REGULAR RESEARCH ARTICLE

Mechanisms Underpinning the Polypharmacy Effects of Medications in Psychiatry

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Abstract

Background: Bipolar disorder is a mental health condition with progressive social and cognitive function disturbances. Most patients' treatments are based on polypharmacy, but with no biological basis and little is known of the drugs' interactions. The aim of this study was to analyze the effects of lithium, valproate, quetiapine, and lamotrigine, and the interactions between them, on markers of inflammation, bioenergetics, mitochondrial function, and oxidative stress in neuron-like cells and microglial cells.
Methods: Neuron-like cells and lipopolysaccharide-stimulated C8-B4 cells were treated with lithium (2.5 mM), valproate (0.5 mM), quetiapine (0.05 mM), and lamotrigine (0.05 mM) individually and in all possible combinations for 24 h. Twenty cytokines were measured in the media from lipopolysaccharide-stimulated C8-B4 cells. Metabolic flux analysis was used to measure bioenergetics, and real-time PCR was used to measure the expression of mitochondrial function genes in neuron-like cells. The production of superoxide in treated cells was also assessed.
Results: The results suggest major inhibitory effects on proinflammatory cytokine release as a therapeutic mechanism of these medications when used in combination. The various combinations of medications also caused overexpression of PGC1α and ATP5A1 in neuron-like cells. Quetiapine appears to have a proinflammatory effect in microglial cells, but this was reversed by the addition of lamotrigine independent of the drug combination.
Conclusion: Polypharmacy in bipolar disorder may have antiinflammatory effects on microglial cells as well as effects on mitochondrial biogenesis in neuronal cells.

Keywords: psychiatry, bipolar disorder, polypharmacy, inflammation, mitochondrial function

Significance Statement

This original research aimed to expand knowledge on why polypharmacy works better to treat bipolar disorder than individual drugs. The cellular and molecular basis for altered brain function in patients with bipolar disorder remains poorly understood. Accordingly, mechanisms underlying the molecular effects of drugs used to treat this disorder are largely unknown. Using neuronal like cells and microglial cells, we accessed the effects of commonly prescribed bipolar drugs alone and in associations to find out possible mechanism of action of this combinations and create new targets for drug discovery in this field.

Introduction

Bipolar disorder (BD) is a potentially neuroprogressive, chronic mental health condition characterized by alternate episodes of mania and depression that has secondary social and cognitive consequences. This disorder is ranked in the top 10 leading causes of lifelong disability worldwide according the World Health Organization and affects approximately 2% of the population (Duong et al., 2016; Rihmer et al., 2016). The pathophysiology of BD is poorly understood. Most useful psychotropic medications produce maximal responses after several weeks, and this observation indicates that chronic adaptive molecular changes are crucial components of successful treatment rather than simple immediate receptor binding effects (Rizig et al., 2012). Operant biological pathways may include mitochondrial dysfunction, alterations in circulating levels of proinflammatory cytokines, neurotrophins, and oxidative stress (OS) (Bhat et al., 2015).

Polypharmacy can be defined as the concurrent use of multiple medications. For individuals with BD, these typically include agents such as mood stabilizers, atypical antipsychotics, antidepressants, and benzodiazepines (Adli et al., 2005). The majority of patients treated for BD receive multiple psychotropic medications concurrently (Goldberg et al., 2009; Sachs et al., 2014). The Systematic Treatment Enhancement Program for Bipolar Disorder showed that monotherapy is applicable to <20% of the patients (Goldberg et al., 2009). Polypharmacy in the management of BD is common given the characteristics of the disorder (e.g., chronicity, comorbidity, uncertainty associated with shifting polarity of illness), characteristics of the drugs (delayed time of action, tolerability, selectivity), and the high frequency of inadequate response to monotherapy (Sachs et al., 2014). Nevertheless, little is known regarding the drug interactions or potential cumulative effects, and there is no clear biological basis for the selection of drug combinations to improve efficacy. Medications prescribed for BD may have plentiful drug-drug interactions, raising safety concerns about polypharmacy and reiterating the need for biologically based evidence (Tsai et al., 2014).

The therapeutic effects of some medications used in the management of the disease have recently been suggested to be related to both mitochondrial and inflammatory mechanisms; however, current literature on this topic is scant. Notwithstanding this, both central and peripheral inflammatory processes seem to play a role in the pathophysiology of BD (Berk et al., 2011). Suppression of microglia-mediated inflammation has been proposed as a strategy in therapy as prolonged immune activation can damage neuronal structure through the production of elevated levels of reactive oxygen species (ROS) and neurotoxins (Block et al., 2007; Czeh et al., 2011). Activation of inflammatory pathways and alterations in glutamate metabolism appear to converge on glial cells and may play a role in mood disorders. In particular, inflammatory mediators might act through glial cells to regulate extracellular glutamate concentrations in both physiological and pathological conditions (Haroon et al., 2017).

Mitochondrial dysfunction is noted in BD driving impaired brain energy metabolism. There is evidence of increased basal metabolic rate in mania (Caliyurt and Altiay, 2009) and decreased bioenergetics in depression, concordant with a biphasic model of energy generation in BD paralleling symptomatology. There are high rates of comorbidity of BD with mitochondrial diseases, and mood stabilizers have documented effects on mitochondria. Mitochondrial dysfunction can increase production of ROS, leading to enhanced OS, causing deleterious consequences on signal transduction, synaptic plasticity, and cellular resilience (de Sousa et al., 2014).

Understanding the actions of medications used to treat BD on these seemingly diverse yet interacting pathways may contribute to our understanding of the underlying pathology of the disease and also allow new, more effective, and targeted treatments to be identified. The aim of this study was to augment the understanding of the mechanisms of action of these medications and, especially, the cumulative effects of these drugs in combination. Therefore, we tested the effects of combinations of lithium, valproate, quetiapine, and lamotrigine on markers of inflammation, bioenergetics, mitochondrial function, and OS in neuron-like cells and microglial cells. These drugs were chosen from diverse pharmacological classes to represent the mechanism of actions of various medications used to treat psychiatric disorders, including BD.

