Attachment 1

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Article

Different Effects of SSRIs, Bupropion, and Trazodone on Mitochondrial Functions and Monoamine Oxidase Isoform Activity

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Abstract: Mitochondrial dysfunction is involved in the pathophysiology of psychiatric and neurodegenerative disorders and can be used as a modulator and/or predictor of treatment responsiveness. Understanding the mitochondrial effects of antidepressants is important to connect mitochondria with their therapeutic and/or adverse effects. Pig brain-isolated mitochondria were used to evaluate antidepressant-induced changes in the activity of electron transport chain (ETC) complexes, monoamine oxidase (MAO), mitochondrial respiratory rate, and ATP. Bupropion, escitalopram, fluvoxamine, sertraline, paroxetine, and trazodone were tested. All tested antidepressants showed significant inhibition of complex I and IV activities at high concentrations (50 and 100 mol/L); complex II + III activity was reduced by all antidepressants except bupropion. Complex I-linked respiration was reduced by escitalopram >> trazodone >> sertraline. Complex II-linked respiration was reduced only by bupropion. Significant positive correlations were confirmed between complex I-linked respiration and the activities of individual ETC complexes. MAO activity was inhibited by all tested antidepressants, with SSRIs causing a greater effect than trazodone and bupropion. The results indicate a probable association between the adverse effects of high doses of antidepressants and drug-induced changes in the activity of ETC complexes and the respiratory rate of mitochondria. In contrast, MAO inhibition could be linked to the antidepressant, procognitive, and neuroprotective effects of the tested antidepressants.

Keywords: oxidative phosphorylation; mitochondrial respiration; reactive oxygen species; ATP; monoamine oxidase; antidepressants

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1. Introduction

Despite progress in neuroscience research over the past decades, major depressive disorder (MDD) pathophysiology has not been fully clarified. Understanding the effects antidepressants and other psychotropic drugs at the molecular level could provide new knowledge on pathophysiology and aid in the search for antidepressants with new mechanisms of action. Historically, depression has been viewed as a multifactorial disorder linked to different biochemical and physiological disturbances in neurotransmitter systems, neuroendocrine and immune functions, circadian rhythms, synaptic and structural neuroplasticity, and neuronal adaptation. Recent evidence includes the association of disturbances in neuroplasticity and brain function with mitochondrial dysfunction and inflammation in the pathogenesis of MDD and the influence of these processes by drugs [1,2].

Mitochondria are the main energy suppliers for all cellular processes in the organism. In addition to being sources of energy, mitochondria are crucial organelles in balancing calcium levels, reactive oxygen species (ROS) production, and apoptosis regulation, key processes in neurodevelopment and neuroplasticity. The mitochondrial electron transport

chain and the structure of ATP synthase are shown in Figure 1 [3]. There is a large body of evidence implying that mitochondrial dysfunction is one of the key players responsi-ble for the development of MDD and other psychiatric and neurodegenerative disorders (including schizophrenia, Parkinson's disease, and Alzheimer's disease). Disruption of mitochondrial functions leads to depletion in ATP production, pushing the brain and other high-energy-demanding tissues toward energetic imbalance, endangering the maintenance of cellular tissue and body homeostasis. Mitochondrial dysfunction can have far-reaching consequences, e.g., increased oxidative stress, inaccurate neuronal signaling, and neuroinflammation, which all inevitably lead to aggravated neuronal adaptability and inefficient maintenance of body homeostasis [1,2,4–6]. Preclinical studies observing rat and mouse models of depression showed decreased ATP production [7], altered brain respiratory rate and membrane potentials, and damaged brain mitochondrial ultrastructure [8]. Rappeneau et al. more closely mapped the correlates of mitochondrial dysfunction in MDD in both clinical and preclinical studies [9]. Multiple studies have shown the presence of mitochondrial dysfunction in MDD patients, observed as abnormal energy metabolism in several brain regions [10–15], decreased mitochondrial respiration and altered activity of mitochondrial electron transport chain (ETC) complexes and other mitochondrial functions [16,17], oxidative imbalance [14], altered mtDNA copy number, and mtDNA mutations [18,19].

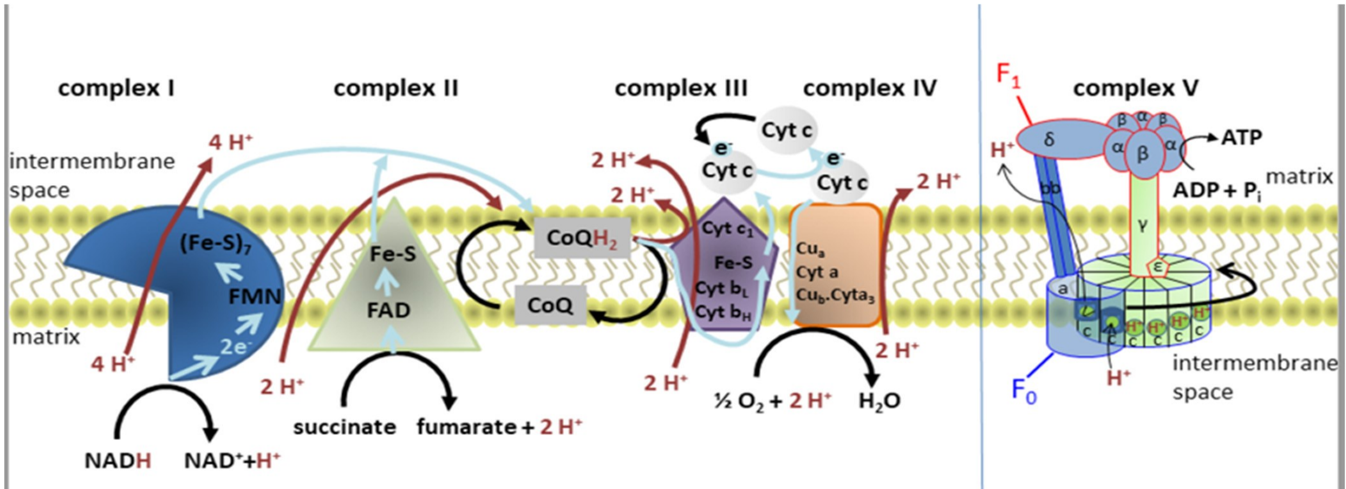


Figure 1. Diagram of the electron transport chain and the structure of ATP synthase. Reduced nicotinamide adenine dinucleotide (NADH) donates electrons (blue arrows) that pass through complex I via flavin mononucleotide (FMN) and iron-sulfur (Fe-S) clusters. Together with two protons, they bind to oxidized coenzyme Q (CoQ) to form reduced coenzyme Q ($CoQH_2$). This electron flow allows four H^+ (red arrows) to be transported into the intermembrane space. Complex II is the electron side entrance, as electrons from succinate pass through oxidized flavin adenine dinucleotide (FAD^+) and Fe-S before forming $CoQH_2$. Electrons then flow from $CoQH_2$ to complex III, and through cytochrome c (Cyt c) to complex IV. In total, ten H^+ are transported into the intermembrane space for each NADH or six H^+ for each $FADH_2$. The stator (blue) and the rotor (green) form complex V. The F_O domain of ATP synthase consists of three a subunits, three b subunits, and ten c subunits forming the c-ring. The a subunit contains the H^+ ion half-channel, which is responsible for mediating proton movement across the membrane. The and subunits of F_1 form a hexamer on the top of the subunit, which is inserted into the c-ring. Adapted from L'upták et al. [3].

Approximately 50% of treated MDD patients achieve remission. It was previously proposed by Emmerzaal et al. that mitochondrial dysfunction could be one of the modulators of treatment response. Evaluating patients' mitochondrial functions and the bioenergetic profile of psychiatric/neurodegenerative disorders might help in understanding the unique bioenergetics of individuals. This would also help with personalized pharmacotherapy of psychiatric diseases when the most appropriate medication (especially for pharmacoresistant patients) could be chosen. Identifying potential modulators of treatment response

(e.g., mitochondrial dysfunction) could be a reasonable step toward personalized pharmacological approaches for psychiatric and neurodegenerative diseases [20]. The impact of antidepressants on mitochondrial functions has shown both positive and negative effects. Fernström et al. showed that mitochondrial markers might help distinguish selective serotonin reuptake inhibitor (SSRI) responders and nonresponders (patients were taking sertraline, fluoxetine, citalopram, and escitalopram). SSRI responders had significantly higher citrate synthase (CS) baseline levels and complex I activity, which decreased with treatment (complex I activity increased with treatment in nonresponders). Treatment-associated changes, evaluated with the Hamilton Depression Rating Scale, correlated with changes in complex I activity. Complex II activity increased with SSRI administration [21].

The mitochondrial sources of ROS include not only complex I and complex III of the electron transport chain (ETC) but also the enzyme monoamine oxidase (MAO), which is located on the outer membrane of mitochondria and catalyzes the oxidative deamination of biogenic amines, including monoamine neurotransmitters [22]. The scheme of enzymatic reactions catalyzed by MAOs is shown in Figure 2. MAO is one of the primary sources of ROS in the brain and can participate in increased oxidative stress leading to neurodegeneration, apoptosis and impaired neuroplasticity and neurogenesis, contributing to psychiatric and neurodegenerative diseases [23]. MAO, as a modulator of monoaminergic neurotransmission, could be a target for drugs used in the treatment of neuropsychiatric disorders [23]. The role of MAO in the pathophysiology of MDD is supported by evidence that MAO-A density is increased during the acute phase of MDD [24].

$$R\text{-}(NH_2) + O_2 + H_2O \xrightarrow{MAO} R\text{-}(=O) + NH_3 + H_2O_2$$

Figure 2. Enzymatic reactions catalyzed by monoamine oxidase (adapted from Fišar et al.) [23]. Monoamines (e.g., dopamine, serotonin, or norepinephrine) are metabolized by oxidative deamination (along with oxygen and water) catalyzed by monoamine oxidase (MAO) to form aldehyde, ammonia, and hydrogen peroxide. Subsequently, aldehyde dehydrogenase oxidizes aldehydes into carboxylic acids, and hydrogen peroxide can be further converted to hydroxyl radical.

It has been shown that long-term therapy with monoamine oxidase inhibitor (MAOI) leads to neuronal adaptive changes such as membrane receptor density and sensitivity modulation, activity of transporters, transcription factors and intracellular signaling pathways, and increases in neuroplasticity and neurogenesis, neurotrophic factor gene expression and antiapoptotic effects [23,25,26]. MAO also participates on amyloid beta aggregation. MAO inhibition leads to decreased monoamine neurotransmitter metabolism and decreased ROS production, both of which are mechanisms of action of antidepressants and drugs for the treatment of Alzheimer's or Parkinson's disease. The neuroprotective properties of some MAOIs are probably linked to their antiapoptotic effects and modulation of gene expression [6,23,27,28]. MAO-B inhibitors (selegiline, rasagiline) are preferentially used in pharmacotherapy for Parkinson's disease; they balance oxidative stress and induce the production of antiapoptotic Bcl-2 and neurotrophic factors. Irreversible MAO-A inhibitors (phenelzine, clorgyline, pargyline) were some of the first antidepressants. Severe adverse effects and frequent interactions led to their withdrawal from the market and their subsequent replacement by safer SSRIs, the first-choice antidepressants [23,27,28]. However, it has been estimated that 15–20% of MDD patients require nonselective MAOIs (phenelzine, tranylcypromine) as a part of antidepressant therapy to achieve an optimal response [29].

SSRIs selectively inhibit serotonin reuptake, and their antidepressant effect is mediated via modulation of serotonergic transmission. In addition to MDD, SSRIs are also indicated for anxiety disorders, posttraumatic stress disorder, obsessive compulsive disorder, and bulimia. These antidepressants share similar adverse effects, including gastrointestinal

irritation, anxiety, sexual dysfunctions, impaired cognition, increased risk of suicidal thoughts and actions, and risk of serotonin syndrome [30]. Escitalopram (ESC) is the therapeutically active S-enantiomer of citalopram and the most selective SSRI; it displays rapid onset of antidepressant action [31]. Fluvoxamine (FLUV) is structurally different from other SSRIs and has higher selective inhibitory properties toward presynaptic uptake of serotonin, making it especially beneficial for obsessive-compulsive and bulimic patients. According to binding studies, paroxetine (PAR) is the most potent inhibitor of serotonin reuptake, with low norepinephrine uptake and low affinity toward cholinergic receptors. It is suitable for use in elderly patients, and in addition to its psychiatric indications, it is also used for the treatment of chronic headache and premenstrual dysphoria disorder. Sertraline (SER) is a highly selective SSRI with low potency toward norepinephrine and dopamine transporters and low affinity to cholinergic, histaminergic, and noradrenergic receptors. It is also used for premenstrual dysphoric syndrome and in premature ejaculation therapy. SER possess the highest risk of suicidality among this group of medications [30].

Bupropion (BUP) and trazodone (TRA) are both non-SSRI antidepressants. BUP acts as a norepinephrine and dopamine reuptake inhibitor and nicotinic acetylcholine receptor antagonist. It is used as an antidepressant and for smoking cessation treatment and for obesity therapy [32]. TRA acts as a serotonin antagonist and reuptake inhibitor; it antagonizes serotonin type 2 and alpha-adrenoreceptors and inhibits serotonin reuptake. It is used for the treatment of MDD, insomnia, anxiety disorders, and sexual dysfunction [33].

The aim of this study was to investigate the effects of selected SSRIs (ESC, FLUV, PAR, and SER), BUP and TRA on mitochondrial functions and the activities of MAO-A and MAO-B. Mitochondrial dysfunction and MAO are connected to the pathophysiology of neurodegenerative and psychiatric disorders. Understanding the effects of the chosen antidepressants on mitochondrial complexes and enzymes could provide deeper insight into their overall influence on mitochondrial function and cell energy metabolism.

2. Materials and Methods

All materials and methods have been described in our previous article [34]; only a summary follows.

2.1. Media and Chemicals

Sucrose 0.32 mol/L and HEPES 4 mmol/L (pH 7.4) were used as the mitochondrial isolation medium. The respiratory medium (MiR05 without BSA) contained sucrose 110 mmol/L, K^+-lactobionate 60 mmol/L, taurine 20 mmol/L, $MgCl_2 6H_2O$ 3 mmol/L, KH_2PO_4 10 mmol/L, EGTA 0.5 mmol/L, and HEPES 20 mmol/L (pH 7.1). The Krebs–Henseleit (KH) buffer consisted of NaCl 118 mmol/L, KCl 4.7 mmol/L, KH_2PO_4 1.2 mmol/L, $NaHCO_3$ 25 mmol/L, and glucose 11.1 mmol/L. 2-phenylethylamine [ethyl-1-^{14}C] hydrochloride ([^{14}C]PEA) and 5-Hydroxytryptamine [^{3}H] trifluoroacetate ([^{3}H]serotonin) were bought from American Radiolabeled Chemicals, (St. Louis, MO, USA), and other substances and chemicals were bought from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Isolation of Pig Brain Mitochondria

The mitochondrial fraction was isolated and purified from the pig brain cortex [35]. For high-resolution respirometry and ATP assays, freshly prepared mitochondria were kept on ice. Part of the mitochondria was frozen, stored at 70 C, and used to determinate mitochondrial enzymes activities (ETC complexes, CS, malate dehydrogenase, and MAO).

2.3. Mitochondrial Enzymes Activities

Ultrasonicated mitochondria were incubated with the tested drugs for 30 min at 30 C, with a corresponding drug-free control for every measurement. The activities of mitochondrial enzymes were determined as the absorbance measured with a GENESYS 180 UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3.1. Activity of Citrate Synthase

Tris, Triton 5,5^{0}-dithiobis-(2-nitrobenzoic) acid, and acetyl coenzyme A were mixed as the reaction mixture. The reaction was started by adding oxaloacetate and the activity of CS was detected as the change in absorbance of 5,5^{0}-dithiobis-(2-nitrobenzoic) acid (measured at 412 nm for 3 min). The final concentration of all tested drugs was 100 mol/L, and the final protein concentration was 20 g/mL [36].

2.3.2. Activity of Malate Dehydrogenase

The conversion of oxaloacetate to malate was used to measure malate dehydrogenase (MDH) activity. The reaction was started by adding oxaloacetate and nicotinamide adenine dinucleotide (NADH) (measured at 340 nm for 3 min). The final concentration of all tested drugs was 100 mol/L, and the final protein concentration was 20 g/mL [37].

2.3.3. Activity of Complex I (NADH Dehydrogenase)

KH_2PO_4, $MgCl_2$, and KCN were mixed as the reaction mixture. The reaction was started by adding decylubiquinone and NADH. Oxidation of NADH was measured for 5 min at 340 nm. The final concentrations of all tested drugs were 2.5, 5, 10, 50, and 100 mol/L, and the final protein concentration was 150 g/mL [38].

2.3.4. Activity of Complex II+III (Succinate Cytochrome c Oxidoreductase)

The activity of complex II+III was measured as the reduction in cytochrome c (measured at 550 nm for 3 min). The reaction was started by adding cytochrome c. KH_2PO_4, EDTA, KCN, and rotenone were mixed as the reaction mixture. The final concentrations of all tested drugs were 2.5, 5, 10, 50, and 100 mol/L, and the final protein concentration was 50 g/mL [39].

2.3.5. Activity of Complex IV (Cytochrome c Oxidase)

Reduced cytochrome c was added to KH_2PO_4 to initiate the reaction. The resulting decrease in absorbance was measured at 550 nm for 3 min. The final concentrations of all tested drugs were 10, 50, and 100 mol/L, and the final protein concentration was 10 g/mL [40].

2.4. ATP Content and Kinetics

ATP content and kinetics were measured using the ATP Bioluminescence Assay Kit CLS II. Luminescence was measured at 562 nm with FluoroMax-3 (Jobin Yvon, Edison, NJ, USA). An ATP standard curve was constructed using ATP standards in the range of 0 to 600 nmol/L. The final concentrations of all tested drugs were 10, 50, and 100 mol/L, and the final protein concentration was 50 g/mL.

2.4.1. Total Complex I- and Complex II-Linked ATP Content

MiR05 buffer was used as the medium and the reaction was started by adding a substrate mix consisting of malate 5 mmol/L and pyruvate 5 mmol/L (for complex I) or succinate 5 mmol/L and rotenone 1 mol/L (for complex II), ADP 60 mol/L and $MgCl_2 6H_2O$ 0.75 mmol/L. The reaction was stopped by heat and the final complex I- and complex II+III-linked ATP content was determined by luminescence (measured for 1 min) [41].

2.4.2. Complex I- and Complex II-Linked ATP Kinetics

MiR05 buffer was used as the medium, the same substrate mixture as above was added, 230 L of luciferase reagent was added and ATP kinetics was measured using the luminescence (measured for 4 min) [42].

2.5. Mitochondrial Respiration

The high-resolution Oxygraph-2k (Oroboros Instruments Corp, Innsbruck, Austria) was used to measure mitochondrial oxygen consumption rate. Malate 2 mmol/L, pyruvate 5 mmol/L, ADP 1.25 mmol/L, and $MgCl_2$ 0.75 mol/L were mixed to form a reaction mixture for complex I-linked respiration; and ADP 1.25 mmol/L, $MgCl_2$ 0.75 mol/L, rotenone 1 mol/L, and succinate 10 mmol/L for complex II-linked respiration. The final protein concentration was 0.05–0.14 mg/mL. Four simultaneous measurements were performed: two oxygraphy chambers were used for a titration up to the final drug concentrations of 0.125–100 mol/L, and two other oxygraphy chambers were used for a titration with the DMSO as a control [43,44].

2.6. Activity of Monoamine Oxidase

KH buffer was used to preincubate mitochondria (final concentration of 800 g/mL) with the tested drugs (concentration range of 0.1–300 mol/L). MAO enzymatic activity was measured using radiolabeled substrates ($[^3H]$serotonin for MAO-A and $[^{14}C]$PEA for MAO-B). The reaction was terminated with hydrochloric acid, the organic phase was separated, and the radioactivity was determined by liquid scintillation counting (LS 6000IC, Beckman Instruments, Inc., Fullerton, CA, USA) [45,46].

2.7. Data Analysis and Statistics

Mitochondrial enzyme activities and ATP kinetics were determined from time-dependent changes in absorbance or fluorescence slope. The ATP content was measured, and the average fluorescence curves over time were calculated. The activity of the control sample was considered to be 100%, and the effect of the drug was expressed as a percentage of the control. An ATP standard curve was constructed.

High-resolution respirometry data were analyzed and real-time oxygen concentrations and flux were displayed using DatLab 7.4 software from Oroboros Instruments (Innsbruck, Austria). Respiration rate (oxygen flux) was quantified as the number of pmol of oxygen consumed per second per mg of a protein.

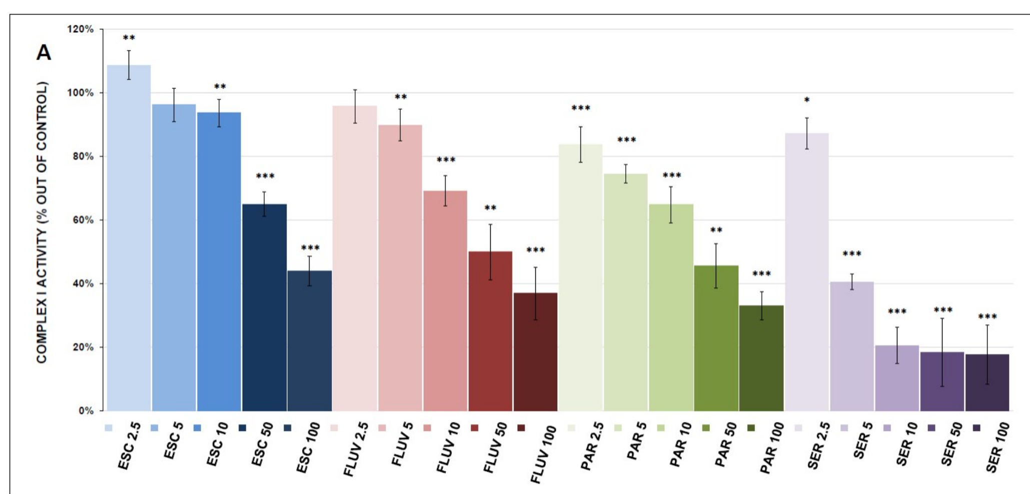
Four-parametric logistic regression was used to analyze inhibition of respiratory rate and MAO activity to determine half-maximal inhibitory concentration (IC_{50}), residual activity, and Hill slope. Prism software from GraphPad (San Diego, CA, USA) was used for this analysis. The IC_{50} indicates the concentration of a drug that is required to reduce the mitochondrial oxygen flux or MAO activity by 50% of the difference between the baseline and the residual value.

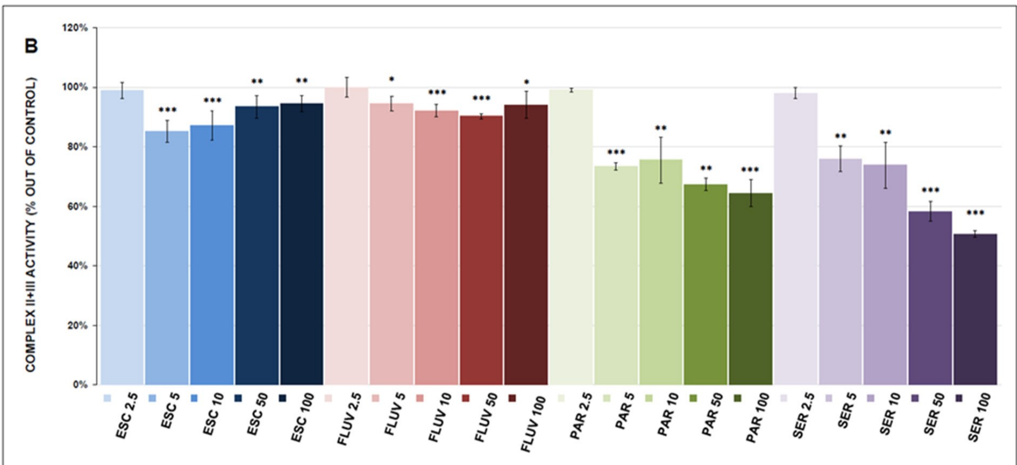
Data were analyzed using one-sample t-tests in the STATISTICA 12 analysis software (TIBCO Software Inc., Palo Alto, CA, USA). All data presented are expressed as either the mean standard deviation (SD) or the mean standard error of the mean (SEM). The Pearson correlation coefficient was used to identify statistically significant correlations, which are presented in the correlation matrix. Correlations with total ATP content and kinetics are part of the supplementary material.

3. Results

3.1. Activity of Mitochondrial Enzymes

The results of the assessments of mitochondrial respiratory complex activity are shown in Figures 3A–C and 4A–C. All tested doses of antidepressants (except ESC 2.5 mol/L) inhibited complex I activity (significantly at most concentrations). SSRI antidepressants showed more potent inhibition. The antidepressants are listed from the most potent to the least potent: SER (17.5 9.3% at 100 mol/L, $p < 0.001$), PAR (33.0 4.4% at 100 mol/L, $p < 0.001$), FLUV (36.8 8.2% at 100 mol/L, $p < 0.001$), ESC (43.9 4.7% at 100 mol/L, $p < 0.001$), TRA (60.9 6.6% at 100 mol/L, $p < 0.001$), and BUP (82.1 6.8% at 100 mol/L, $p = 0.045$).





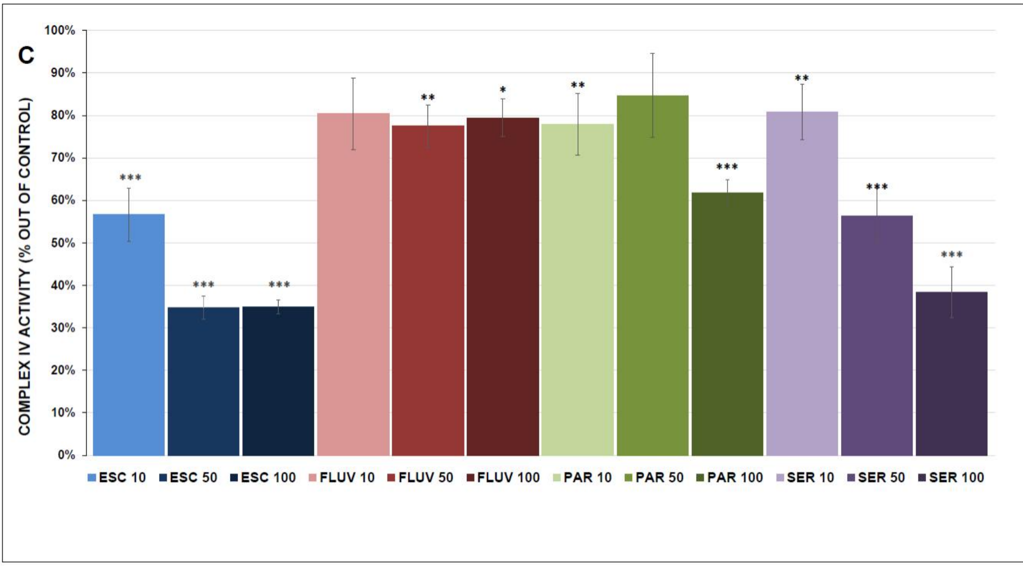
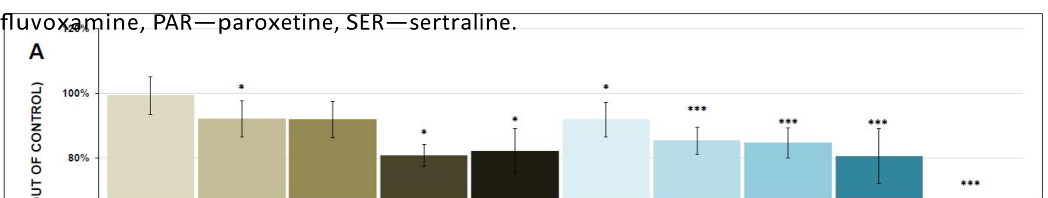
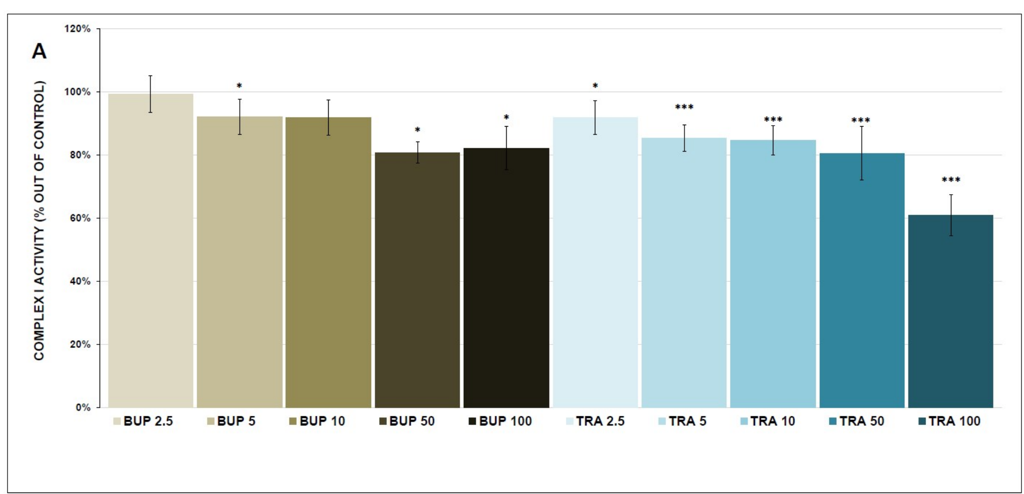
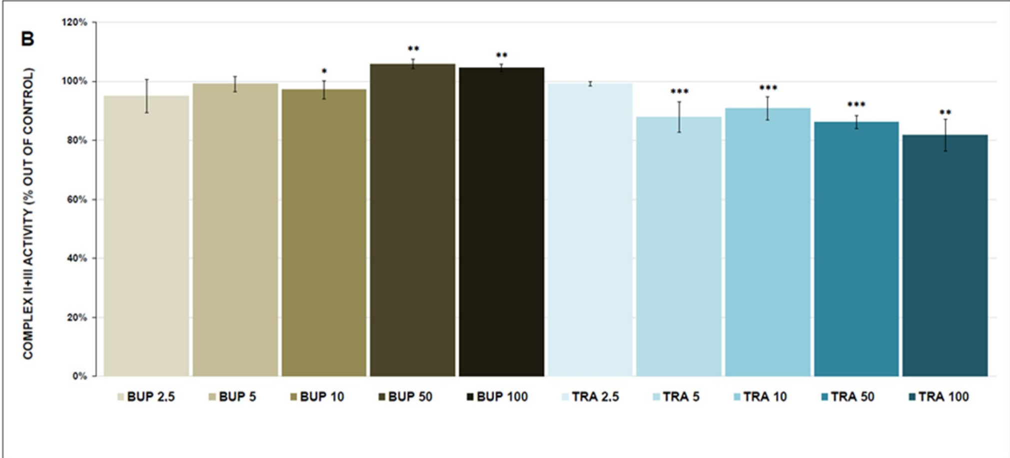


Figure 3. SSRI-induced changes in the activity of electron transport chain (ETC) complexes (complex I, complex II + III and complex IV, (**A**–**C**), respectively). Relative activity is expressed as the percentage difference from the activity of the control sample, with the mean value and standard deviation (SD) calculated from at least three independent measurements. A one-sample t-test was performed to assess statistical significance, with the mean control value set at 100% and is expressed as * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. Drug concentrations are expressed in m/L. ESC—escitalopram, FLUV—fluvoxamine, PAR—paroxetine, SER—sertraline.







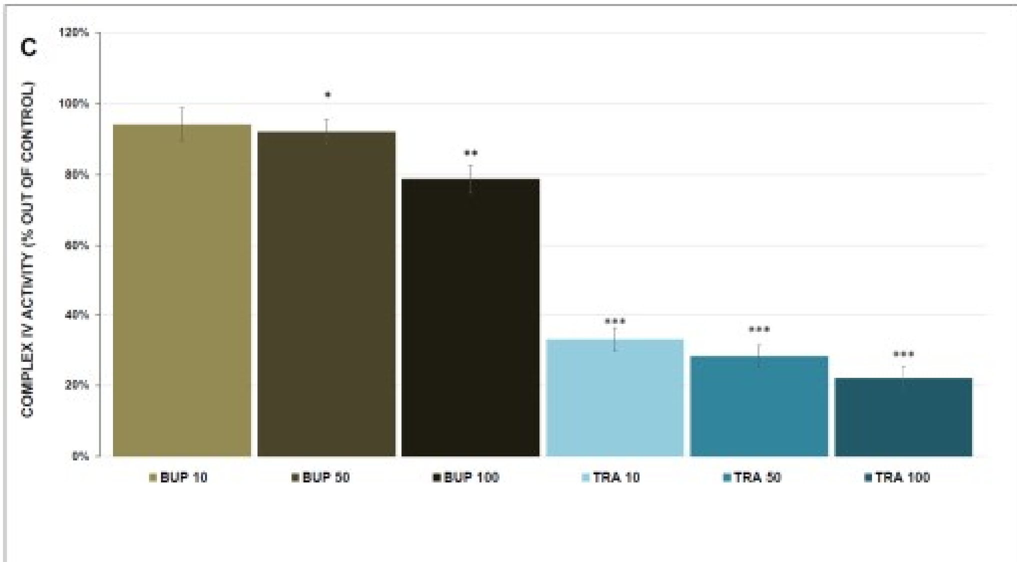


Figure 4. Bupropion- and trazodone-induced changes in the activity of electron transport chain (ETC) complexes (complex I, complex II + III and complex IV, (**A**–**C**), respectively). Relative activity is expressed as the percentage difference from the activity of the control sample, with the mean value and standard deviation (SD) calculated from at least three independent measurements. A one-sample t-test was performed to assess statistical significance, with the mean control value set at 100% and is expressed as * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. Drug concentrations are expressed in m/L. BUP—bupropion, TRA—trazodone.

Complex II+III activity was inhibited by all tested antidepressants except BUP, which showed weak stimulatory activity (104.5 1.2% at 100 mol/L, $p = 0.013$). The most potent inhibitor was SER (50.7 1.2% at 100 mol/L $p < 0.001$), followed by PAR (64.4 4.5% at 100 mol/L, $p < 0.001$), TRA (81.7 5.5% at 100 mol/L, $p = 0.002$), FLUV (94.1 4.6% at 100 mol/L, $p = 0.044$), and ESC (94.4 2.7% at 100 mol/L, $p = 0.002$).

Complex IV activity was inhibited by all tested antidepressants: TRA (22.0 3.3% at 100 mol/L, $p < 0.001$), ESC (34.9 1.7% at 100 mol/L, $p < 0.001$), SER (38.4 1.7% at 100 mol/L, $p < 0.001$), PAR (61.1 3.1% at 100 mol/L, $p < 0.001$), BUP (78.6 3.8% at 100 mol/L, $p = 0.001$), and FLUV (79.4 4.4% at 100 mol/L, $p = 0.015$).

None of the tested antidepressants affected CS and MDH activities (the results are shown in Table S1 of the Supplementary Material).

3.2. Mitochondrial Respiration

The drug-induced inhibition of complex I- and complex II-linked respiration is shown in Figure 5A,B, and the evaluated parameters (IC_{50}, Hill slope and residual activity) are summarized in Table 1 (for partial inhibitors only). All tested substances acted as partial inhibitors of complex I-linked respiration. SER was the most potent complex I-linked respiration inhibitor, with a respiration rate of 24.5 10.4% (mean SD) at 50 mol/L ($p < 0.001$), an IC_{50} of 12.4 0.8 mol/L (mean SEM), and a residual activity of 17.9% 0.03 (mean SEM). As mentioned previously, drug titration was stopped at the point of sudden increase in the mitochondrial respiratory rate, which occurred at high drug concen-trations [34].

Table 1. Inhibitory parameters of antidepressant-induced changes in complex I- and complex II-linked respiration in isolated mitochondria.

Drug	IC_{50} (mol/L)	Hill Slope	Residual Activity (rel.u.)	Inhibition
Complex I-Linked Respiration				
escitalopram	0.14 0.08	1.39 1.05	0.774 0.015	partial
fluvoxamine	15.84 2.57	3.09 1.51	0.817 0.017	partial
paroxetine	26.71 6.73	1.27 0.36	0.672 0.029	partial
sertraline	12.38 0.75	2.05 0.23	0.179 0.031	partial
trazodone	0.45 0.19	1.10 0.43	0.815 0.015	partial
complex II-linked respiration				
bupropion	10.58 4.49	1.17 0.44	0.690 0.045	partial

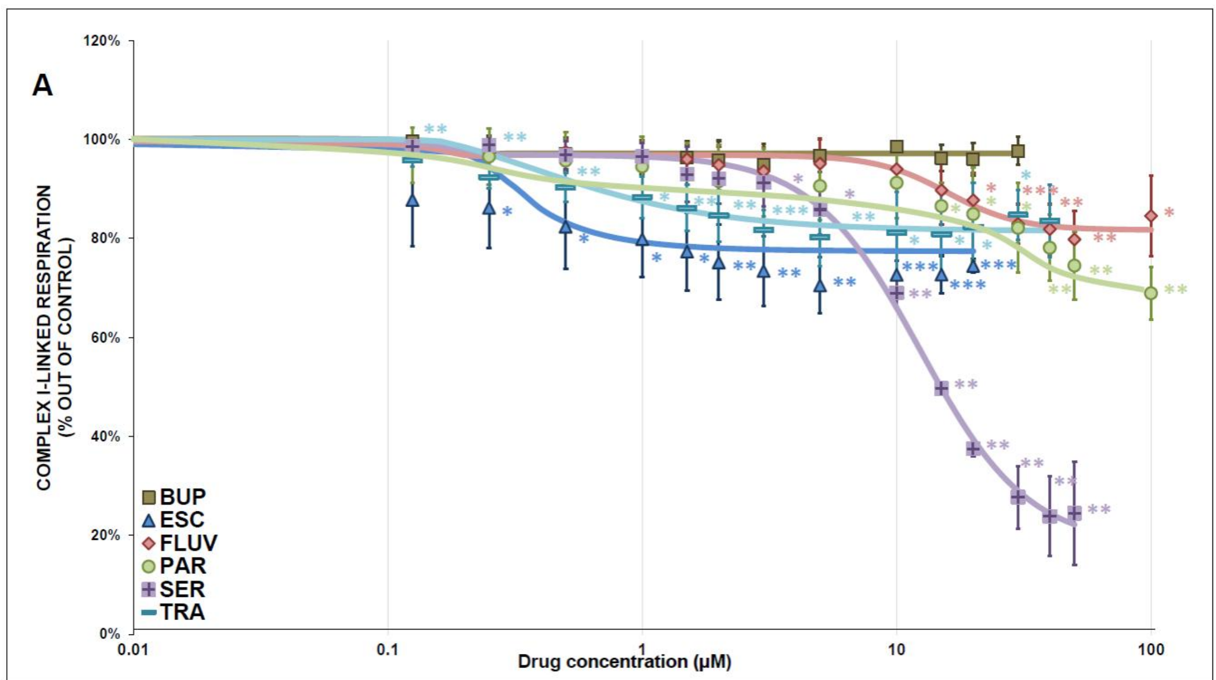
The mean SEM is used to express the values obtained from four independent measurements. IC_{50} refers to the half-maximal inhibitory concentration.

