoptotic stimuli related to CIA. Clozapine itself is apparently not directly toxic to neutrophils or their progenitors at therapeutic concentrations (Williams et al., 1997, 2000; Gardner et al., 1998). However, bioactivation/oxidation of clozapine in neutrophils produces reactive and unstable clozapine metabolites which induce toxic oxidative stress leading to neutrophil apoptosis (Williams et al., 2000; Fehsel et al., 2005; Husain et al., 2006) and may be cytotoxic to bone marrow stroma (Pereira and Dean, 2006; Lahdelma et al., 2010) potentially leading to accelerated neutrophil or myelocyte precursor apoptosis (Flanagan and Dunk, 2008; Iverson et al., 2010; Nooijen et al., 2011). In support of this, the functional term 'Apoptosis of bone marrow cell lines' was associated with clozapine-exposed cells.

Metabolic and neurological pathways

The APD-associated changes in genes associated with oxidative stress and mitochondrial function, altered here in T-lymphocytes, may provide insight into the molecular effects of APDs in other cellular contexts. Disruption of these pathways in other cell types/tissues could underlie the pathophysiology of diverse side effects. The current findings could be relevant to APD-associated metabolic side effects such as weight gain, metabolic syndrome, dyslipidemia and insulin resistance (Newcomer, 2007; Miljevic et al., 2010). The most significant canonical pathway for genes dysregulated by 'acute AND/OR subacute haloperidol and clozapine' was 'Fatty acid β-oxidation I' and the category 'Lipid metabolism' was among the top five molecular and cellular functions for haloperidol-exposed cells. This is consistent with our previous study in which differentially expressed mRNA:miRNA in mouse whole brain following exposure to olanzapine and clozapine were associated with altered lipid metabolism (Santarelli et al., 2013). Moreover, others report differential expression of genes associated with fatty acid biosynthesis and lipid metabolism after APD exposure in cell culture (Ferno et al., 2005; Polymeropoulos et al., 2009) and rodent brain (Thomas et al., 2003; Duncan et al., 2008). Similarly, we observed a number of terms and pathways associated with neural development and function. 'Neurological disease' was the top Disease/Disorder for genes altered by both acute and subacute exposure to haloperidol and clozapine and included several terms consistent with APD-induced EPS including 'Appendicular dystonia', 'Quadrupedal gait', Huntington's disease' and 'Movement disorder'. While APD-induced EPS and movement disorders are principally thought to arise through nigrostriatal dopaminergic receptor inhibition, there is evidence that altered redox balance/oxidative neurotoxic stress may also be involved (Andreassen and Jorgensen, 2000; Lohr et al., 2003; Thelma et al., 2007; Cho and Lee, 2012). The aforementioned glutaredoxin family has been associated with neurodegenerative disease (Akterin et al., 2006; Diwakar et al., 2007; Saeed et al., 2008). Similarly, APD-induced differential expression of BCL2 family members resulted in both neuroprotective and neurotoxic effects in rat brains, as well as in human neuronal cell lines (Lezoualc'h et al., 1996; Post et al., 2002; Wei et al., 2003; Fatemi et al., 2012). We also observed up-regulation of the stress-responsive gene *PRKRA*, which controls the apoptotic PKR pathway, after clozapine and haloperidol exposure (Patel et al., 2000; Donze et al., 2004; Taylor et al., 2005; Lee et al., 2007; Singh and Patel, 2012). Abnormalities in *PRKRA* have been associated with deficits in nervous system development and neuromuscular function (Bennett et al., 2008) as well as dystonia-parkinsonism (Camargos et al., 2008; Seibler et al., 2008; Bragg et al., 2011).

Conclusion

While APDs have revolutionized the treatment of psychotic and behavioral disorders, much of the fine detail underlying the neuropsychopharmacology remains to be determined, particularly in regards to side effects in peripheral tissue. In this study we examined mRNA–miRNA interactions in APD treated T-lymphocyte cultures and revealed several pathways with significance to T-cell function and CIA, such as cellular metabolism and oxidative stress, which may also offer insight into the molecular mechanisms that underlie APD-induced metabolic and neurological side effects in other cell types.

Supplementary material

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145713001752.

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Statement of Interest

The authors declare no conflict of interest.

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