Materials And Methods

NT2 Cell Culture

NTera2/cloneD1 (NT2) human teratocarcinoma cells (ATCC) were cultured in media comprising Dulbecco's modified Eagle's Medium (Life Technologies) including 10% fetal bovine serum (Thermo Fisher Scientific), and 1% antibiotic/antimycotic solution (Life Technologies). These cells are known to express a neuron-like phenotype following retinoic acid-induced differentiation (Pleasure et al., 1992). To achieve that, the cells were treated with retinoic acid (Sigma-Aldrich) at 1×10^{-5} M for 28 days with media refreshed every 2 to 3 days. Neuronal markers such as NeuroD (neuronal differentiation), GluR (glutamate receptor), and Tau (cytoskeletal protein) were measured by real-time PCR (RT-PCR) to confirm the neuronal-like state of the cells (data not shown) (Megiorni et al., 2005). Cells were seeded onto 24-well and 96-well plates coated with 10 μg/mL poly-D-lysine (Sigma-Aldrich) and 10 μg/mL laminin (Sigma-Aldrich) at 2×10^{5} cells/well (24-well plates) and 8×10^{4} cells/well (96-well plates and 24-well XF24 cell culture microplates, Seahorse Bioscience) with further addition of mitotic inhibitors (1 μM cytosine and 10 μM uridine; Sigma-Aldrich) for a total of 7 days with media refreshed every 2 to 3 days to maintain an enriched culture of differentiated neuronal cells (NT2-N) for the drug treatments.

C8-B4 Cell Culture Conditions

C8-B4 microglial cells (ATCC CRL-2540) were cultured in Dulbecco's modified Eagle's Medium (Life Technologies) with

10% fetal bovine serum (Life Technologies) and seeded onto 24-well plates at 1.3 x 10^5 cells/well for the measurement of cytokine release.

Drug Treatments

The NT2-N cells were treated with lithium (2.5 mM), valproate (0.5 mM), quetiapine (0.05 mM), and lamotrigine (0.05 mM) individually and in all possible combinations. All drugs were purchased from Sigma-Aldrich. C8-B4 cells were stimulated with 1 ng/mL lipopolysaccharide (LPS) per well and then received the same treatments as for NT2-N cells. Vehicle control cells were treated with an equal volume of MilliQ water for lithium or valproate controls, 0.2% DMSO for lamotrigine or quetiapine controls, or the combination of both (for various drug combinations as required). Dose response studies were performed to find optimal doses to balance the effects of each individual drug on the expression of a number of candidate gene targets, such that the effect of one drug did not dominate the overall effect and did not affect viability of the cells (data not shown). Each treatment had n=6 replicates.

Following the 24-h treatment, the cells were harvested and RNA was extracted using RNeasy mini kits (Qiagen) and reverse-transcribed to produce cDNA using Maxima H Minus first strand cDNA synthesis kit (Thermo Fisher Scientific) following the manufacturer's instructions.

Inflammatory Biomarkers

A panel of 20 cytokines (interleukin [IL]-1a, IL-1b, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17a, Eotaxin, granulocyte-colony stimulating factor [GCSF]), granulocyte macrophage colony-stimulating factor, interferon-gamma, keratinocyte chemoattractant, chemokine ligand 5, tumor necrosis factor-alpha) was measured with a Bioplex Pro mouse cytokine assay kit (Bio-Rad) according to the manufacturer's protocol, using media from LPS-stimulated C8-B4 cells following 24-h drug treatments and normalized by the protein content (Pierce™ BCA protein assay kit; Thermo Fisher Scientific).

Gene Expression

RT-PCR was used to measure the expression of specific genes relating to mitochondrial function in NT2-N cells following drug treatments: peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α: forward primer, AAC CAC ACC CAC AGG ATC AGA; reverse primer, TCT TCG CTT TAT TGC TCC ATG A; efficiency: 95.30%), NADH:ubiquinone oxidoreductase subunit B8 (forward primer, CAG CCT CCC ACA TGA CCA AG; reverse primer, GCC ATC ATC CGG GTA AGG TT; efficiency: 94.52%), succinate dehydrogenase subunit B (forward primer, ATG TGG CCC CAT GGT ATT GG; reverse primer, TGG TGT CAA TCC TTC GGG TG; efficiency: 96.70%), ubiquinol-cytochrome C reductase core protein II (forward primer, GGG AAA GTG TTA GCG GGG AA; reverse primer, TGG CTT TAA CTT TGG GGG CA; efficiency: 97.31%), mitochondrially encoded cytochrome C oxidase II (forward primer, CCG TCT GAA CTA TCC TGC CC; reverse primer, GAG GGA TCG TTG ACC TCG TC; efficiency: 95.94%), ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1 (ATP5A1: forward primer, TCA GTC TAC GCC GCA CTT AC; reverse primer, ATG TAC GCG GGC AAT ACC AT; efficiency: 89.63%), and hexokinase II (HK2: forward primer, CCA ACC TTA GGC TTG CCA TT; reverse primer, CTT GGA CAT GGG ATG GGG TG; efficiency: 104.16%).

The experiments were carried out in a PikoReal™ qPCR machine (Thermo Fisher Scientific) using the following protocol: 95℃ for 7 minutes, 4 cycles of 95℃ for 30 seconds and 60℃ for 1 minute and then data acquisition, 60℃ for 30 seconds, 55℃ to 95℃, data acquisition and 20℃ for 10 seconds. Resultant melt curves were used as an indicator of amplification specificity. The Quant-iT OliGreen ssDNA Assay Kit (Life Technologies) was used to quantify the cDNA concentration in each sample as per the manufacturer's instructions. Gene expression data were quantified using the 2ΔCT method normalized to the derived cDNA concentration of each sample.

Mitochondrial Function Quantitation

The cellular bioenergetic profile of NT2-N-treated cells was assessed using a Seahorse XF24 Flux Analyzer (Seahorse Bioscience). Three basal oxygen consumption rate (OCR) measurements were performed, and measurements were repeated following injection of oligomycin (1 mM), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (1 mM) and Antimycin A (1 mM). Basal extracellular acidification rate (ECAR) was determined from data collected at basal measurement points. Calculations of respiratory parameters of mitochondrial function were performed as previously described (Martin et al., 2014). The protein concentration from each well was quantified by Pierce BCA protein assay (Thermo Fisher Scientific) to account for differences in cell density during data analysis. Each treatment had n=4 to 6 replicates.

Mitochondrial Superoxide Production

The production of superoxide in NT2-N cells was measured using MitoSOX™ (Thermo Fisher Scientific) according to the manufacturer's instructions. The increase in fluorescence intensity was measured with a FlexStation II Scanning Fluorometer (Molecular Devices; λex=510 nm, λem=580 nm). Results are presented relative to vehicle treated cells.