BUP was the only tested antidepressant that acted as a weak partial inhibitor of complex II-linked mitochondrial respiration. Other antidepressants showed very weak or no inhibitory activity.

3.3. ATP Content and Kinetics

The complex I-linked ATP content was significantly decreased by 50 mol/L TRA (87.8 0.4%, $p = 0.013$) and increased by SER (118.0 6.0% at 50 mol/L, $p = 0.009$) and 10 mol/L TRA (111.3 3.0%, $p = 0.022$). The complex I-linked ATP kinetics were significantly inhibited by ESC (81.6 5.1% at 50 mol/L, $p = 0.006$), FLUV (80.7 10.7% at 50 mol/L, $p = 0.037$), and PAR (80.4 5.1% at 10 mol/L, $p = 0.022$).

The complex II-linked ATP content was significantly decreased by PAR (88.6 4.0% at 100 mol/L, $p = 0.040$). The complex II-linked ATP kinetics were significantly stimulated by BUP (105.7 0.4% at 50 mol/L, $p = 0.032$) and inhibited by ESC (81.5 4.5% at 50 mol/L, $p = 0.019$), FLUV (76.6 5.4% at 50 mol/L, $p = 0.017$), PAR (82.1 0.1% at 100 mol/L, $p = 0.004$), SER (82.9 2.0% at 10 mol/L, $p = 0.005$), and TRA (87.6 3.0% at 10 mol/L, $p = 0.019$).



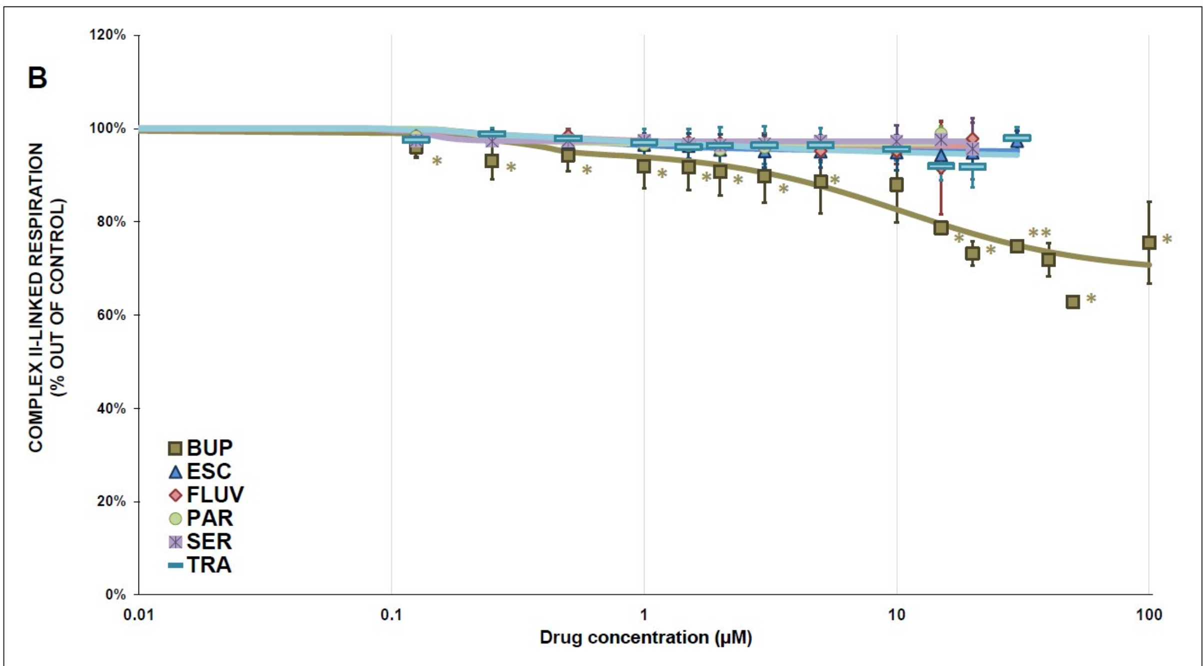
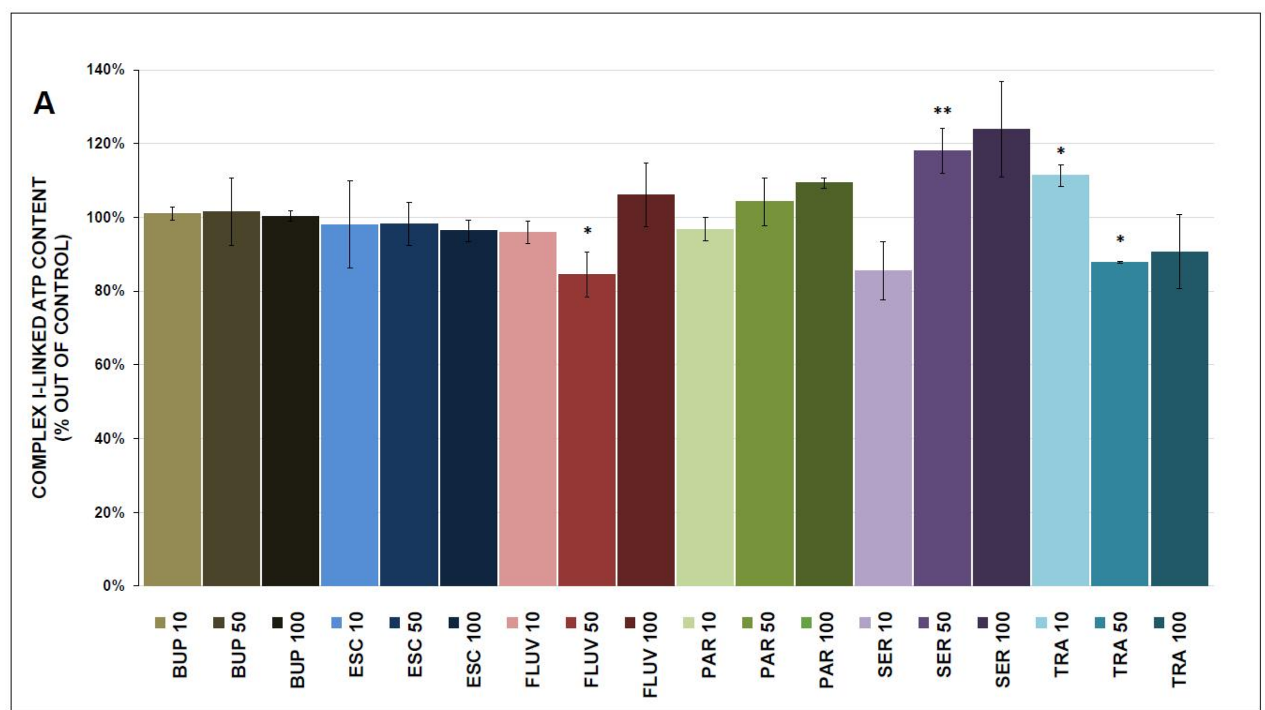


Figure 5. Effect of antidepressants on respiration in isolated mitochondria (complex I-linked respiration, complex II-linked respiration, (**A**,**B**), respectively). The dose-response curves are represented by the oxygen consumption rate plotted against drug concentration. The respiratory rate of the sample titrated with the antidepressants is relative to the control sample titrated with the DMSO (solvent). Four different measurements were used to calculate the plot points. Statistical significance was tested using a one-sample t-test with a mean control value of 100%, indicated as * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. Table 1 shows the half-maximal inhibitory concentration (IC_{50}), Hill slope, and the resid-ual activity calculated using a four-parameter logistic function. BUP—bupropion, ESC—escitalopram, FLUV—fluvoxamine, PAR—paroxetine, SER—sertraline, TRA—trazodone.

The results of the ATP content and kinetics measurements are shown in Figure 6A,B and Figure 7A,B.



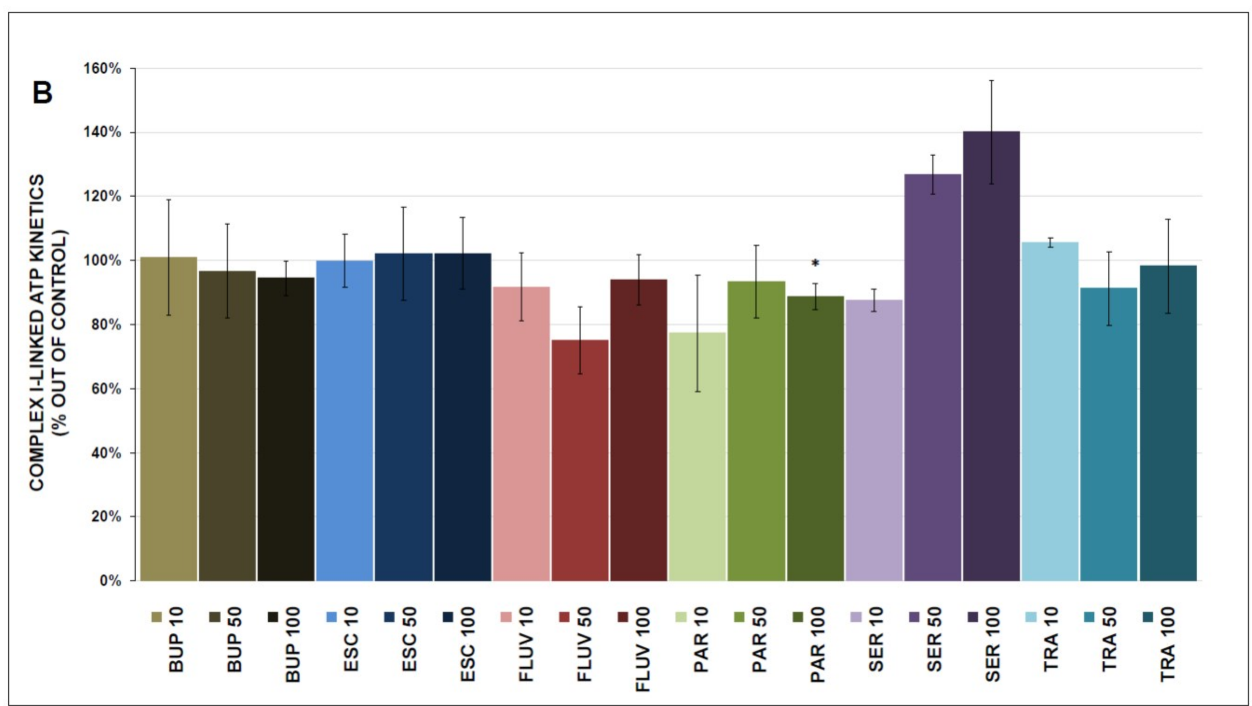
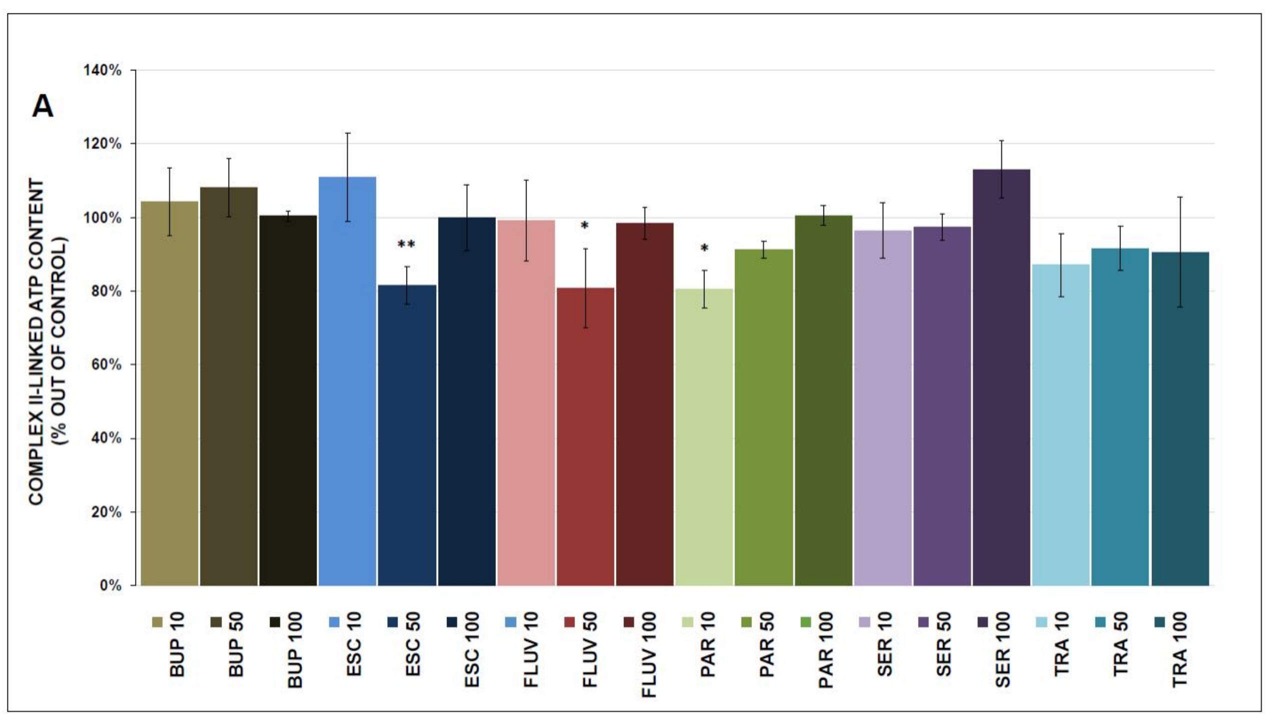


Figure 6. Antidepressant-induced changes in complex I-linked ATP content and kinetics (ATP content, ATP kinetics, (**A**,**B**), respectively). Relative activity is expressed as the percentage difference from the activity of the control sample (100% corresponded to the production of (**A**) 160 nmol of ATP per 1 mg of protein and (**B**) 282 nmol of ATP per 1 mg of protein per 1 min), with the mean value and standard deviation (SD) calculated from at least six independent measurements. A one-sample t-test was performed to assess statistical significance, with the mean control value set at 100% and is expressed as * $p < 0.05$. ** $p < 0.01$. Drug concentrations are expressed in m/L. BUP—bupropion, ESC—escitalopram, FLUV—fluvoxamine, PAR—paroxetine, SER—sertraline, TRA—trazodone.



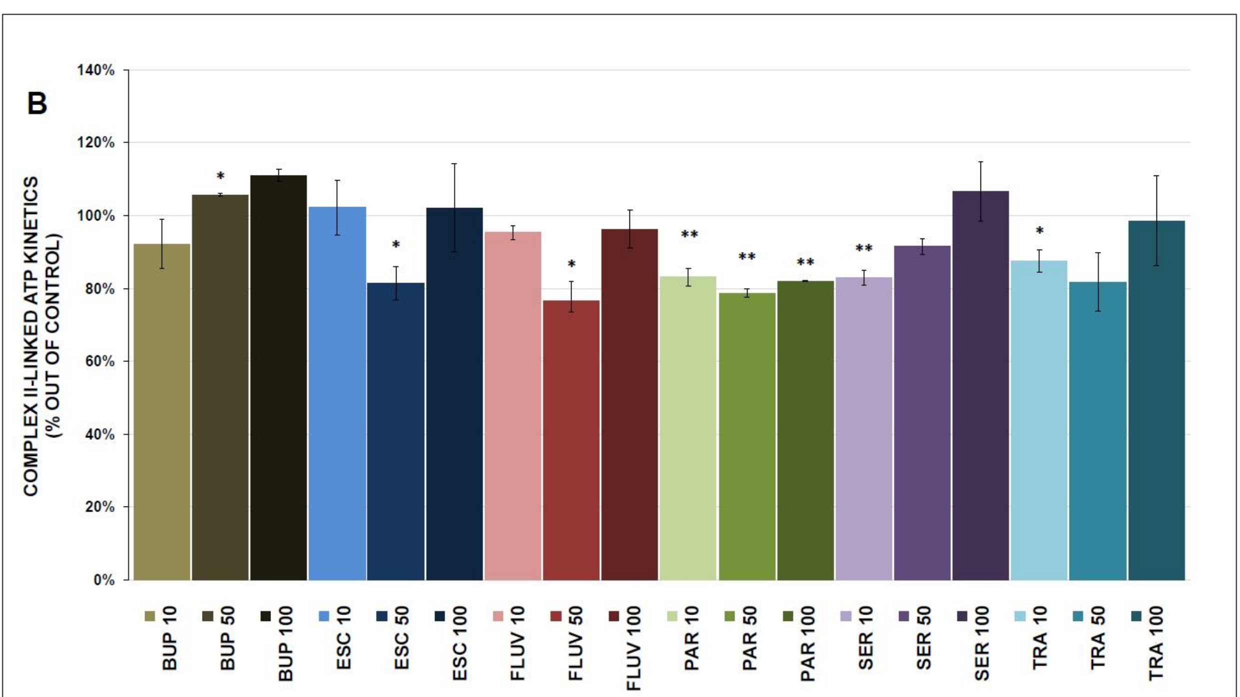
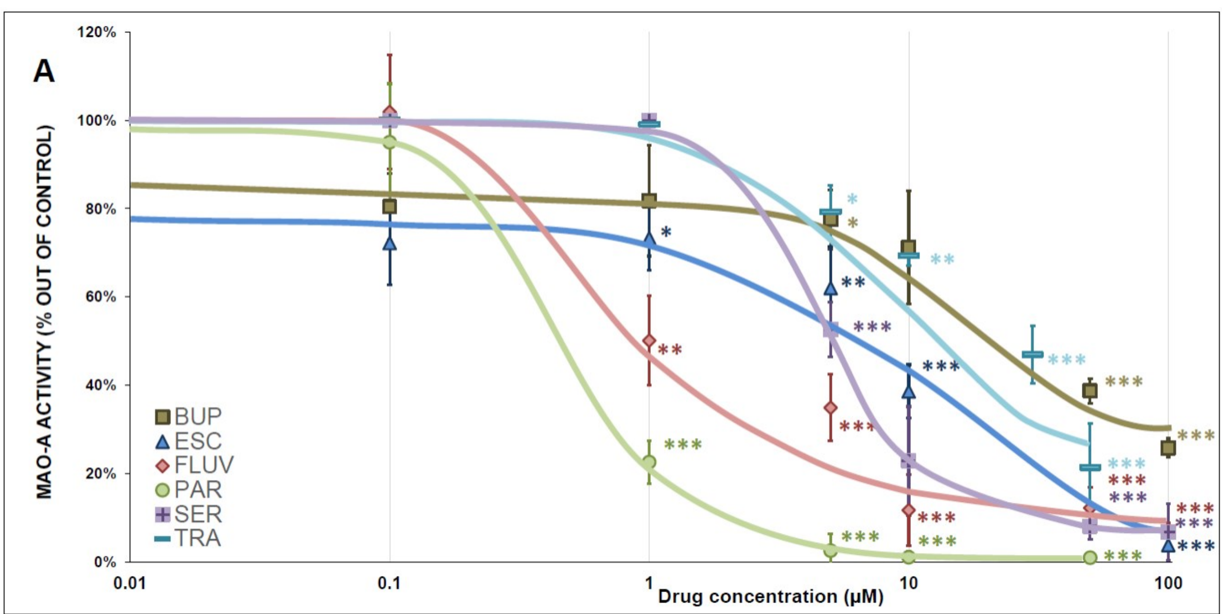


Figure 7. Antidepressant-induced changes in complex II-linked ATP content and kinetics (ATP content, ATP kinetics, (**A**,**B**), respectively). Relative activity is expressed as the percentage difference from the activity of the control sample (100% corresponded to the production of (**A**) 291 nmol of ATP per 1 mg of protein and (**B**) 1289 nmol of ATP per 1 mg of protein per 1 min), with the mean value and standard deviation (SD) calculated from at least six independent measurements. A one-sample t-test was performed to assess statistical significance, with the mean control value set at 100% and is expressed as * p < 0.05. ** p < 0.01. Drug concentrations are expressed in m/L. BUP—bupropion, ESC—escitalopram, FLUV—fluvoxamine, PAR—paroxetine, SER—sertraline, TRA—trazodone.

3.4. MAO Activity

All tested antidepressants significantly inhibited MAO-A activity: ESC, FLUV, and PAR acted as full inhibitors, and BUP, SER, and TRA acted as partial inhibitors. MAO-B activity was also significantly inhibited by all tested antidepressants: ESC and PAR acted as full inhibitors, and BUP, FLUV, SER, and TRA acted as partial MAO-B inhibitors. The antidepressant-induced MAO-A and MAO-B inhibition curves are shown in Figure 8A,B, and the kinetic parameters (IC_{50}, Hill slope, and residual activity) are summarized in Table 2.



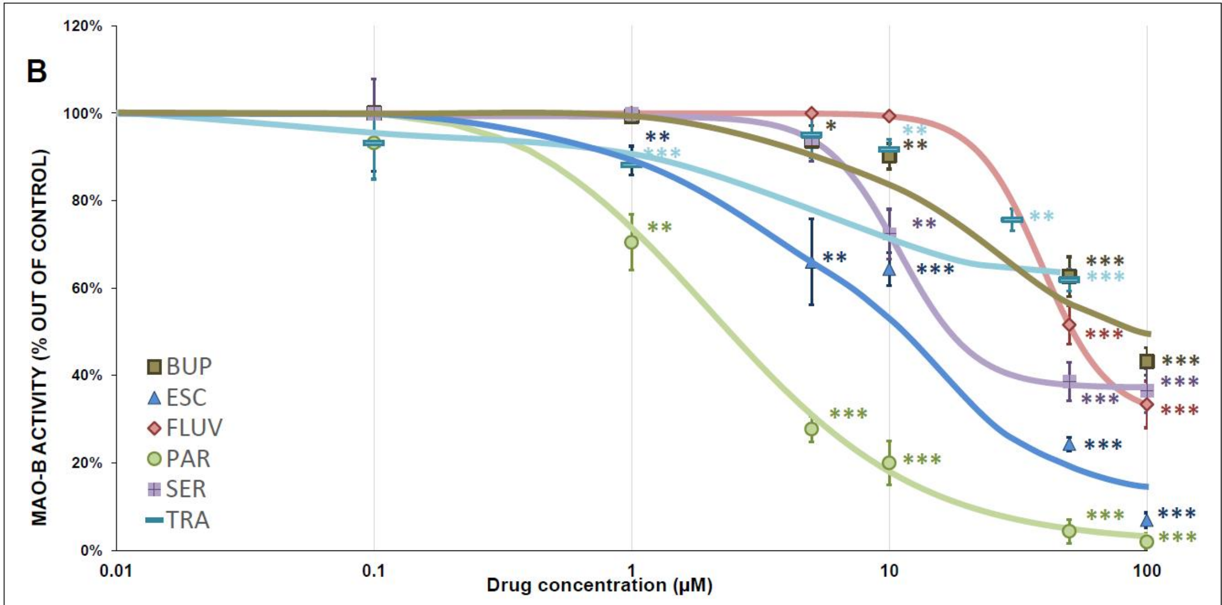


Figure 8. Antidepressant-induced inhibition of MAO activity (MAO-A, MAO-B, (**A**,**B**), respectively). Relative activity is expressed as the percentage difference from the activity of the control sample. A one-sample t-test was performed to evaluate statistical significance with the mean control value set at 100% and is expressed as * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. Table 2 shows the calculated half-maximal inhibitory concentration (IC_{50}), Hill slope, and the residual activity. Drug concentrations are expressed in mol/L. BUP—bupropion, ESC—escitalopram, FLUV—fluvoxamine, PAR—paroxetine, SER—sertraline, TRA—trazodone.

Table 2. The inhibition of monoamine oxidase activity (MAO-A and MAO-B) induced by antidepressants.

Drug	IC_{50} (mol/L)	Hill Slope	Residual Activity (rel.u.)	Inhibition
		MAO-A		
bupropion	20.15 5.68	1.36 0.33	0.235 0.050	partial
escitalopram	8.78 2.27	0.99 0.29	0.005 0.057	full
fluvoxamine	0.99 0.23	0.91 0.17	0.079 0.041	full
paroxetine	0.47 0.06	1.65 0.22	0.007 0.017	full
sertraline	4.91 0.23	2.37 0.39	0.074 0.017	partial
trazodone	13.16 2.55	1.23 0.22	0.184 0.042	partial
		MAO-B		
bupropion	31.54 3.32	1.68 0.20	0.410 0.018	partial
escitalopram	15.16 2.23	1.13 0.14	0.038 0.032	full
fluvoxamine	39.04 2.63	3.39 1.35	0.306 0.034	partial
paroxetine	2.19 0.21	1.06 0.10	0.015 0.021	full
sertraline	10.95 0.59	3.00 0.68	0.372 0.014	partial
trazodone	29.73 5.77	2.75 1.35	0.560 0.079	partial

The mean SEM is used to express the values obtained from four independent measurements. IC_{50} refers to the half-maximal inhibitory concentration.

3.5. Correlations

Several statistically significant correlations were found between measured mitochondrial parameters (complex I, II + III, and IV activities; complex I-linked respiration; complex II-linked respiration; MAO-A and MAO-B activities; complex I-linked ATP content and kinetics; and complex II-linked ATP content and kinetics) using Pearson correlation coefficients. Strong and statistically significant correlations were found between the activities of individual complexes I, II + III, and IV, between the activities of complexes I, II + III, and IV and complex I-linked respiration, between complex II + III and complex II-linked respiration, between complex II-linked ATP kinetics and complex II-linked ATP content, between complex II-linked ATP kinetics and complex II-linked respiration, and between MAO and complex I activities or complex I-linked respiration. Table 3 summarizes the results of the correlation analysis.

Table 3. Correlation coefficients for antidepressant-induced changes in mitochondrial parameters.

		Complex I Activity	Complex II + III Activity	Complex IV Activity	Complex I-Linked ATP Content	Complex I-Linked ATP Kinetics	Complex II-Linked ATP Content	Complex II-Linked ATP Kinetics	Complex I-Linked Respiration	Complex II-Linked Respiration
complex II + III activity	r	*** 0.58	-	-	-	-	-	-	-	-
	N	153								
complex IV activity	r	*** 0.75	*** 0.66	-	-	-	-	-	-	-
	N	64	64							
complex I-linked ATP content	r	0.16	0.03	* 0.32						
	N	53	53	53	-	-	-	-	-	-
complex I-linked ATP kinetics	r	* 0.35	0.14	0.22	0.08					
	N	46	46	46	44	-	-	-	-	-
complex II-linked ATP content	r	0.12	0.19	0.00	0.06	0.11				
	N	56	56	56	52	46	-	-	-	-
complex II-linked ATP kinetics	r	0.04	** 0.42	0.03	0.12	0.19	*** 0.50			
	N	45	45	45	45	43	45	-	-	-
complex I-linked respiration	r	*** 0.51	*** 0.57	*** 0.66	0.16	0.16	0.01	0.10		
	N	82	82	55	52	43	52	45	-	-
complex II-linked respiration	r	0.05	*** 0.61	* 0.43	0.03	0.21	0.26	*** 0.75	0.09	
	N	54	54	27	24	21	25	21	294	-
MAO-A activity	r	*** 0.48	0.02	0.06	0.04	0.12	0.26	0.28	* 0.23	0.12
	N	71	71	52	50	42	51	44	100	74
MAO-B activity	r	*** 0.58	0.04	* 0.30	0.12	0.20	0.06	0.06	*** 0.37	0.15
	N	87	87	63	53	46	56	45	104	76

The Pearson correlation coefficient (r) was used to assess the linear correlation between mitochondrial parameters measured at different concentrations of antidepressants. Statistical significance is indicated as * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. The number of measurements is indicated as N.

4. Discussion

We evaluated the antidepressant-induced changes in mitochondrial energy metabolism and MAO-A and MAO-B activities. Any potential mitochondrial impairment (disease- or drug-related) could have an impact on neurotransmission, neuroplasticity, and neurodevelopment. Inefficient energy supply together with increased MAO-A and MAO-B activities could participate in the pathophysiology of psychiatric and neurodegenerative disorders and influence the adverse effects of drugs [23,47,48]. In vitro studies of the mitochondrial effects of antidepressants are essential for improving our knowledge of the pathophysiology of depression, elucidating the mechanisms of the therapeutic and adverse effects of antidepressants, and discovering potential mitochondrial targets for new drugs.

4.1. Mitochondrial Enzyme Activity and Respiration

All tested antidepressants (except BUP in complex II + III) significantly inhibited the activities of all ETC complexes. All tested SSRIs were more potent inhibitors of complex I (inhibiting complex I activity by more than 50%) than BUP and TRA. The inhibitory effect of SSRIs was less distinctive in complex II+III activity, with significant inhibition only by PAR (64.3 4.4% at 100 mol/L, $p < 0.001$) and SER (50.7 1.2% at 100 mol/L, $p < 0.001$). Overall, the most vulnerable complex was complex IV, and all tested antidepressants decreased its activity below 80% of the control. These findings are in accordance with our previous studies with pharmacologically different antidepressants that potently inhibited mitochondrial respiration and the activities of ETC complexes, especially at high concentrations [34,49].

Complex I-linked respiration was significantly inhibited by all tested antidepressants except BUP, which is in accordance with the inhibition of complex I. Complex I is the main entry and first control point to oxidative phosphorylation (OXPHOS) and is greatly vulnerable to lipophilic molecules and oxidative stress [50]. It is also a rate-limiting unit for oxygen consumption. Inhibition of complex I activity could affect mitochondrial respiration, cause ineffective OXPHOS, and impair ATP production [51]. Inhibition of mitochondrial complexes I and III can be linked to higher ROS production and oxidative damage; moreover, it causes a shift in the production of ATP from mitochondria toward glycolysis, causing increased lactate production [52].

BUP was the only tested antidepressant that partially inhibited complex II-linked respiration and mildly inhibited complex I and IV activities. Our group previously reported that BUP acted as a partial inhibitor of complex I-linked mitochondrial respiration, but the final tested drug concentration reached 1 mmol/L and had no significant effect on the activity of ETC complexes, CS activity, or complex II-linked respiration [49]. There have been very few studies observing the effect of bupropion on cell energy metabolism using cell lines instead of isolated mitochondria, which makes these studies difficult to compare with our results [52,53].

SER and PAR were the strongest inhibitors of complex I, complex II + III, and complex I-linked respiration. They were also very potent inhibitors of complex IV but had no effect on complex II-linked respiration. These findings are partially in accordance with a study observing isolated rat liver mitochondria, where SER (25–100 mol/L) inhibited the activity of complexes I and V and acted as an OXPHOS uncoupler. The origin of mitochondria as well as applied assays can explain these differences [54]. SER also showed a significant and dose-dependent decrease in oxygen consumption in mitochondria isolated from rat liver, in accordance with our results [55]. Similar to our study, PAR displayed inhibitory properties toward all ETC complexes (nonsignificantly for complex IV) in mitochondria isolated from bovine heart [56].

ESC and FLUV were also very potent complex I activity inhibitors, inhibiting complex IV (FLUV only weakly) and complex I-linked respiration, though with no effect on complex II-linked respiration. ESC showed a similar inhibitory effect in rat brain mitochondria isolated after chronic ESC administration (10 mg/kg for 14 days). The activities of complexes

I and II + III were inhibited, while no effect was found on the activity of complex IV, CS, or MDH [50]. The different results for complex IV activity could be explained by chronic ESC administration and the evaluation of different animal tissue.

Study results for FLUV are in accordance with our findings: FLUV caused strong complex I inhibition and mild complex IV inhibition and acted as a partial inhibitor of complex I-linked respiration; it was found to alter the activity of CS, dose dependently affect complex I, and inhibit complex II+II, complex IV, and MDH activities in different rat brain regions [57].

TRA was the most potent inhibitor of complex IV and also inhibited complex I and II+III activities and complex I-linked respiration. Complex IV initiates the final step in the ETC, and its activity has been previously linked with neuronal activation [58]. In another study, TRA acted as an uncoupler of OXPHOS and inhibited oxygen consumption in rat brain homogenate and slices [59].

4.2. ATP Production

ATP production and kinetics were determined separately for complex I and complex II to better understand the molecular mechanisms of drug action. Mild but significant decreases in ATP content and kinetics were observed with ESC, FLUV, PAR, and TRA treatments (all at 50 mol/L); SER treatment resulted in mild increases in ATP content and kinetics.

It was reported previously that different antidepressants decreased the production of mitochondrial ATP: TRA (200 mol/L) and BUP (0.2–1 mmol/L) significantly decreased ATP contents in HepG2 cells [52,60]; PAR and SER caused collapses in mitochondrial membrane potential and depletion of ATP in H9c2 cells [61,62]; and SER (25–100 mol/L) was reported to decrease total ATP contents in rat primary hepatocytes, human placental BeWo cells, and human platelets [54,63,64]. Conversely, PAR had no effect on ATP content in murine and human endothelial cells [65]. There are no available studies observing the effect of the chosen antidepressants on isolated mitochondria.

In agreement with the inhibition of complex I activity, all tested antidepressants also inhibited complex I-linked respiration. In contrast, antidepressant-induced changes in complex I-linked ATP content or ATP kinetics were much smaller and were both inhibitory and stimulatory. These findings indicate that ATP production is less sensitive to the in vitro effects of antidepressants than complex I activity and the complex I-linked rate of oxygen consumption in isolated mitochondria. These results support earlier observations that mitochondrial oxygen consumption alone does not appear to be a measure of actual ATP production and that environmental factors can induce variation in mitochondrial efficacy [66]. The cause of variability in the amount of ATP generated per unit of oxygen consumed is not well understood. In the case of proton pumping using complex I, slippage of the proton pumps and the activity of uncoupling proteins can be applied, leading to a change in the coupling efficiency and the attenuation of mitochondrial ROS production [67].

Our results show that, from the point of view of testing the mitochondrial effects of drugs, the measurement of mitochondrial respiratory rates appears to be a more appropriate/sensitive parameter than the measurement of ATP production. Although some antidepressants significantly inhibit the activity of ETC complexes at therapeutically achievable concentrations in the brain, their effects on cellular bioenergetics may be relatively small. It can be speculated that mitochondria are able to use the capacity reserve in the activity of ETC complexes and/or compensatory mechanisms are applied in the OXPHOS system. Knowledge of these mechanisms is necessary to evaluate changes in mitochondrial respiration as markers of drug-induced mitochondrial dysfunction leading to the adverse or therapeutic effects of antidepressants.

4.3. MAO Inhibition

All tested antidepressants significantly inhibited both MAO-A and MAO-B activities. Significant levels of inhibition of MAO-A at therapeutically achievable brain concentrations

of antidepressants were observed for PAR, FLUV, and ESC, indicating the possibility of enhancing their antidepressant effects through inhibition of the metabolism of monoamine neurotransmitters such as serotonin. Inhibition of MAO-B may enhance the therapeutic effects of PAR and ESC through the reduction in hydrogen peroxide production and perhaps neuroprotective effects unrelated to their primary mechanisms of action [68]. Our results indicate that MAO inhibition could participate in both the antidepressant and neuroprotective effects of the tested antidepressants, which are not classified as MAOIs.

There are no available data describing the effects of the chosen antidepressants on brain-isolated mitochondria. There are studies observing antidepressant-induced MAO-A and MAO-B activity inhibition in rat brain tissue and in female patients with MDD; however, these results are difficult to compare with ours because of the completely different methods employed [69–75]. Our group previously reported that fluoxetine and citalopram, other SSRI antidepressants, functioned as noncompetitive MAO-A inhibitors and mixed and uncompetitive MAO-B inhibitors. This is in accordance with our current results, considering the structural similarities among the SSRI groups (except FLUV), with the result that MAO-inhibitory properties are expected among these drugs [76].

4.4. Correlations

Correlations between the measured mitochondrial parameters reflect the interconnection and balance of the individual components of the OXPHOS system. The significant positive correlations between the activities of complexes I, II + III, and IV confirmed that the activity of a certain ETC complex reflects drug-induced changes in the activities of other complexes and/or that the activities of individual complexes are modified by the antidepressant in the same manner.

The significant positive correlations between the activities of individual ETC complexes and complex I-linked respiration and no or negative correlations between the activities of individual ETC complexes and complex II-linked respiration confirmed that complex I-linked respiration is primarily regulated by the activities of respiratory complexes, while complex II-linked respiration appears to be more regulated by other components of the OXPHOS system (most likely influencing the availability of the electron donor $FADH_2$). This approach is also supported by the observation of a significant negative correlation between complex II-linked ATP kinetics and complex II-linked respiration, reflecting the finding that the observed antidepressant-induced reduction in complex II-linked ATP kinetics is not accompanied by reduced complex II-linked respiration. The negative correlation between complex II-linked respiration and complex II-linked ATP kinetics is most likely correlation without causation, because: (i) the only antidepressant that slightly inhibited complex II-linked respiration was BUP, which had no Inhibitory effect towards complex II-linked ATP kinetics; and (ii) the inhibitory effects of the other antidepressants tested were not associated with changes in complex II-linked respiration.

The lack of correlation between complex I-linked respiration and complex I-linked ATP content supports the existence of variability between oxygen consumption and ATP production, as discussed above (Section 4.2). The significant correlations between MAO activity and complex I activity and between MAO activity and complex I-linked respiration, but not between MAO activity and complex II+III or complex IV activity, indicate the possible existence of an association between MAO activity and complex I activity. It can be assumed that similar changes in the activities of MAO and complex I are a consequence of drug-induced conformational changes of the enzyme and/or affecting protein–protein interactions between MAO units or between subunits of complex I.

4.5. Study Limitations

In this study, isolated pig brain mitochondria were used as previously described; this in vitro model is suitable for investigating mitochondrial drug effects and allows more accurate and closer recognition of the mitochondrial mechanisms of action of an-

tidepressants [35]. However, the model cannot cover regulatory and compensatory brain mechanisms that could be observed in in vivo measurements.

Significant changes in the measured mitochondrial parameters were often observed even with high concentrations of antidepressants. Therefore, measurements of the effects of drugs on mitochondrial parameters had to be performed over wide concentration ranges, including therapeutic plasma/brain concentrations of antidepressants. The recommended target plasma levels are 3.50–6.30 M for BUP, 0.05–0.23 M for ESC, 0.47–0.94 M for FLUV, 0.21–0.36 M for PAR, 0.03–0.16 M for SER, and 1.75–4.03 M for TRA [77,78]. In our study, the final concentration range of antidepressants used in the experiments was 0.125–100 mol/L. Most antidepressants are molecules that are amphiphilic and cationic, and they tend to accumulate in subcellular organelles, membranes, and the brain. Moreover, the mitochondrial matrix facilitates the accumulation of xenobiotics inside mitochondria. The rates of mitochondrial replacement might vary from days to weeks, providing enough time for xenobiotic accumulation to cause drug-induced mitochondrial changes [60,79,80]. Almost all tested antidepressants (no PAR data were found) showed brain accumulations in animal and human studies of brain-to-plasma ratios [81–86]. Significant changes in mitochondrial function observed at high concentrations of the tested antidepressants should be interpreted with caution, recognizing that the results cannot be directly extrapolated to clinical situations. However, the findings from these in vitro studies provide important preliminary information for further in vivo research using appropriate animal models and clinical trials, which are necessary for clinical validation and understanding of the relevance of the observed in vitro effects of high doses of antidepressants.

The rationale for studying the in vitro mitochondrial effects of tested antidepressants in a wide range of concentrations, significantly exceeding the therapeutic plasma concentrations of these drugs, includes: (i) safety and toxicity assessment (recognition of mitochondrial adverse effects that may occur after overdose or local accumulation of the drug); (ii) elucidation of the mechanisms by which the tested drugs exert their effects on cellular function with a focus on mitochondrial function; (iii) comparative analysis of mitochondrial changes at low and high concentrations of antidepressants in order to recognize and understand dose-dependent effects and to determine potential toxic doses; and (iv) theoretical contributions in the field of revealing the mitochondrial response to extremely high concentrations of antidepressants.

A limitation of this study is the lack of information on the effects of antidepressants on mitochondrial morphology and oxidative stress. Antidepressant-induced changes that may be related to changes in mitochondrial morphology have been described [54,87–89]. This suggests a potential effect of antidepressants on mitochondrial morphology, which is associated with mitochondrial dysfunction and increased oxidative stress. The effect of antidepressants on oxidative stress, measured as increased production of ROS, lipid peroxidation, or decreased activity of antioxidant enzymes has been described [90–92]. Therefore, antidepressants have the potential to affect mitochondrial morphology and regulate the oxidative stress, and these effects should be further investigated for a full understanding of their therapeutic effects or side effects.

4.6. Possible Clinical Impact

The most frequent adverse effect of SSRIs is nausea, and the overall incidence was similar for all SSRIs. PAR probably has the worse tolerability profile of all SSRIs [93,94]. Considering that SER and PAR were the most potent inhibitors of mitochondrial function and, according to the literature, also the drugs with the lowest tolerability profiles among the tested SSRIs, this mitochondrial inhibition could be connected to their adverse effects, especially through complex II+III activity inhibition [30,93].

BUP has similar efficacy but a better tolerability profile than SSRIs, usually causes lower rates of sexual dysfunction, and it is more likely to cause weight loss [95,96]. TRA is generally considered to be as effective as SSRIs and less likely to cause sexual dysfunction or insomnia [97].

Mixed results have been found for the effect of antidepressants on cognition, probably through complex mechanisms involving monoamine transporters, receptors, and degradative enzymes (including MAO). A meta-analysis showed that antidepressant treatment had a modest beneficial effect on cognitive function in depressed patients, with the largest effect mediated by SSRIs. This was not shown for non-depressed participants [98]. It was published that long-term SSRI treatment of depressed patients with mild cognitive impairment significantly delayed progression to Alzheimer's dementia compared with short-term SSRI treatment, other antidepressants, or no treatment, and compared with patients with mild cognitive impairment without a history of depression [99]. A systematic review confirmed the procognitive effect of newer SSRIs, with the best results for ESC. Cognitive improvement was also confirmed for BUP, whose procognitive effect is mainly attributed to its noradrenergic mechanism of action [100]. TRA is useful in the control of agitation and insomnia in AD. A systematic review analyzing the effect of TRA on cognition found that acute TRA treatment may be associated with cognitive impairment, but long-term treatment tends to prevent cognitive decline. It is important to note, that this effect is related to the effect of TRA on the unfolded protein response pathway [101]. A cohort study found no cognitive benefit in patients with dementia taking TRA compared to other antidepressants [102].

5. Conclusions

The results of this in vitro study showed that SSRIs, BUP, and TRA significantly altered OXPHOS system functioning. All tested antidepressants (except BUP in complex II + III) significantly decreased ETC complexes activities at all tested concentrations. The most potent inhibitors of complex I and II + III activities were PAR and SER, and the strongest complex IV inhibitor was TRA. All tested antidepressants except BUP significantly reduced complex I-linked respiration (PAR and FLUV at very high concentrations). The SSRIs were the strongest inhibitors of complex I and II+III activities and complex I-linked respiration compared to BUP and TRA. BUP was the only tested antidepressant with some inhibitory activity against complex II-linked respiration. Based on our results, SSRIs affect mitochondrial ETC complexes and respiration differently than BUP and TRA. Considering that all tested antidepressants showed inhibitory properties against OXPHOS, they can participate in drug-induced mitochondrial dysfunction, which can endanger neuronal adaptation and body homeostasis, especially at high doses. Antidepressants did not significantly affect total ATP content or kinetics, suggesting that ATP production is not directly dependent only on ETC enzymes activities; engagement of mitochondrial capacity reserve or potential OXPHOS compensatory mechanisms may occur.

Understanding the subcellular mechanisms of action of antidepressants and evaluating their effects on mitochondrial parameters is a very important step in elucidating how drug-related mitochondrial changes contribute to their therapeutic and/or adverse effects. Mitochondrial dysfunction could be a treatment response modulator and/or a predictor of patient responsiveness, which might help pharmacoresistant patients in choosing an effective pharmacotherapy. Based on our study results, it is evident that these drugs have MAO-inhibiting properties that could participate in their antidepressant, procognitive, and neuroprotective effects. These mechanisms should be further investigated, especially in in vivo studies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox12061208/s1. Table S1. The changes in citrate synthase (CS) and malate dehydrogenase (MDH) activities induced by antidepressants.

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Abbreviations

BUP	bupropion
CS	citrate synthase
ESC	escitalopram
ETC	electron transport chain
FLUV	fluvoxamine
IC_{50}	half-maximal inhibitory concentration
MAOI	monoamine oxidase inhibitor
MAO	monoamine oxidase
MDD	major depressive disorder
MDH	malate dehydrogenase
NADH	Reduced nicotinamide adenine dinucleotide
OXPHOS	oxidative phosphorylation
PAR	paroxetine
ROS	reactive oxygen species
SD	standard deviation
SEM	standard error of the mean
SER	sertraline
SSRI	selective serotonin reuptake inhibitor
TRA	trazodone

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Attachment 2

Ľupták M, Fišar Z, Hroudová J. Different effects of SSRIs, bupropion, and trazodone on mitochondrial functions and monoamine oxidase isoform activity. Antioxidants. 2023; 12(6):1208. doi: 10.3390/antiox12061208.

Article

Agomelatine, Ketamine and Vortioxetine Attenuate Energy Cell Metabolism—In Vitro Study

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Abstract: This determination of the mitochondrial effect of pharmacologically different antidepressants (agomelatine, ketamine and vortioxetine) was evaluated and quantified in vitro in pig brain-isolated mitochondria. We measured the activity of mitochondrial complexes, citrate synthase, malate dehydrogenase and monoamine oxidase, and the mitochondrial respiratory rate. Total hydrogen peroxide production and ATP production were assayed. The most potent inhibitor of all mitochondrial complexes and complex I-linked respiration was vortioxetine. Agomelatine and ketamine inhibited only complex IV activity. None of the drugs affected complex II-linked respiration, citrate synthase or malate dehydrogenase activity. Hydrogen peroxide production was mildly increased by agomelatine, which might contribute to increased oxidative damage and adverse effects at high drug concentrations. Vortioxetine significantly reduced hydrogen peroxide concentrations, which might suggest antioxidant mechanism activation. All tested antidepressants were partial MAO-A inhibitors, which might contribute to their antidepressant effect. We observed vortioxetine-induced MAO-B inhibition, which might be linked to decreased hydrogen peroxide formation and contribute to its procognitive and neuroprotective effects. Mitochondrial dysfunction could be linked to the adverse effects of vortioxetine, as vortioxetine is the most potent inhibitor of mitochondrial complexes and complex I-linked respiration. Clarifying the molecular interaction between drugs and mitochondria is important to fully understand their mechanism of action and the connection between their mechanisms and their therapeutic and/or adverse effects.

Keywords: oxidative phosphorylation; mitochondrial respiration; reactive oxygen species; ATP; monoamine oxidase; antidepressants; agomelatine; ketamine; vortioxetine

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1. Introduction

The primary role of mitochondria is as a source of energy in the form of adenosine triphosphate (ATP) for cellular processes. In addition, mitochondria are involved in calcium homeostasis, redox signaling, apoptosis regulation and heat production. Consequently, mitochondria have a key role in neurodevelopment and neuroplasticity. Thus, mitochondrial dysfunction and the consequent depletion of ATP production may play an important role in the pathophysiology of psychiatric disorders, including depression and may be a target for antidepressants. This is supported by many findings, including impaired mitochondrial membrane potential and damaged mitochondrial brain ultrastructure in a preclinical mouse model of chronic mild stress or reduced glucose utilization in certain brain areas of patients suffering from depression. Increased concentrations of oxygen and nitrogen species and lowered antioxidant protection lead to damage to nucleic acids, proteins and lipids. Increased markers of oxidative DNA damage, together with lowered DNA repair ability, have been found in patients with depression [1,2]. Based on these findings, the mitochondrial hypothesis has been postulated. It has been proven that mitochondria play a key role in

neuroplasticity and neurodevelopment. The consequence of mitochondrial dysfunction is not only insufficient energy production but also impairment of neuronal communication, neuroinflammation, oxidative stress (as a result of redox imbalance) and reduced neuronal adaptation toward internal and external signals—neuroplasticity. Different studies have observed a stress-induced decrease in hippocampal neurogenesis and oxidative stress is a contributing factor. All these changes can participate in the development and progression of depression [1,3].

Currently, there is no single hypothesis covering all the signs and symptoms of depression, suggesting that depression pathophysiology has multiple mechanisms that are linked and lead to the same symptoms of the disease. The most currently discussed biological hypotheses potentially linked to mitochondrial dysfunction are the biogenic amine hypothesis, the genetic hypothesis, the environmental hypothesis, the immunological-inflammatory hypothesis, the abnormal glutamate receptor hypothesis and the neurotrophic hypothesis [4].

The current pharmacotherapy of depression relies especially on selective serotonin reuptake inhibitors (SSRIs) [5]. However, approximately 30% of patients treated with first-line SSRI treatment do not achieve full remission [6]. Many patients also suffer from SSRI side effects such as gastrointestinal irritation, sexual dysfunction, sleep disturbances and emotional blunting. [7,8]. Undoubtedly, there is a need to discover an antidepressant with a novel mechanism of action that would be efficacious for SSRI-unresponsive and/or SSRI-intolerant patients. Recently, the FDA/WHO approved antidepressants with different mechanisms of action, e.g., serotonin (5-HT) modulators and stimulators (vortioxetine), N-methyl-D-aspartate (NMDA) glutamate receptor antagonists (ketamine) and melatonin agonists and selective serotonin antagonists (agomelatine) [5,9].

Agomelatine (AGO) is a melatonergic antidepressant that is structurally similar to melatonin and affects circadian rhythms. It is a well-tolerated antidepressant, despite its hepatotoxicity. The most frequent adverse effects of AGO are headache, nasopharyngitis, back pain and upper respiratory tract infections [9–11].

Ketamine (KET), originally used as an anesthetic, has shown antidepressant effects at low subanesthetic doses. It is a potent antidepressant with a rapid onset that is effective in severe, drug-resistant and suicidal depression. Due to its complex mechanism of action and its effects, KET provides new insight into the pathophysiology of depression [12]. It has been suggested that the antidepressant effect of KET is mediated by energy metabolism and effects on antioxidant defense [13]. Moreover, it was shown that KET can increase BDNF expression and synthesis [14].

In general, low-dose and short-term KET administration is well tolerated; mild adverse effects include dizziness, vertigo, nausea, short-term dissociation and a blood pressure increase. High doses of KET administered for a prolonged time carry a risk of severe adverse effects, including acute anxiety, panic attack and a prolonged psychomimetic or dissociative effect [14–17].

Vortioxetine (VOR) is a multimodal antidepressant that acts as a serotonin transporter inhibitor and modulator of 5-HT receptors. The most common side effects were nausea and vomiting, dizziness, insomnia and sexual dysfunction. There are very little data available showing the effect of VOR on mitochondrial functions and cellular energy metabolism.

There is a growing body of evidence showing the relationship between mitochondrial dysfunction and depression. For example, patients with mitochondrial disorders are 3.9 times more prone to develop depressive comorbidity [18]. Muscle biopsy in patients with major depressive disorder (MDD) found significantly decreased mitochondrial ATP production rates, complex I activity and other enzyme ratios compared with healthy controls [18–21]. Many altered proteins identified in MDD are linked to oxidative phosphorylation (OXPHOS), e.g., the meta-analysis revealed decreased expression of complex I subunits in MDD and bipolar affective disorder. Our group reported impaired mitochondrial respiration in intact platelets from depressive patients, manifesting mostly as a decreased respiratory rate and maximal capacity of the electron transport chain (ETC). Similar findings were reported by Karabatsiakis et al. in peripheral blood mononuclear

cells from patients with MDD; moreover, these results were negatively correlated with the severity of symptoms [22,23]. Interestingly, increased protein expression of complex I and ATP synthase was found in certain brain areas of depressive patients, which might be a compensatory mechanism for reduced energy supply [21]. It was previously shown that patients diagnosed with MDD have higher levels of 8-oxoguanine, a marker of oxidative DNA damage, pointing toward impaired mitochondrial function and oxidative imbalance [2]. Patients with MDD consistently show signs of oxidative DNA damage and increased lipid peroxidation compared to healthy controls, when both parameters improve with antidepressant treatment [24]. Peripheral blood mononuclear cell mitochondrial DNA from patients with MDD is more vulnerable to oxidative damage than DNA from controls [25]. It is believed that mitochondria might try to compensate for mtDNA damage by enhancing mitochondrial biogenesis.

In this study, we investigated the in vitro effects of three currently used antidepressants on mitochondrial energy metabolism and reactive oxygen species (ROS) production using isolated pig brain mitochondria as a biological model. Based on the mitochondrial dysfunction hypothesis, it can be assumed that the effects of some antidepressants can be targeted at mitochondrial dysfunction, primarily at the disruption of bioenergetics and oxidative stress.

2. Results

2.1. Activity of Mitochondrial Enzymes

The results of the mitochondrial ETC complexes activities are depicted in Figure 1A–C. VOR caused the dose-dependent and statistically significant inhibition of complex I (Figure 1A) at all concentrations (5.5 1.8% at 100 M, $p < 0.001$). VOR was also inhibited complex II+III activity (Figure 1B) (43.9 1.2% at 100 M, $p < 0.001$) and significantly inhibited complex IV activity (Figure 1C) (9.3 3.8% at 100 M, $p < 0.001$).

KET-induced complex I inhibition (Figure 1A) reached statistical significance only at a concentration of 100 M (91.2 6.6%, $p = 0.007$). Nevertheless, KET inhibited complex II+III activity (Figure 1B) (93.6 2.6% at 100 M, $p < 0.001$) and complex IV activity (Figure 1C) (3.9 3.0% at 100 M, $p < 0.001$).

AGO inhibited the activity of complex I (Figure 1A) (73.7 5.1% at 100 M, $p < 0.001$), complex II+III (Figure 1B) (94.9 4.3 at 100 M, $p = 0.013$) and also dose-dependently inhibited complex IV activity (Figure 1C) (45.0 3.6% at 10 M, $p < 0.001$; 17.9 3.4% at 50 M, $p < 0.001$; 12.8 4.1% at 100 M, $p < 0.001$).

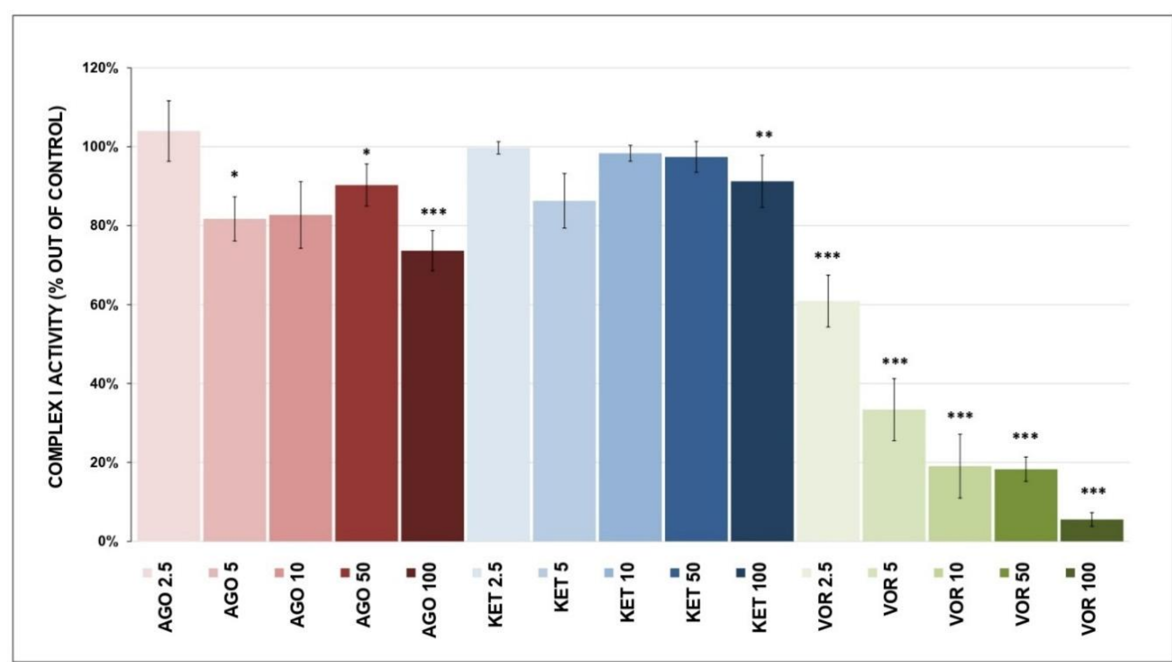
Tested substances showed no or very little inhibitory/inductive properties toward CS and MDH (Table 1).

Table 1. The effect of antidepressants on citrate synthase (CS) and malate dehydrogenase (MDH) activity.

Drug	Concentration (M)	CS (% of Control)		N	MDH (% of Control)		N
agomelatine	10	** 108.8	1.06	3	101.7	1.99	3
	100	** 95.7	0.55	3	101.0	2.31	3
ketamine	10	107.3	3.91	3	97.4	2.98	3
	100	97.4	1.08	3	101.7	2.61	3
vortioxetine	10	109.3	6.11	3	106.9	6.80	3
	100	* 96.9	1.08	3	100.2	1.82	3

The values are expressed as the mean SD for 3 independent measurements. Statistical significance was tested using a one sample t-test that control value is equal to 100% and is expressed as * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. N—number of measurements.

(**A**)



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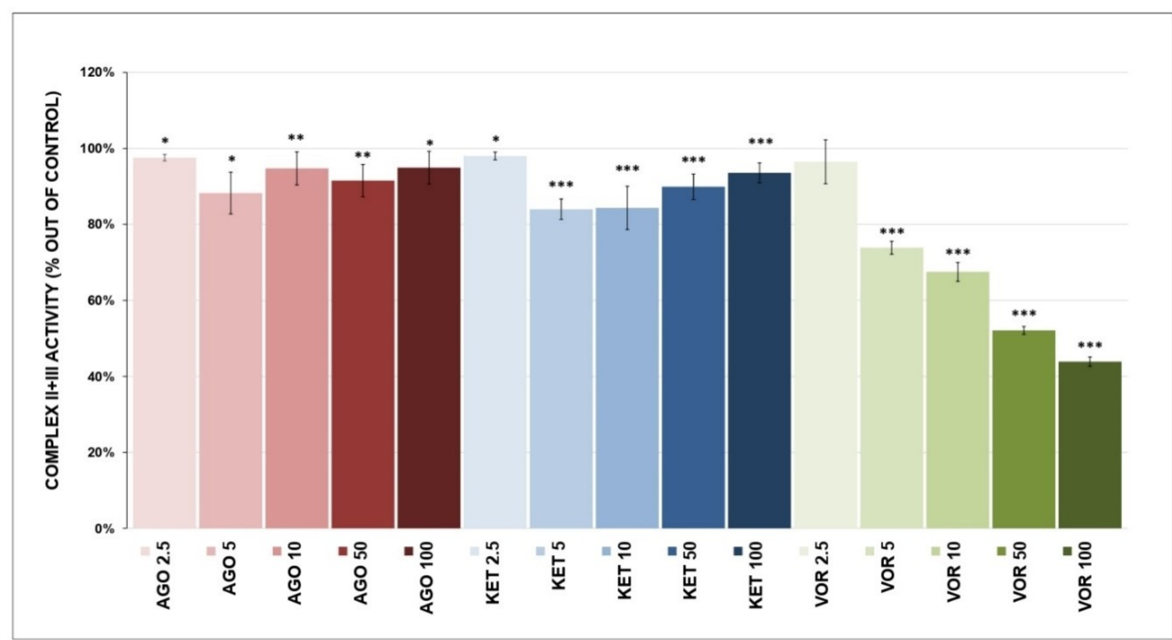


Figure 1. Cont.

(**C**)

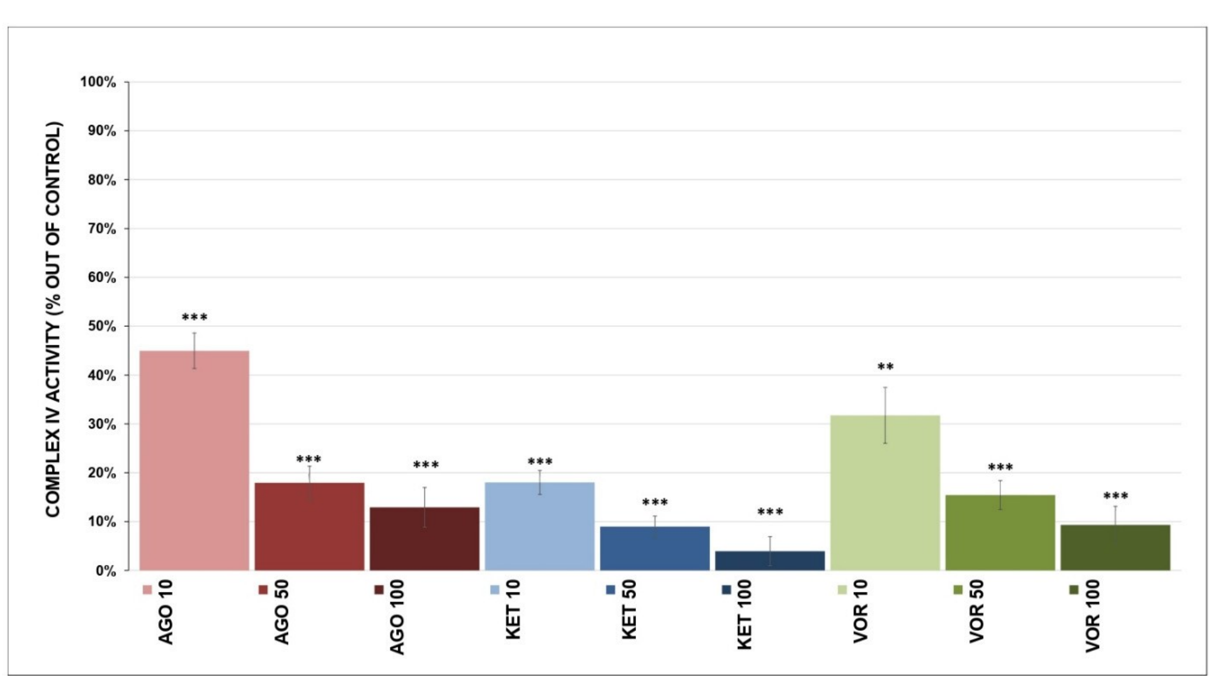


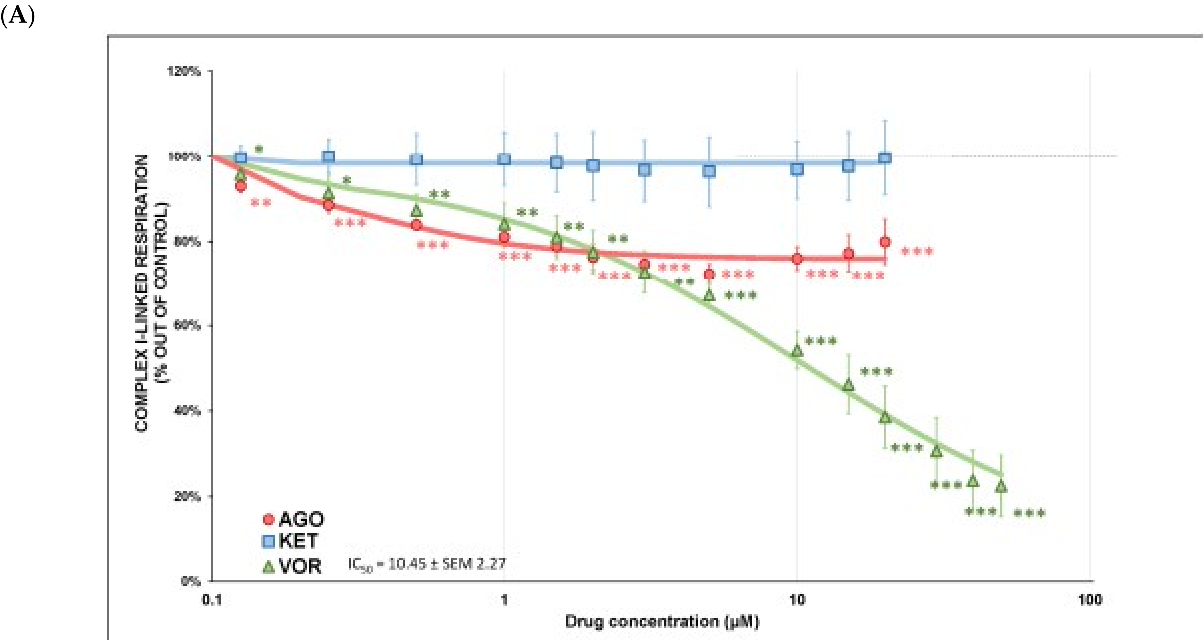
Figure 1. (**A**) Antidepressant-induced inhibition of complex I activity. Relative activity is displayed as 100% activity of control sample. The values are expressed as the mean SD for at least three independent measurements. Statistical significance was tested using a one sample t-test that mean value is equal to 100% and is expressed as * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. Drugs concentrations are expressed in M. AGO—agomelatine, KET—ketamine, VOR—vortioxetine. (**B**) Antidepressant-induced inhibition of complex II+III activity. Relative activity is displayed as 100% activity of control sample. The values are expressed as the mean SD for at least three independent measurements. Statistical significance was tested using a one sample t-test that mean value is equal to 100% and is expressed as * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. AGO—agomelatine, KET—ketamine, VOR— vortioxetine. (**C**) Antidepressant-induced changes in complex IV activity. Relative activity is displayed as 100% activity of control sample. The values are expressed as the mean SD for at least three independent measurements. Statistical significance was tested using a one sample t-test that mean value is equal to 100% and is expressed as * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. Drugs concentrations are expressed in M. AGO—agomelatine, KET—ketamine, VOR—vortioxetine.

2.2. Mitochondrial Respiration

Drug-induced changes in complex I-linked and complex II-linked respiration are depicted in Figure 2A,B. VOR was the only full inhibitor of complex I-linked respiration (Figure 2A) with a respiration rate of 22.5 7.1 % (mean SD) at 50 M ($p < 0.001$), IC_{50} = 10.5 2.3 M (mean SEM) and residual activity of 4.7 % 0.07 (mean SEM). KET inhibited complex I-linked respiration very weakly and AGO did not affect complex I-linked respiration at all (Figure 2A). In higher concentrations of all tested drugs, we observed the phenomenon of a rapid increase in the respiratory rate, which was probably caused by drug accumulation in mitochondrial membranes changing the lipid-protein interactions rather than by a direct drug-respiratory complex interaction [26–29]. Drug titration was terminated at the point of a sudden increase in the mitochondrial respiratory rate at high drug concentrations.

None of the three substances tested affected complex II-linked respiration (Figure 2B).

(A)



(B)

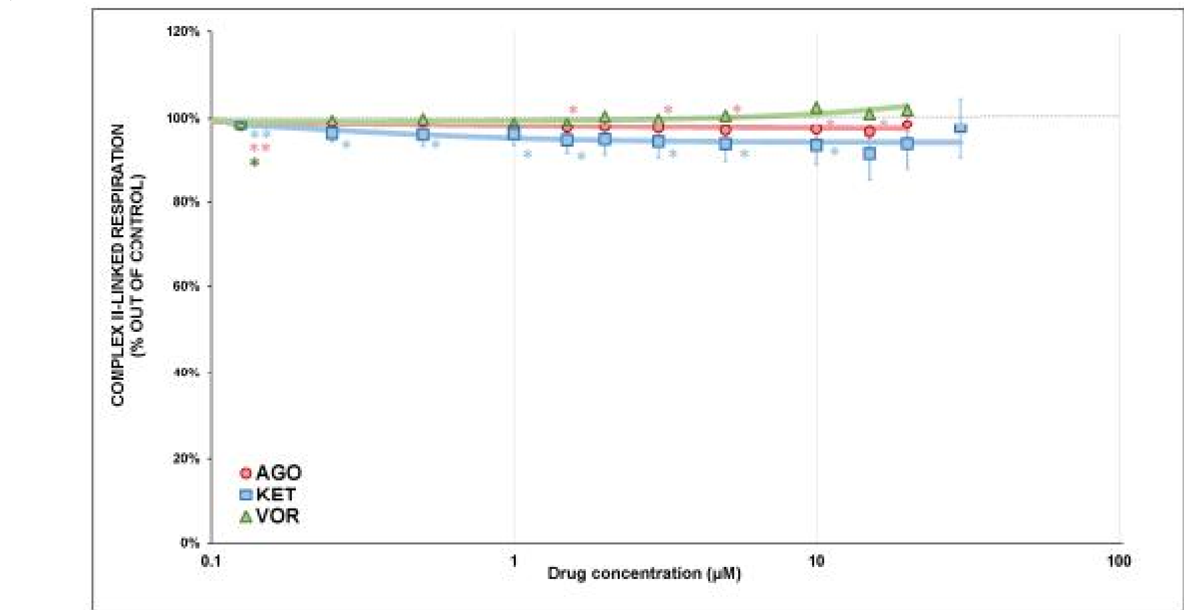


Figure 2. (**A**) Antidepressant-induced inhibition of complex I-linked respiration. Dose-response curves are displayed as plots of the respiration rate against drug concentration. Relative activity is displayed as 100% activity of control sample. Points are the mean of four independent measurements and lines represent the best/fitted curves using a four-parameter logistic function. Statistical signif-icance was tested using a one sample t-test that mean value is equal to 100% and is expressed as * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. The half-maximal inhibitory concentration (IC_{50}) was calculated for VOR. Drugs concentrations are expressed in M. AGO—agomelatine, KET—ketamine, VOR— vortioxetine. (**B**) Antidepressant-induced inhibition of complex II-linked respiration. Dose-response curves are displayed as plots of the respiration rate against drug concentration. Relative activity is displayed as 100% activity of control sample. Points are the mean of four independent measurements and lines represent the best/fitted curves using a four-parameter logistic function. Statistical signif-icance was tested using a one sample t-test that mean value is equal to 100% and is expressed as * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. Drugs concentrations are expressed in M. AGO—agomelatine, KET—ketamine, VOR—vortioxetine.

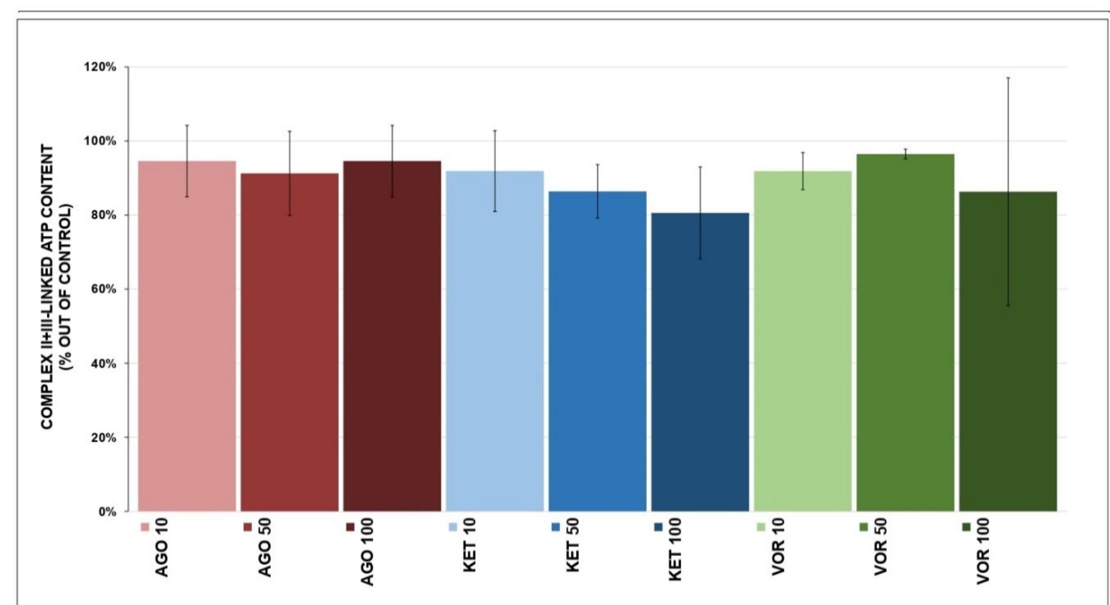
2.3. ATP Content and Kinetics

The ATP content and kinetics results are depicted in Figure 3A,B and Figure 4A,B, respectively.

(**A**)

(**B**)

Figure 3. (**A**) Antidepressant-induced changes in complex I-linked ATP content. Relative activity is displayed as 100% activity of control sample (100% corresponded to a production of 160 nmol of ATP per 1 mg of protein). The values are expressed as the mean SD for at least six independent measurements. Statistical significance was tested using one sample t-test that mean value is equal to 100% and is expressed as * $p < 0.05$. Drugs concentrations are expressed in M. AGO—agomelatine, KET—ketamine, VOR—vortioxetine. (**B**) Antidepressant-induced changes in complex I-linked ATP kinetics. Relative activity is displayed as 100% activity of control sample (100% corresponded to a production of 282 nmol of ATP per 1 mg of protein per 1 min). The values are expressed as the mean SD for at least six independent measurements. Statistical significance was tested using one sample t-test that mean value is equal to 100%. Drugs concentrations are expressed in M. AGO—agomelatine, KET—ketamine, VOR—vortioxetine.



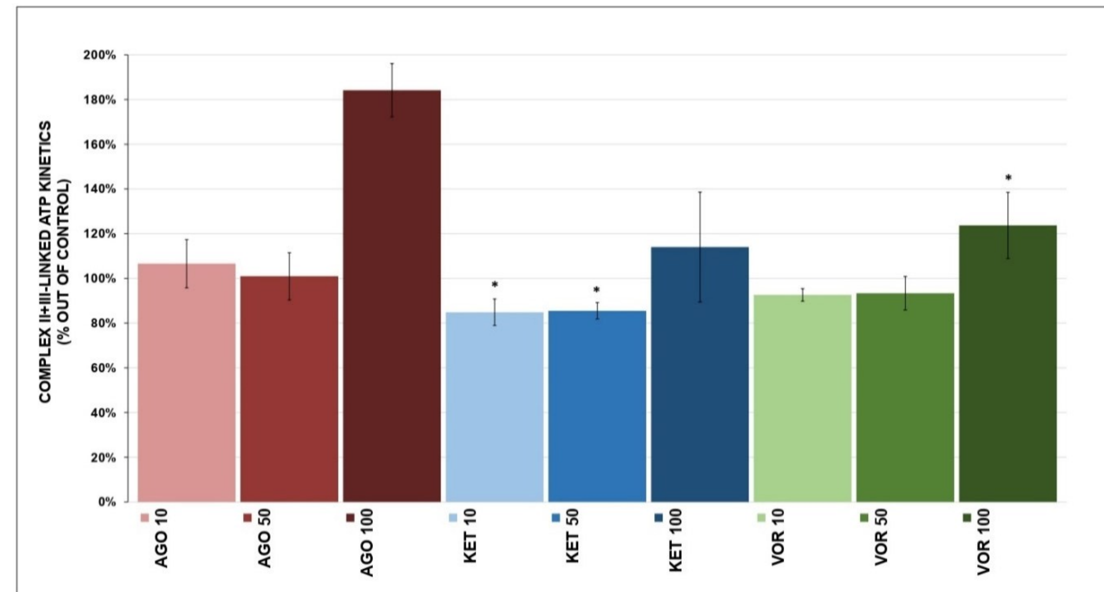


Figure 4. (**A**) Antidepressant-induced changes in complex II-linked ATP content. Relative activity is displayed as 100% activity of control sample (100% corresponded to a production of 291 nmol of ATP per 1 mg of protein). The values are expressed as the mean SD for at least six independent measurements. Statistical significance was tested using one sample t-test that mean value is equal to 100%. Drugs concentrations are expressed in M. AGO—agomelatine, KET—ketamine, VOR—vortioxetine. (**B**) Antidepressant-induced changes in complex II-linked ATP kinetics. Relative activity is displayed as 100% activity of control sample (100% corresponded to a production of 1289 nmol of ATP per 1 mg of protein per minute). The values are expressed as the mean SD for at least six independent measurements. Statistical significance was tested using one sample t-test that mean value is equal to 100% and is expressed as * $p < 0.05$. Drugs concentrations are expressed in M. AGO—agomelatine, KET—ketamine, VOR—vortioxetine.

Complex I-linked ATP content (Figure 3A) was increased after VOR (119.0 10.6% at 100 M, $p = 0.037$, mean SD) and AGO (106.4 4.0% at 10 M, $p = 0.044$), but KET had no significant effect. Complex I-linked ATP kinetics (Figure 3B) were not affected by any tested drug. None of the tested drugs significantly affected complex II+III-linked ATP content

(Figure 4A). Complex II+III-linked ATP kinetics (Figure 4B) was significantly affected by KET (84.9 5.9% at 10 M, p = 0.048) and VOR (92.6 2.8% at 10 M, p = 0.046).

2.4. Hydrogen Peroxide Production

Changes in H_2O_2 production were observed in all tested drugs, total H_2O_2 content is depicted in Figure 5. The statistically significant increase in H_2O_2 content was caused by AGO (130.1 2.7% at 10 M, p = 0.041) and by VOR (124.5 2.5% at 50 M, p = 0.046). However, VOR at 100 M decreased the total H_2O_2 content (85.4 5.0%, p = 0.037). KET showed no statistically significant effect on total H_2O_2 production.