Statistical Analysis

Shapiro-Wilk (inflammatory biomarkers) or Kolmogorov-Smirnov (all the remain analysis) tests were used to check data sets for normality of distribution. In addition, biomarker data were visually inspected using box-plots for presence of outliers and influential data. Levene's test was used to determine whether equal variances could be assumed between groups. Since there were no outliers in biomarker data, and there was no alarming heterogeneity, parametric tests were performed for biomarker data analyses.

For the mitochondrial function and oxidative stress analysis, drug treatment groups were compared against their respective controls using independent samples t tests. Differences were considered statistically significant when $P \leq .05$.

The cytokine levels and mitochondrial gene expression were analyzed using 1-way ANOVA followed by pairwise comparisons. For each ANOVA test the overall F-test significant level of 0.05 was considered. The 1-way ANOVA tests have 7 numerator degrees of freedom (df); we are therefore entitled to investigate 6 (i.e., 1-df) orthogonal contrasts of the treatment means without the need to consider the so-called "family-wise" Type I error rate for these multiple t tests. However, due to a relatively large number of pairwise comparisons (4 comparisons) within each ANOVA, the significance level for pairwise between group comparisons was set at $P \leq .01$.

Statistical analysis was performed using Statistical Package for the Social Sciences version 22 (SPSS) software.

Results

Inflammation Biomarkers

Microglial cells are the resident macrophages of the central nervous system. They perform key signaling and scavenging roles, interact with neurotransmission, and mediate neuroplasticity. Microglia are activated by various neuropathological states and can, in response, secrete proinflammatory cytokines (Derecki and Kipnis, 2013). LPS strongly upregulated the secretion of most cytokines measured from C8-B4 cells (Figure 1). The effects of the tested drugs on the release of cytokines from LPS-stimulated C8-B4 cells are shown in Table 1. The inflammatory role of microglia in the brain justifies the use of this cell line to assess the effects of these drugs on inflammation.

Lithium (alone) had no effect on cytokine release from LPS-stimulated C8-B4 cells. Valproate and lamotrigine alone had relatively minor effects. Valproate and lamotrigine had no significant effect on cytokine levels. These data suggest that these drugs when acting alone have little overall effect on cytokine release from activated glial cells. Quetiapine, however, robustly increased the release of all cytokines measured except for GCSF, suggesting a proinflammatory effect on glial cells. Lithium combined with either lamotrigine or quetiapine slightly increased cytokine release, suggesting a proinflammatory effect. Valproate when combined with either lamotrigine or quetiapine also increased release of some cytokines, suggesting an overall proinflammatory effect. On the other hand, the combination of lamotrigine and quetiapine robustly reduced cytokine release, suggesting a strong antiinflammatory effect.

When 3 drugs were used in combination, a clear pattern emerged. The combinations of lithium/valproate/lamotrigine and lithium/valproate/quetiapine increased cytokine production and release, while the combinations of lithium/lamotrigine/quetiapine and valproate/lamotrigine/quetiapine robustly reduced cytokine release. The 4 drugs used in combination also robustly lowered cytokine release.
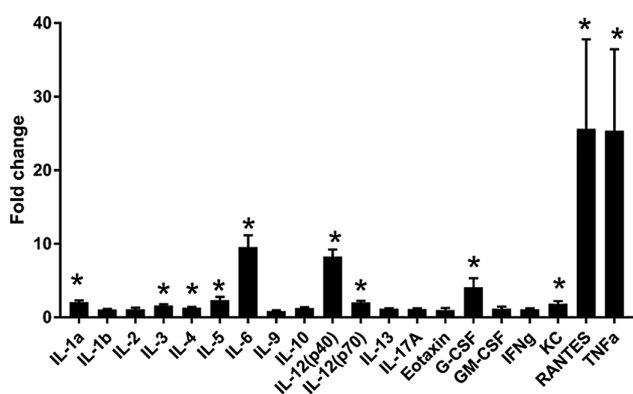

Figure 1. Effects of lipopolysaccharide (LPS) on C8-B4 cells (fold change relative to vehicle treated cells). LPS treatment increased the release of a number of proinflammatory cytokines, including interleukin (IL)-1a, IL-3, IL-4, IL-5, IL-6, IL-12, GCSF, KC, RANTES, and tumor necrosis factor (TNF)α. These data confirm that LPS had a proinflammatory effect on C8-B4 cells. n = 3 samples in duplicate. *P < .05 compared with vehicle treated cells (descriptive statistical test).

Mitochondrial Gene Expression

Table 2 shows an overview of expression, relative to control groups, of genes related to mitochondrial function in treated NT2-N cells. ATP5A1 expression was significantly increased following most treatments (except for lithium, lamotrigine, valproate/quetiapine, lamotrigine/quetiapine, and lithium/valproate/lamotrigine). PGC1α expression was increased by most treatments, including a 60% increase in response to treatment with the combination of all 4 drugs. HK2, succinate dehydrogenase subunit B, mitochondrially encoded cytochrome C oxidase II, NADH:ubiquinone oxidoreductase subunit B8, and ubiquinol-cytochrome C reductase core protein II expression had minor changes across the different treatments. The RT-PCR data suggest a tendency for reduced expression of genes related to mitochondrial function in cells treated with lithium relative to control. This is consistent with the aerobic respiration data of lithium-treated cells, which appeared to be reduced (Figure 3A). Quetiapine significantly increased mRNA levels of ATP5A1. This result was unexpected given that flux bioanalysis revealed significantly decreased basal respiratory rate in cells treated with quetiapine.

Mitochondrial Function

Figure 2 shows an overview of mitochondrial function and basal bioenergetics in NT2-N treated cells. Lithium-treated cells (n = 6) exhibited a 27% lower basal respiratory rate (P = .032) and a 25% lower rate of ATP turnover (P = .032) relative to the control group (n = 5) (Figure 3A). The cells also appeared to exhibit increased basal and maximal glycolytic rates, which is consistent with decreased capacity for aerobic respiration as shown in lithium-treated NT2-N cells (Figure 3B). Cells treated with valproate tended to have the same direction of changes, although these were not significant. Quetiapine appeared to decrease respiratory activity and capacity in NT2-N cells. Quetiapine-treated cells (n = 6) exhibited a 20.1% lower basal respiratory rate (P = .026) compared with the control group (n = 4) (Figure 3A).