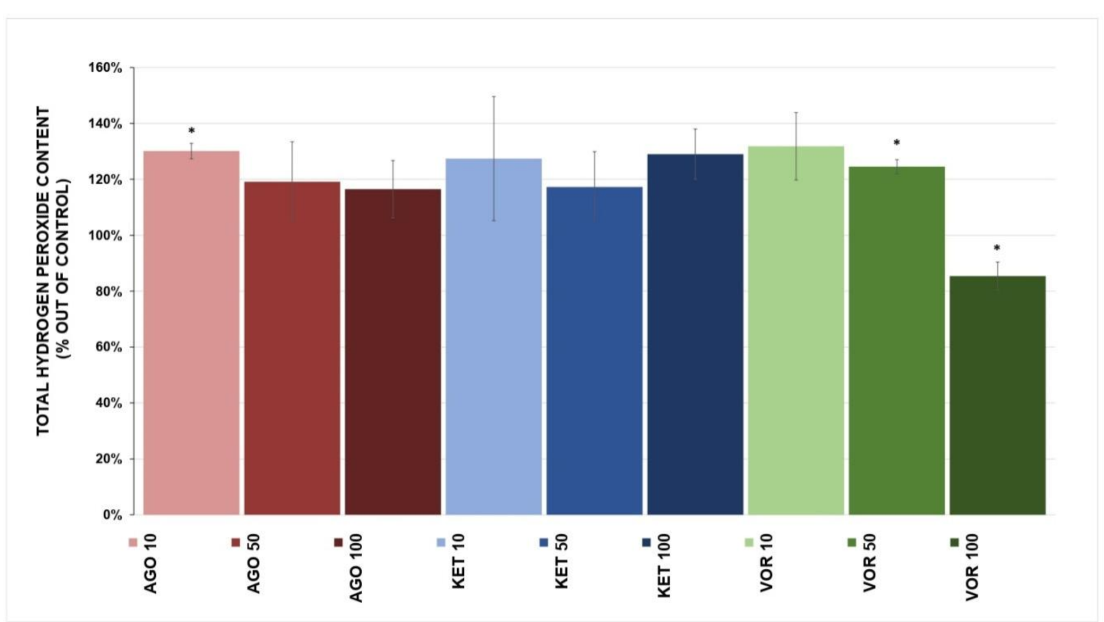


Figure 5. Antidepressant-induced changes in total hydrogen peroxide (H_2O_2) content. Relative activity is displayed as 100% activity of control sample (100% corresponded to a production of 450 pmol of H_2O_2 per 1 mg of protein). The values are expressed as the mean SD for at least three independent measurements. Statistical significance was tested using a one sample t-test that mean value is equal to 100% and is expressed as * $p < 0.05$. Drugs concentrations are expressed in M. AGO—agomelatine, KET—ketamine, VOR—vortioxetine.

2.5. MAO Activity

All three tested substances were found to be partial inhibitors of MAO-A (Figure 6A). The strongest MAO-A inhibitor was VORlinebreak (IC_{50} = 7.33 1.1 M), followed by AGO (IC_{50} = 8.20 1.4 M) and KET (IC_{50} = 10.36 8.3 M). MAO-B activity (Figure 6B) was partially inhibited by VOR (IC_{50} = 18.24 3.5 M); KET showed only a weak MAO-B inhibition (IC_{50} = 51.16 25.5 M); AGO did not affect its activity at all (kinetic parameters were not calculated). Drug-induced MAO-A and MAO-B inhibition curves are depicted in Figure 6A,B and the kinetic parameters are summarized in Table 2.

Table 2. Drug-induced monoamine oxidase (MAO-A and MAO-B) inhibition.

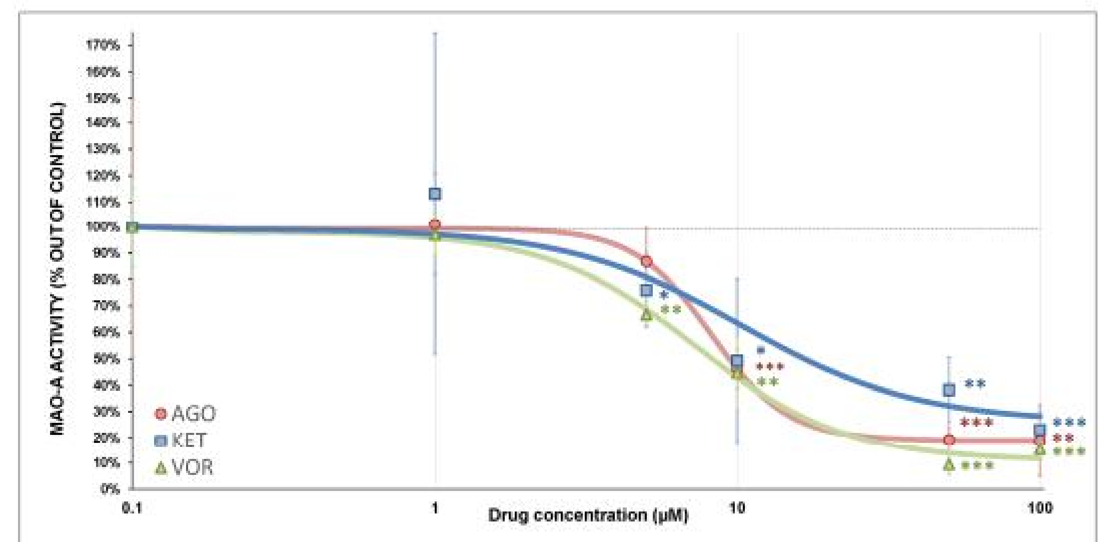
	MAO-A						
Drug	IC_{50} (M)		Hillslope		Residual Activity (rel.u.)		Inhibition
agomelatine	8.20	1.41	3.40	1.72	0.190	0.070	partial
ketamine	10.36	8.28	1.50	1.83	0.259	0.214	partial
vortioxetine	7.33	1.10	1.76	0.61	0.109	0.050	partial

Table 2. Cont.

MAO-B											
agomelatine		-			-				-		none
ketamine	51.16	25.54	2.12		2.04		0.707		3.50	0.161	weak
vortioxetine	18.24			2.58		0.68		0.229		0.041	partial

The values are expressed as the mean SEM for 4 independent measurements. IC_{50} is half maximal inhibitory concentration.

(**A**)



(**B**)

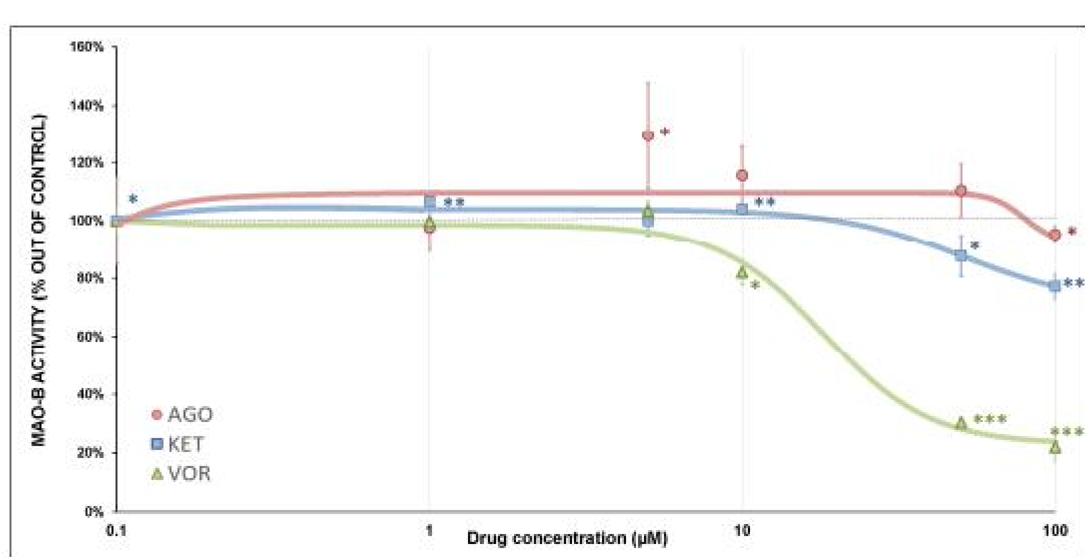


Figure 6. (**A**) Antidepressant-induced MAO-A inhibition. The relative activity is displayed as 100% activity of the control sample. Statistical significance was tested using a one sample t-test that mean value is equal to 100% and is expressed as * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. The half-maximal inhibitory concentration (IC_{50}), Hill slope and residual activity was calculated (Table 2). Drugs concentrations are expressed in M. AGO—agomelatine, KET—ketamine, VOR—vortioxetine. (**B**) Antidepressant-induced MAO-B inhibition. The relative activity is displayed as 100% activity of the control sample. Statistical significance was tested using a one sample t-test that mean value is equal to 100% and is expressed as * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. The half-maximal inhibitory concentration (IC_{50}), Hill slope and residual activity was calculated (Table 2). Drugs concentrations are expressed in M. AGO—agomelatine, KET—ketamine, VOR—vortioxetine.

2.6. Correlations

Statistically significant correlations were identified between measured mitochondrial parameters using the Pearson correlation coefficient. Strong and statistically significant correlations were found between the activity of complex I and the activity of complex II+III, between the activity of complex II+III and the activity of complex IV, between the activity of complex II+III and total ROS content, between the activity of complex II+III and complex I-linked respiration, and between the complex II-linked ATP kinetics and complex I-linked respiration for VOR. For AGO, there were found to be significant correlations between the activities of complex IV and complex I-linked ATP kinetics and between complex I-linked ATP kinetics and complex II-linked ATP kinetics. The complete results of the correlation analysis are summarized in Tables 3 and 4.

Table 3. Correlation coefficients for agomelatine-induced changes in mitochondrial parameters.

Agomelatine	Complex I Activity		Complex IV Activity		Complex I-Linked ATP Kinetics		Complex II-Linked ATP Kinetics	
	r	N	r	N	r	N	r	N
complex IV activity	0.01	11	-	-	-	-	-	-
complex I-linked ATP kinetics	0.12	7	* 0.87	7	-	-	-	-
complex II-linked ATP kinetics	0.76	6	0.47	6	* 0.81	6	-	-
total H_2O_2 content	0.12	8	0.48	8	0.77	6	-	-
complex I-linked respiration	0.12	13	0.17	4	0.86	3	0.59	6

The Pearson correlation coefficient (r) was used as a measure of linear correlation between mitochondrial parameters measured at various concentrations of antidepressants and statistical significance and it is expressed as * $p < 0.05$. H_2O_2—hydrogen peroxide, N—number of measurements. Respiratory parameters unaffected by drugs were not included in the correlation analysis.

Table 4. Correlation coefficients for vortioxetine-induced changes in mitochondrial parameters.

Vortioxetine	Complex I Activity		Complex II+III Activity		Complex IV Activity		Complex I-Linked ATP Content		Complex II-Linked ATP Kinetics	
	r	N	r	N	R	N	r	N	r	N
complex II+III activity	*** 0.91	19	-	-	-	-	-	-	-	-
complex IV activity	0.41	10	*** 0.91	11	-	-	-	-	-	-
complex I-linked ATP content	* 0.69	11	** 0.78	11	0.55	10		-	-	-
complex II-linked ATP kinetics	0.11	8								-
total ROS content	0.73	7	0.48	8	0.55	8	0.03	8	-	-
complex I-linked respiration	0.46	11	* 0.86	7	0.74	7	0.42	7	0.39	6
			*** 0.86	12	* 0.67	9	0.27	8	* 0.83	8

The Pearson correlation coefficient (r) was used as a measure of linear correlation between mitochondrial parameters measured at various concentrations of antidepressants and statistical significance and it is expressed as * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. ROS—reactive oxygen species, N—number of measurements. Respiratory parameters unaffected by drugs were not included in the correlation analysis.

3. Discussion

3.1. Mitochondrial Enzyme Activity and Respiration

All three tested drugs significantly inhibited the activity of mitochondrial complexes I, II+III and IV (Figure 1A–C); complex IV was the most affected. VOR was the most potent inhibitor of individual ETC complexes; complex I was inhibited to 5.5% (at 100 M) and complex I-linked respiration (Figure 2A) was fully inhibited by VOR (IC_{50} = 10.5 M). Complex I is usually the most vulnerable part of OXPHOS, and there are more than

60 well-known inhibitors of complex I, especially lipophilic molecules [30]. Complex I is also a potent ROS producer; its functional impairment can lead to both insufficient ATP production and increased oxidative damage. VOR also significantly decreased the activity of complexes II+III and IV (Figure 1B,C). Because complex II+III is an alternative electron input to OXPHOS, this inhibitory effect on mitochondrial respiratory complexes could lead to OXPHOS impairment and insufficient energy supply. Although VOR administration lowered the activity of all individual complexes, it was no surprise that it also acted as a potent inhibitor of mitochondrial complex I-linked respiration (Figure 2A). However, complex II-linked respiration (Figure 2B) remained unaffected by VOR, suggesting that OXPHOS is still able to perform respiration through complex II even at very high VOR concentrations. Borhannejad et al. found no significant difference in adverse events between a group of patients taking VOR and a group of patients taking sertraline. Since the sample size of this study was rather small (40 patients in total), the clinical relevance of these results may be questionable. Rare adverse reactions may also not be visible with this small sample size [31]. Another study shared similar results comparing drug-induced adverse effects and therapy discontinuations in patients taking escitalopram, desvenlafaxine and VOR [32]. Overall, VOR is considered to be safe and tolerable. Meta-analyses showed higher rates of treatment discontinuation in the VOR group than in the placebo group but lower rates than in the active control group. It was reported that its tolerability worsens with increasing doses [33–35]. However, we can speculate that its inhibitory effect on ETC complexes and mitochondrial respiratory rate might play a part in the adverse effects of high VOR concentrations.

These results are in accordance with our previous data for tricyclic antidepressants and SSRIs, which extensively inhibited mitochondrial respiration and the activity of respiratory chain complexes at high drug concentrations when the most affected complexes were complexes I and IV [36]. It can be assumed that this conformity in antidepressant-induced mitochondrial dysfunction could be a consequence of similar drug-induced changes in mitochondrial functions. In the search for new biological markers of treatment response, it was found that higher basal complex I and CS activity and a higher treatment-induced complex I activity decrease were directly linked to better response to SSRI treatment in patients with MDD [20].

KET and AGO had almost no effect on the activity of complexes I, II+III (Figure 1A,B) and the mitochondrial respiratory rate (Figure 2A,B). However, they were very potent inhibitors of complex IV (Figure 1C). Complex IV is the rate-limiting player in OXPHOS, initiating the final step in the ETC and its activity is also coupled with neuronal activation. It was previously reported that antidepressants might have both stimulating and inhibiting properties toward complex IV activity, while other psychoactive drugs mostly tend to increase their activity [37]. The current results for KET are in accordance with our previous results as follows: KET is only a very weak inhibitor of complex I-linked respi-ration (Figure 2A) and shows no inhibitory activity against complex II-linked respiration (Figure 2B) [30]. Other studies describing KET-induced mitochondrial changes reported the following several inconsistent results: reversed chronic mild stress-induced inhibition of complexes I, II and IV in rats; increased complex IV activity in rats after subchronic KET administration; no significant effect on mouse brain and macrophage mitochondria [38–40].

Our results for AGO are in accordance with Kumar et al., who showed impaired activity of mitochondrial complexes after subchronic AGO administration to rats as follows: complex I activity was increased mostly by a 10 mg/kg dose, whether higher doses decreased complex I activity; complex II activity was increased by the highest dose of 50 mg/kg; complex IV activity was decreased by lower doses and increased by the highest dose [41]. In in vitro studies, AGO was able to normalize the impaired activity of mitochondrial complexes and ROS increase in rats induced by prenatal exposure to valproic acid and reverse galactose-induced mitochondrial dysfunction [42,43]. The inconsistency of our results compared to these studies is most likely the result of different methodologies (in vivo vs. in vitro). We observed a direct molecular effect of antidepressants on mitochon-

drial parameters without the involvement of other biological pathways or an effect on the whole organism.

3.2. ATP Production

We separately studied complex I- and complex II-linked ATP content and kinetics to better understand the molecular mechanisms of drug action. VOR significantly increased complex I-linked ATP content (Figure 3A) and complex II-linked ATP kinetics (Figure 4A). This was quite surprising that VOR showed the most potent inhibitory properties toward individual ETC complexes and complex I-linked respiration. We hypothesize that due to this inhibition, some adaptation mechanisms could be triggered to preserve ATP formation through complex II-linked respiration. There are no comparative data describing the effect of VOR on ATP kinetics.

Complex II-linked ATP kinetics (Figure 4B) were significantly decreased by KET, which might be linked to the strongest KET-induced inhibition of complex IV as a rate-limiting process in ATP formation. It was previously reported that KET treatment reduced ATP/ADP metabolic ratios in rodents, which correlated with a forced-swim test time. It was also suggested that KET might cause energetic deficits by stimulating anabolic processes that consume ATP and require higher ATP production, which could lead to OXPHOS stimulation and ROS increases [13]. Moreover, KET-treated pluripotent stem cell-derived neurons produced less ATP than untreated controls [44].

AGO-mediated insignificant stimulation of both complex I- and complex II-linked ATP kinetics (Figures 3B and 4B) was also observed. We suggest that higher inhibitory complex I activity and complex I-linked respiration (compared to KET) lead to potential adaptive mechanisms, similar to VOR.

All these findings suggest that mitochondrial ATP production is a very complex process that cannot be simply derived from the activity of isolated ETC complexes or the oxygen consumption rate.

3.3. Hydrogen Peroxide Production

We did not observe a trend in increased H_2O_2 production by mitochondria (Figure 5) with increasing concentrations of the tested antidepressants. The highest significant H_2O_2 content increase was caused by AGO at a concentration of 10 M. In a rodent study, subchronic AGO administration did not significantly affect oxidative stress markers, but there was a small effect on antioxidant systems [41]. AGO normalized galactose-induced ROS increase in a rat model of hippocampal aging and reduced oxidative stress and damage in vitro in PC12 cells [43,45].

VOR, similar to AGO, stimulates H_2O_2 production at lower concentrations. At a concentration of 100 M, VOR was able to decrease total H_2O_2 production, which might be linked to the inhibition of complex I activity as the most potent producer of ROS.

The effect of KET on mitochondrial H_2O_2 production was insignificant, showing a mild stimulation of its production. In stem cell-derived neurons, KET treatment significantly increased ROS production, which might not be directly linked to mitochondrial ROS [44].

In addition to cellular oxidative damage, ROS play important signaling functions, including activating guanylate cyclase and are also essential for long-term neuronal plasticity by modulating the activities of several kinases and phosphatases. The peroxide anion reacts with nitric oxide, creating peroxinitrite, a compound that disrupts the enzymatic func-tion of tyrosine residues, which could decrease monoamine neurotransmitter production, worsening depressive symptoms.

Direct binding of antidepressants to ETC complexes might lead to an increase in ROS production, as was seen with cannabinoid drugs [46]. However, there are also data that indicate that antidepressants, regardless of their mechanism of action, can improve oxidative stress parameters in a subpopulation of patients, thus showing a capacity to improve antioxidant mechanisms. An increased level of oxidative stress was also found to be a marker of a poorer response to SSRI treatment [20]. Because ROS are also important

signaling molecules, it is difficult to determine the consequences of antidepressant-induced changes in ROS levels. We observed a drug-induced increase in total ROS production of approximately 30%, which is not an increase of approximately hundreds of percent that would reliably indicate an increase that would lead to increased oxidative damage. This mild an ROS increase could lead to signaling changes, and this hypothesis should be further tested. There is a possibility that ROS increase might participate in adverse effects associated with treatment. More studies observing lipid peroxidation, mtDNA damage and other markers of increased oxidative stress are needed to understand whether this ROS increase is on the "physiological" or the "pathophysiological" side.

3.4. MAO Inhibition

All tested drugs were partial inhibitors of monoamine oxidase A (Figure 6A) (MAO-A). The MAO-A isoform is an important metabolic enzyme of serotonin, norepinephrine and dopamine, which are neurotransmitters closely linked to depression. This mechanism could participate in their antidepressive effect, and MAO inhibitors were used as the first antidepressants [47].

MAO-B activity was significantly inhibited by VOR (Figure 6B). MAO-B is preferentially responsible for dopamine, benzylamine, phenethylamine and tyramine oxidative deamination. MAO-B inhibition is therapeutically used for the treatment of neurodegenerative disorders, e.g., Alzheimer's or Parkinson's disease, whereby the reduced production of H_2O_2 during MAO-catalyzed oxidation of monoamine neurotransmitters may be decisive [48]. In three large placebo-controlled studies, VOR significantly improved memory impairment in patients with MDD. Our results indicate that VOR-induced MAO-B inhibition may participate in the VOR-induced decrease in H_2O_2 production and might be one of the mechanisms by which VOR improves cognitive deficits [49].

3.5. Correlations

Strong and significant positive correlations were found between complex I and complex II+III activity and between complex II+III and complex IV activity for VOR. The correlation between individual complexes is logical; both complex II+III and complex IV are the "next steps" in the ETC and should be affected by previous complexes. Another strong correlation was found between the activity of complex II+III and the total ROS content. ROS production should be linked to individual ETC complex activity, and this finding suggests a causal correlation between these two parameters. A strong correlation was also found between complex II+III activity and complex I-linked respiration, indicating that the activity of complex II+III might also affect complex I-linked mitochondrial respiration, but this is a highly complex process that is not strictly dependent only on ETC complex activities.

The correlations between complex II-linked ATP kinetics and complex I-linked respiration (VOR) and between complex II-linked ATP kinetics and complex IV activity/complex I-linked ATP kinetics (AGO) might involve the previously mentioned activation of adaptation mechanisms but also might not be causal. Further research observing the connection between these parameters is needed.

3.6. Study Limitations

We investigated the effect of currently used antidepressants (AGO, KET and VOR) on mitochondrial parameters in the purified mitochondrial fraction, which allows more accurate recognition of drug effects on compensatory and regulatory mechanisms in mitochondria than in in vivo measurements. However, it must be noticed, that the investigation of the regulatory and compensatory brain mechanisms and other pathways that can influence the effects of the tested drugs on mitochondrial functions in vivo is beyond the experimental approach of this study.

We used a wide range of drug concentrations (units to tens of M) in our measurements to observe their effect on mitochondrial functions, some of which were much higher than

therapeutic plasma concentrations in vivo (tenths to units of M). All tested antidepressants are lipophilic molecules with a high probability of accumulation in neuronal membranes and subcellular structures [50–55]. A high antidepressant concentration might be expected in the mitochondria due to several mechanisms of xenobiotic accumulation [56]. High drug concentrations allowed us to determine the correlation between individual parameters at a concentration achievable in the brain at overdose, where significant drug-induced mitochondrial dysfunction occurred. Pig brain mitochondria were used as a biological model and were established and evaluated previously as a suitable model for studies investigating mitochondrial drug effects [57]. Purified mitochondria allow for studying direct drug-induced changes in mitochondrial parameters using suitable substrates, inhibitors and uncouplers. The further transition of this research to cell cultures is expected, especially for drugs that showed significant effects in therapeutic concentrations.

3.7. Possible Clinical Impact

The results of this study showed that AGO, KET and VOR significantly affected mitochondrial parameters in different ways, which indirectly supports the neurotrophic hypothesis of depression. Decreased levels of neurotrophic factors such as BDNF could directly affect mitochondrial performance because they act as mitochondrial respiratory couplers on complex I, which enhance mitochondrial energy production. Both a lack of BDNF and decreased mitochondrial performance contribute to disturbances in neuroplasticity and neurodevelopment. However, there is clear evidence of a connection between mitochondrial and neurotrophic hypotheses at this level [58].

It can be hypothesized that long-term drug-induced inhibition of individual respiratory complexes (especially complex IV, in this case) may cause mitochondrial toxicity manifesting as adverse drug effects [59]. In addition to lowered ATP formation and increased ROS production, mitochondrial dysfunction could activate immune and inflammatory processes, which likely contribute to adverse drug effects. Mitochondria can release oxidized mitochondrial DNA, and other factors act as proinflammatory mediators. This could serve as evidence connecting the mitochondrial and immune-inflammatory hypotheses [58].

Mitochondrial functions and cell energy metabolism are tightly linked with the pathophysiology of psychiatric diseases; therefore, measuring the mitochondrial functions of patients and considering the bioenergetic profile of individual psychiatric diseases should be involved in clinical practice in advance to choose the most appropriate medication. This is relevant, especially in patients who do not respond to treatment, are pharmacoresistant, or have an unidentified mitochondrial disease. Identifying potential modulators of treatment response (e.g., mitochondrial dysfunction) could help to optimize and personalize the pharmacological treatment of psychiatric diseases [60].

4. Materials and Methods

All materials and methods have been described in our previous article; only brief summary follows [59]. New protocol for ATP kinetics and content was established.

4.1. Media and Chemicals

The composition of the media used was the following: mitochondrial isolation medium: 0.32M sucrose and 4 mM HEPES (pH 7.4); respiratory medium (MiR05 without BSA):110 mM sucrose, 60 mM K^+-lactobionate, 20 mM taurine, 3 mM $MgCl_2 6H_2O$, 10 mM KH_2PO_4, 0.5 mM EGTA and 20 mM HEPES (7.1); Krebs-Henseleit (KH) buffer: 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 11.1 mM glucose. Chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The 5-Hydroxytryptamine [^{3}H] trifluoroacetate ([^{3}H]serotonin) and 2-phenylethylamine [ethyl-1-^{14}C] hydrochloride ([^{14}C]PEA) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). AGO, KET and VOR were dissolved in DMSO in corresponding concentrations.

4.2. Isolation of Pig Brain Mitochondria

Pig brains were obtained from a slaughterhouse. Mitochondrial fraction isolation and purification from brain cortex were performed as previously described [59]. Briefly, grey matter from pig brains was homogenized and crude mitochondrial fraction was isolated and then centrifuged on the sucrose gradient to obtain purified mitochondrial fraction. The freshly purified mitochondria were kept on ice until the assays were performed and were used for measurements of the mitochondrial oxygen consumption rate, measurements of ATP and ROS formation. Frozen mitochondria (stored at 70 C) were used for the following enzyme activity measurements: ETC complexes, citrate synthase (CS) and malate dehydrogenase (MDH) activity.

4.3. Activities of Mitochondrial Enzymes

Ultrasonication and incubation of mitochondria with the tested drugs for 30 min at 30 C was performed before measurement with a corresponding drug-free control (DMSO) for every measurement. Mitochondrial enzymes activities were determined spectrophotometrically as absorbance using a GENESYS 180 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

4.4. Citrate Synthase Activity

The reaction mixture consisted of Triton, Tris, 5,5^0-dithiobis-(2-nitrobenzoic) acid and acetyl coenzyme A. The reaction was started by adding oxaloacetate and the CS activity was measured by detecting the color change of 5,5^0-dithiobis-(2-nitrobenzoic) acid at 412 nm with a duration of 3 min. The final protein concentration was 20 g/mL and final drug concentration 100 M. Each measurement had a corresponding drug-free control (DMSO).

4.5. Malate Dehydrogenase Activity

The conversion of oxaloacetate to malate was used to measure the activity, and it was measured at 340 nm for 3 min. The reaction was initiated by adding oxaloacetate and nicotinamide adenine dinucleotide (NADH). The final protein concentration was 20 g/mL and final drug concentration 100 M. Each measurement had a corresponding drug-free control (DMSO).

4.6. Complex I (NADH Dehydrogenase) Activity

The reaction mixture consisted of KH_2PO_4, $MgCl_2$ and KCN and the reaction was started by adding decylubiquinone and NADH. The rotenone-sensitive NADH oxidation reaction was measured at 340 nm for 5 min. The final protein concentration was 150 g/mL and final drugs concentrations 2.5, 5, 10, 50 and 100 M. Drug-free control (DMSO) was used as a control for each measurement.

4.7. Complex II+III (Succinate Cytochrome c Oxidoreductase) Activity

The activity of complex II+III was measured with an antimycin A-sensitive cyt c reduction at 550 nm for 3 min, and it was initiated by the addition of cyt c. The medium consisted of KH_2PO_4, EDTA, KCN and rotenone. The final protein concentration was 50 g/mL and final drugs concentrations 2.5, 5, 10, 50 and 100 M. Drug-free control (DMSO) was used as a control for each measurement.

4.8. Complex IV (Cytochrome c Oxidase) Activity

The medium consisted of KH_2PO_4, and the reaction was initiated by reduced cyt c. The decrease in absorbance was measured at 550 nm for 3 min. The final protein concentration was 10 g/mL and final drugs concentrations 10, 50 and 100 M. Drug-free control (DMSO) was used as a control for each measurement.

4.9. ATP Content and Kinetics

The following protocol has been modified and optimized [61–64]. An ATP Bioluminescence Assay Kit CLS II was used to measure ATP content and kinetics. The luminescence was measured using FluoroMax-3 (Jobin Yvon, Edison, NJ, USA) at 562 nm. Standard curve for ATP determination was prepared from ATP standard in the range from 0 to 600 nM. Tested substances were used at final concentrations 10, 50 and 100 M, with drug-free control (DMSO) for each measurement and the final protein concentration was 50 g/mL.

4.10. Total Complex I- and Complex II+III-Linked ATP Content

Mitochondria were incubated for 30 min on ice with the MiR05 buffer, tested substances and mitochondria. Consequently, the substrate mix was added, consisting of 5 mM malate and 5 mM pyruvate (for complex I) or 5 mM succinate and 1 M rotenone (for complex II), 60 M ADP and 0.75 mM $MgCl_2 6H_2O$ and incubated for 30 min in 30 C. Reaction was stopped by heating vials at 100 C for 2 min. Background luminescence was measured. In total, 230 L of luciferase reagent was added and total ATP content for complex I and complex II+III was determined by measuring the luminescence for 1 min. Drug-free control (DMSO) was part of each measurement.

4.11. Complex I- and Complex II+III-Linked ATP Kinetics

For ATP kinetics determination MiR05 buffer, mitochondria and drugs were incubated on ice for 20 min and another 10 min at room temperature. Background luminescence was measured. Consequently, the same substrate mixture as mentioned above, and 230 L of luciferase reagent were added and ATP kinetics was determined by measuring the luminescence for 4 min. Each measurement had a corresponding drug-free control (DMSO). Previous study confirmed high correlation between the rate of polarographic- and bioluminescence-derived ATP production. Thus, further agreement between polarographic-based and luciferase-based measurements of ATP production was not verified in this study [63].

4.12. Hydrogen Peroxide Production

Hydrogen peroxide formation was determined using an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit. The reaction mixture consisted of 27 mM HEPES (pH 7.4), 114 mM sucrose, 100 mM KCl, 1.3 mM K_2HPO_4, 5 mM malate, 5 mM pyruvate, 1 M rotenone, 10 mM succinate, 0.75 mM $MgCl_2$, 50 mM ADP, 15 M Amplex Red and horseradish peroxidase 0.09 U/ML. Mitochondria were incubated with the tested substances for 30 min at 30 C at final concentrations 10, 50 and 100 M and each measurement had a corresponding drug-free (DMSO) control. Fluorescence of Amplex Red was observed at an excitation of 571 nm and an emission of 585 nm for 1 min. The reaction was stopped by the addition of antimycin A. The standard curve for hydrogen peroxide was prepared using H_2O_2 standard.

4.13. Mitochondrial Respiration

The mitochondrial oxygen consumption rate was measured by high-resolution respirometry using Oxygraph-2k (Oroboros Instruments Corp, Innsbruck, Austria). The final protein concentration was 0.05–0.14 mg/mL and the reaction mixture consisted of 2 mM malate, 5 mM pyruvate, 1.25 mM ADP and 0.75 M $MgCl_2$ for complex I-linked respiration; 1.25 mM ADP, 0.75 mM $MgCl_2$, 1 M rotenone and 10 mM succinate for complex II-linked respiration. The following four simultaneous measurements were assessed: a titration with the drug to the final drug concentrations of 0.125–100 M in one chamber and a titration with a drug-free control (DMSO) in the second oxygraph chamber.

4.14. Monoamine Oxidase Activity

Mitochondria in KH buffer at final concentration of 800 g/mL were preincubated with the tested drugs in a final concentration range of 0.1–300 M for 60 min at 37 C. For

each measurement, there was a corresponding drug-free control (DMSO). Radiolabeled substrates ([^{3}H]serotonin for MAO-A and [^{14}C]PEA for MAO-B) were added to initiate the reaction. The reaction was carried out at 37 C for 20 min for MAO-A and for 1 min for MAO-B, and the reaction was stopped by hydrochloric acid. The radioactivity of the organic phases of the extracts were measured by liquid scintillation counting (LS 6000IC, Beckman Instruments, Inc., Fullerton, CA, USA) [65].

4.15. Data Analysis and Statistics

The mitochondrial enzyme activities and ATP kinetics data were measured and calculated as the slope of the time dependence of absorbance or fluorescence. ATP and ROS contents were measured, and the means of the time-dependent fluorescence curves were calculated. The control sample activity was 100%, and the drug effect was expressed as a % of the control. ATP and H_2O_2 standard curves were constructed.

DatLab 7.4 software (Oroboros Instruments, Innsbruck, Austria) was used for high-resolution respirometry data collection and analysis it displays oxygen flux and the real-time oxygen concentration. The respiration rate was expressed as pmol O_2 consumed/second/mg of a protein.

The inhibition of respiration rate and MAO activity were analyzed by A four-parameter logistic regression with Prism software (GraphPad Software, San Diego, CA, USA) was used to analyze the respiratory rate inhibition and activity of MAO. These data were used to establish the half-maximal inhibitory concentration (IC_{50}), residual activity and the Hill slope. The IC_{50} represents the drug concentration required to inhibit the difference between the baseline and the residual value of the mitochondrial oxygen flux or MAO activity by 50%.

Data analysis was performed with STATISTICA 12 analysis software (TIBCO Software Inc., Palo Alto, CA, USA) using one-sample t test. Data are expressed as the mean standard deviation (SD) or the mean standard error of the mean (SEM). The correlation matrix and Pearson correlation coefficient were used to identify and display statistically significant correlations between individual parameters. Respiratory parameters, which were not affected by drugs, were excluded from the correlation analysis (for this reason all KET data were excluded).

5. Conclusions

Our in vitro study with currently used antidepressants revealed important and statistically significant drug-induced changes in OXPHOS. All three tested antidepressants decreased the activity of ETC complexes at higher concentrations. Reduced activity of ETC complexes should cause reduced ATP generation in the OXPHOS system. All three antidepressants were very potent inhibitors of complex IV activity, which is the rate-limiting complex for ATP generation. It can be speculated that the drug-induced maintenance or increase in ATP kinetics might be an adaptation mechanism to address insufficient respiration through complex I or by switching primarily to complex II-linked respiration, which remained preserved despite the drug-induced inhibition of complex II+III. Likely, long-term inhibition of OXPHOS could be linked to a neuronal ATP deficit, which could negatively contribute to neuronal damage at very high concentrations of the drug.

Considering the overall drug-induced changes in mitochondrial parameters in relation to adverse effects, long-term inhibition of complex IV likely compromises physiological processes and could be involved in adverse drug reactions. The most pronounced drug-induced mitochondrial dysfunction-related adverse effect could be linked to VOR as the most potent inhibitor of individual ETC complexes and complex I-linked respiration.

We also noticed an effect on ROS production. The VOR-induced decrease in H_2O_2 concentrations suggests the activation of antioxidant mechanisms. Drug-induced changes in H_2O_2 concentration affect redox balance and signaling cascades. Further research is needed to describe whether drugs increase oxidative damage (which is involved in undesirable effects) or promote the activation of antioxidant defense.

Monoamine oxidase inhibition likely plays a part in the desirable effects of these substances. All three tested antidepressants acted as partial MAO-A inhibitors, suggesting additional antidepressive effects in addition to their primary mechanism of action. VOR also partially inhibited MAO-B, which might be linked to its ability to decrease total H_2O_2 content, lower oxidative damage and improve cognitive deficits in MDD patients. In vitro studies have limitations and further cell culture, and in vivo research is needed to clarify the connections among the pathophysiology of MDD, mitochondrial activity and the mitochondrial effect of psychoactive drugs.

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Abbreviations

5-HT	serotonin
AGO	agomelatine
AMPA	-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
CS	citrate synthase
cyt c	cytochrome c
ETC	electron transport chain
FDA	Food and Drug Administration
H2O2	hydrogen peroxide
KET	ketamine
MDD	major depressive disorder
MDH	malate dehydrogenase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NADH	nicotinamide adenine dinucleotide
NMDA	N-methyl-d-aspartate
OXPHOS	oxidative phosphorylation
PFC	prefrontal cortex
ROS	reactive oxygen species
SSRIs	selective serotonin reuptake inhibitors
TrkB	tropomyosin receptor kinase B
VOR	vortioxetine
WHO	World Health Organization

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Attachment 3

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REVIEW

Novel approaches in schizophrenia-from risk factors and hypotheses to novel drug targets

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Abstract

Schizophrenia is a severe psychiatric disorder characterized by emotional, behavioral and cognitive disturbances, and the treatment of schizophrenia is often complicated by noncompliance and pharmacoresistance. The search for the pathophysiological mechanisms underlying schizophrenia has resulted in the proposal of several hypotheses to explain the impacts of environmental, genetic, neurodevelopmental, immune and inflammatory factors on disease onset and progression. This review discusses the newest insights into the pathophysiology of and risk factors for schizophrenia and notes novel approaches in antipsychotic treatment and potential diagnostic and theranostic biomarkers. The current hypotheses focusing on neuromediators (dopamine, glutamate, and serotonin), neuroinflammation, the cannabinoid hypothesis, the gut-brain axis model, and oxidative stress are summarized. Key genetic features, including small nucleotide polymorphisms, copy number variations, microdeletions, mutations and epigenetic changes, are highlighted. Current pharmacotherapy of schizophrenia relies mostly on dopaminergic and serotonergic antagonists/partial agonists, but new findings in the pathophysiology of schizophrenia have allowed the expansion of novel approaches in pharmacotherapy and the establishment of more reliable biomarkers. Substances with promising results in preclinical and clinical studies include lumateperone, pimavanserin, xanomeline, roluperidone, agonists of trace amine-associated receptor 1, inhibitors of glycine transporters, AMPA allosteric modulators, $mGLUR_{2-3}$ agonists, D-amino acid oxidase inhibitors and cannabidiol. The use of anti-inflammatory agents as an add-on therapy is mentioned.

Key Words: Schizophrenia; Immune system; Inflammation; Genetics; Novel antipsy-

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Core Tip: This review discusses the newest insights in the pathophysiology and risk factors for schizophrenia and points out the novel approaches of antipsychotic treatment, potential diagnostic and theranostic biomarkers. The hypotheses focusing on neuromediators (dopamine, glutamate, serotonin), neuroinflammation, cannabinoid hypothesis, gut brain axis model, and other currently discussed hypotheses are summarized. Key genetic features and new findings in the pathophysiology of schizophrenia support the expansion of novel approaches in pharmacotherapy and development of non-dopaminergic antipsychotics.

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INTRODUCTION

Schizophrenia is a serious mental disorder with a lifelong prevalence of approximately 1% and a peak age of onset of 23-34 years in women and the early twenties in men. It is a very complex syndrome that involves widespread brain multi-dysconnectivity. It is characterized by cognitive, behavioral and emotional dysfunctions. To fulfil the diagnostic criteria for schizophrenia, patients must exhibit two or more negative, disorganized or positive symptoms that persist for a minimum of six months, and at least one symptom must be disorganized speech or a positive symptom[1]. Positive symptoms include hallucinations and delusions; negative symptoms are characterized by deficits in normal behavior, including asociality, alogia, anhedonia, blunted affect, and avolition[2]. There is a wide range of treatment possibilities; however, the effectiveness and/or adverse effects of antipsychotics with different pharmacological profiles vary. Successful treatment of schizophrenia is complicated by noncompliance and pharmacoresistance. The prevalence of pharmacoresistant schizophrenia is estimated to range from 12.9% to 48%[3]. It has been estimated that approximately 20% of patients with schizophrenia receive combination treatment and/or antipsychotic polypharmacy[4]. Augmentation strategies used in clinical practice include the addition of another antipsychotic, concurrent administration of benzo-diazepines or mood stabilizers, repetitive transcranial magnetic stimulation or electroconvulsive therapy.

The pathophysiological mechanism of the onset and progression of schizophrenia, the diagnostic neuropathology, and sensitive and specific biomarkers have not yet been identified. Several different hypotheses have been proposed to explain the neuropathology of schizophrenia that focus on environmental, genetic, neurodevelopmental, and neurochemical effects. Research and development in imaging methods and in preclinical studies have led to the improvement of these theories. Positron emission tomography (PET) and single photon emission computer tomography enable *in vivo* quantification of dopaminergic functions in the brain and dopamine synthesis, release, and availability in postsynaptic dopaminergic neurons and transporters.

The targeting of existing and new drugs is based primarily on the dopamine and glutamate hypotheses of schizophrenia. All current antipsychotics modulate the function of the dopamine D_2 receptor. A nonlinear relationship between D_2 receptor occupancy, clinical response, and adverse effects of current antipsychotics was found. A small response to antipsychotic treatment appears at 50% dopamine receptor occupancy; as receptor occupancy increases, the response increases as well as the risk of extrapyramidal adverse effects[5]. These findings were proven in a double-blind study in patients with first episode schizophrenia; 65% occupancy of D_2 receptors was the borderline between responders and nonresponders[6]. Recently, research has focused on the prodromal phase of schizophrenia. Dopamine synthesis increases

during the acute phase of the disease. Stress and other risk factors affect the dopamine systems, leading to their dysregulation and consequently to the development of psychotic disorder[7].

Excitatory glutamate neurotransmission occurs though ionotropic and metabotropic glutamate receptors. The glutamate hypothesis of schizophrenia is based on the dysfunction of the N-methyl-D-aspartate (NMDA) receptor. Currently, the effects of ketamine on brain function in healthy volunteers are being examined; studies are focused on glutamate concentrations in the brains of patients with prodromal symptoms during the first episode and other episodes of schizophrenia. Dysfunction of both NMDA receptors and presynaptic synthesis of dopamine has been implicated in the clinical symptoms of schizophrenia. Relationships between presynaptic dopamine dysfunction and positive symptoms and between glutamate dysfunction and negative and cognitive symptoms are expected[7].

To improve the diagnosis of schizophrenia, predict the therapeutic response to antipsychotics, develop new drugs, and personalize treatment, it is necessary to identify new specific and sensitive biomarkers of the disease[8]. Blood-based biomarkers are regarded as a feasible option because the dysregulation of gene expression, epigenetic patterns, protein quantities, and metabolic and inflammatory molecules in peripheral blood have been shown to have distinct patterns in patients with schizophrenia[8]. The aim of this review is to provide the newest insights into the pathophysiology and risk factors of schizophrenia and novel approaches to antipsychotic treatment.

GENETICS AND SCHIZOPHRENIA

Schizophrenia is closely linked to genetic factors, including small nucleotide polymorphisms (SNPs), copy number variations and changes in gene expression. Combinations of different pathogenic mechanisms, including aberrant DNA methylation, altered histone code, dysregulated long noncoding RNA (lncRNA)-dependent tethering of epigenetic complexes to DNA, aberrant polyadenylation of pre-mRNAs, and mis-splicing, have been reported to play a role in schizophrenia development[9]. The hereditary burden of schizophrenia is estimated to be approximately 80%. Genome-wide association studies (GWAS) have identified more than 100 loci, many of which contain multiple genes that are significantly associated with schizophrenia. The assessment of polygenic scores allows us to determine the risk of schizophrenia based on the number of risk alleles weighted by the odds ratio of each allele.

DNA methylation, an epigenetic process that produces 5-methylcytosine, is mediated by DNA methyltransferases and has a key role in several processes, such as imprinting, inactivation of the X-chromosome, silencing of transposons or regulation of genomic stability and chromatin structure. Schizophrenia is linked to pathophysiological DNA methylation of several genes, including those encoding reelin, catechol-O-methyltransferase (COMT), monoamine oxidase A, serotonin receptor 2A, the transcription factor SOX-10, and others. Unfortunately, no schizophrenia-specific "methylation panel" has been proposed, and it has not yet been clarified whether these changes represent causes or consequences of schizophrenia development[9].

Approximately 70%-80% of the genome is transcribed into noncoding transcripts, and the majority of schizophrenia-associated risk variants have been found in noncoding regions. LncRNAs can interact with DNA, RNA, and proteins, influencing transcription and posttranscriptional processes such as splicing, polyadenylation and/or regulation of transcript stability. MicroRNAs (miRNAs) are small noncoding RNAs that regulate more than 50% of protein-coding genes by acting as promoter or enhancer elements; miRNAs might participate in histone, DNA, or chromatin methylation and modification. Both lncRNAs and miRNAs can be affected by different genetic variants, especially SNPs, which could increase the risk of schizophrenia onset [9,10].

Microdeletions in chromosomal region 22q11.2 are one of the well-established genetic risk factors for schizophrenia and increase the risk of schizophrenia development to 30%-40%[11,12]. COMT is a major dopamine catabolic enzyme, and its gene is located in this microdeletion region. In addition, a functional COMT polymorphism [valine/methionine (VAL/MET) substitution at codon 108] causes differences in its catabolic activity, dopamine baselines and stress-induced cortical dopamine release[13]. The MET version of the allele is not as stable as the VAL version, causing decreased COMT activity and an increase in dopamine levels,

especially in the prefrontal cortex[14].

The major histocompatibility complex (MHC) locus located on chromosome 6, which contains genes encoding proteins essential for adaptive immunity, has one of the strongest links to schizophrenia. Specifically, there was increased expression of complement component 4A (C4A). Sex differences in the *C4* gene could explain the higher male susceptibility to schizophrenia. Schizophrenia patients with higher C4 Levels were characterized as low responders or nonresponders to antipsychotic medication. The expression of the genes encoding CSMD1 and CSMD2, which are important regulators of C4, has been found to be decreased in schizophrenia and connected with reduced cognition and executive function[15,16]. Other immune receptors, including toll-like receptors (TLRs), which take part in microbe-derived molecular signaling, early brain development, synaptic plasticity, and neurogenesis, have been identified as schizophrenia susceptibility genes by GWAS. Both TLR2 and TLR4 were altered in the blood and brain tissue of schizophrenic patients[15].

The genes encoding for neuregulin 1 and neuregulin 3 are candidate schizophrenia genes and produce several possible proteins that influence neuronal differentiation and migration. The role of neuregulin 1 in schizophrenia is not well known, but increased neuregulin 1 signaling led to NMDA receptor hypofunction (in accordance with the glutamate hypofunction hypothesis of schizophrenia). There is no evidence of hyperexpression of neuregulin 1 itself; however, the possibility of mutations causing the production of proteins with enhanced function is still present[14]. Neuregulin 3 is a ligand for receptor tyrosine-protein kinase erbB-4 (ErbB4), and different genetic variants of the neuregulin 3 gene, especially the rs10748842 allele, are connected with higher schizophrenia risk and cognitive impairment[17]. Mutant mice with ErbB4 deletion from fast-spiking interneurons exhibited increased cortical excitability and oscillatory activity and desynchronized neurons in the cortical region, probably caused by the disruption of the proper function of inhibitory GABA circuits in interneurons. These functional changes manifested in increased locomotion, impaired social and emotional behavior and cognitive dysfunction, which are common symptoms of schizophrenia[18,19].

The gene encoding dystrobrevin-binding protein 1 (also referred to as dysbindin or DTNBP1) has been identified as a gene associated with schizophrenia; however, no specific protein coding mutations increasing the risk of schizophrenia have been identified. Decreased dysbindin expression has been found in the brains of schizophrenia patients, and dysbindin risk haplotypes have been associated with increased negative symptomatology in schizophrenia[14].

The gene most closely linked to schizophrenia is probably the gene encoding the protein disrupted in schizophrenia 1 (DISC1), which has been associated with schizophrenia mainly due to a mutation causing a translocation between exons 8 and 9. The molecular mechanism of this mutation is not known, but the shortened mutant DISC1 protein is incapable of dimerization, and it may interact with other proteins. DISC1 expression is especially high during neurodevelopment in the late fetal and early postnatal phases, during which it participates in hippocampal development; however, DISC1 expression continues into adulthood. In schizophrenia pathophysiology, not only DISC1 itself but also its binding and interaction partners, such as microtubule-associated protein 1A, glycogen synthase kinase 3β, phosphodiesterase 4 and fasciculation and elongation protein zeta-1, might play a crucial role[14,20-22].

The synaptosomal-associated protein SNAP25 is involved in synaptic vesicle docking and fusion during neurotransmitter release. The promoter variant rs6039769 with the C risk allele caused an increase in SNAP25 expression, probably causing a larger amygdala and greater functional connectivity between the amygdala and ventromedial prefrontal cortex in male schizophrenic patients. This modulation in the plasticity of the prefrontal cortex-limbic connection caused higher schizophrenia risk [23].

The gene encoding transcription factor 4 (TCF4) is another GWAS-confirmed gene associated with schizophrenia. It encodes class I basic helix-loop-helix transcription factors and plays a role in neurodevelopment. Altered expression of TCF4 in the forebrain of a transgenic mouse caused altered cognition and long-term depression increased the density of immature spines[24]. Many other genes have been associated with schizophrenia diagnosis and have been reported in the literature[25-27]; description of all schizophrenia-linked genes is beyond the scope of this review.

TRIGGERS AND RISK FACTORS

Environmental model of schizophrenia

The onset and severity of schizophrenia are always modulated by an interplay between genetic and environmental risk factors[28]. Many epidemiological studies have investigated putative environmental risk factors for schizophrenia and peripheral biomarkers of the disease[29,30]. According to an umbrella review of meta-analyses on risk factors and peripheral biomarkers for schizophrenia[31], history of obstetric complications, exposure to stressful events in adulthood or to childhood adversity, cannabis use, and serum folate level showed robust evidence of association with schizophrenia.

The prenatal and perinatal periods are characterized by great neural vulnerability to environmental insults. A recent systematic review and meta-analysis of 152 studies revealed numerous prenatal and perinatal risk factors, calculated with odds ratios (ORs), that were statistically linked to schizophrenia onset[32]. The biggest risk factors for schizophrenia onset are any familial psychopathology, especially maternal psychosis (OR: 7.61). Maternal infections (herpes simplex 2, OR: 1.35; unspecified infections, OR: 1.27), a suboptimal number of antenatal care visits (OR: 1.83), or maternal stress (OR: 2.4) can lead to a higher prevalence of obstetric events (OR: 1.52), which are the longest-studied and best replicated environmental risk factors for schizophrenia. Significantly relevant obstetric events include maternal hypertension (OR: 1.4), hypoxia (OR: 1.63), premature rupture of membranes (OR: 2.29) and polyhydramniosis (OR: 3.05). There is experimental and clinical evidence showing significant risks of prenatal infection and inflammation for the later development of schizophrenia. According to the viral model of schizophrenia, prenatal viral and bacterial infections and inflammation play an important role in the development of schizophrenia[33].

Nutritional deficits or famine in pregnancy (OR: 1.4) or more than two pregnancies (OR: 1.3) can be associated with reduced allocation or lower socioeconomic status. Another risk factor is congenital malformations (OR: 2.35)[32]. The most relevant postnatal environmental risk factors are childhood trauma (OR: 2.87), urban living (OR: 2.19), migration (2.10) and cannabis use (OR: 5.17), and these stress factors lead to the sensitization of the subcortical dopamine system[11].

Many genes relevant to schizophrenia, especially immune genes, can be altered by air pollution. Children with greater exposure to traffic-related air pollution had increased levels of proinflammatory cytokines. It is not yet clear whether air pollution itself causes brain changes or inflammatory changes caused by air pollution contribute to the pathology of schizophrenia[15].

A study of the roles of both genetic and environmental influences on the development of schizophrenia is necessary to explain the fact that in approximately 40%-55% of cases, monozygotic twins do not share a diagnosis of schizophrenia[34]. How genetic and environmental factors interact and the related neurobiological mechanisms that induce schizophrenia are not yet known.

Stress and schizophrenia

The vulnerability-stress model of schizophrenia proposes that when stress exceeds the vulnerability threshold, an individual is likely to develop a psychotic episode[35]. Stressful life events or psychological stress, especially in key periods of neurodevelopment, increase the risk of schizophrenia. These events include physical or mental abuse, lower socioeconomic status, urban environment, and neglect. The molecular mechanisms connecting these stressful situations with schizophrenia remain unclear. It was proven that patients with schizophrenia have altered cortisol function, and its release is linked to the inflammatory response rather than the anti-inflammatory response. Observation of HPA axis activation and cortisol release as a result of stress events in individuals with schizophrenia has produced inconsistent results; however, HPA axis dysfunction has been observed[15].

Neurons are extremely sensitive to redox imbalance during neurodevelopment and differentiation, mostly because of their high lipid content and metabolic rate. Increased reactive oxygen species (ROS) production and/or lowered antioxidant system capacity are considered risk factors for schizophrenia development. Increased protein and lipid oxidation and lowered levels of vitamin C and E, catalase, glutathione peroxidase and superoxide dismutase have been detected in schizophrenia patients. A study revealed that participants with low vitamin D3 Levels in the first year of life were at two times higher risk of schizophrenia. Glutamate-cysteine ligase is the rate-limiting biosynthetic enzyme of glutathione. One allelic variant of the GCLC gene is linked to the decreased

activity of glutamate-cysteine ligase and schizophrenia. NMDA receptors are regulated by the redox state, and glutathione deficiency induces NMDA receptor hypofunction, which leads to cortical oxidative stress and glutathione decrease[36,37].

Neurodevelopmental model

The neurodevelopmental model postulates that an increased risk of schizophrenia development is the result of abnormal brain neurodevelopment caused by genetic and environmental factors years before the onset of the disease[38]. The hypothesis is based on clinical, epidemiological, brain imaging, and genetic studies[39,40]. Schizophrenia is supposed to be a developmental disorder of the brain, and changes in brain neuroplasticity are involved. The disconnection hypothesis[41] presumes the involvement of abnormal synaptic connections in the pathophysiology of schizophrenia. Impaired synaptic plasticity and synaptic efficacy, mainly in areas of the brain responsible for learning, memory, and emotion, participate in schizophrenia pathophysiology. Modulation of ascending neurotransmitter systems and consolidation of synaptic connections during learning are implicated in schizophrenia neuropsychology, especially in impaired adaptive behavior and disintegrative aspects [42].

The unitary hypothesis of schizophrenia includes different types of pathophysiological models[43]; according to the hypothesis, early brain insults can lead to dysplasia of selective neural circuits, which is responsible for premorbid cognitive and psychosocial dysfunction in patients with schizophrenia. The onset of psychosis in adolescence may be associated with the excessive elimination of synapses with subsequent dopaminergic over activity. Decreased glutamatergic neurotransmission can predispose the brain to these processes. After the onset of the disease, these neurochemical changes can lead to further neurodegenerative processes. Brain plasticity includes both synaptic and nonsynaptic plasticity. The dysplastic model of schizophrenia suggests that impaired neuroplasticity during brain development may underlie cognitive and deficit symptoms and may lead to reorganization in other neuronal circuits, which may lead to affective and psychotic symptoms[44].

The multiple hit theory of schizophrenia[45] presumes that schizophrenia can be conceptualized as a process involving multiple vulnerability factors across numerous neurodevelopmental windows in which some hits are applied prenatally, in childhood, in adolescence, and in adulthood. Thus, the development of schizophrenia is driven by the interactions between genetic vulnerability and environmental influences (including prenatal vitamin D, nutrition, childhood trauma, viral infections, IQ, smoking, cannabis use, and social defeat), which are cumulative and interact with each other. The neurodevelopmental phase involves changes in synaptogenesis, synaptic enhancement, and myelination, leading to excessive elimination of synapses and loss of neuroplasticity.

An extension of the neurodevelopmental model[46] proposes that the abnormal formation and maturation of connectomes (an extensive network of interconnected neurons) is central to the etiology of the disease. That is, abnormal anatomical architecture and functional organization of the connectome may be a final common pathway leading to the manifestation of schizophrenia symptoms. To further refine the developmental hypothesis of schizophrenia, progress in our understanding of brain connectivity during development and dysconnectivity resulting from genetic and environmental factors is necessary.

Oxidative stress and apoptosis

Disconnection of the prefrontal cortex in schizophrenic patients is associated with abnormalities in white matter, oligodendrocytes, and myelin. Myelin is produced by mature oligodendrocytes, and oligodendrocyte precursor cells are extremely sensitive to oxidative stress. A redox-induced prefrontal oligodendrocyte precursor cell-dysfunctioning hypothesis of cognitive symptomatology in schizophrenia has been proposed[47]. According to this hypothesis, the combination of environmental factors and genetic predisposition causes oxidative stress due to the excessive generation of ROS and reactive nitrogen species in oligodendrocyte precursor cells. Oxidative stress can lead to the downregulation of myelin-related genes in oligodendrocytes, decreased expression of myelin basic protein, and a reduced number of oligodendrocytes in the rat brain. During adolescence, a high concentration of ROS impairs the proliferation and differentiation of oligodendrocytes and their precursors. This leads to their dysfunction and hypomyelination and consequently to the disruption of connectivity in the prefrontal cortex. The resulting cognitive symptoms coincide with the onset of schizophrenia.

Additionally, oxidative stress induces dysregulation of the immune system and favors a proinflammatory response. Inflammation and disruption of immunity are other factors contributing to the pathogenesis of schizophrenia, as described in the following sections.

Mitochondria play a major role in cellular bioenergetics, oxidative stress, and apoptosis. According to the mitochondrial hypothesis of schizophrenia, mitochondrial dysfunction leads to distorted neuronal activity and plasticity, causing imbalanced brain circuitry and finally abnormal behavior[48]. Massive loss of white matter oligodendrocytes is a hallmark of schizophrenia. Therefore, it has been hypothesized that mitophagy is increased in oligodendrocytes in schizophrenia, which contributes to disease-related white matter neuropathology.

The intrinsic pathway of apoptosis is activated by intracellular signals generated during cellular stress and is triggered by the release of proapoptotic factors from mitochondria. Thus, consistent with the mitochondrial hypothesis, the apoptotic hypothesis postulates that apoptosis contributes to the pathophysiology of schizophrenia. The data indicate a dysregulation of apoptosis in several cortical areas in schizophrenia. The potential involvement of nonlethal localized apoptosis in the early stages of the disease is presumed[49].

NEUROCHEMICAL HYPOTHESES

Dopamine hypotheses

According to the classic (receptor) dopamine hypothesis of schizophrenia, psychotic symptoms are related to dopaminergic hyperactivity in the brain. Hyperactivity of dopaminergic systems during schizophrenia is the result of increased sensitivity and density of dopamine 2 (D_2) receptors. This increased activity can be localized in specific brain regions[50,51]. The dopamine hypothesis does not assume that dopamine hyperactivity fully explains schizophrenia. Over activation of D_2 receptors appears to be only one effect of the overall dysregulation of chemical synapses in this disease.

The modified dopamine hypothesis assumes that schizophrenia is characterized by abnormally low prefrontal dopamine activity (causing negative symptoms) that leads to excessive dopamine activity in mesolimbic dopamine neurons (causing positive symptoms). Thus, this hypothesis presumes the co-occurrence of high and low dopamine activity in different neuronal circuits, which could explain the concurrent presence of positive and negative symptoms[52].

The unifying dopamine hypothesis of schizophrenia, called "the final common pathway", proposes that multiple environmental, genetic, and other risk factors (such as stress, drugs, or frontotemporal dysfunction) interact and result in striatal dopamine dysregulation, which alters signal transmission and leads to psychosis[53]. This hypothesis combines dopamine dysfunction with other risk factors, including pregnancy and obstetric complications, stress and trauma, drug abuse, genetic predisposition and environment–gene interactions, with both increased presynaptic striatal dopaminergic function and other brain functions that underlie negative and cognitive symptoms.

A model has been presented of how genes and environmental factors may sensitize the dopamine system so that it is vulnerable to acute stress, leading to progressive dysregulation and the onset of psychosis[13]. The main steps of this model are as follows: genetic risk factors lead to impaired glutamatergic regulation, followed by increased striatal dopamine release, aberrant salience, and psychotic symptoms. Acute psychosocial stress can activate increased striatal dopamine release both directly and indirectly *via* blunted cortical dopamine release and impaired glutamatergic regulation. The dopaminergic system interacts also with muscarinic cholinergic system and closely related muscarinic hypothesis of schizophrenia.

Glutamate hypotheses

The glutamate hypothesis assumes that schizophrenia is caused by developmental abnormalities in glutamate synapse formation at specific sites, particularly at GABA interneurons in the cerebral cortex. These abnormalities may lead to subsequent excessive glutamate signaling to the ventral tegmental area (VTA), and excessive activation of this pathway may result in an excess of dopamine in the ventral striatum *via* the mesolimbic pathway[54]. The role of dysregulation of glutamatergic neurotrans -mission in the pathophysiology of schizophrenia is supported by evidence from genetics, pharmacological, postmortem, and brain imaging studies[55]. The conver-

gence of GABA impairment and glutamate neurotransmission in the dorsolateral prefrontal cortex could explain the impairment of certain cognitive functions in schizophrenia[56].

The NMDA receptor hypofunction hypothesis[57] assumes that genetic and other risk factors induce epigenetic alterations leading to NMDA receptor hypofunction in schizophrenia. NMDA receptor hypofunction induces a cascade of downstream disturbances in neuronal activity, calcium entry, and epigenetic machinery, leading to abnormal synaptic development and dopaminergic and GABAergic dysfunction. These changes in neurotransmission result in the cognitive and social deficits found in schizophrenia. According to this hypothesis, changes in the dopamine system are secondary to NMDA receptor hypofunction.

Antagonists of NMDA receptors (*e.g.*, phencyclidine) have been shown to cause symptoms similar to the positive and negative symptoms and cognitive defects in schizophrenia[58]. According to increasing evidence, deficits in NMDA transmission are linked to cognitive defects and negative symptomatology[59].

Serotonin hypothesis

There are 3 interconnected pathways hypothetically associated with hallucinations and delusions: (1) Dopamine hyperactivity at D_2 dopamine receptors in the mesolimbic pathway, which extends from the VTA to the ventral striatum; (2) NMDA receptor hypoactivity on GABAergic interneurons in the prefrontal cortex; and (3) Serotonin (5-HT) hyperactivity of 5-HT_{2A} receptors on glutamate neurons in the cerebral cortex. All 3 pathways can lead to hyperactivity of the mesolimbic dopamine pathway[54].

According to the serotonin hypothesis[60], the basic cause of schizophrenia is stress-induced serotonergic hyperfunction in the cerebral cortex, especially in the anterior cingulate cortex and the dorsolateral frontal lobe. The serotonin hypothesis assumes hyperfunction of 5-HT_{2A} receptors on glutamate neurons in the cerebral cortex. This overactivation of 5-HT_{2A} receptors may be due to an excess of serotonin, upregulation of 5-HT_{2A} receptors, or the effects of 5-HT_{2A} receptor agonists. Subsequent release of glutamate in the VTA may activate the mesolimbic pathway, resulting in excess dopamine in the ventral striatum[54].

Cannabinoid hypothesis

According to the cannabinoid hypothesis[61-63], changes in the endocannabinoid system may contribute to the pathogenesis of schizophrenia. This hypothesis proposes that increased activation of the endocannabinoid system through CB_1 receptors on GABAergic interneurons in the ventral tegmental area, basolateral amygdala, and medial prefrontal cortex may lead to a hyperdopaminergic and hypoglutamatergic status, which may cause schizophrenia. The hypothesis was supported by evidence that cannabis use in adolescence is an independent risk factor for schizophrenia development (OR: 3.90)[31] and by the confirmation of interactions between the cannabinoid and dopamine systems that may be related to the processes associated with drug addiction or schizophrenia[64].

BLOOD BRAIN BARRIER

The pathophysiology of many central nervous system (CNS) disorders, including schizophrenia, includes altered function of the blood brain barrier (BBB), as shown by evidence from neuroimaging studies, research of both cerebrospinal fluid (CSF) and blood-based biomarkers, and postmortem studies[65]. It remains to be elucidated whether BBB dysfunction is the cause or consequence of schizophrenia pathology[65]. P-glycoprotein is highly expressed in capillary endothelial cells. P-glycoprotein limits the accumulation of psychotropic drugs in the brain and is responsible for the efflux of drugs from the CNS by using the energy from ATP hydrolysis to return the compound to the bloodstream.

According to increasing evidence, malfunction of the BBB and microvascular abnormalities contribute to the pathophysiology of schizophrenia[66]. In a postmortem study, the cellular expression of ABCB1 [the gene encoding P-glycoprotein 1 (P-gp); multidrug resistance protein 1] was examined in patients with schizophrenia. A reduced density of P-gp-expressing neurons was found in the medial habenula of patients with schizophrenia compared to that of controls[66]. Furthermore, polymorphisms of *ABCB1* have been associated with changes in drug disposition and pharmacotherapy response[67].

P-gp is not the only efflux protein; multiple drug resistance (MRP) and breast cancer resistance protein (BCRP) also facilitate the efflux of ATP-dependent substrates. In addition to P-gp, BCRP (ABCG2) and the multidrug resistance proteins MRP1 (ABCC1) and MRP2 (ABCC2) are ATP-dependent efflux transporters present in the BBB[68].

Claudin-5 is a component of tight junctions and is specifically expressed in endothelial cells in the CNS. Polymorphism of claudin-5 has been associated with schizophrenia risk[65], and serum claudin-5 Levels were decreased in patients with schizophrenia[69]. The expression of claudin-5 in the hippocampus was reduced in patients with schizophrenia; the levels of claudin-5 correlated with the duration and age of onset of the disease[70]. The BBB impedes the transfer of many drugs, including antipsychotics, as well as some inflammatory molecules, such as cytokines, which play an important role in the pathophysiology of schizophrenia (see below).

Other explanations of pharmacoresistance in schizophrenia involve abnormal structure of the BBB, downregulation of genes encoding ion transport proteins, impaired immune system, dysfunctional glutamatergic transmission, *etc.*

NEUROINFLAMMATION

Based on the observation that schizophrenia is often associated with chronic neuroinflammation in the CNS[71], the vulnerability-stress model has been expanded into the vulnerability-stress-inflammation model[72], which suggests that the symptoms of schizophrenia are associated with specific changes in dopaminergic, serotonergic, noradrenergic, and glutamatergic neurotransmission following neuroinflammation and microglial activation. The hypothesis is based on the following findings: (1) Stress can increase proinflammatory cytokines and may even contribute to a chronic proinflammatory condition; (2) The typical changes in neurotransmission observed in schizophrenia have also been found in low-level neuroinflammation; (3) Risk factors for schizophrenia include genes whose expression promotes inflammation, environmental stressors, alterations of the immune system, severe infections, and autoimmune disorders; and (4) Antipsychotics also provide anti-inflammatory and immunomodulatory effects.

The vulnerability-stress-inflammation model of schizophrenia suggests that genetic vulnerability and infection during pregnancy may induce a proinflammatory response in the mother, causing deleterious effects on the neurodevelopment of the fetus and increasing the risk of developing schizophrenia. The development of the glutamate system may be disrupted. Re-exposure to stress at a later age may be followed by increased cytokine release, astrocyte activation or loss, dopaminergic hyperactivity, and NMDA antagonism, leading to the positive, negative, and cognitive symptoms of schizophrenia. Immune conditioning and immune sensitization can elicit a repeated response to stress leading to the symptoms of the disease.

Immunologic processes in schizophrenia

Currently, the immune system, immunological processes and inflammation are believed to have a significant role in the neurobiology of schizophrenia[73]. Evidence of immune etiology in schizophrenia comes from GWAS, where a significant association between schizophrenia and the expression of MHC, located on chromosome 6, was observed[16,74].

The relationship between neurotransmitters and mediators of the inflammatory response can be reciprocal; an immunoregulatory function of dopamine has been described. Increased expression of dopamine D_3 receptors and increased synthesis of interferon gamma (IFNγ) in lymphocytes were observed in nonmedicated patients suffering from schizophrenia[75]. An important finding from PET studies of inflammation with elevation of proinflammatory cytokines produced by microglia was an elevated microglial activity in subjects with subclinical symptoms and patients with schizophrenia[64].

Numerous studies have found immune dysregulation in patients with schizophrenia compared to healthy controls, and several meta-analyses have concluded that patients with schizophrenia exhibit signs of low-grade peripheral inflammation characterized by upregulated proinflammatory cytokines and acute phase proteins[76-78]. A recent meta-analysis of postmortem brain studies evaluating histological alterations of cellular composition and those assessing molecular parameters strengthened the immunologic hypothesis of schizophrenia[79]. The authors found significant increases in the density of microglia (especially in the temporal cortex) and

the overall expression of pro-inflammatory genes but no difference in the expression of anti-inflammatory genes in patients with schizophrenia compared to those in controls. However, it is important to note that these immunological alterations have been found only in a subgroup of patients with schizophrenia: approximately 40% of studied patients have exhibited some level of inflammation[80,81]. As schizophrenia is seen as a syndrome consisting of several disease phenotypes with different underlying pathologies, it is crucial to define robust immune biomarkers that would help in the identification of patient groups that might benefit from anti-inflammatory therapy[76,82]. Cytokines represent a broad category of signaling molecules produced by a wide range of cells, including immune cells such as B and T lymphocytes, macrophages, and mastocytes, as well as endothelial cells and fibroblasts. A meta-analysis of 18 studies found alterations in both proinflammatory and anti-inflammatory cytokines, and these disturbances were stage dependent[83]. In patients with first-episode psychosis, elevated levels of proinflammatory cytokines were found, whereas the level of interleukin (IL)-4 was significantly reduced. In acutely ill patients, increased pro-inflammatory cytokines were observed, and lower levels of IL-4 and IL-10 Levels were found than in controls. In chronically ill patients, augmented levels of IL-1β, sIL-2R, IL-6, and tumor necrosis factor alpha (TNF-α) and reduced IFNγ levels were observed compared to those in controls. Details are summarized in Table 1.

Moreover, a study evaluating the gene expression of cytokines in peripheral blood mononuclear cells reported increased mRNA levels of IL-6, IL-8 and TNF-α and decreased anti-inflammatory IL-2 mRNA[81]. Alterations in these cytokines were also found in CFS[84], and a meta-analysis of 16 studies found significantly higher CSF levels of IL-1β, IL-6 and IL-8 in patients with schizophrenia compared to healthy controls. Interleukins (*e.g.*, IL-1β and IL-6) play important roles in neurotransmitter systems in schizophrenia. A relationship exists between increased concentrations of IL-6 in childhood and a higher risk of subclinical psychotic symptoms in young adulthood. Increased concentrations of IL-6 and other proinflammatory cytokines, such as TNF-α, IL-1β, and IFNγ, are normalized in episodes of remission after antipsychotic treatment. Some studies have suggested an association among increased serum concentrations of cytokines, including IL-6, severity of the disease, and duration and antipsychotic therapy[74].

Alterations in the immune system influence the neurotransmission of dopamine, 5-HT, norepinephrine, and glutamate. The immune system can activate indoleamine 2,3-dioxygenase, an enzyme involved in tryptophan/kynurenine metabolism[73]. Kynurenic acid acts as a naturally occurring NMDA antagonist in the human brain. Increased levels of kynurenic acid were found in the CSF of patients with schizophrenia[85,86]; however, no changes in kynurenic acid levels were observed in the peripheral blood of patients with schizophrenia[73]. Proinflammatory cytokines increase the concentration of kynurenic acid. Approximately 10% of nonmedicated patients in acute episodes of schizophrenia produce NMDA receptor antibodies, and this finding supports the hypothesis of NMDA receptor antagonism in schizophrenia [72,73].

C-reactive protein (CRP) appears to be the most promising theranostic marker for inflammation, and patients with increased CRP might benefit from anti-inflammatory therapy[76,87]. CRP is synthesized in the liver in response to IL-1β, IL-6 and TNF-α and is released from macrophages and adipocytes[76]. CRP has been reported to correlate both with positive and negative symptoms of schizophrenia[88] and with cognitive dysfunction[89]. Recently, “ultraresistance” to treatment in schizophrenia (defined as current clozapine treatment and a mean positive and negative syndrome scale (PANSS) score ≥ 70) was found to be associated with abnormal CRP levels (> 3 g/L), providing further justification for treating ultra-resistant patients with anti-inflammatory agents[90]. Additionally, other acute phase proteins, including haptoglobin, alpha-1 antitrypsin, and alpha-2-macroglobulin, were also found to be elevated in a subgroup of individuals with schizophrenia and other psychoses[91,92].

Additional biomolecules and metabolites essential for inflammation and endothelial cell function (*e.g.*, creatine kinase m/B, angiotensin-converting enzyme, matrix metalloproteinase, thyroid-stimulating hormone, thyroxine-binding globulin, intercellular adhesion molecule 1, cortisol, α-2-macroglobulin, and thrombopoietin) have been found in lower concentrations in drug-naïve patients than in controls[93]. The authors commented that most of these proteins are involved. Additionally, upregulation of leucocyte adhesion molecules was described in psychotic disorders, increased levels of soluble L-selectin were detected in the serum of drug-naïve patients with schizophrenia, and the serum levels of L-selectin and P-selectin in patients with schizophrenia did not differ from those in healthy controls[94]. In another study, P-selectin plasma levels were found to be increased in patients with acute psychosis[95].

Table 1 Possible diagnostic/theranostic immunologic biomarkers in schizophrenia

Parameter	Serum/plasma/peripheral blood	CSF
Pro-inflammatory cytokines	↑ IL-6, IFN-γ, IL-1RA, IL-1β, IL-6, IL-8, IL-12, sIL-2R, TGF-β, and TNF-α	↑ IL-1β, IL-6 and IL-8
Anti-inflammatory cytokines	↓ IL-10 and IL-4	
Acute phase proteins	↑ CRP, haptoglobin, α-1 antitrypsin, and α-2 macroglobulin	
Antibodies	↑ Anti-cardiolipin IgG and anti-NMDA receptor titers	
Immune cells	↑ CD4+, CD3+ and CD56+	
Other biomolecules/metabolites	↓ Creatine kinase m/B, MMP3, ACE, cortisol, TBG, α-2 macroblobulin, thrombopoietin, TSH, and ICAM-1, P-selectin	

ACE: Angiotensin-converting enzyme; CRP: C-reactive protein; CSF: Cerebro-spinal fluid; ICAM: Intercellular adhesion molecule; IFNγ: Interferon gamma; IL: Interleukin; MMP3: Matrix metalloproteinase 3; NMDA: N-methyl-D-aspartate; TBG: Thyroxine-binding globulin; TGF-β: Transforming growth factor-beta; TNF-α: Tumor necrosis factor-alpha; TSH: Thyroid-stimulating hormone.

Certain studies have also found changes in the number of immune cells in patients with schizophrenia compared to healthy controls. A meta-analysis of 16 studies evaluating blood lymphocyte counts found a significant increase in the percentages of CD4+ (T-helper lymphocytes) and CD56+ (natural killer cells) lymphocytes in acutely relapsed inpatients and a significant increase in the absolute numbers of total lymphocytes and CD3+ (T-lymphocytes) and CD4+ cells[96]. However, a significant decrease in the percentage of CD3+ cells was found in drug-naïve patients in the first episode of schizophrenia. Additionally, some autoimmune responses were also found in schizophrenic patients, but their clinical relevance remains elusive. Regarding the pathogenesis of schizophrenia, different autoantibodies, as well as antibodies against diet antigens, *e.g.*, gliadin and casein, were investigated in different parts of the brain, serum, and CSF. A systematic quantitative review of 81 studies found significantly increased anti-cardiolipin IgG and anti-NMDA receptor autoantibody titers in patients in the first episode of schizophrenia[97]. The authors also reported increased titers of anti-cardiolipin IgG and IgM and nerve growth factor in patients with schizophrenia compared with controls.

As mentioned, there is a strong link between oxidative stress and the immune system; therefore, by counteracting oxidative stress, antioxidants reduce inflammation and the overactive immune response. Glutathione is an antioxidant that is essential in the myelinization and maturation of white matter and can be supplemented as the amino acid precursor N-acetyl cysteine. N-acetyl cysteine possesses antioxidant properties and mild anti-inflammatory effects and regulates synaptic NMDA receptors. NAC supplementation for 6 mo ameliorated positive symptoms and improved neurocognition (processing speed) in patients with schizophrenia with high peripheral oxidative stress[98]. Omega-3-type polyunsaturated fatty acids have also exerted antioxidative capacity and anti-inflammatory effects[99].

Non-steroidal anti-inflammatory drugs in pharmacotherapy

Anti-inflammatory agents have shown some benefits as adds-ons to antipsychotic treatment in schizophrenia, as reported by two recent meta-analyses[100,101]. A meta-analysis of 62 double-blind randomized clinical trials studying aspirin, celecoxib, omega-3 fatty acids, estrogens, pregnenolone, minocycline, N-acetyl cysteine, and erythropoietin in 2914 patients found an overall significant effect of a decrease in the PANSS score[100]. Additionally, cognitive improvement was significantly associated with minocycline and pregnenolone therapy. Another meta-analysis of 70 randomized clinical trials including 4104 subjects investigated either primarily non-steroidal anti-inflammatory drugs (NSAIDs), minocycline and monoclonal antibodies, or drugs with potential anti-inflammatory properties (N-acetyl cysteine, melatonin, neurosteroids, estrogens, fatty acids, statins, and glitazones) as adjunctive therapies to antipsychotics. The analysis also found a decrease in the PANSS score[101]. Small but significant effect sizes were observed on both negative and positive symptoms, general psychopathology and working memory. Interestingly, primarily anti-inflammatory drugs were not found to be superior to potential anti-inflammatory drugs. However, the authors highlighted that the reported effects might be overestimated due to the many small study samples included in the analysis.