Conversely, lamotrigine was the only drug tested that tended to increase respiratory rate and capacity. However, the variability in the data rendered these increases statistically insignificant. Basal and maximal glycolytic rates appeared to be reduced in cells treated with quetiapine and lamotrigine; however, no significant differences were found compared with control cells.

Cells simultaneously treated with lithium and quetiapine exhibited a 31% lower basal respiratory rate (P = .029) and a 32% lower rate of ATP turnover (P = .041) compared with the control group (Figure 3C), with no significant change in basal or maximal glycolytic rate (Figure 3D).

No difference was found in the OCR values, basal or maximal glycolytic rates following treatment with the various combinations of 3 or 4 drugs relative to the control groups (Figure 2). Overall, using the drugs in combinations appeared to suppress any effect on mitochondrial function caused by the treatment with individual drugs in NT2-N cells.

Mitochondrial Superoxide Production

Mitochondrial superoxide is the major intracellular source of ROS. Cells treated with lithium plus quetiapine showed significantly decreased superoxide production (P = .05), and the combination of lamotrigine and quetiapine increased superoxide production (P = .027) (Figure 4).

Table 1. Effects of BD Drugs Alone and in Combination on Cytokine Production and Release from C8-B4 Cells

	IL-1A	IL-1B	IL-3	IL-4	IL-5	IL-6	IL-10	IL-12(P40)	IL-12(P70)
Li	99.67±0.12	94.42±0.12	96.77±0.25	93.29±0.16	86.89±0.16	106.85±0.39	95.94±0.16	76.99±0.28	95.57±0.20
Val	78.68±0.04	81.67±0.00	63.14±0.03	74.09±0.04	68.44±0.04	47.46±0.03	71.98±0.04	20.93±0.03	72.38±0.08
Lam	166.07±0.17	87.40±0.05	166.38±0.26	86.43±0.06	84.70±0.07	37.37±0.07	98.02±0.07	55.78±0.18	83.86±0.10
Quet	284.69±0.23	140.47±0.02* (P=.004)	496.43±0.28* (P<.001)	182.73±0.09* (P=.003)	223.20±0.03* (P<.001)	277.90±0.22* (P=.006)	192.63±0.07* (P=.004)	917.69±1.80* (P<.001)	289.46±0.34* (P=.003)
Li/Val	96.25±0.02	101.62±0.01	101.19±0.05	98.38±0.03	93.40±0.04	82.07±0.13	97.12±0.02	30.49±0.04	88.83±0.05
Li/Lam	299.46±0.61* (P=.010)	114.37±0.03	313.87±0.41	131.09±0.19	158.86±0.10	382.98±0.46* (P<.001)	140.15±0.18	521.52±1.82	224.56±0.54
Li/Quet	50.07±0.02	111.53±0.07	41.26±0.02	126.77±0.15	210.69±0.41	233.86±0.52	123.58±0.05	305.26±0.68	153.00±0.17
Val/Lam	47.41±0.00	115.47±0.07	46.10±0.06	113.89±0.08	191.64±0.06	259.39±0.10	104.89±0.06	125.90±0.19	119.98±0.13
Val/Quet	49.70±0.02	110.76±0.07	37.26±0.02	106.34±0.06	197.85±0.35	220.97±0.55	114.08±0.09	155.10±0.48	155.63±0.24
Lam/Quet	18.17±0.01* (P<.001)	76.13±0.02	66.58±0.21*	70.64±0.17* (P<.001)	57.27±0.04* (P<.001)	15.78±0.03* (P<.001)	58.12±0.02* (P=.002)	31.68±0.03* (P<.001)	37.65±0.02
Li/Val/Lam	94.17±0.14	149.15±0.06* (P=.006)	79.13±0.16* (P=.001)	173.92±0.25* (P<.001)	427.60±0.27* (P<.001)	634.05±0.72* (P<.001)	164.93±0.17	460.83±0.94	287.94±0.45* (P=.010)
Li/Val/Quet	50.58±0.07	130.30±0.12	76.15±0.16	163.64±0.23	372.68±0.30* (P<.001)	337.26±0.31	143.90±0.20	331.43±0.76	236.05±0.68
Li/Lam/Quet	15.74±0.01* (P<.001)	68.84±0.05* (P=.002)	45.31±0.07* (P<.001)	49.75±0.07* (P<.001)	55.64±0.02* (P<.001)	16.87±0.02* (P<.001)	54.46±0.06* (P=.001)	23.85±0.06* (P<.001)	27.02±0.07* (P<.001)
Val/Lam/Quet	16.39±0.00* (P<.001)	76.92±0.00	47.21±0.07* (P=.003)	53.25±0.04* (P=.001)	61.55±0.04* (P=.001)	12.12±0.01* (P<.001)	59.08±0.02* (P=.003)	15.69±0.02* (P<.001)	39.20±0.02* (P=.002)
Li/Val/Lam/Quet	17.41±0.02* (P<.001)	77.08±0.06	63.33±0.10	61.99±0.06* (P=.002)	67.37±0.03* (P=.008)	19.32±0.02* (P<.001)	62.17±0.11* (P=.007)	24.68±0.07* (P<.001)	45.98±0.13* (P=.008)

	IL-13	IL-17A	EOTAXIN	GCSF	GMCSF	IFNG	KC	RANTES	TNFA
Li	101.51±0.15	98.28±0.20	99.28±0.33	84.31±0.27	99.09±0.23	99.54±0.14	104.63±0.25	68.30±0.20	129.32±0.49
Val	80.42±0.07	71.37±0.04	44.92±0.05	30.59±0.01	66.43±0.06	81.09±0.03	62.56±0.05	60.71±0.08	33.58±0.03
Lam	96.10±0.04	80.87±0.09	87.09±0.41	45.79±0.07	117.27±0.30	113.38±0.09	85.67±0.10	72.67±0.25	58.67±0.09
Quet	182.05±0.09* (P=.004)	163.03±0.09	209.88±0.05	120.48±0.07	207.34±0.01	181.82±0.08	297.50±0.23* (P<.001)	1928.86±7.1* (P=.007)	753.62±1.00* (P=.006)
Li/Val	103.13±0.01	107.20±0.01	125.82±0.05	48.74±0.03	118.33±0.01	106.99±0.01	101.24±0.03	67.98±0.12	88.49±0.11
Li/Lam	148.23±0.12	109.34±0.11	76.98±0.07	66.26±0.08	118.52±0.14	129.22±0.12	223.62±0.27* (P=.009)	801.71±3.85	750.64±2.58* (P=.006)
Li/Quet	143.54±0.06	121.81±0.01	304.55±1.01	52.62±0.06* (P=.002)	204.03±0.20	143.19±0.02	186.98±0.46	253.87±0.68	159.99±0.89
Val/Lam	116.51±0.07	100.25±0.10	308.97±0.55	49.98±0.01* (P=.002)	153.81±0.29	115.64±0.04	103.06±0.04	213.46±0.14	116.15±0.11
Val/Quet	126.97±0.13	121.44±0.11	166.89±0.24	34.93±0.03* (P<.001)	138.71±0.23	130.52±0.23	110.98±0.10	273.76±0.86	42.50±0.23
Lam/Quet	70.77±0.03	47.69±0.02* (P=.001)	122.50±0.25	8.64±0.00* (P<.001)	28.39±0.10* (P=.007)	46.57±0.05* (P=.007)	40.19±0.04* (P<.001)	13.92±0.01* (P=.001)	1.55±0.00* (P<.001)
Li/Val/Lam	204.12±0.21	168.25±0.17	347.42±0.58	83.98±0.03	315.84±0.35* (P<.001)	237.39±0.27* (P=.003)	470.21±0.18* (P<.001)	1360.39±5.4	2377.16±6.1* (P<.001)
Li/Val/Quet	169.39±0.34	149.17±0.27	312.24±0.28	48.79±0.10* (P=.001)	233.78±0.26	190.51±0.40	307.22±0.39* (P<.001)	984.03±2.67	821.81±2.47
Li/Lam/Quet	52.99±0.02	42.00±0.06* (P<.001)	103.07±0.24	7.36±0.01* (P<.001)	39.84±0.09* (P<.001)	38.54±0.08* (P=.001)	63.56±0.03* (P=.010)	12.38±0.02* (P=.001)	1.36±0.00* (P<.001)
Val/Lam/Quet	68.71±0.03	51.72±0.02* (P=.002)	120.55±0.21	6.47±0.00* (P<.001)	44.78±0.02* (P<.001)	53.98±0.05	35.16±0.01* (P<.001)	16.86±0.01* (P<.001)	0.82±0.00* (P<.001)
Li/Val/Lam/Quet	73.88±0.13	56.90±0.11* (P=.006)	124.74±0.40	7.06±0.01* (P<.001)	51.91±0.06* (P=.002)	53.76±0.15	64.14±0.06 (P=.010)	26.05±0.08* (P=.007)	2.34±0.01* (P<.001)

Abbreviations: BD, bipolar disorder; Lam, lamotrigine; Li, lithium; Quet, quetiapine; Val, valproate.

Percent of vehicle treated cells + SEM. n=3 samples in duplicate except for DMSO vehicle, n=6. One-way ANOVA and pairwise comparison corrected for multiple testing *P<.01 compared with vehicle treated cells.

Table 2. Effects of BD Drugs Alone and in Combination on Mitochondrial Gene Expression in NT2-N Cells

	SDHB	ATP5A1	MTCO2	NDUFB8	HKII	UQCRC2	PGC1α
Li	95.28±20.34	111.58±16.03	73.14±17.20	89.63±19.27	92.90±14.42	96.70±10.81	95.04±10.12
Val	130.31±6.22	189.18±36.41* (P=.009)	146.88±6.09* (P=.003)	85.35±12.20	116.52±3.48	60.11±11.49	145.29±11.45* (P=.010)
Quet	88.54±4.48	333.82±11.18* (P<.001)	115.59±6.28	92.85±3.50	111.29±3.35	90.31±5.39	97.79±5.63
Lam	84.38±6.11	139.43±18.42	103.98±18.98	103.84±4.24	128.31±9.04	89.21±5.12	130.57±8.73* (P=.010)
Li/Val	122.94±14.29	140.81±11.42* (P=.010)	113.40±14.60	97.71±7.20	104.36±9.01	128.54±17.04	142.28±14.90
Li/Quet	91.29±3.68	594.17±74.09* (P=.010)	92.44±15.00	87.45±9.04	93.09±8.00	104.87±6.49	102.27±5.97
Li/Lam	80.55±7.12	163.76±29.11* (P=.010)	107.68±12.61	99.44±8.38	92.41±11.20	101.56±10.76	91.22±8.04
Val/Quet	87.46±8.56	126.54±22.67	99.96±6.87	90.17±7.06	111.91±3.20	84.75±5.57	126.54±2.15
Val/Lam	76.29±14.44	143.55±41.98* (P=.010)	98.44±22.62	95.52±18.96	92.19±6.83	88.65±12.37	105.81±13.26
Lam/Quet	146.37±33.53	442.98±141.97	118.50±9.33	118.53±30.37	123.96±10.45	130.47±36.14	126.96±12.46
Li/Val/Quet	105.50±1.58	454.76±2.10* (P=.005)	95.56±7.61	99.48±1.68	113.09±4.45	86.29±2.85	153.87±6.13* (P=.010)
Li/Val/Lam	104.45±18.49	216.69±6.84	115.12±20.09	94.76±18.72	125.50±13.05	95.23±17.46	142.63±19.53
Li/Quet/Lam	95.93±4.62	362.43±23.76* (P=.010)	71.97±15.57	91.38±3.55	95.33±5.89	86.88±6.46	117.39±5.52
Val/Quet/Lam	93.44±3.71	424.39±50.41* (P=.010)	93.41±7.96	88.83±4.06	118.81±5.90	78.79±6.25	148.32±5.87
Li/Val/Quet/Lam	98.26±9.88	575.31±51.74* (P=.002)	124.89±13.45	98.78±4.24	104.03±14.95	98.09±5.31	160.34±9.45* (P<.001)

Abbreviations: BD, bipolar disorder; Lam, lamotrigine; Li, lithium; Quet, quetiapine; Val, valproate.

Percent of vehicle treated cells + SEM. n=6 samples in duplicate. *P<.05 compared with vehicle treated cells (descriptive statistical test).