In conclusion, changes in the frequencies of immune cells, the levels and expression of cytokines, and the levels of acute phase proteins in the blood and CSF were observed in patients with schizophrenia compared to healthy controls, and CRP seems to be a promising theranostic biomarker for schizophrenia. Moreover, larger studies with longer treatment durations and the inclusion of only schizophrenic patients with proven inflammation (CRP levels > 3 g/L) are warranted to elucidate the efficacy of anti-inflammatory treatment in schizophrenia.

The challenges of drug development include the development of novel molecules affecting the immune system and of immunotherapy using autoantibodies as well as the stratification of patients with schizophrenia according to their immune phenotypes to enable the selection of effective pharmacotherapeutic agents[74].

MICROBIOME-BRAIN AXIS MODEL

The microbiome-gut-brain axis model postulates that bidirectional communication between the central and enteric nervous systems ensures the connection of the brain with peripheral intestinal functions[102,103]. It is presumed that the gut microbiome can program brain function during early development; in other words, active signals from the microbiome play a critical role in brain development. It is thought that the microbiome may affect brain development through epigenetic mechanisms[104]. Signal pathways from the gut microbiome to the brain include: (1) The direct activation of the vagus nerve; (2) The production or induction of various metabolites that may cross the BBB to regulate neurological functions; and (3) An immune system whose cytokines affect neurophysiology[105].

Probiotics modulate the immune responses of the host and could be beneficial for schizophrenia patients[106]. The immunomodulatory effects of probiotic supplementation were examined after 14 wk in chronic patients with schizophrenia, and *Lactobacillus* and *Bifidobacterium* were administered as adjuvant treatments. Increased levels of brain-derived neurotrophic factor, chemokine ligand 5 (RANTES), monocyte chemotactic protein and macrophage inflammatory protein-1 beta were found[106]. Gut-brain communication suggests the direct secretion of some neuroactive substances, and some intestinal bacteria can produce mediators, *e.g.*, GABA, acetylcholine.

INNOVATIVE DRUG APPROACHES AND TARGETS

All antipsychotics for schizophrenia treatment are based on dopamine agonism/antagonism, and no nondopaminergic antipsychotics have yet been developed or approved (Figure 1). Lumateperone is characterized as a partial D_2 receptor agonist/antagonist and a 5-HT_{2A} antagonist, and it is a serotonin reuptake inhibitor that indirectly modulates glutamatergic transmission (effect on NMDA receptor subtype 2B, NR2B)[107,108]. It has no metabolic or extrapyramidal adverse effects and positively affects cognition[109]. Another study found that lumateperone was well tolerated with minimal extrapyramidal and cardiometabolic adverse effects and maintained or reduced the symptoms of schizophrenia after a switch from a previous antipsychotic[110].

A promising therapeutic drug strategy for schizophrenia is the use of antipsychotics without D_2 receptor binding[111]. Agonists of trace amine-associated receptor 1 (TAAR1) seem to selectively affect dopamine and may represent a new class of psychotropic drugs[112,113]. A novel compound, SEP-363856, was described as a TAAR1 and 5-TH_{1A} agonist and was tested in a pilot trial with 120 patients with an acute exacerbation of schizophrenia. The reduction in the PANSS score of the treatment group was significant compared with that of the placebo group; longer and larger trials are necessary to prove the efficacy and safety of this TAAR1 agonist[113]. Other TAAR1 agonists, *e.g.*, RO5263397, have been developed and are being tested for use in schizophrenia. RO5263397 was found to be safe, but a great variety in metabolism and plasma levels was found that depended on ethnicity and genotype [112,114].

Novel drugs indirectly targeting glutamate neurotransmission are among the strategies being pursued for antipsychotic development. Inhibitors of glycine transporters (*e.g.*, sarcosine and bitopertin), coadministration of NMDA agonists, allosteric modulators of AMPA receptors (ampakines), and allosteric modulators of $mGluR_5$ are being tested; novel drugs affecting the glutamate ionotropic and meta-

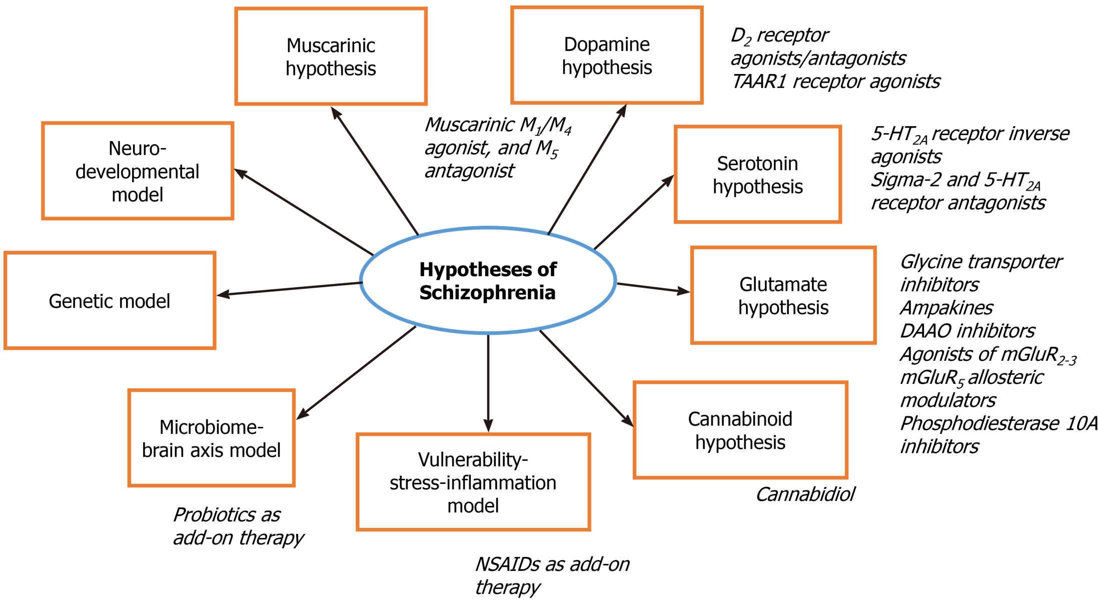

Figure 1 Illustration of current hypotheses and novel approaches in treatment of schizophrenia. DAAO: D-Amino acid oxidase; TAAR1: Trace amine-associated receptor 1; NSAIDs: Non-steroidal anti-inflammatory drugs.

botropic receptors $mGluR_{2-3}$ have been developed[115].

Agonists of $mGluR_{2-3}$ have anxiolytic properties and reverse the effects of stress; agonists of the $mGluR_3$ receptor antagonize the effect of phencyclidine and amphetamine. Novel drugs directly affecting receptors were found to be ineffective and/or to have adverse effects. Pomaglumetad methionil is an $mGluR_{2-3}$ agonist[58]. It was tested as an adjunctive therapy for patients with negative symptoms of schizophrenia, and the study did not find a difference between the pomaglumetad group and the placebo group[116]. Post hoc analyses suggested the efficacy of pomaglumetad in patients suffering from schizophrenia for less than or equal to 3 years or in patients previously treated with antipsychotics predominantly acting as D_2 antagonists [117,118]. Thus, the potential of novel $mGluR_{2-3}$ agonists is suggested for treating psychosis and aggression/agitation associated with neurodegenerative diseases (Parkinson’s disease, Alzheimer’s disease, dementia with Lewy bodies, *etc.*)[117].

D-Amino acid oxidase (DAAO) inhibitors were found to modulate NMDA transmission. Sodium benzoate, a DAAO inhibitor, has been tested as an add-on therapy and improved symptoms in clozapine-resistant patients[119]. In another study, sodium benzoate was administered to patients with schizophrenia as an add-on therapy compared to placebo. The adjunctive therapy was well tolerated, and chronic schizophrenia patients showed improved function, especially neurocognition[120].

Cannabidiol seems to be a promising candidate for the treatment of schizophrenia. Increased function of the endocannabinoid system was observed in schizophrenic patients; cannabidiol was found to decrease mesolimbic dopaminergic activity. There is evidence that chronic and acute administration of cannabidiol led to improvement of schizophrenia symptomatology[121].

Based on the serotonin hypothesis, pimavanserin was developed and characterized as an inverse agonist at 5-HT_{2A} receptors, and it has binding affinity for sigma-1 receptors. This antipsychotic has been FDA-approved for the treatment of psychosis associated with Parkinson’s disease. Further randomized controlled trials are needed to consider pimavanserin as a drug for schizophrenia treatment[122].

Antagonism at sigma-2 receptors was described for roluperidone, which was developed as a novel antipsychotic affecting 5-HT_{2A} receptors and sigma-2 receptors [58]. Roluperidone has been found to be effective in the treatment of negative symptoms[123].

Xanomeline is a muscarinic M_1/M_4 agonist and M_5 antagonist that was originally developed for Alzheimer´s disease. Currently, it is being tested in combination with trospium, which reduces peripheral adverse effects (nausea and vomiting), as a new antipsychotic with a novel mechanism of action.

Phosphodiesterase inhibitors have been investigated as drugs to enhance cognition in schizophrenia[124]. Phosphodiesterase 10A inhibitors likely modulate D_1 (directly) and D_2 (indirectly) striatal pathways and regulate glutamate receptors[112]. TAK-063, a

phosphodiesterase 10A inhibitor, was tested, but the clinical trial did not meet the primary endpoint, and extrapyramidal syndromes occurred more often in the TAK-063 group than in the placebo group[125].

CONCLUSION

Genetic predisposition and neurodevelopmental and environmental risk factors for schizophrenia were summarized. Nevertheless, the understanding of schizophrenia pathophysiology is limited, and current pharmacotherapy is complicated by adverse effects, pharmacoresistance, and low compliance of patients. Novel targets and approaches of antipsychotic treatment are being developed with the aim of covering the wide range of schizophrenia symptoms, especially negative symptomatology, cognitive impairment, and residual and treatment-resistant symptoms. Novel drug targets based on current schizophrenia hypotheses include molecules indirectly targeting glutamate neurotransmission, DAAO inhibitors, 5-HT_{2A} receptor inverse agonists, sigma-2 receptor antagonists, phosphodiesterase inhibitors, *etc.* In pharmacoresistant patients, possible comorbidities may be related to inflammation or a disrupted microbiome-gut-brain axis, and augmentation of antipsychotic treatment *via* NSAID or probiotic administration can be considered. Further research on schizophrenia pathophysiology, genetic predisposition (based on GWAS), regulatory mechanisms in impaired mediator transmission and other factors is needed to improve the clinical outcomes of pharmacotherapy.

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Attachment 4

Ľupták M, Fišar Z, Hroudová J. Effect of Novel Antipsychotics on Energy Metabolism - In Vitro Study in Pig Brain Mitochondria. Mol Neurobiol. 2021; 58(11):5548-5563. doi: 10.1007/s12035-021-02498-4.

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Effect of Novel Antipsychotics on Energy Metabolism — In Vitro Study in Pig Brain Mitochondria

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Abstract

The identification and quantification of mitochondrial effects of novel antipsychotics (brexpiprazole, cariprazine, loxapine, and lurasidone) were studied in vitro in pig brain mitochondria. Selected parameters of mitochondrial metabolism, electron transport chain (ETC) complexes, citrate synthase (CS), malate dehydrogenase (MDH), monoamine oxidase (MAO), mitochondrial respiration, and total ATP and reactive oxygen species (ROS) production were evaluated and associated with possible adverse effects of drugs. All tested antipsychotics decreased the ETC activities (except for complex IV, which increased in activity after brexpiprazole and loxapine addition). Both complex I- and complex II-linked respiration were dose-dependently inhibited, and significant correlations were found between complex I-linked respiration and both complex I activity (positive correlation) and complex IV activity (negative correlation). All drugs significantly decreased mitochondrial ATP production at higher concentrations. Hydrogen peroxide production was significantly increased at 10 μM brexpiprazole and lurasidone and at 100 μM cariprazine and loxapine. All antipsychotics acted as partial inhibitors of MAO-A, brexpiprazole and loxapine partially inhibited MAO-B. Based on our results, novel antipsychotics probably lacked oxygen uncoupling properties. The mitochondrial effects of novel antipsychotics might contribute on their adverse effects, which are mostly related to decreased ATP production and increased ROS production, while MAO-A inhibition might contribute to their antidepressant effect, and brexpiprazole- and loxapine-induced MAO-B inhibition might likely promote neuroplasticity and neuroprotection. The assessment of drug-induced mitochondrial dysfunctions is important in development of new drugs as well as in the understanding of molecular mechanism of adverse or side drug effects.

Keywords Oxidative phosphorylation · Mitochondrial respiration · Reactive oxygen species · ATP · Monoamine oxidase · Dopamine system stabilizers

Abbreviations

BREX	Brexpiprazole
CAR	Cariprazine
CS	Citrate synthase
cyt *c*	Cytochrome *c*
ETC	Electron transport chain
LOX	Loxapine
LUR	Lurasidone
MDH	Malate dehydrogenase
MAO-A	Monoamine oxidase A
MAO-B	Monoamine oxidase B
NAD^+/NADH	Nicotinamide adenine dinucleotide
OXPHOS	Oxidative phosphorylation
ROS	Reactive oxygen species

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Introduction

It has been suggested that impaired mitochondrial functions are related to the pathophysiology of a range of neuropsychiatric and neurodegenerative disorders, including schizophrenia, depression, bipolar affective disorder, and anxiety. In addition to their crucial role in energy production, mitochondria participate in cellular signaling, regulation of calcium buffering, and take part in the intrinsic pathway of apoptosis. Mitochondrial dysfunction includes impaired oxidative phosphorylation (OXPHOS), which is

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due to the altered activity of mitochondrial enzymes (e.g., citrate synthase (CS) and electron transport chain (ETC) complexes), decreased mitochondrial respiration, disrupted calcium homeostasis, and increased production of reactive oxygen species (ROS), and these dysfunctions consequently lead to cellular damage and apoptosis. These processes also participate in immunological and inflammatory responses which might lead to neurodevelopmental disruption [1–3].

Postmortem studies, brain imaging, and positron emission tomography scans of patients with schizophrenia revealed alterations in brain energy, including the following: decreased activity of complex I + III in the basal ganglia and temporal cortex, increased activity of complex II and complex IV in the putamen and nucleus accumbens, and decreased activity of complex IV in the caudate nucleus and frontal cortex [4–7]. Hyperplasia and decrease in mitochondrial volume or number were also observed in the brain mitochondria of patients with schizophrenia [8].

Almost no data are available to examine the effects of novel antipsychotics on mitochondrial functions. In a study on pig brain mitochondria, the inhibition of complex I was observed after incubation with both the typical and atypical antipsychotics [9].

As a third-generation of antipsychotics, dopamine system stabilizers, use partial agonism or antagonism of dopamine $D_{2/3}$ receptors to reduce psychosis without extrapyramidal adverse effects [10]. In addition to aripiprazole, brexpiprazole (BREX) and cariprazine (CAR) have been registered as novel antipsychotics.

BREX acts as a partial agonist at serotonin 5-HT_{1A} and $D_{2/3}$ receptors and an antagonist at $5\text{-HT}_{2A/2B/7}$ and adrenergic α_1 receptors. Compared to aripiprazole, BREX has lower intrinsic activity at D_2 receptors and is supposed to cause less akathisia [11]. The most common side effect is weight gain; the akathisia, sleep disturbance, and sedation side effects are comparable to placebo.

CAR is a third-generation antipsychotic similar to the BREX agonist at 5-HT_{1A} and $D_{2/3}$ receptors and the antagonist at serotonin $5\text{-HT}_{2B/7}$ receptors. Compared to aripiprazole, CAR has 3- to tenfold greater activity on D_3 receptors. It was suggested that its electivity to D_3 receptors contributes to its pro-cognitive effects [12]. The most common side effects are extrapyramidal symptoms and akathisia [13].

Loxapine (LOX) is a first-generation antipsychotic that acts as an antagonist at $D_{1\text{-}4}$ and 5-HT_{2A} receptors with a higher affinity to the 5-HT_{2A} than D_2 receptors. This pharmacodynamic profile is closer to that of atypical antipsychotics. LOX is classified as first-generation probably because of its metabolite 7-hydroxyloxapine has similar D_2 blocking properties to those of haloperidol [14]. Similar to clozapine, LOX binds D_4 receptors and has a higher affinity for D_3 than D_2 receptors [15]. Antagonism at noradrenergic, histaminergic H_1, and cholinergic M_1 receptors is responsible for the most frequent adverse effects of LOX, sedation, somnolence, and xerostomia [14, 16].

Lurasidone (LUR) is a second-generation antipsychotic that acts as a full antagonist at 5-HT_{2A} and D_2 receptors [17] and 5-HT_7 as antagonist and 5-HT_{1A} agonist, which could lead to cognitive improvement and antidepressant effects; it also acts as a 5-HT_{2A} antagonist. The most frequent side effects are somnolence, akathisia, nausea, parkinsonism, and insomnia [13].

The chemical structures of novel antipsychotics are depicted in Figure S1 (in supplementary material).

Currently, there are no valid data about the effect of BREX, CAR, LOX, or LUR on the selected mitochondrial parameters and cell energy metabolism. We hypothesize that antipsychotics are able to modulate cellular energy metabolism through their influence on the mitochondrial enzyme activity, total respiration, and ROS and ATP production of the cell. The mitochondrial effects of novel antipsychotics may contribute to both their therapeutic (probably related to the promotion of positive neuroplasticity and neuroprotectivity) and undesirable effects (probably related to decreased ATP production and increased oxidative stress). To verify this hypothesis, we primarily focused on identifying and quantifying the mitochondrial effects of the novel antipsychotics BREX, CAR, LOX, and LUR in terms on the possible associations of these effects with drugs adverse effects. The effects of tested antipsychotics on the activity of mitochondrial monoamine oxidases, as important modulators of monoamine brain activity, are also discussed in terms of possible drugs side effects related to the known antidepressant effects of MAO-A inhibitors and neuroprotective effects of MAO-B inhibitors. In in vitro experiments, we determined the effect of these psychopharmaca on the activity of mitochondrial ETC complexes, the activity of CS and malate dehydrogenase (MDH), MAO activity, mitochondrial respiration rate, and total ATP and ROS production, and the positive or negative correlations between the tested parameters were analyzed.

Materials and Methods

Media and Chemicals

Mitochondrial isolation medium consisted of 0.32 M sucrose and 4 mM HEPES and was buffered to pH 7.4. Respiratory medium (MiR05) for high-resolution respirometry and ATP production consisted of 110 mM sucrose, 60 mM K^+-lactobionate, 20 mM taurine, 3 mM $MgCl{\cdot}6H_2O$, 10 mM KH_2PO_4, 0.5 mM EGTA, and 20 mM HEPES, adjusted to pH 7.1 (with KOH). Krebs–Henseleit (KH) buffer (pH 7.4) consisted of 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 25 mM NaHCO3, and 11.1 mM glucose.

5-Hydroxytryptamine [^{3}H] trifluoroacetate ([^{3}H]serotonin) and 2-phenylethylamine [ethyl-1-^{14}C] hydrochloride ([^{14}C] PEA) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA), and the other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Isolation of Pig Brain Mitochondria

Pig brains were obtained from a slaughterhouse and mitochondria were isolated from the brain cortex, as described previously [18]. The purified mitochondrial fraction was prepared by centrifugation with a sucrose gradient; the Lowry method was used to determine the protein concentration [19]. Freshly prepared mitochondria were kept on ice and used for high-resolution respirometry and measurements of ATP and ROS formation. The activity of ETC complexes, CS, and MDH was assayed with frozen mitochondria stored at − 70 °C.

Activity of Mitochondrial Enzymes

Mitochondria were resuspended in hypotonic buffer (25 mM KH_2PO_4, 5 mM $MgCl_2$, adjusted to pH 7.2) and ultrasonicated three times to achieve the highest enzymatic activity. Mitochondria were incubated with the tested substances at final concentrations of 10, 50, and 100 µM for 30 min at 30 °C, and each measurement had a corresponding control. The activity of mitochondrial enzymes was determined spectrophotometrically as absorbance using a GENESYS 180 UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Citrate Synthase and Malate Dehydrogenase Activity

CS

The reaction mixture consisted of Triton, 0.1%; Tris, 0.1 M; 5,5′-dithiobis-(2-nitrobenzoic) acid, 0.2 mM; and acetyl coenzyme A, 0.1 mM, adjusted to pH 7.4, and the reaction was started by adding oxaloacetate (0.2 mM in final concentration) [20]. The CS activity was measured by detecting the color change of 5,5′-dithiobis-(2-nitrobenzoic) acid at 412 nm with a duration of 3 min. The final protein concentration was 20 µg/mL.

MDH

The conversion of oxaloacetate to malate was used to measure the activity, and it was measured at 340 nm for 3 min. The reaction was initiated by adding oxaloacetate and nicotinamide adenine dinucleotide (NADH) at final concentrations of 0.5 mM and 0.15 mM, respectively [21]. The final protein concentration was 20 µg/mL.

Complex I (NADH Dehydrogenase) Activity

The reaction mixture consisted of KH_2PO_4, 25 mM; $MgCl_2$, 5 mM; and KCN, 2 mM (pH 7.2) and the reaction was started by adding decylubiquinone and NADH, and both had final concentrations of 0.1 mM [22]. The rotenone-sensitive NADH oxidation reaction was measured at 340 nm for 5 min. The final protein concentration was 150 µg/mL.

Complex II + III (Succinate Cytochrome c Oxidoreductase) Activity

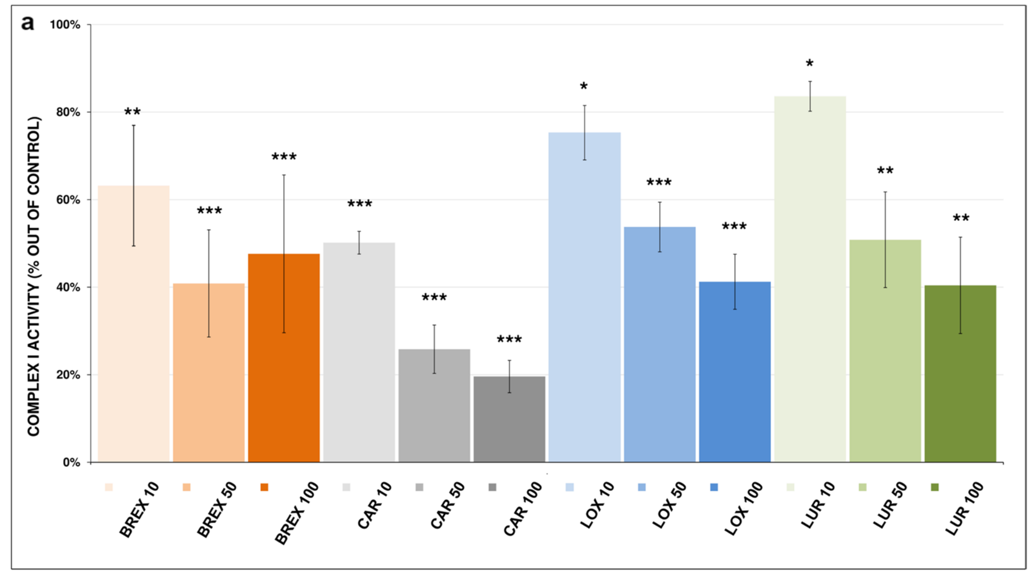
The activity of complex II + III was measured with an antimycin A-sensitive cytochrome *c* (cyt *c*) reduction at 550 nm for 3 min, and it was initiated by the addition of cytochrome *c* at final concentration 30 µM [23]. The medium consisted of KH_2PO_4, 50 mM; EDTA, 0.625 mM; KCN, 2 mM; and rotenone, 0.1 mM, adjusted to pH 7.4 and the final protein concentration was 50 µg/mL.

Complex IV (Cytochrome c Oxidase) Activity

The medium consisted of KH_2PO_4 (10 mM) adjusted to pH 7.4, and the reaction was initiated by reduced cyt *c* at final concentration of 35.7 µM. The decrease in absorbance was measured at 550 nm for 3 min. The final protein concentration was 10 µg/mL.

ATP Production

Mitochondria were resuspended in MiR05 buffer and incubated for 30 min with the final concentrations of the tested substances at 10, 50, and 100 µM and the final protein concentration at 150 µg/mL. All used substances were dissolved in DMSO, and each measurement had a corresponding control. ATP formation was determined by measuring the luminescence using FluoroMax-3 (Jobin Yvon, Edison, NJ, USA). The substrate mix used for ATP formation consisted of 5 mM malate, 5 mM pyruvate, 10 mM succinate, 5 mM glutamate, and 1 mM ADP. An ATP Bioluminescence Assay Kit CLS II was used to measure ATP production. The reaction was started by adding the substrate mix and luciferase reagent to a volume of 230 µL. Luminescence was observed at 532 nm for 6 min [24]. The agreement between polarographic-based and luciferase-based measurement of ATP production was not verified in this study, whereas the rate of ATP production derived from both the polarographic and bioluminescence methods was previously found highly correlated [25].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COMPLEX I ACTIVITY (% OUT OF CONTROL)
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80%
60%
40%
20%
0%
BREX 10
BREX 50
BREX 100
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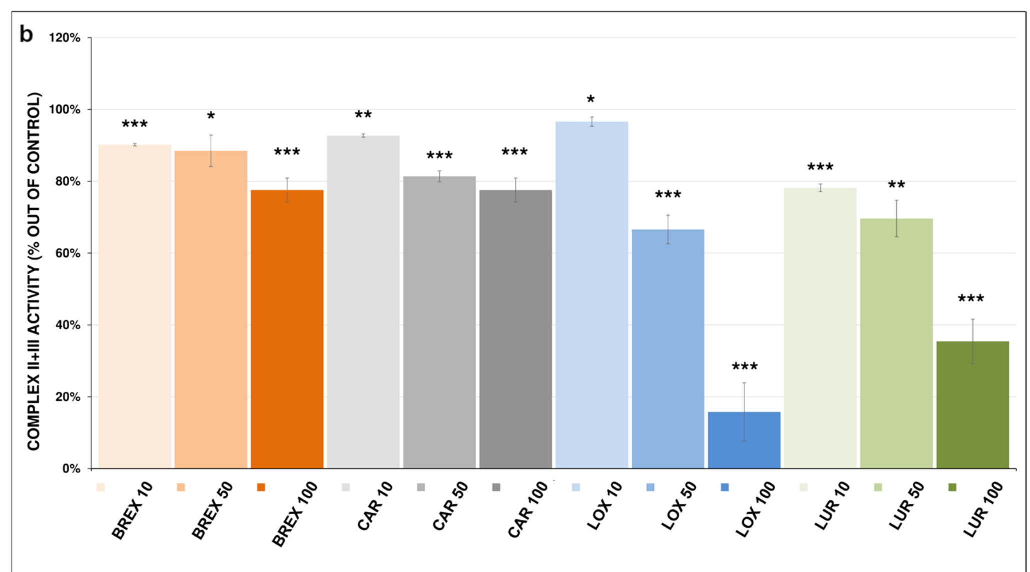
CAR 100
LOX 10
LOX 50
LOX 100
LUR 10
LUR 50
LUR 100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80%
60%
40%
20%
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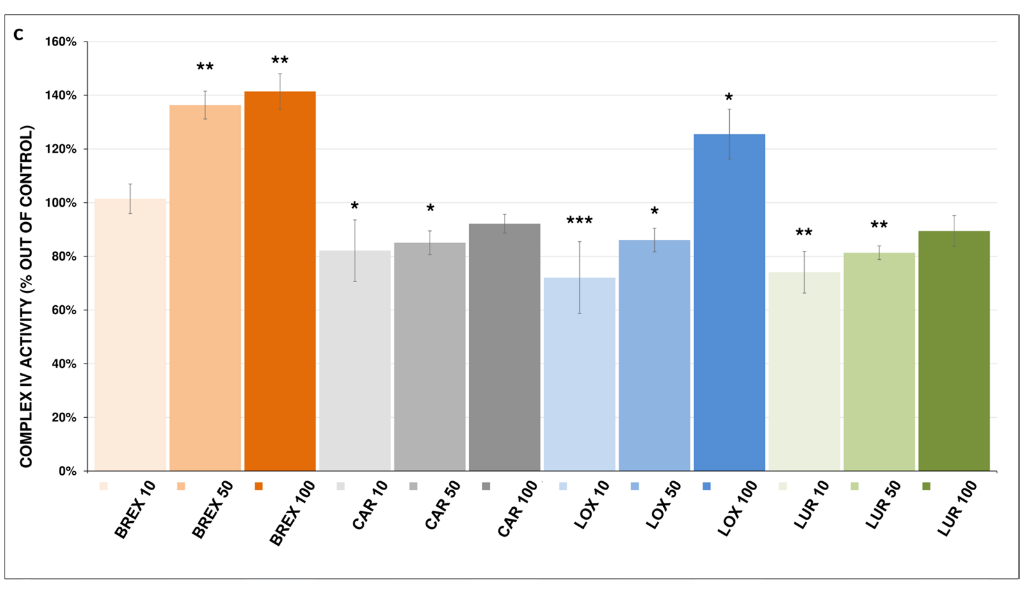
LUR 100


c
COMPLEX IV ACTIVITY (% OUT OF CONTROL)
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140%
120%
100%
80%
60%
40%
20%
0%
BREX 10
BREX 50
BREX 100
CAR 10
CAR 50
CAR 100
LOX 10
LOX 50
LOX 100
LUR 10
LUR 50
LUR 100

◂**Fig. 1** **a** Antipsychotic-induced inhibition of complex I activity. Relative activity is displayed as 100% — activity of control sample. The values are expressed as the mean ± SD for at least three independent measurements. Statistical significance was tested using a one sample *t*-test that mean value is equal to 100% and is expressed as $*p < 0.05$. $**p < 0.01$. $***p < 0.001$. Drug concentrations are expressed in µM. **b** Antipsychotic-induced inhibition of complex II + III activity. Relative activity is displayed as 100% — activity of control sample. The values are expressed as the mean ± SD for at least three independent measurements. Statistical significance was tested using a one sample *t*-test that mean value is equal to 100% and is expressed as $*p < 0.05$. $**p < 0.01$. $***p < 0.001$. **c** Antipsychotic-induced changes in complex IV activity. Relative activity is displayed as 100% — activity of control sample. The values are expressed as the mean ± SD for at least three independent measurements. Statistical significance was tested using a one sample *t*-test that mean value is equal to 100% and is expressed as $*p < 0.05$. $**p < 0.01$. $***p < 0.001$. Drug concentrations are expressed in µM. BREX, brexpiprazole; CAR, cariprazine; LOX, loxapine; LUR, lurasidone

Reactive Oxygen Species Production

For ROS production, mitochondria were prepared and incubated with the tested substances as mentioned above. Hydrogen peroxide formation was determined using an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit. The reaction medium consisted of HEPES, 27 mM; sucrose, 114 mM; KCl, 100 mM; K_2HPO_4, 1.3 mM; rotenone, 1 µM; succinate, 10 mM; and ADP, 1 mM, adjusted to pH 7.3. The reaction was initiated by adding Amplex Red at a final concentration of 15 µM and horseradish peroxidase at a final concentration of 0.09 U/mL. Fluorescence of Amplex Red was observed at an excitation of 571 nm and an emission of 585 nm for 6 min [26].

Mitochondrial Respiration

The mitochondrial oxygen consumption rate was measured at 37 °C using a high-resolution Oxygraph-2 k (Oroboros Instruments Corp, Innsbruck, Austria) with Clark-type electrodes and titration-injection micropump. MIR05 (oxygen solubility factor = 0.92) was used to fill the oxygraph chambers to a final volume of 2 mL, and mitochondria were added at the final protein concentration of 0.05–0.14 mg/mL. The following specific mitochondrial substrates were added: malate, 2 mM; pyruvate, 5 mM; ADP, 1.25 mM; and $MgCl_2$, 0.75 mM (for complex I-linked respiration); ADP, 1.25 mM; $MgCl_2$, 0.75 mM; rotenone, 1 µM; and succinate, 10 mM (for complex II-linked respiration). Rotenone (complex I inhibitor) was used to monitor the complex I-linked respiration background (residual oxygen consumption); similarly, antimycin A (complex III inhibitor) was used for complex II-linked respiration background. For a determination of the drug-induced inhibition, two simultaneous measurements were assessed: a titration with the drug to the final drug concentrations of 0.125–100 µM in one chamber and a titration with the DMSO in the second oxygraph chamber [9].

Monoamine Oxidase Activity

The activities of both isoforms of monoamine oxidase, MAO-A and MAO-B, were determined radiochemically, as described previously [27]. Mitochondria in KH buffer at final concentration of 800 µg/mL were preincubated with the tested drugs in a final concentration range of 0.1–300 µM for 60 min at 37 °C. Radiolabeled substrates were added to initiate the reaction: 3.2 µM [^{3}H]serotonin for MAO-A and 10 µM [^{14}C]PEA for MAO-B. The reaction was carried out at 37 °C for 20 min for MAO-A and for 1 min for MAO-B, and the reaction was stopped by adding 250 µL of 2 N hydrochloric acid. The reaction products were extracted with benzene:ethyl acetate 1:1 (v/v), and the radioactivity of the organic phases of the extracts was measured by liquid scintillation counting (LS 6000IC, Beckman Instruments, Inc., Fullerton, CA, USA).

Data Analysis and Statistics

The mitochondrial enzyme activities data were calculated as the slope of the time dependent absorbance curve. Data obtained from ATP and ROS measurement were calculated as the mean of the time dependent fluorescence curve. The control sample activity was 100%, and the drug effect was expressed as a % of the control.

High-resolution respirometry data were collected and analyzed using DatLab 4.3 software (Oroboros Instruments, Innsbruck, Austria) and they displayed the real-time oxygen concentration and oxygen flux. The oxygen flux (respiration rate) was expressed as a pmol O_2 consumed per second per mg of a protein.

The inhibition of respiration rate and MAO activity was analyzed by a four-parameter logistic regression with Prism software (GraphPad Software, San Diego, CA, USA) in order to establish the half-maximal inhibitory concentration (IC_{50}), residual activity, and the Hill slope. The IC_{50} represents the concentration of a drug that is required to inhibit the difference between the baseline and the residual value of the mitochondrial oxygen flux or MAO activity by 50%.

STATISTICA 12 analysis software (TIBCO Software Inc., Palo Alto, CA, USA) was used for data analysis using a one-sample *t*-test. All data presented are expressed as the mean ± standard deviation (SD) or as the mean ± standard error (SE). Several statistically significant correlations of our data were identified using correlation matrix displayed as the Pearson correlation coefficient.

Results

Activity of Mitochondrial Enzymes

The mitochondrial ETC complexes activity results are depicted in Fig. 1a–c. All four drugs inhibited significant and dose-dependently complex I activity. The strongest inhibitor of complex I was CAR at all concentrations (19.6 ± 3.7% at 100 μM). Complex II + III was also significantly and dose-dependently inhibited by all substances, and LOX measured the strongest inhibition of complex II + III (15.8 ± 8.1% at 100 μM). Complex IV was significantly inhibited by CAR (85.1 ± 4.4% at 50 μM) and LUR (74.1 ± 7.8% at 10 μM); increased complex IV activity was observed after incubation with BREX (141.4 ± 6.6% at 100 μM). LOX significantly decreased complex IV activity at lower concentrations (72.1 ± 13.4% at 10 μM, 86.1 ± 4.4% at 50 μM); however, increased complex IV activity was found at the highest concentration (125.6 ± 9.3 at 100 μM). The activity of CS and MDH was not affected by the tested antipsychotics (Table 1).