Discussion

The major finding in this study is the broadly antiinflammatory effects of polypharmacy in BD, as opposed to monotherapy, where less consistent patterns were seen. BD is associated with inflammation in the periphery and in the central nervous system, and there is increasing evidence to suggest that activation of microglia is a key element in this process (Block et al., 2007; Reus et al., 2015). Microglial proinflammatory cytokine release can lead to oligodendrocyte cell death by apoptosis, blood brain barrier destruction, neuronal damage, and mitochondrial impairment (Naaldijk et al., 2016). Our results demonstrated effects on proinflammatory cytokine release that may play a role in the therapeutic mechanisms of some commonly prescribed drugs when used in combination. To our knowledge, this is the first study investigating differences in cytokine production between these drugs alone and in combination in a microglial cell line. It should be noted that this study was conducted in a mouse microglial cell line, and it would be interesting to see whether these results can be replicated in human microglial cells. The results are indicative of additive antiinflammatory effects of specific psychopharmacological combinations on cytokine production, which could provide a protective effect in neurons.

The literature regarding human cytokine levels after treatment for BD is conflicting. Some studies showed a reduction in IFN-γ peripherally within 6 weeks of treatment (Kim et al., 2007; Uyanik et al., 2015). Recently, 2 meta-analyses on the effects of medications on cytokine profile were published. Modabbernia et al. (2013) in a meta-analysis provided evidence for a lack of medication effect on plasma concentrations of IL-2, IL-4, sIL-6R, and INF-γ (Modabbernia et al., 2013). In another meta-analysis, Munkholm et al. (2013) reviewed 10 studies on the association between plasma cytokine levels and medicines; of these, 6 studies did not find any significant correlation between medication status and cytokine levels, and 4 studies demonstrated correlations for individual cytokine parameters, of which none were replicated (Munkholm et al., 2013). Ricken et al. (2018) recently published a study showing the treatment with lithium does not change cytokine serum levels (IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, INF-γ), even after augmentation (Ricken et al., 2018). In addition, a systematic review showed 2 studies that report no effect of valproate on cytokine levels (van den Ameele et al., 2016).

It is important to note that most studies analyzed a heterogeneous group of patients regardless of medication status.

Considering every drug separately in BD patients could offer a clearer picture of medication effect on cytokines, but that will be limited to small sample sizes. Analyzing these effects in cell culture can clarify the possible mechanism of action of these drugs. In addition, it should be pointed out that the effects of the drugs in the central nervous system may differ from the peripheral effects in circulating cells, that way being more informative than the systemic concentrations of these cytokines.

Quetiapine was the most cytokine-active drug tested in our study, showing strong proinflammatory effects that could in theory contribute to the low-level inflammatory state during antipsychotic treatment reported in previous studies (Sarvari et al., 2014). However, when combined, especially with lamotrigine, this effect was suppressed and an antiinflammatory effect appeared to emerge.

Multiple drug combinations that include both lamotrigine and quetiapine appeared to have strong antiinflammatory effects on glial cells. The reduction of proinflammatory cytokines observed in this study could reduce the activated state of microglial cells. It has been recently proposed that glial inflammation can lead to increased release of glutamate into the extrasynaptic space by decreasing the capacity of glial transporters to buffer and clear glutamate (Haroon et al., 2017). The effects of inflammation on glial cells can further affect glutamate neurotransmission. Our data suggest that the antiinflammatory effects from the combination of lamotrigine with quetiapine may be driven by lamotrigine playing a pivotal role in the inhibition of glutamate release in glial cells.

Considering antiinflammatory effects, in an association of lithium plus valproate, it is important to add lamotrigine and quetiapine rather than just one of them. Both drugs together had the power to shift the direction of the effect, which did not happen when they are added individually to the lithium plus valproate combination.

Our results demonstrate differential effects of these particular four drugs, alone and in combinations, on inflammatory processes. Given that inflammation is thought to play a role in BD pathophysiology, at least in some patients, these data could provide a foundation for developing a new treatment strategy to improve outcomes in some BD patients. It should be noted that lithium alone, which does not appear to affect cytokine release, is efficacious in some patients, suggesting that antiinflammatory activity is not required in some patients but is likely required for efficacy in others. The concept that a component of the mechanism of action
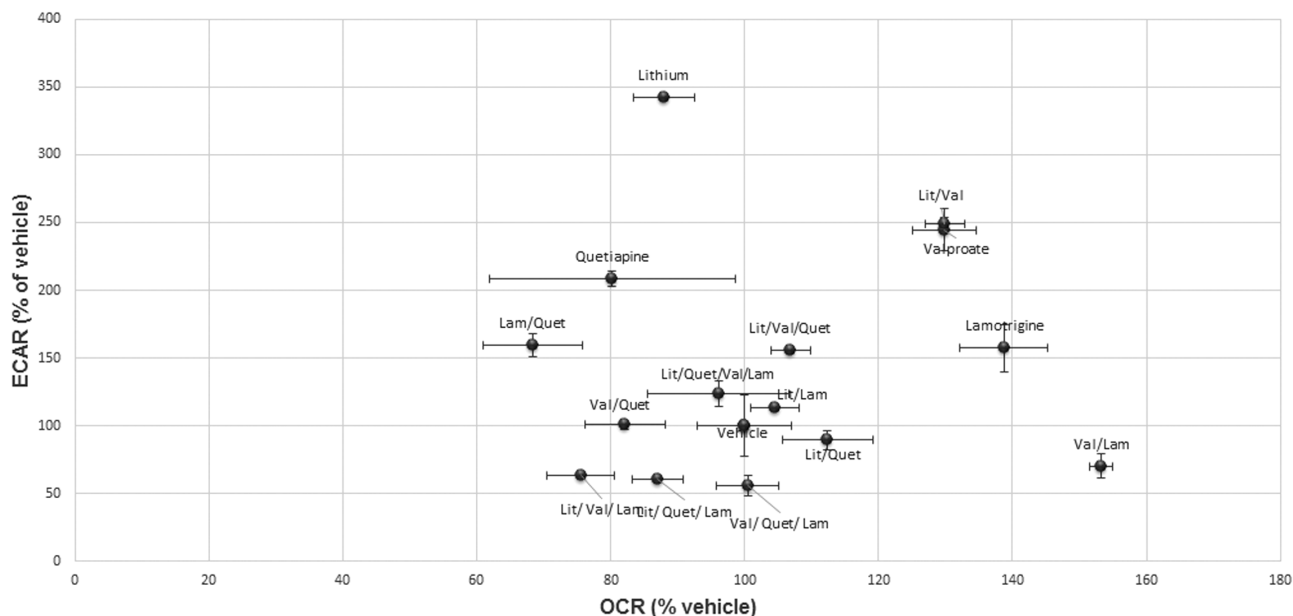
Figure 2. Mitochondrial function and basal bioenergetics in NT2-N treated cells. Basal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of NT2-N cells exposed for 24 h to either vehicle or bipolar disorder (BD) drugs alone and in combinations. All values are reported as perecent of vehicle±SEM (n=6/group).
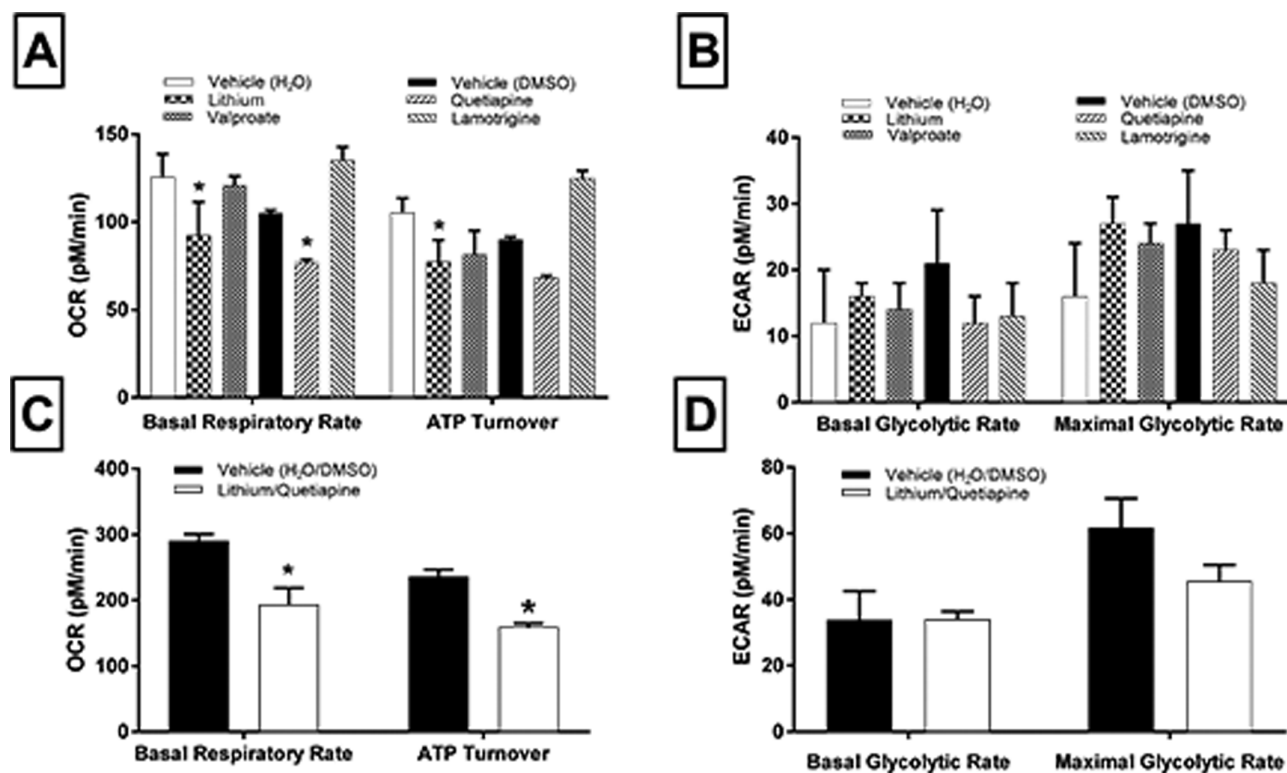

Figure 3. Aerobic respiration and glycolysis in differentiated NT2-N cells as determined by oxygen consumption rate flux in response to mitochondrial probes (for oxygen consumption rate [OCR]) and by flux bioanalysis (for extracellular acidification rate [ECAR]). Data represented as mean OCR (pmol/min) or ECAR (pM/min)±SEM; n=4 to 6 per group. *$P < .05$ compared with vehicle treated cells (descriptive statistical test).

of combination of these drugs includes an antiinflammatory effect is supported by a recent meta-analysis showing that nonsteroidal antiinflammatory drugs have a moderate antidepressant effect in subjects with bipolar disorder (Rosenblat et al., 2016).

Mitochondrial dysregulation associated with decreased oxidative phosphorylation shifts metabolism toward anaerobic energy production via glycolysis, increasing lactate levels and pH and leading to production of ROS, glutamate excitotoxicity, and apoptosis (Konradi et al., 2004). There is a body of evidence showing an essential dysfunction of mitochondria, such as decreases in mitochondrial respiration, high-energy phosphates and pH; changes in mitochondrial morphology; increases in
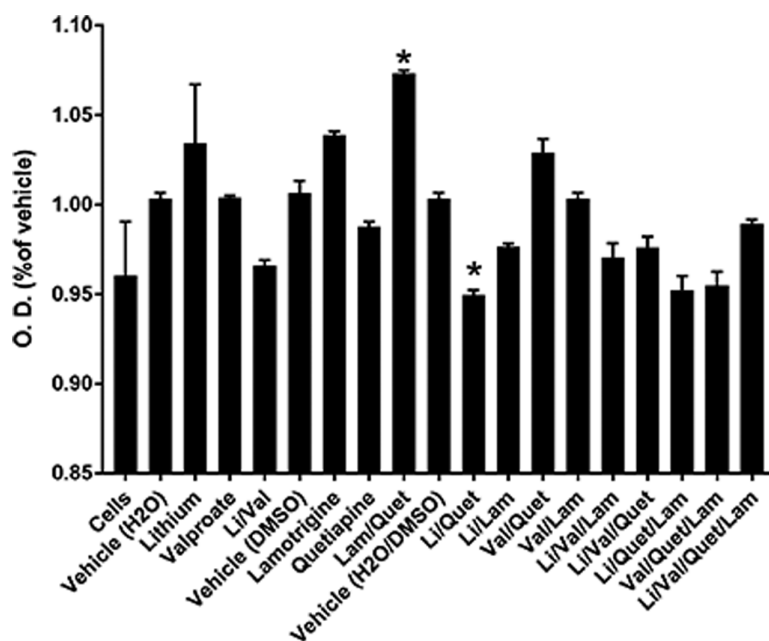
Figure 4. Effects of bipolar disorder (BP) drugs alone and in combination on the intracellular reactive oxygen species (ROS) production in NT2-N cells (relative to vehicle treated cells). n = 5 samples. *P < .05 compared with vehicle treated cells (descriptive statistical test).

mitochondrial DNA polymorphisms; and downregulation of nuclear mRNA molecules and proteins involved in mitochondrial respiration (Scaini et al., 2016). Other findings in BD include the dysregulation of mitochondria-related genes, increase of lactate in the brain, decrease of complex I in postmortem brains, abnormal mitochondrial structure in cells of bipolar patients, and elevation of isocitrate in cerebrospinal fluid associated with impaired function of isocitrate dehydrogenase (Kato, 2017).