ATP and Reactive Oxygen Species Production

The ATP and ROS production results are depicted in Figs. 2 and 3, respectively. The inhibition of ATP production was observed for BREX, CAR, and LUR, at 100 μM,

Table 1 The effect of antipsychotics on citrate synthase (CS) and malate dehydrogenase (MDH) activity

Drug	Concentration	CS (% of control)	*N*	MDH (% of control)	*N*
Brexpiprazole	10 μM	98.6 ± 0.85	3	100.5 ± 1.88	3
Cariprazine	10 μM	98.3 ± 2.46	3	98 ± 3.98	3
Loxapine	10 μM	***109.8 ± 0.49	3	*99.3 ± 0.15	3
Lurasidone	10 μM	101.1 ± 1.13	3	100.6 ± 0.83	3

The values are expressed as the mean ± SD for 3 independent measurements. Statistical significance was tested using a one sample *t*-test that mean value is equal to 100% and is expressed as $*p < 0.05$. $**p < 0.01$. $***p < 0.001$. *N* is number of measurements

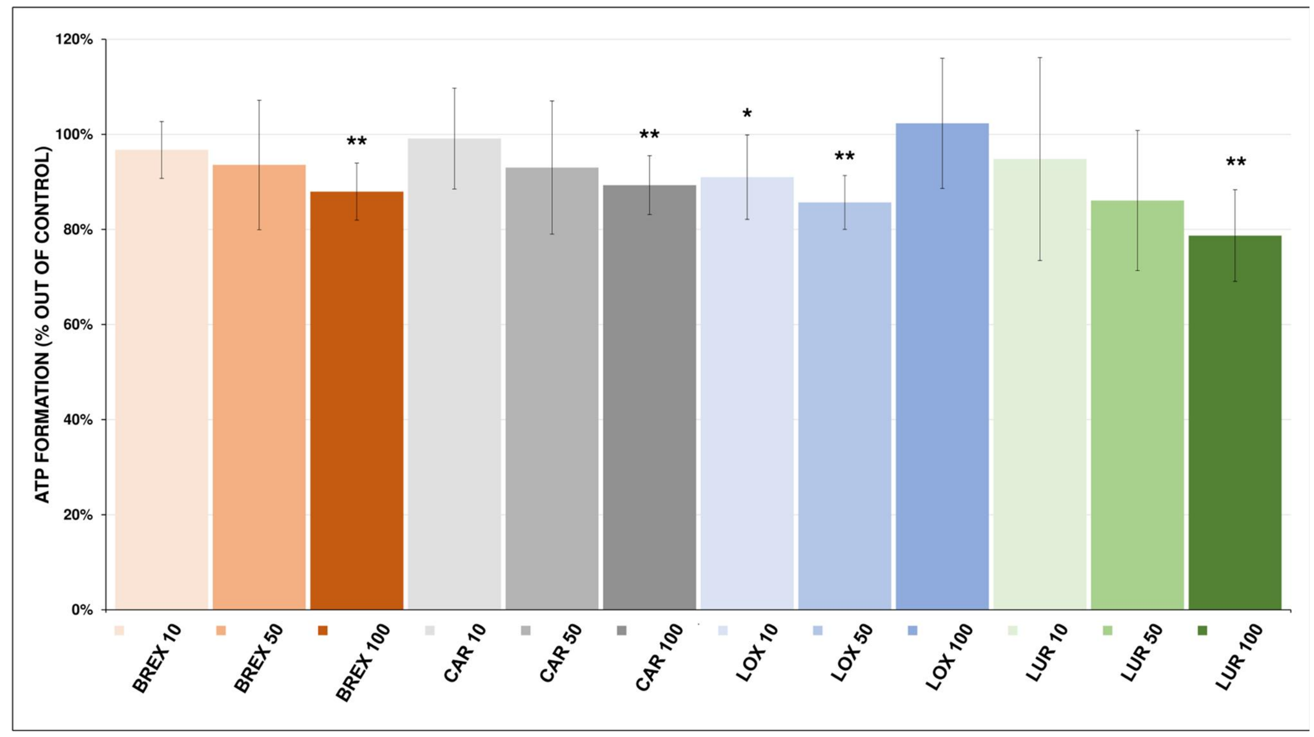


Fig. 2 Antipsychotic-induced changes in ATP production. Relative activity is displayed as 100% — activity of control sample (100% corresponded to a production of 8.5–10.5 nmol of ATP per 1 mg of protein, depending on the used kit). The values are expressed as the mean ± SD for at least six independent measurements. Statistical significance was tested using one sample *t*-test that mean value is equal to 100% and is expressed as $*p < 0.05$. $**p < 0.01$. $***p < 0.001$. Drug concentrations are expressed in μM. BREX, brexpiprazole; CAR, cariprazine; LOX, loxapine; LUR, lurasidone

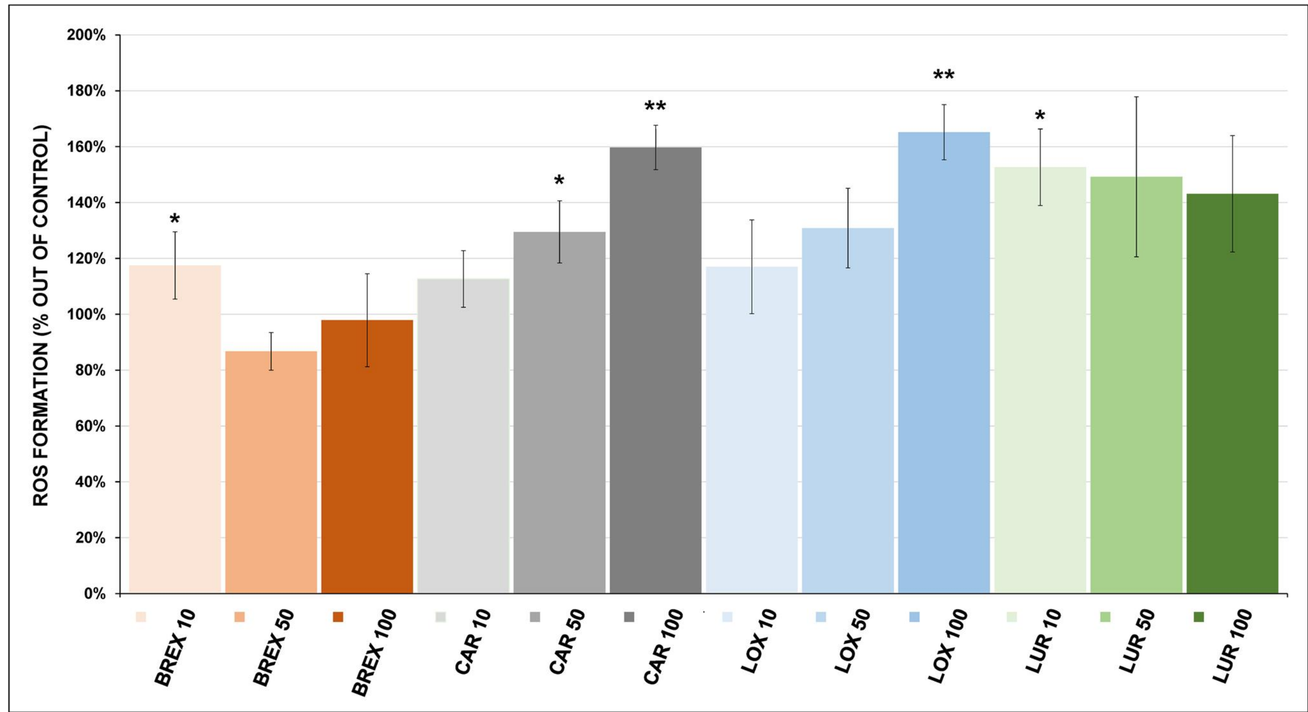


Fig. 3 Antipsychotic-induced changes in ROS production. Relative activity is displayed as 100% — activity of control sample (100% corresponded to a production of 350 pmol of H_2O_2 per 1 mg of protein per minute). The values are expressed as the mean ± SD for at least three independent measurements. Statistical significance was tested using a one sample *t*-test that mean value is equal to 100% and is expressed as *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. Drug concentrations are expressed in µM. BREX, brexpiprazole; CAR, cariprazine; LOX, loxapine; LUR, lurasidone

and at 10 µM and 50 µM for LOX. LUR was found the strongest inhibitor of ATP production (78.7 ± 9.6% at 100 µM). A statistically significant increase in ROS production was observed for the all tested drugs, and the greatest increase in ROS production was observed for LOX (165.2 ± 9.9% at 100 µM).

Mitochondrial Respiration

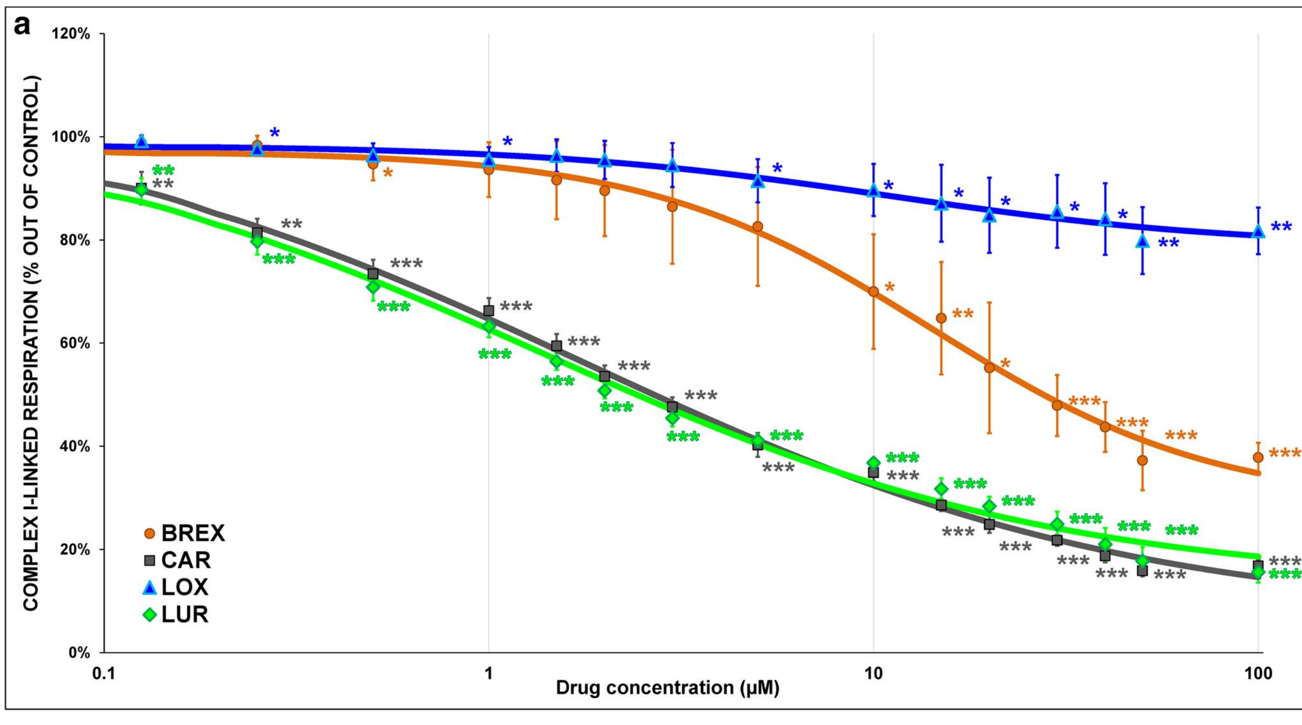
Drug-induced changes in complex I-linked and complex II-linked respiration are depicted in Fig. 4a and b, and the evaluated parameters (IC_{50}, hill slope, and residual activity) are summarized in Table 2. Complex I-linked respiration was partially inhibited by BREX, LOX, and LUR and fully inhibited by CAR, and had malate, pyruvate, and ADP as substrates. CAR (IC_{50} = 1.76, residual activity = 0.07) and LUR (IC_{50} = 1.38, residual activity = 0.13) had the strongest inhibition. Complex II-linked respiration was partially and weakly inhibited by LOX and fully inhibited by LUR, which was also the most potent inhibitor (IC_{50} = 39.94, residual activity = 0.08). BREX and CAR did not inhibit complex II-linked respiration.

MAO Activity

MAO-A activity was partially inhibited by BREX, CAR, and LOX; LUR exhibited only very weak inhibitory properties. BREX and LOX acted as partial MAO-B inhibitors, CAR and LUR caused only very weak MAO-B inhibition. A drug-induced MAO-A and MAO-B inhibition curves are depicted in Fig. 5a and b, and the evaluated parameters are summarized in Table 3.

Correlations

Several statistically significant correlations were identified between measured mitochondrial parameters using the Pearson correlation coefficient. The correlation coefficients are summarized in Tables S1–S4 as a supplementary material. All tested substances had a significantly positive correlation between the activity of complex I and complex I-linked respiration as well as between complex I activity and complex II-linked respiration. Significant negative correlation was found between complex IV activity and complex I-mediated respiration. Significant positive correlation was found



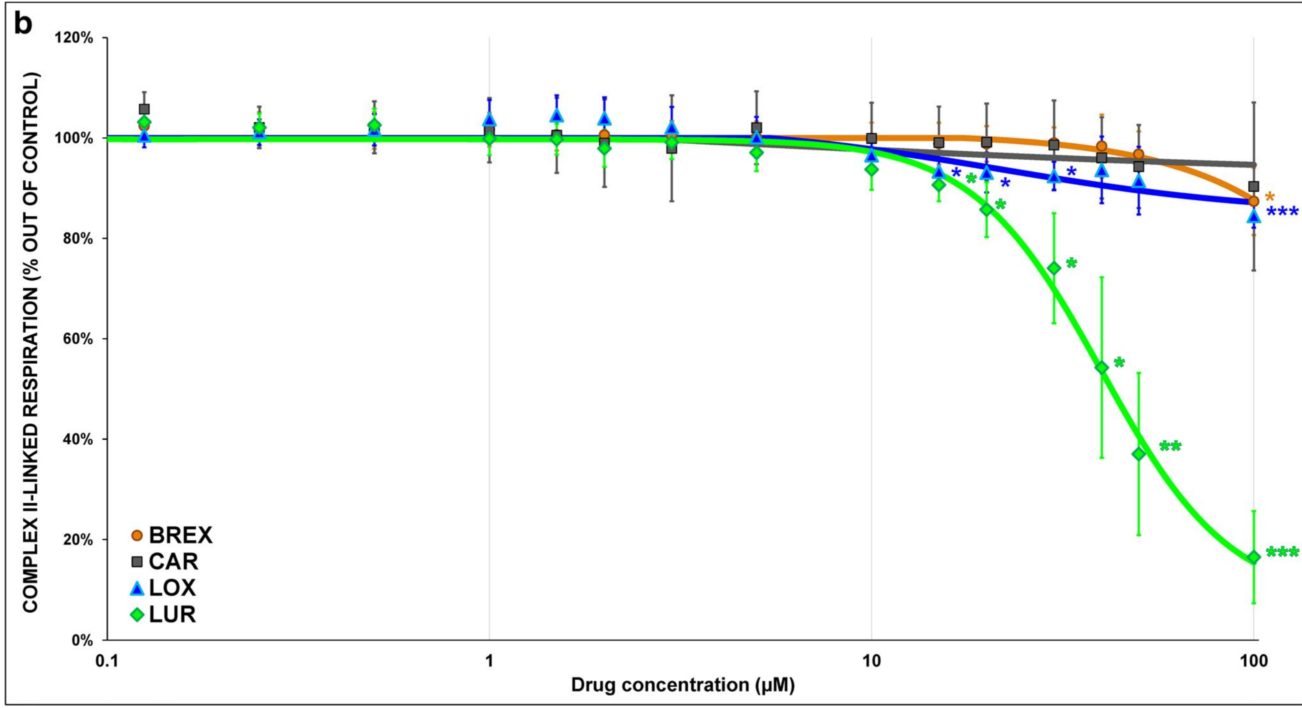


Fig. 4 **a** Antipsychotic-induced inhibition of complex I-linked respiration. Dose–response curves are displayed as plots of the respiration rate against drug concentration. Relative activity is displayed as 100% — activity of control sample. Points are the mean of four independent measurements and lines represent the best/fitted curves using a four/parametric logistic function. Statistical significance was tested using a one sample *t*-test that mean value is equal to 100% and is expressed as $*p < 0.05$. $**p < 0.01$. $***p < 0.001$. The half-maximal inhibitory concentration (IC_{50}), hill slope, and residual activity were calculated (Table 2). **b** Antipsychotic-induced inhibition of complex II-linked respiration. Dose–response curves are displayed as plots of the respiration rate against drug concentration. Relative activity is displayed as 100% — activity of control sample. Points are the mean of four independent measurements and lines represent the best/fitted curves using a four/parametric logistic function. Statistical significance was tested using a one sample *t*-test that mean value is equal to 100% and is expressed as $*p < 0.05$. $**p < 0.01$. $***p < 0.001$. The half-maximal inhibitory concentration (IC_{50}), hill slope, and residual activity were calculated (Table 2). BREX, brexpiprazole; CAR, cariprazine; LOX, loxapine; LUR, lurasidone

Table 2 Antipsychotic-induced inhibition of complex I- and complex II-linked respiration

Drug	IC_{50} (μM)	Hill slope	Residual activity (rel.u.)	Inhibition
Complex I-linked respiration				
Brexpiprazole	14.14 ± 2.99	1.17 ± 0.23	0.284 ± 0.068	Partial
Cariprazine	1.76 ± 0.27	0.60 ± 0.07	0.066 ± 0.032	Full
Loxapine	10.98 ± 6.64	0.96 ± 0.46	0.787 ± 0.049	Partial
Lurasidone	1.38 ± 0.25	0.65 ± 0.08	0.133 ± 0.027	Partial
Complex II-linked respiration				
Brexpiprazole	-	-	-	None
Cariprazine	-	-	-	None
Loxapine	21.78 ± 12.16	1.22 ± 0.59	0.848 ± 0.053	Partial
Lurasidone	39.94 ± 3.19	2.57 ± 0.39	0.075 ± 0.068	Full

The values are expressed as the mean ± SE for 4 independent measurements. IC_{50} is half maximal inhibitory concentration

between complex I-/II-linked respiration and ATP production for all tested antipsychotics except LOX.

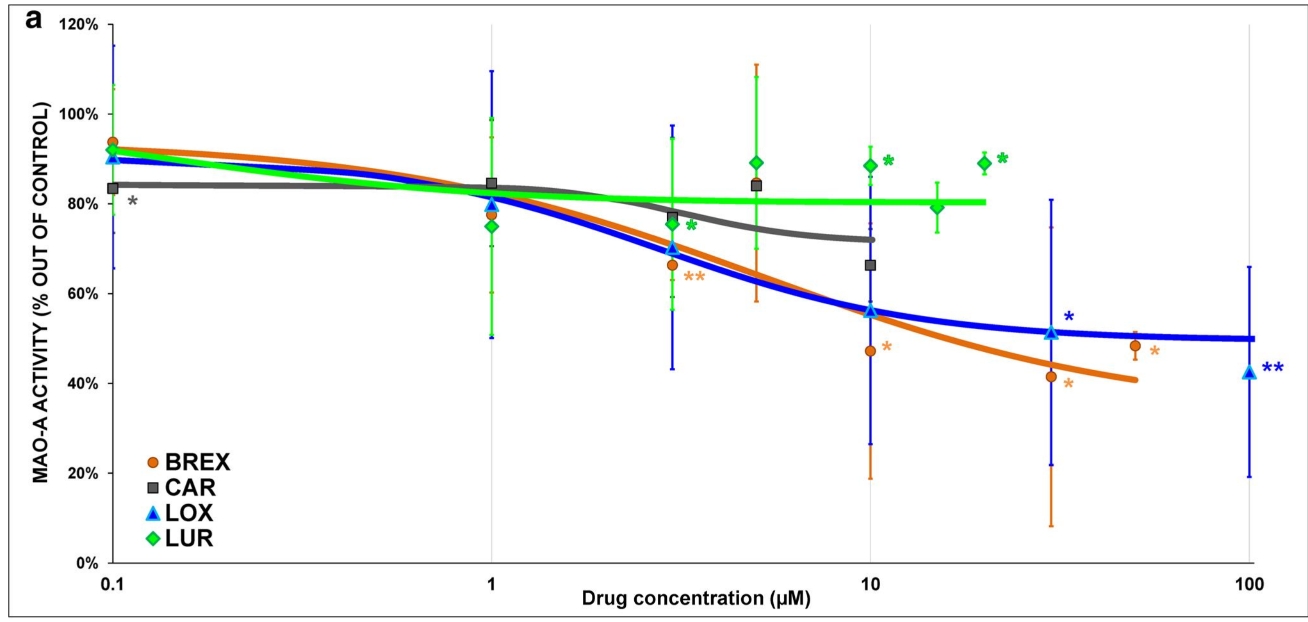
Discussion

We determined the effects of the tested antipsychotics on the parameters selected for cellular energy metabolism. The optimal activity of ETC complexes resulting in sufficient mitochondrial respiration is crucial for OXPHOS to function correctly and perform its primary goal: supply energy in the form of ATP for whole organisms. In addition, OXPHOS is involved in synaptic signaling. Mitochondria are a major source of intracellular ROS, and excessive production of ROS might lead to cellular damage, which may be related to psychiatric and neurodegenerative diseases [1, 2]. Both CS and MDH are components of the citric acid cycle, localized in the matrix of mitochondria and included in cellular energy pathways [28, 29]. Monoamine oxidases are enzymes bound to the outer mitochondrial membrane. They metabolize monoamines and are involved in Alzheimer's and Parkinson's diseases, depression, and other psychiatric disorders, and their involvement is mainly due to their overexpression [30].

Concentrations of antipsychotics were extrapolated from the monitored therapeutic plasma concentrations of individual antipsychotics: 13–80 ng/mL (30–185 nM) BREX, 8.5–33 ng/mL (18–71 nM) CAR, 24–177 ng/mL (73–540 nM) LOX, and 15–132 ng/mL (28–250 nM) LUR [31–36]. We decided to use higher than plasmatic concentration to more easily observe the in vitro effect of the chosen antipsychotics on the selected parameters of energy metabolism, and because of the lipophilic character and high volume of the distribution of the tested antipsychotics, the accumulation of the antipsychotics in tissues and organs is presumed. For example, plasma concentration of CAR was 91 ng/ml after perioral CAR administration to rats (1 mg/kg); with a brain/plasma AUC ratio to be 7.6:1 [37]. Moreover, concentrations of tested drugs in intracellular space (affecting directly mitochondria) are not known. Due to submicromolar plasma concentrations of the tested antipsychotics, no tissue and intracellular accumulation can be expected higher than 1–10 μM [38]. The final concentrations of antipsychotics used in the in vitro experiment ranged from 0.125 to 100 μM. The highest drug concentrations exceeded plasma drug concentrations, which could be used to determine the correlations between mitochondrial parameters in order to specify which drug interactions with individual mitochondrial proteins are responsible for disruption of complex mitochondrial functions (especially oxygen consumption kinetics in OXPHOS). Whether the effects of tested antipsychotics on mitochondrial functions were mostly significant only at high drug concentrations, it can be assumed that these effects might occur rather after antipsychotic overdose and are related to the adverse effects of these drugs. Our study confirmed that the antipsychotic-induced inhibition of mitochondrial respiratory rate is largely dependent on the inhibition of individual ETC complexes. Different correlation coefficients found for individual drugs between the change in the activity of individual ETC complexes and complex mitochondrial function suggesting some specificity of the mitochondrial effects of individual psychopharmaca. Pig brain mitochondria were used as a biological model and were evaluated previously as a suitable model for studies that aim observing the mitochondrial drug effects [18].

Mitochondrial Enzyme Activity and Respiration

Complex I activity was significantly affected by all tested antipsychotics; the most potent inhibitor was CAR. These results are consistent with other studies, in which most antipsychotics inhibited complex I activity, whereas atypical antipsychotics did not alter complex I activity as much as the typical antipsychotics did [9, 39, 40]. Complex I is



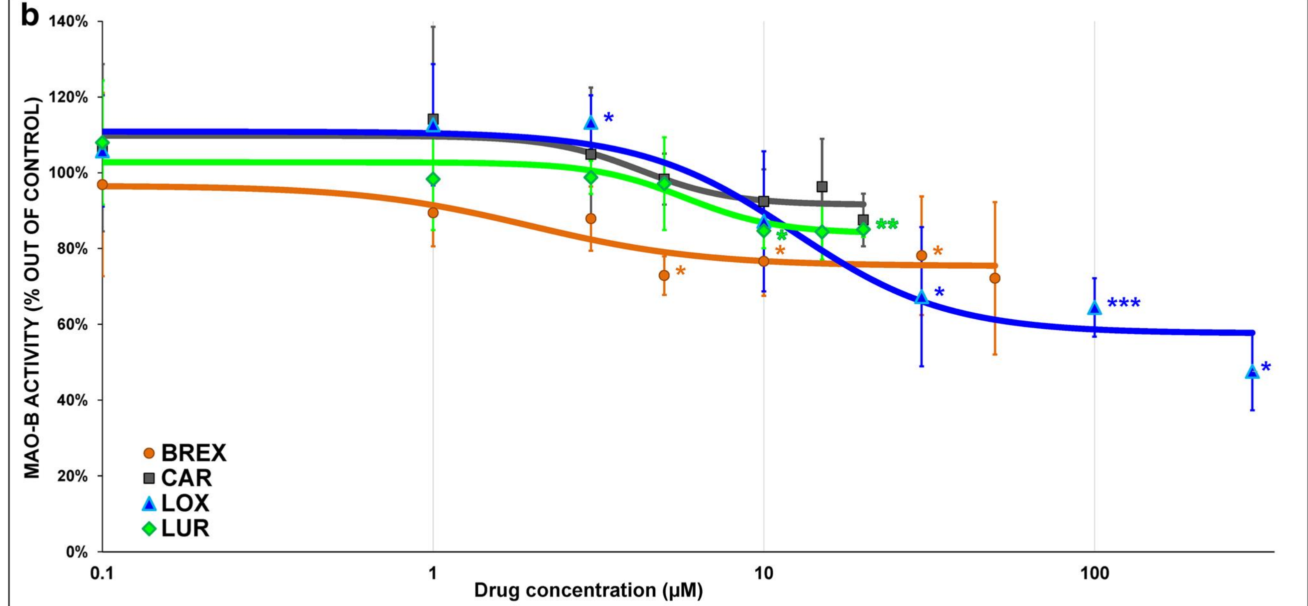


Fig. 5 **a** Antipsychotic-induced MAO-A inhibition. The relative activity is displayed as 100% — activity of the control sample. Statistical significance was tested using a one sample *t*-test that mean value is equal to 100% and is expressed as $*p < 0.05$. $**p < 0.01$. $***p < 0.001$. The half-maximal inhibitory concentration (IC_{50}), hill slope, and residual activity were calculated (Table 3). **b** Antipsychotic-induced MAO-B inhibition. The relative activity is displayed as 100% — activity of the control sample. Statistical significance was tested using a one sample *t*-test that mean value is equal to 100% and is expressed as $*p < 0.05$. $**p < 0.01$. $***p < 0.001$. The half-maximal inhibitory concentration (IC_{50}), hill slope, and residual activity were calculated (Table 3). BREX, brexpiprazole; CAR, cariprazine; LOX, loxapine; LUR, lurasidone

the main entry to the OXPHOS and rate limiting point for oxygen consumption. Consequently, complex I inhibition can affect complex I-linked respiration and ATP formation. We found a significant positive correlation between complex I activity and complex I-linked respiration for all the antipsychotics tested. Complex I-linked respiration was partially inhibited by all the antipsychotics (fully by BREX), whereas LOX-induced inhibition was very small. Taking the relevance of therapeutic concentrations (below 1 μM), CAR and LUR significantly inhibited complex I-linked respiration even in these concentrations, reducing the respiration by around 30%; no inhibition was observed in BREX. Several studies found decreased complex I activity and decreased complex I-linked respiration in peripheral blood

Table 3 Antipsychotic-induced monoamine oxidase (MAO-A and MAO-B) inhibition

Drug	IC_{50} (μM)	Hill slope	Residual activity (rel.u.)	Inhibition
MAO-A				
Brexpiprazole	5.22 ± 8.22	0,87 ± 1.20	0.334 ± 0.390	Partial
Cariprazine	3.22 ± 3.13	2.58 ± 8.31	0.713 ± 0.070	Partial
Loxapine	2.77 ± 3.16	1.25 ± 1.65	0.495 ± 0.102	Partial
Lurasidone	-	-	-	None
MAO-B				
Brexpiprazole	1.97 ± 2.02	1.68 ± 2.38	0.754 ± 0.059	Partial
Cariprazine	4.24 ± 2,46	3.14 ± 5.82	0.915 ± 0.067	Weak
Loxapine	12.37 ± 3,82	1.88 ± 1.05	0.576 ± 0.061	Partial
Lurasidone	5.79 ± 2,34	3.10 ± 3.32	0.840 ± 0.044	Weak

The values are expressed as the mean ± SE for 4 independent measurements. IC_{50} is half maximal inhibitory concentration

cells, lymphoblastoid cells, and brain tissue of schizophrenic patients medicated with antipsychotics [41–43]. Compared to typical antipsychotics, the new antipsychotics have been associated with a lower incidence of tardive dyskinesia. Patients treated with antipsychotics have demonstrated positive correlations between the inhibition of complex I and frequency of extrapyramidal symptoms [6]. Our data are consistent with these findings because among the antipsychotics, BREX seems to have the lowest tendency cause akathisia and there is almost no need to use antiparkinsonian comedication; the risk ratios of CAR, LOX, and LUR in both parameters were comparable with and higher than those in BREX [44]. Our data support the assumption that long-term complex I inhibition could likely lead to the drug-induced mitochondrial toxicity that manifests as adverse drug effects.

Minor but significant inhibition of complex II + III activity was observed for all tested antipsychotics, and the strongest inhibitor was LOX. Complex II is a side entry point to OXPHOS and connects with the citric acid cycle. It does not create proton motive force; thus, it could be involved in ATP production indirectly [45, 46]. However, the positive correlation between complex II + III activity and the mitochondrial oxygen consumption (Tables S1–S4 of supplementary material) confirms that drug-induced changes in complex II + III activity can significantly contribute to the drug-induced changes in the OXPHOS activity. The complex II-linked respiration was inhibited only at the highest LOX and LUR concentrations, thus, no drug-induced inhibition can be presumed at therapeutic drug concentrations. Complex IV activity was shown to increase, and did so most significantly at high concentrations of BREX and LOX. At lower concentrations of the antipsychotics, the activity of complex IV was slightly inhibited or unaffected. Previously, our results confirmed that complex IV stimulation occurred after incubation with quetiapine, other antipsychotics did not alter its activity, and typical antipsychotics (chlorpromazine, zotepine, and levomepromazine) acted as inhibitors. [47]. A consistently negative correlation between complex IV activity and the mitochondrial respiration or ATP production (Tables S1–S4 of supplementary material) suggests that direct inhibition or stimulation of complex IV by the antipsychotics is not decisive for the resulting oxygen consumption and ATP production.

The observation that drugs inhibited the activity of individual ETC complexes significantly more than the overall activity of mitochondrial respiration or the production of ATP and ROS can be explained by the way these mitochondrial parameters were measured. While respiratory rate, ATP, and ROS production were measured in intact isolated mitochondria, the activities of individual complexes were measured in disrupted mitochondria (freeze–thaw, after sonication). Disrupted mitochondria enable better access of drugs to complexes and interactions with individual complexes may be more potent and robust. In case on LUR, we speculate that the inconsistency between high inhibition of complex I- and II-linked respiration and relatively small inhibition of ATP production might be the results of LUR-induced increase in the P/O rations (the amount of ATP produced per molecule of oxygen consumed by mitochondria). The oxygen consumption alone or ATP production alone are though unlikely to be sufficient marker of energy metabolism; drug-induced changes in multiple mitochondrial functions should be measured simultaneously [48, 49]. These unexplained LUR effects will be addressed in another study focusing on the different concentrations of LUR on extended panel of mitochondrial functions, including not yet evaluated parameters such as mitochondrial membrane potential, mitochondrial permeability transition, swelling, calcium retention capacity, release of pro-apoptotic factors, and MTT test [50]. We confirmed that the measurement of the individual mitochondrial enzyme activity cannot reflect the overall function of the organelle as complex as the mitochondria. However, the high correlation between drug-induced inhibition of mitochondrial respiration and inhibition of respiratory chain complexes indicates that the inhibition of individual ETC complexes is responsible for the inhibition of respiratory rate.

ATP and ROS Production

At high concentrations, all four antipsychotics slightly but significantly decrease ATP production, which might lower the energy available for cellular processes and disrupt neuronal functions. In particular, neurons are extremely

dependent on their own ATP production. ATP also serves as a coenzyme and signaling molecule, and it is necessary for the neuronal function and regulation of the neuroplasticity in health and disease [51–53]. In contrast, inhibitors of the F_0 domain of ATP synthase have been proven to prolong cell viability under the hypoxic conditions of ischemia in rat myocardium [54]. This illustrates that ATP synthase and ATP production are involved in the cell compensatory mechanisms under critical conditions.

We observed no change or increased mitochondrial H_2O_2 production due to the tested antipsychotics. Significant increase in H_2O_2 production was not found at high concentrations of BREX and LUR; however, high concentrations of CAR and LOX induced significantly increased H_2O_2 production. It can be presumed that BREX can minimally induce the adverse effects that are related to oxidative stress.

Complex I and III are considered to be a major source of ROS in the mitochondria [55, 56]. The oxidation of NADH is a reversible reaction in which electrons are reversibly transferred back to NAD^+ to produce superoxide. ROS can serve as signaling molecules, and their modulation can trigger compensating mechanisms, such as increased the activity of superoxide dismutase and normalized redox imbalance caused by clozapine in animals exposed to social isolation [57]. Further research is necessary to determine whether antipsychotic-induced ROS increase causes direct cellular damage and adverse effects or normalizes impaired redox imbalance as a compensatory mechanism.

Other Correlations

Statistically significant positive correlations were found between complex II + III activity and the ATP formation for BREX, CAR, and LUR (complex I activity correlated positively with ATP formation for CAR and LUR, complex IV correlated negatively with ATP formation for CAR and LUR). These findings are consistent with the correlation between ATP production and complex I-/complex II-linked respiration. Our results suggest that the activity of complex II + III has a high impact on ATP production.

The correlation analysis showed that the increased ROS production by CAR and LOX was significantly negatively associated with complex II + III activity. The inhibition of complex II + III may decrease the OXPHOS efficacy and increase ROS formation rather than passing the electrons to complex IV. However, this was not proven for CAR at the highest concentration that also significantly increased ROS production but only mildly inhibited complex II + III activity. These differences might be due to the binding of substances to different sites of complex II + III. We suppose that all tested antipsychotics are unlikely to act as OXPHOS uncouplers, because uncouplers decrease ROS production and increase the respiration rate in the isolated mitochondria [58]. The effects of tested antipsychotics on mitochondrial ROS production may be due to the direct inhibition of complexes I and II + III.

Inhibition of MAO Isoforms

All tested antipsychotics (except LUR) acted as partial inhibitors of MAO-A. MAO-A inhibition might contribute to the antipsychotic antidepressant properties in addition to their more important 5-HT receptors partial agonism/antagonism, which probably carries majority of antidepressant effects [13, 59]. Three out of the four tested antipsychotics are approved for treatment of different depressive states: BREX and LOX have been approved for augmentation treatment of major depressive disorder. However, some studies denied the efficacy of BREX in nonresponding patients with major depressive disorders [60, 61].

BREX and LOX were partial MAO-B inhibitors, CAR and LUR had only very weak inhibitory effect on MAO-B activity. Due to the low inhibitory properties of some tested drugs and insufficient solubility, the inhibition parameters were only partially determined. Note that MAO-B inhibition may be a positive effect of drugs because MAO-B inhibitors can be used in the treatment of neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases [62]. Systematic review identified studies describing neuroprotective properties of LUR. Several mechanisms were involved in neuroprotection and some of them might be related to mitochondrial functions [63].

Possible Clinical Impact of Molecular Effects

Compared with age-matched healthy controls, patients with Alzheimer's disease had reduced complex IV activity and higher complex I/CS and complex II/CS activities [64]; impaired complex I, II, and IV activity might be associated with cognitive decline. Clinical studies support the expected benefits of BREX and LUR, e.g., the improvement of cognitive functions and negative and depressive symptoms of patients with schizophrenia [13]. LOX on the other hand might worsen cognitive impairment due to its anticholinergic properties [65]. This might correlate with our findings that LOX is the most potent inducer of ROS formation, and causes further neuronal damage.

Compared to healthy controls, patients with DM-2 had decreased complex IV activity [66]. Clozapine and olanzapine, which are high-metabolic-risk antipsychotics, decreased all functional parameters of the mitochondrial oxygen consumption and the activity of mitochondrial complexes in both patients and healthy controls but did not alter the respiratory control ratio, which is an indicator of OXPHOS efficacy, suggesting that these substances do not act as uncouplers but rather as metabolic inhibitors [67]. Complex

IV activity could be linked to the adverse metabolic effects of psychopharmaca. The risk of weight gain is relatively low for all tested substances and decreases in the following order: BREX = CAR > LUR > LOX [44, 68–71]. No evidence about increased blood glucose or total cholesterol was found in patients with long-term treatment with BREX or CAR, proving that these drugs are metabolically safe [71].

A decrease in ATP production was previously observed in vitro in different cell types after they were incubated with clozapine, risperidone, chlorpromazine, and haloperidol [72, 73].

In addition to the previously mentioned weight gain and extrapyramidal symptoms, there are several other adverse effects linked to antipsychotic treatment. The prolactin level increase caused by the tested psychopharmaca was considered to be a low decrease in the following order: LUR > BREX > CAR. No QT interval prolongation was associated with the administration of these drugs [33, 44].

Study Limitations

The main focus of this study is to measure the effect of novel antipsychotics on complex mitochondrial parameters (mitochondrial respiration, ATP, and ROS production) in isolated mitochondria, allowing more accurate recognition of drugs effect on mitochondrial compensatory and regulatory mechanisms than is possible in in vivo measurements. We are unable to cover the regulatory and compensatory brain mechanisms that may influence the effects of tested drugs on mitochondrial functions in vivo in our experimental approach. For example, the effects of drugs on mitochondrial dynamics (fusion and fission) or intracellular processes such as interactions with the endoplasmic reticulum and other organelles or the effect of drugs on NAD + /NADH or NADP/NADPH ratio cannot be observed in the model of isolated mitochondria.

Conclusions

Our in vitro study of novel antipsychotics revealed important and statistically significant drug-induced changes in selected mitochondrial parameters at high concentrations. All four substances that were tested showed a decrease in the ETC complexes activities (except complex IV activity, which increased in BREX and LOX), mitochondrial respiration rate, and ATP formation. Decreased ETC complexes activity leads to an ineffective OXPHOS system, decreased ATP formation, and excessive ROS production. A lack of energy and higher oxidative stress would probably lead to neuronal damage at very high drug concentrations. Increased ROS production was observed for all the tested substances. CAR and LUR showed little but significant inhibition of complex I-linked respiration also in the range of therapeutic drug concentrations. The inhibition of complex II-linked respiration by therapeutic drug concentrations of all tested antipsychotics is unlikely. Though decreased ATP production and increased ROS production, as well as long-term complex I inhibition, are likely linked to the adverse effects of antipsychotics, the tested antipsychotics acted as partial or weak MAO-A inhibitors, suggesting they could have a possible antidepressive effect in augmentation therapy for affective disorders. MAO-B was partially inhibited by BREX and LOX indicating their participation on neuroplasticity and neuroprotectivity. The inhibition of monoamine oxidases might play a part in desirable effects of novel antipsychotics.

Further research, using cell cultures and especially in vivo animal brain studies, is needed to determine the connection of mitochondrial activity, schizophrenia pathophysiology, and the mitochondrial impact of novel antipsychotics in vivo.

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Code Availability Not applicable.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Authors consent for the publication of the manuscript.

Conflict of Interest The authors declare no competing interests.

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Attachment 5

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ORIGINAL ARTICLE

In vitro effects of antipsychotics on mitochondrial respiration

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Abstract
Assessment of drug-induced mitochondrial dysfunctions is important in drug development as well as in the understanding of molecular mechanism of therapeutic or adverse effects of drugs. The aim of this study was to investigate the effects of three typical antipsychotics (APs) and seven atypical APs on mitochondrial bioenergetics. The effects of selected APs on citrate synthase, electron transport chain complexes (ETC), and mitochondrial complex I- or complex II-linked respiratory rate were measured using mitochondria isolated from pig brain. Complex I activity was decreased by chlorpromazine, haloperidol, zotepine, aripiprazole, quetiapine, risperidone, and clozapine. Complex II + III was significantly inhibited by zotepine, aripiprazole, quetiapine, and risperidone. Complex IV was inhibited by zotepine, chlorpromazine, and levomepromazine. Mitochondrial respiratory rate was significantly inhibited by all tested APs, except for olanzapine. Typical APs did not exhibit greater efficacy in altering mitochondrial function compared to atypical APs except for complex I inhibition by chlorpromazine and haloperidol. A comparison of the effects of APs on individual respiratory complexes and on the overall mitochondrial respiration has shown that mitochondrial functions may not fully reflect the disruption of complexes of ETC, which indicates AP-induced modulation of other mitochondrial proteins. Due to the complicated processes associated with mitochondrial activity, it is necessary to measure not only the effect of the drug on individual mitochondrial enzymes but also the respiration rate of the mitochondria or a similar complex process. The experimental approach used in the study can be applied to mitochon-drial toxicity testing of newly developed drugs.