Evidence from animal studies and cellular models on the molecular pharmacology of mood stabilizing drugs has implicated mitochondrial energy metabolism as a potential target. There is evidence that lithium and valproate modulate mitochondrial function by affecting the expression and activity of mitochondrial complexes. A recent study showed that valproate reversed the methamphetamine-induced inhibition of complexes I, II, III, and IV in rat brains (Valvassori et al., 2010). Similarly, lithium has been shown to increase the activities of mitochondrial complexes I, II, and III in the frontal cortex tissue of human brains (Maurer et al., 2009). The activity of complex IV was unaffected, suggesting that lithium selectively increases the activity of specific enzymes, rather than acting as a general stimulator of the electron transport chain (Maurer et al., 2009). A more recent study detected no difference in complex I activity between the lymphoblasts of bipolar patients and healthy control subjects (Huzayyin et al., 2014). It is proposed that the effects of lithium on complex I may be specific to neurons. Quetiapine has been shown to differentially affect genes for complex I subunits causing upregulation of NDUFV2 and downregulation of NDUFA10 (Young, 2007).

This evidence is consistent with the idea that commonly prescribed bipolar disorder drugs influence mitochondrial function. However, more data are required to demonstrate the precise nature of this association. The present findings showed relatively minor changes in mitochondrial function in NT2-N cells after treatment with BD drugs. The major finding is the combination of lithium and quetiapine affected mitochondrial function in NT2-N-treated cells compared with vehicle. Lithium significantly reduced basal respiratory rate and ATP turnover in NT2-N cells and tended to reduce other parameters of mitochondrial function as well.

Quetiapine significantly decreased basal respiratory rate in NT2-N cells and tended to reduce ATP turnover. But when both drugs were used together, we observed a significant further reduction in basal respiratory rate and ATP turnover in NT2-N cells. Together with this, a significant reduction in mitochondrial superoxide production and an increase in ATP5A1 gene expression was observed in NT2-N cells treated with lithium plus quetiapine. In contrast, only lamotrigine increased respiratory rate and capacity, which is noteworthy given that clinically it is the most robust antidepressant of the drugs studied here. Following the polarity index of drugs, anticonvulsants (particularly lamotrigine) seem more effective for prevention of depressive episodes, while atypical antipsychotics and lithium may have a manic polarity index of efficacy (Popovic et al., 2012). That observed efficacy in mania may be related to the decreased bioenergetics highlighted in this study.

Quetiapine has previously been reported to have antioxidant properties (Shao et al., 2005; Wang et al., 2005), so it is likely that the decrease in basal oxygen consumption seen in the present study was associated with decreased ROS production. However, this hypothesis of redox modulation by quetiapine was not supported by the results of the mitochondrial superoxide production analysis. This result could possibly be explained by the fact that the cells were not under a redox stress condition when treated, and the drugs were therefore unable to cause a downward shift in the baseline ROS levels. Our results showed a trend for reduced glycolysis in cells following treatment with quetiapine or lamotrigine, indicating a potential to reverse mitochondrial dysregulation. When both drugs were used together, the opposite effect was observed, suggesting an increase in mitochondrial dysregulation confirmed by the significant increase in mitochondrial superoxide production in NT2-N cells followed by the treatment with lamotrigine plus quetiapine.

Gene expression was measured for one subunit of each of the mitochondrial complexes and also measured for HK2, an important regulator of glycolysis, and PGC1α, a transcription factor involved in mitochondrial biogenesis. PGC1α is expressed at high levels in mitochondria rich cells with high energy demands, such as neurons, and has been shown to be a master regulator of mitochondrial

biogenesis and cellular energy metabolism (Wareski et al., 2009; Markham et al., 2014). The PGC1 family of coactivators controls mitochondrial density in primary neurons (Wareski et al., 2009). The upregulation of PGC1α by the drug combinations observed in our study could compensate for neuronal mitochondrial loss. Enhancing mitochondrial biogenesis regulates cellular oxidative capacity, and this may be useful for neuronal recovery and survival in neurodegenerative disorders (Wareski et al., 2009). It would also be expected that an increase in PGC1α expression would lead to augmented respiration as a result of increased mitochondrial biogenesis, but this effect may not be prominent within 24 h of commencing treatment. Overall, there was a clear effect on mitochondrial biogenesis, as indicated by PGC1α gene expression, that may contribute to a delayed effect of treatment with these drugs.

The use of human NT2-N cells and microglial cells provided an element of physiological relevance in the study of psychiatric drugs. The doses of the drugs used in this study are not all consistent with expected plasma concentrations when the drugs are used therapeutically; however, the main purpose of this study was to observe potential interactions between the various drug combinations, so the doses were modified empirically to ensure that any effects were not dominated by any one drug. Some limitations should be considered, including the fact that the duration of treatment may not be sufficient to elicit changes in mitochondrial function, oxidative stress, and cytokines. Monocultural cell lines may not reflect the status in the brain, with many interacting cellular elements. In the clinical context, additional factors such as the patient's age, weight, co-medications, gender, liver and renal function, and specific physiological states may also modulate the individual or combinatorial properties of these drugs.

In summary, the hypothesis that multiple combinations of psychiatric drugs affect inflammatory cytokine levels and mitochondrial function are partially validated by the results of this study. Drugs that work better in depression tend, in combination, to boost mitochondrial biogenesis, and those that work better in mania tend to inhibit biogenesis. The results suggest that the various combinations of drugs may be controlling mitochondrial capacity in neuronal cells due to an overexpression of PGC1α and ATP5A1. Quetiapine appears to have a strong proinflammatory effect in microglial cells, but this can be reversed when used together with lamotrigine or other drug combinations that include lamotrigine. Polypharmacy is the norm in BD with the average patient taking over 4 medicines. Our findings add to the understanding of how these drugs work together and shed light on why polypharmacy is usually more efficacious in BD, taking into account the significant role of inflammation in the disorder. This insight may help us better understand the therapeutic mechanisms of these drugs and identifies several targets for further research on the genetic and metabolic effects of mood stabilizers, especially in combination therapy.

Acknowledgments

Statement of Interest

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