Keywords Antipsychotics · Citrate synthase · Electron transport chain complexes · Mitochondrial respiration

Abbreviations

AP	Antipsychotic
COX	Complex IV, cytochrome c oxidase
CS	Citrate synthase
ETC	Electron transport chain
MARTA	Multi-acting receptor targeted antipsychotics
OXPHOS	Oxidative phosphorylation
ROS	Reactive oxygen species

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Introduction

Antipsychotics (APs) are commonly used and widely prescribed medications for both psychiatric and non-psychiatric indications. The main molecular mechanism of action of APs, except for aripiprazole, is the blockade of dopamine D_2 receptors. Antagonism of D_2 receptors in mesolimbic dopamine pathway is responsible for treatment of positive symptoms of schizophrenia; however, the high and non-specific occupation of D_2 receptors through the CNS exhibits a wide range of adverse effects, most remarkably extrapyramidal syndrome and hyperprolactinemia (Sangani and Saadabadi 2019). The effects of individual drugs depend on affinity for the affected neurotransmitter receptors and transporters.

The first generation of APs (typical) strongly antagonizes D_2 receptors in cortical and striatal areas. Sedative typical APs, e.g., chlorpromazine and levomepromazine (syn. methotrimeprazine), affect dopaminergic and other mediator systems (cholinergic and histaminergic system) and have significant tranquilizing and anticholinergic effects (Church and Young 1983; Trabucchi et al. 1974). Haloperidol is characterized as a typical incisive AP and affects D_2 receptors in ventral striatum with higher specificity. It exhibits distinctive side effects, and haloperidol-induced movement disorders include parkinsonism, dyskinesia, dystonia, and akathisia (Wirshing 2001).

The second generation of APs (atypical) have a higher affinity for diverse neurotransmitter systems (5-HT, dopamine, muscarinic, adrenergic, and histamine receptors) (Kapur and Seeman 2001; Seeman 2002). Serotonin and dopamine antagonist (SDA) class of APs is represented by risperidone and ziprasidone. Risperidone has a higher binding affinity to 5-HT_2 receptors than it does to dopamine D_2 receptors (Keegan 1994); ziprasidone displays partial agonism at 5-HT_{1A} receptors and has a relatively high affinity for the H_1 receptor (Nasrallah 2008). Multi-acting receptor-targeted antipsychotics (MARTA) act as antagonists on numerous neurotransmitter receptors such as dopamine (D_1, D_2, D_3, or D_4), serotonin (5-HT_{2A}, 5-HT_{2C}, 5-HT_6, or 5-HT_7), adrenergic receptors (α_1 and α_2), muscarinic receptors (M_1, M_2, M_3, M_4, and M_5), and histamine H_1 receptors (Nasrallah 2008). MARTA are represented by clozapine, olanzapine, zotepine, and quetiapine. Aripiprazole differs from other atypical APs by partial agonism at D_2 receptors (Bolonna and Kerwin 2005), and it acts also as a partial 5-HT_{1A} receptor agonist and 5-HT_{2A} receptor antagonist. Although atypical APs are well tolerated, hyperglycemia, dyslipidemia, weight gain, and hypertension are risk factors of metabolic syndrome, which should be a concern in long-term antipsychotic treatment (Masand et al. 2005). Furthermore, QT interval prolongation, cardiac effects, seizures, etc. can accompany treatment with APs.

Disturbed brain bioenergetics, described as defect in oxidative phosphorylation (OXPHOS) in specific brain areas of patients with schizophrenia (Maurer et al. 2001), indicates the need to study the effects of antipsychotics on mitochondrial dysfunction. Treatment-responsive schizophrenia patients showed a significant reduction in the number of mitochondria at synapses in certain areas of the brain compared to treatment-resistant cases (Roberts 2017). Although the primary biochemical mechanisms of AP action are well described, their effect on mitochondrial dysfunction is not sufficiently recognized. Mitochondrial impairment appears to be related to some adverse effects of APs due to inhibition of the mitochondrial respiratory chain complexes. Haloperidol, chlorpromazine, and fluphenazine, classical APs, have long been reported to inhibit complex I in rat or mouse brain mitochondria more strongly than clozapine, an atypical AP (Balijepalli et al. 1999; Burkhardt et al. 1993; Prince et al. 1997). It has been suggested that reduced complex I activity may correlate with the extrapyramidal side effects of APs. The hypothesis that extrapyramidal side effects of APs may be caused by inhibition of mitochondrial electron transport chain (ETC) was supported by observing the different effects of haloperidol and chlorpromazine and atypical APs risperidone, zotepine, and clozapine on the activity of ETC complexes and citrate synthase (CS) activity in human brain tissue (Maurer and Möller 1997). However, the therapeutic and side effects of antipsychotics seem unrelated (Maurer and Volz 2001). The extrapyramidal side effects of APs appear to be caused by mitochondrial impairment, which is more noticeable for the typical APs than for the atypical APs (Casademont et al. 2007). Tardive dyskinesia correlated with the inhibition of the electron transport chain (ETC) complexes and the production of reactive oxygen species (ROS) during the antipsychotic treatment (Elkashef and Wyatt 1999; Goff et al. 1995).

The metabolic side effects of APs could be connected with alterations in mitochondrial homeostasis, which leads to an imbalance in the mitochondrial fusion/fission ratio and to an inefficient mitochondrial phenotype of muscle cells (Del Campo et al. 2018). For example, olanzapine induced a downregulation of genes involved in the mitochondrial enzymes of the ETC, as well as decreased enzyme activity, ATP synthesis, and oxygen consumption in blood cells of patients at elevated risk for metabolic syndrome (Scaini et al. 2018). The other effect of olanzapine was assessed on freshly isolated rat hepatocytes, and the results of the study showed that cytotoxicity of olanzapine leads to hepatotoxicity, which are mediated by mitochondrial potential collapse and oxidative stress (Eftekhari et al. 2016).

In recent years, the new atypical antipsychotics have opened new ways to therapy. While the effects of APs on the activity of individual mitochondrial enzymes are relatively well described, little is known about the effects of APs on complex mitochondrial processes such as respiratory rate. It is hypothesized that various APs modulate synaptic activity and cell energy metabolism, especially OXPHOS and activities of ETC complexes. To verify this hypothesis, we determined the effect of both older and new APs on mitochondrial energy metabolism. We investigated the in vitro effects of three typical APs (chlorpromazine, levomepromazine, and haloperidol) and seven atypical APs (risperidone, ziprasidone, zotepine, aripiprazole, clozapine, olanzapine, and quetiapine) on complex I- and complex II-linked mitochondrial respiration and activities of individual mitochondrial enzymes, CS, and ETC complexes I, II + III, and IV.

Materials and methods

Media and chemicals

The media used for isolation and preservation of mitochondria consisted of sucrose 0.32 mol/L and HEPES 4 mmol/L, buffered to pH 7.4. The Krebs-Henseleit buffer (KH buffer) was composed of 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4 \cdot 7H_2O$, 25 mmol/L $NaHCO_3$, and 11.1 mmol/L glucose (pH 7.4). Mitochondrial respiratory medium (MiR05) was applied in mitochondrial respiration measurements and consisted of sucrose 110 mmol/L, K^+-lactobionate 60 mmol/L, taurine 20 mmol/L, $MgCl_2 \cdot 6H_2O$ 3 mmol/L, KH_2PO_4 10 mmol/L, EDTA 0.5 mmol/L, BSA essentially fatty acid free 1 g/L, and HEPES 20 mmol/L, adjusted to pH 7.1 with KOH (Pesta and Gnaiger 2012). Other media are specified in the chapters describing the measurement of enzyme activities. All chemicals were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA).

Isolation of brain mitochondria

Pig brains were obtained from a slaughterhouse and the mitochondria for the assays were isolated from brain cortex and prepared by a previously described method (Fišar and Hroudová 2016; Fišar et al. 2010; Pinna et al. 2003). Mitochondria were purified on a sucrose gradient and kept on ice until the assay. The freshly purified mitochondria were used for measurements of the mitochondrial oxygen consumption rate (Fišar and Hroudová 2016). The enzyme activity measurements were assayed with the frozen mitochondria (stored at − 70 °C). The Lowry method was applied in order to determine the concentration of proteins; bovine serum albumin was taken as a standard (Lowry et al. 1951).

Activity of citrate synthase and complexes of electron transport chain

The suspension of purified mitochondria with hypotonic buffer (25 mmol/L potassium phosphate and 5 mmol/L $MgCl_2$, pH 7.2) was ultrasonicated three times in order to reach the highest activity of the enzymes. Prepared samples were incubated with particular drugs for 30 min at a temperature of 30 °C. The examined drugs were added at a concentration of 50 μmol/L. Every single measurement was compared with a control set of solvent instead of the drug; the total sample volume was 1 mL. The temperature during the measurement was 30 °C. All the measurement of enzyme activities was carried out spectrophotometrically using Uvicon XL spectrophotometer (SECOMAM, Alès, France).

Complex I (NADH dehydrogenase (ubiquinone), EC 1.6.5.3)

The activity of mitochondrial complex I was measured as the rotenone-sensitive rate of NADH oxidation at 340 nm. The oxido-reductive reaction was initiated by adding coenzyme Q_1 (to a final concentration of 33 μmol/L) and NADH (1 mmol/L) and measured for 3 min (Folbergrová et al. 2010; Hroudova and Fisar 2010). The final protein concentration was higher than that used for other complexes (1 mg/mL) to achieve good quality of the measurement.

Complex II + III (succinate cytochrome c oxidoreductase, EC 1.8.3.1)

The activity of complex II + III was measured as the antimycin A-sensitive rate of cytochrome c reduction (Trounce et al. 1996). The reduction of cytochrome c was monitored by a spectrophotometer at 550 nm for 1 min. Succinate was used as a substrate. The final protein concentration was 500 μg/mL.

Complex IV (cytochrome c oxidase, EC 1.9.3.1)

The principle of the measurement of the cytochrome c oxidase (COX) activity was based on monitoring the decrease in absorbance during the oxidation of reduced cytochrome c at 550 nm for 3 min (Rustin et al. 1994). The final protein concentration was 2.2 μg/mL.

Citrate synthase (EC 2.3.3.1)

In the spectrophotometric measurement of CS activity, 5,5′-dithiobis-(2-nitrobenzoic) acid (DNTB) was observed for a color change. The reaction was started after the addition of oxaloacetate (0.5 mmol/L) and measured at 412 nm for 3 min (Srere 1969). The final protein concentration was 20 μg/mL.

Mitochondrial respiration

Mitochondrial respiration was measured as oxygen consumption rate at 37 °C using the O2k-Respirometer (Oroboros Instruments Corp, Innsbruck, Austria) equipped with Clark-type electrodes. Protocols of the experiments were designed on the basis of experience from previous studies (Fišar et al. 2016b; Hroudová and Fišar 2012; Pesta and Gnaiger 2012). The chambers of the oxygraph were filled by the respiration medium MiR05 (oxygen solubility factor = 0.92) to a final volume of 2 mL. The isolated mitochondria purified on a sucrose gradient were used; the final protein concentration was 0.05–0.20 mg/mL. The specific mitochondrial substrates 2 mmol/L malate, 5 mmol/L pyruvate, and 1 mmol/L ADP were utilized to assess the respiration rate of mitochondria linked to complex I; 0.5 μmol/L rotenone was used to inhibit complex I. For complex II-linked respiration, 1 mmol/L ADP,

0.5 μmol/L rotenone, and 10 mmol/L succinate were added to mitochondria; and 1.25 μg/mL antimycin A was used to inhibit complex III. Two simultaneous measurements were assessed—titration with drug and titration with solvent. The final drug concentrations were in the range 10^0–10^2 μmol/L.

Data analysis and statistics

Activities of mitochondrial enzymes were measured as the slope of time dependence of absorbance using LabPower Junior software (SECOMAM). Relative changes of enzyme activities induced by the drugs were determined assuming that the control sample activity was equal to 100%. High-resolution respirometry data were recorded and analyzed using DatLab software version 4.3 (Oroboros Instruments Corp, Innsbruck, Austria). Respiration rates were expressed as mass-specific oxygen flux (pmol O_2 consumed per second per mg of protein in the sample).

The inhibition of respiration rate was analyzed using the four-parameter logistic regression with SigmaPlot software (Systat Software Inc., Richmond, CA, USA) to establish the half maximal inhibitory concentration (IC_{50}), the Hill slope, and the residual activity at high drug concentration.

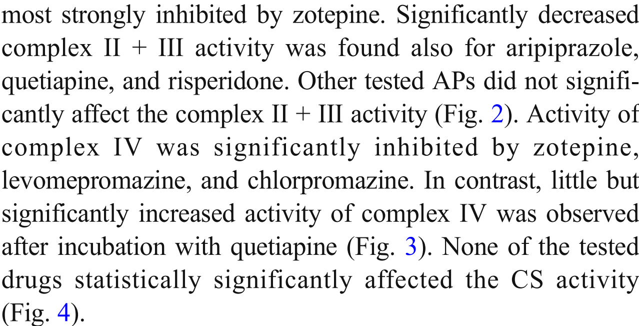
Thea Statistica data analysis software, version 13 (TIBCO Software Inc., Palo Alto, CA, USA) was used for statistical analyses. One-sample t tests for single means were used to determine whether enzyme activity in the sample with drug was significantly different from the control. The data are expressed as the mean ± standard deviation.

Results

The different AP structures seem to be responsible for their various therapeutic and side effects (Jafari et al. 2012). The structures of the compounds analyzed in the present study are shown in Table 1. Therapeutic plasma concentrations of the tested APs (Hiemke et al. 2018) are listed in Table 2.

Activity of CS and respiratory chain complexes

Activities of both CS and ETC complexes were evaluated after 30 min of incubation with 10 pharmacologically different antipsychotics at concentration 50 μmol/L. The most significantly affected was complex I (Fig. 1). Significant inhibitory effects were observed after the incubation with haloperidol, zotepine, aripiprazole, risperidone, clozapine, and quetiapine. Chlorpromazine completely blocked the reaction. Levomepromazine, ziprasidone, and olanzapine did not significantly inhibit complex I. Complex II + III activity was


most strongly inhibited by zotepine. Significantly decreased complex II + III activity was found also for aripiprazole, quetiapine, and risperidone. Other tested APs did not significantly affect the complex II + III activity (Fig. 2). Activity of complex IV was significantly inhibited by zotepine, levomepromazine, and chlorpromazine. In contrast, little but significantly increased activity of complex IV was observed after incubation with quetiapine (Fig. 3). None of the tested drugs statistically significantly affected the CS activity (Fig. 4).

Mitochondrial respiration

Inhibitory effects of tested antipsychotics on mitochondrial respiration rate are shown in Figs. 5 and 6, except for chlorpromazine, whose inhibitory curves were published in our previous work (Hroudová and Fišar 2012). The parameters characterizing the strength and type of inhibition are summarized in Tables 2 and 3.

Complex I-linked respiration was strongly inhibited by aripiprazole, zotepine, and haloperidol. These drugs were full inhibitors of mitochondrial respiration with IC_{50} 13.1, 39.5, and 64.9 μmol/L, respectively. Risperidone and quetiapine were also found as full inhibitors, and the inhibitory effects were observed at higher concentrations (IC_{50} 263 and 424 μmol/L, respectively). Other examined antipsychotics (ziprasidone, clozapine, and levomepromazine) were revealed as partial inhibitors of complex I-linked respiration with IC_{50} in the range 10^{-4} mol/L (Table 2). The AP/rotenone ratio represents the ratio of IC_{50} values determined for AP and for rotenone (Fišar et al. 2017) and approximately expresses the relative inhibitory capacity of the AP relative to rotenone; it should be noted that this ratio does not capture partial inhibition.

Full inhibition of complex II-linked respiration was observed after exposition by zotepine, quetiapine, and clozapine with IC_{50} 107, 491, and 650 μmol/L, respectively. Other antipsychotics were partial inhibitors of complex II-linked respiration (Table 3). The AP/antimycin ratio represents the ratio of IC_{50} values determined for AP and for antimycin A and approximately expresses the relative inhibitory capacity of the AP relative to antimycin A.

Correlations

Correlation between relative complex I activity and complex I-linked respiration in isolated pig brain mitochondria after the addition of atypical AP at a final concentration of 50 μmol/L to the sample (Fig. 7) was found rather weak ($r = 0.753$, $p = 0.051$, $N = 7$).

No significant correlation was found between the therapeutic concentration of AP calculated as mean of lower and upper limit of the therapeutic reference range (Table 2) and the AP-

Table 1 Structures of examined typical and atypical antipsychotics

Structure	Antipsychotic *Chemical class*	Structure	Antipsychotic *Chemical class*
	Chlorpromazine *Phenothiazines (with aliphatic side-chain)*		Aripiprazole *Phenylpiperazines*
	Levomepromazine *Phenothiazines (with aliphatic side-chain)*		Zotepine *Dibenzothiepines*
	Haloperidol *Butyrophenones*		Clozapine *Dibenzodiazepines (tetracycle)*
	Risperidone *Benzisoxazoles*		Olanzapine *Thienobenzodiazepines (clozapine-like)*
	Ziprasidone *Indoles*		Quetiapine *Dibenzothiazepines*

induced inhibition of individual respiratory complexes (Figs. 1, 2, and 3), including the complex I activity at 50 μmol/L AP concentration ($r = 0.280$, $p = 0.43$, $N = 10$). Similarly, there was no statistically significant correlation between therapeutic concentration and IC_{50} for both the complex I-linked respiration ($r = 0.369$, $p = 0.29$, $N = 10$) and complex II-linked respiration ($r = 0.457$, $p = 0.18$, $N = 10$).

Discussion

The in vitro effects of pharmacologically different APs on mitochondrial energy metabolism were examined in isolated pig brain mitochondria. Activities of both CS and ETC complexes and mitochondrial respiration rate were studied with the aim of supporting the hypothesis that AP-induced mitochondrial dysfunction can result in impaired cell energy metabolism and likely in adverse effects of APs.

Antipsychotics and complex I activity

Seven of the 10 APs tested showed a significant inhibitory effect on the complex I. Chlorpromazine and haloperidol, conventional APs, have been found to be the strongest inhibitors of complex I (Fig. 1), which confirmed earlier findings that conventional APs are more potent inhibitors of complex I than atypical APs (Balijepalli et al. 1999; Burkhardt et al. 1993; Prince et al. 1997). However, this is not generally the case since levomepromazine showed less complex I inhibito-ry effects than some atypical APs. Our results closely correspond to an in vitro study in which effects of APs were examined in rat liver mitochondria (Modica-Napolitano et al. 2003). In that study, (1) the typical APs (chlorpromazine, haloperidol, and thioridazine) inhibited complex I enzyme activity more strongly than the atypical APs (risperidone, quetiapine, clozapine, and olanzapine) with the least inhibitory effect observed for olanzapine and clozapine, and (2) complex I-linked mitochondrial respiration was inhibited by chlorpromazine, haloperidol, risperidone, and quetiapine but not by clozapine, olanzapine, or thioridazine.

Complex I plays a decisive role in the control of OXPHOS and its altered activity could lead to impairment in cell energy metabolism and subsequently to changes in neuronal activity (Pathak and Davey 2008). Based on the observation that conventional antipsychotics with a higher risk of causing undesirable side

Table 2 Antipsychotic-induced inhibition of complex I-linked mitochondrial respiration

Drug	IC_{50} (μmol/L)	Residual (rel.u.)	Hill slope	N	Inhibition	Range of drug concentrations (μmol/L)	AP/ rotenone	Therapeutic reference range[b] (μmol/L)
Chlorpromazine[a]	116 ± 11	0.530 ± 0.025	1.67 ± 0.19	8	Partial	0.5–500	3222	0.09–0.94
Levomepromazine	336 ± 17	0.296 ± 0.041	− 3.34 ± 0.49	3	Partial	5–750	9333	0.09–0.49
Haloperidol	64.9 ± 4.6	− 0.015 ± 0.045	− 2.25 ± 0.31	3	Full	5–225	1803	0.003–0.026
Risperidone	263 ± 26	− 0.010 ± 0.029	− 0.84 ± 0.06	3	Full	5–750	7305	0.05–0.15
Zotepine	39.5 ± 2.7	− 0.004 ± 0.056	− 2.43 ± 0.32	7	Full	5–188	1097	0.03–0.45
Ziprasidone	188 ± 27	0.209 ± 0.066	− 1.52 ± 0.28	3	Partial	5–750	5222	0.12–0.48
Aripiprazole	13.1 ± 1.3	− 0.016 ± 0.056	− 1.85 ± 0.29	3	Full	0.6–150	364	0.22–0.78
Clozapine	281 ± 26	0.167 ± 0.071	− 3.09 ± 0.71	3	Partial	5–750	7805	1.07–1.84
Olanzapine	198 ± 221	0.964 ± 0.024	1.55 ± 1.50	9	Partial, very little	0.5–750	5500	0.06–0.26
Quetiapine	424 ± 17	− 0.045 ± 0.028	− 1.95 ± 0.11	3	Full	5–750	11,778	0.28–1.30

Values are means ± standard error. The AP/rotenone ratio represents the ratio of IC_{50} values determined for the antipsychotic (AP) and for rotenone. The IC_{50} value of 0.036 μmol/L for rotenone was used from our previous study (Fišar et al. 2017)

IC_{50} half maximal inhibitory concentration, N number of measurement, Residual residual activity at high drug concentration

[a] Inhibitory parameters of chlorpromazine were used from our previous study (Hroudová and Fišar 2012)

[b] Therapeutic reference range represents a range of drug concentrations in blood (below lower limit: a drug-induced therapeutic response is relatively unlikely to occur; above upper limit: tolerability decreases or further therapeutic improvement is unlikely) (Hiemke et al. 2018)

effects are stronger inhibitors of the complex I than atypical antipsychotics, it has been suggested that the degree of complex I inhibition is likened, at least partially, to potency for the extrapyramidal effect, tardive dyskinesia included (Burkhardt et al. 1993; Maurer and Möller 1997; Maurer and Volz 2001; Modica-Napolitano et al. 2003). Our observation that levomepromazine, which causes side effects such as akathisia, does not significantly inhibit the complex I could be explained by the fact that some of the adverse effects of APs can be attributed to mitochondrial dysfunction and other direct changes in neurotransmitter systems.

Spectrophotometric measurement of a complex I activity refers to a high-resolution respirometry measurement of mitochondrial respiration. All significant inhibitors of complex I were full (haloperidol, risperidone, zotepine, aripiprazole, and quetiapine), or almost full (clozapine), inhibitors of complex I-linked respiration

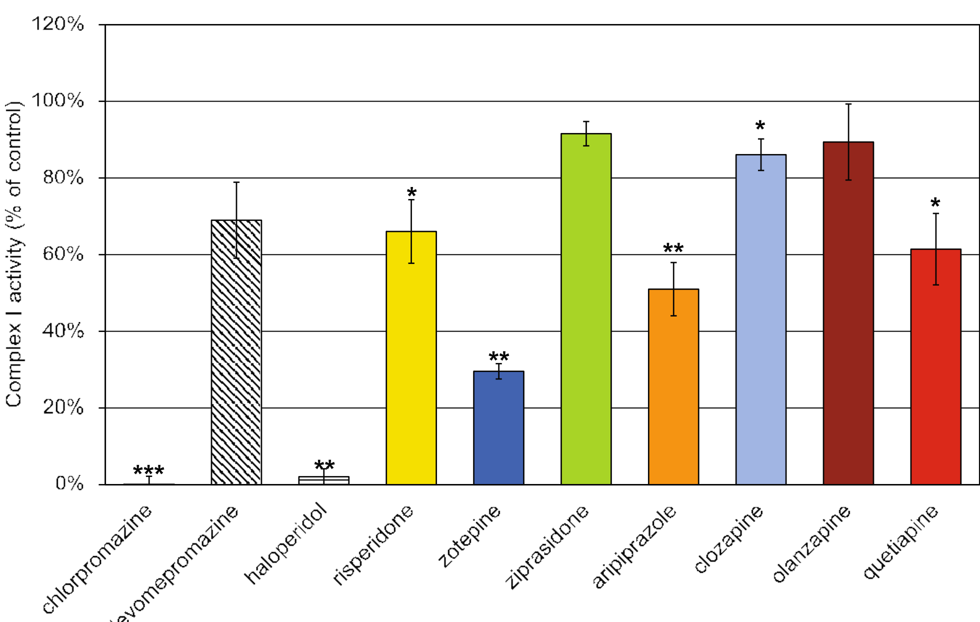


Fig. 1 Effects of antipsychotics on respiratory chain complex I activity in brain mitochondria. The isolated mitochondria purified on a sucrose gradient were incubated with a drug at 30 °C for 30 min and enzyme kinetic was measured spectrophotometrically as described in the section BMaterials and methods.^ Relative activity is displayed (100% = activity of control sample without the drug). Values are means ± standard deviation of at least three independent measurements. Comparison between control and sample with drug was performed using the Wilcoxon matched pairs test (*p < 0.05, **p < 0.01, ***p < 0.001)

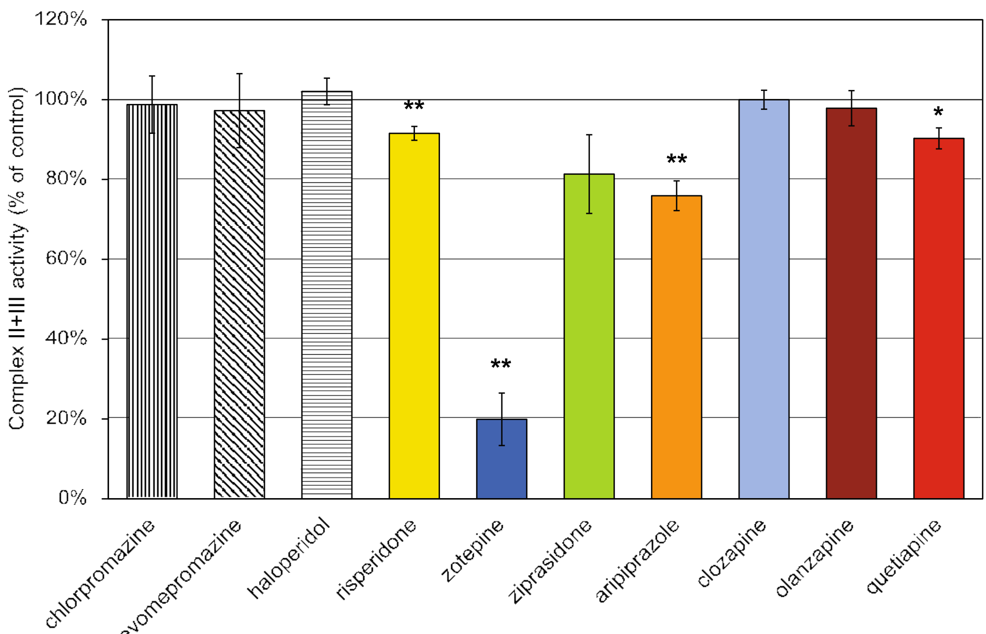


Fig. 2 Effects of antipsychotics on respiratory chain complex II + III activity in brain mitochondria. The isolated mitochondria purified on a sucrose gradient were incubated with a drug at 30 °C for 30 min and enzyme kinetic was measured spectrophotometrically as described in the section BMaterials and methods.^ Relative activity is displayed (100% = activity of control sample without the drug). Values are means ± standard deviation of at least three independent measurements. Comparison between control and sample with drug was performed using the Wilcoxon matched pairs test (*p < 0.05, **p < 0.01)

rate, confirming the causal association of inhibition of complex I activity and inhibition of the overall mitochondrial respiratory rate. However, correlation between complex I activity and complex I-linked respiration at high AP concentration (Fig. 7) indicates that association between complex I activity and complex I-linked respiration is not trivial.

Our results support another study, where chlorpromazine, haloperidol, risperidone, and clozapine affected ATP production (depending on the AP concentration), led to a significant increase in lactate production, led to a significant reduction in mitochondrial complex I activity, and lastly decreased oxygen consumption rates and mitochondrial membrane potential in rat ovarian theca cells (Elmorsy et al. 2017).

Antipsychotics and complex II + III activity

Significant inhibitors of complex II + III, zotepine and quetiapine, were full inhibitors of complex II-linked respiration rate. Olanzapine, which showed no inhibitory

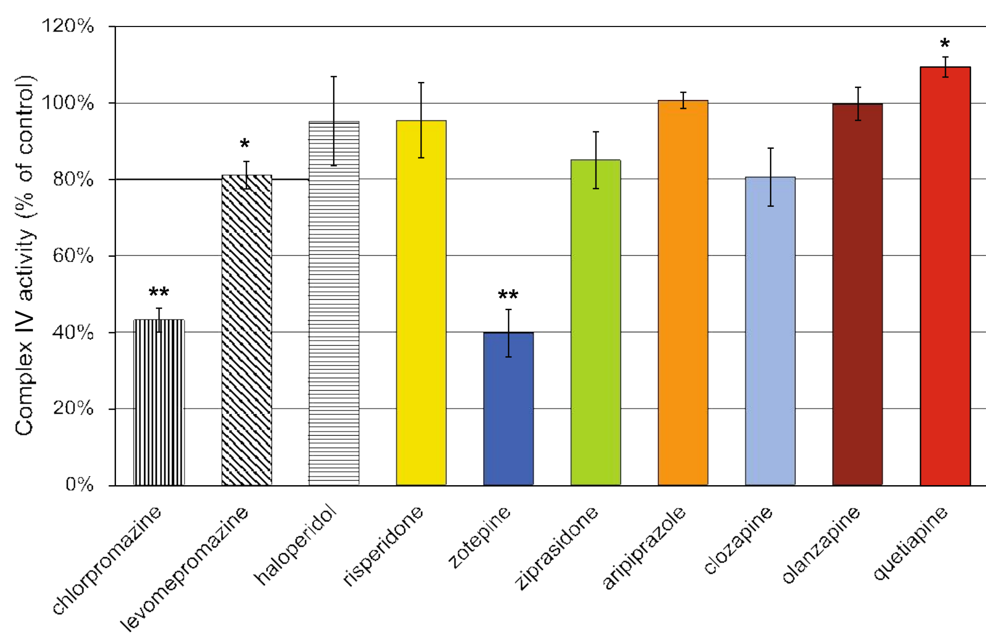


Fig. 3 Effects of antipsychotics on respiratory chain complex IV activity in brain mitochondria. The isolated mitochondria purified on a sucrose gradient were incubated with a drug at 30 °C for 30 min and enzyme kinetic was measured spectrophotometrically as described in the section BMaterials and methods.^ Relative activity is displayed (100% = activity of control sample without the drug). Values are means ± standard deviation of at least three independent measurements. Comparison between control and sample with drug was performed using the Wilcoxon matched pairs test (*p < 0.05, **p < 0.01)

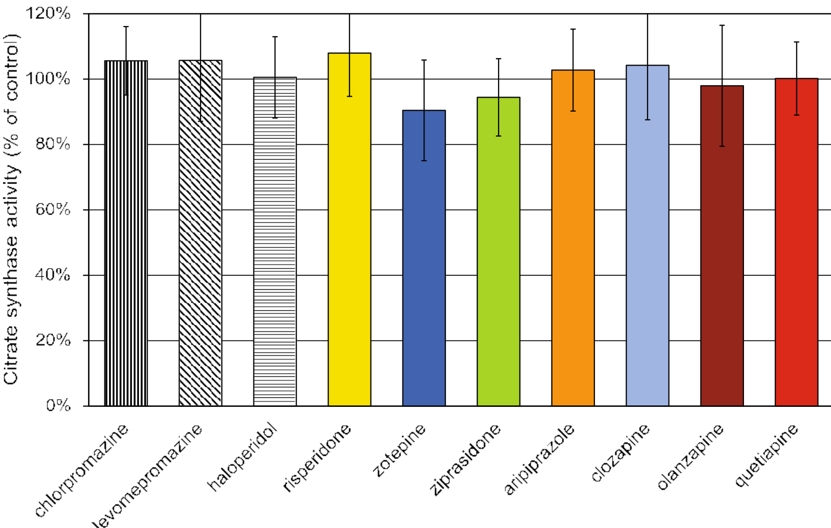


Fig. 4 Effects of antipsychotics on citrate synthase (CS) activity in brain mitochondria. The isolated mitochondria purified on a sucrose gradient were incubated with a drug at 30 °C for 30 min and enzyme kinetic was measured spectrophotometrically as described in the section BMaterials and methods.^ Relative activity is displayed (100% = activity of control sample without the drug). Values are means ± standard deviation of at least three independent measurements. Comparison between control and sample with drug was performed using the Wilcoxon matched pairs test (*$p < 0.05$, **$p < 0.01$)

effects on the activities of the complexes I, II + III, and IV, also did not substantially inhibit the mitochondrial respiratory rate. Risperidone and aripiprazole inhibited complex II + III, however, were only partial inhibitors of complex II-linked respiration, indicating that the association of complex II + III inhibition with inhibition of mitochondrial respiration is less tight than that of complex I inhibition.

Antipsychotics and complex IV activity

Chlorpromazine, levomepromazine, and zotepine significantly inhibited the activity of complex IV. Other antipsychotic drugs tested showed no inhibitory effects on complex IV; quetiapine showed a stimulatory effect (Fig. 3). Our data are consistent with another study in which haloperidol, olanzapine, clozapine, and aripiprazole did not significantly

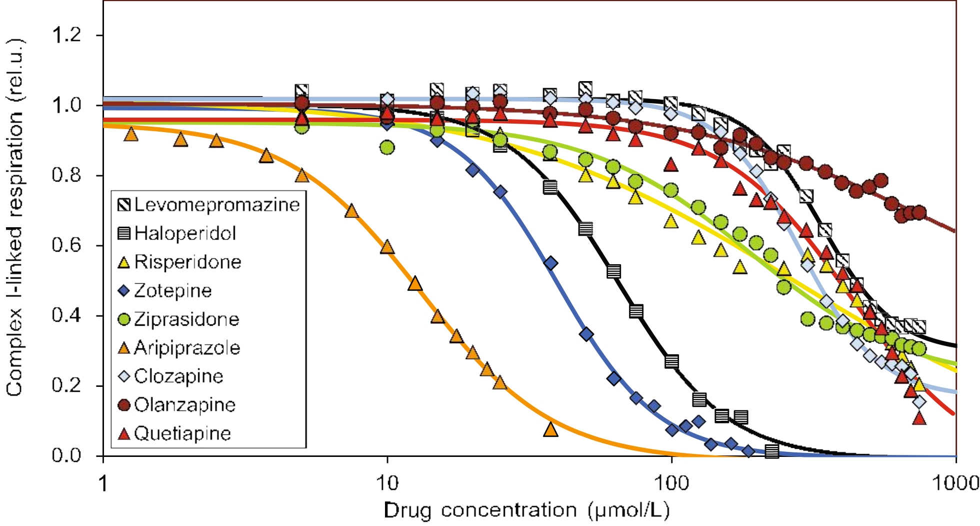


Fig. 5 Drug effect on complex I-linked mitochondrial respiration in isolated pig brain mitochondria. Dose-response curves are displayed as plots of the relative respiratory rate against the drug concentration. The samples were continuously stirred and incubated at 37 °C in two chambers: drug titration in one chamber and buffer titration in the second chamber were performed at 2–3 min intervals. Following subtraction of residual oxygen consumption from all respiratory rates, the time change in respiratory rate during titration was corrected and relative drug-induced changes in respiratory rate were determined, presuming that the relative respiratory rate equals to one at zero drug concentration. Points represent means from at least three independent measurements. Lines represent the best-fitted curves using the four-parameter logistic function. Half maximal inhibitory concentration (IC_{50}), residual activity, and Hill slope were calculated (Table 2)

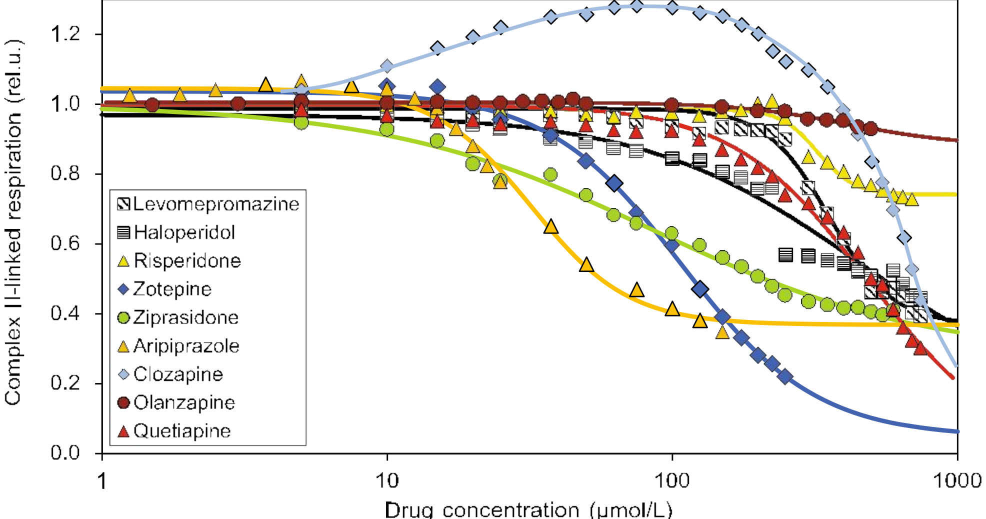


Fig. 6 Drug effect on complex II-linked mitochondrial respiration in isolated pig brain mitochondria. Dose-response curves are displayed as plots of the relative respiratory rate against the drug concentration. The samples were continuously stirred and incubated at 37 °C in two chambers: drug titration in one chamber and buffer titration in the second chamber were performed at 2–3 min intervals. Following subtraction of residual oxygen consumption from all respiratory rates, the time change in respiratory rate during titration was corrected and relative drug-induced changes in respiratory rate were determined, presuming that the relative respiratory rate equals to one at zero drug concentration. Points represent means from at least three independent measurements. Lines represent the best-fitted curves using the four-parameter logistic function. Half maximal inhibitory concentration (IC_{50}), residual activity, and Hill slope were calculated (Table 3)

affect the activity of complex IV (Streck et al. 2007) or haloperidol and olanzapine did not significantly affect changes in COX subunit expression in rat brains after chronic administration (Rice et al. 2014). Complex IV represented by cytochrome c oxidase is often considered as an endogenous metabolic marker of neuronal activity (Wong-Riley 1989). Chlorpromazine selectively and significantly inhibited the growth and proliferation of glioma cells by switching the expression of the COX4-1 regulatory subunit. Thus, chlorpromazine could be used in the future for treating chemoresistant glioma on the basis of antiproliferative activity relating to the inhibition of complex IV activity (Oliva et al. 2017).

We confirmed that chlorpromazine acts as an eminent inhibitor of mitochondrial complex I and complex IV (Figs. 1 and 3). However, both complex I- and complex II-linked

Table 3 Antipsychotic-induced inhibition of complex II-linked mitochondrial respiration

Drug	IC_{50} (μmol/L)	Residual (rel.u.)	Hill slope	N	Inhibition	Range of drug concentrations (μmol/L)	AP/ antimycin
Chlorpromazine[a]	263 ± 33	0.395 ± 0.072	2.38 ± 0.45	10	Partial	0.5–600	42,147
Levomepromazine	359 ± 36	0.365 ± 0.071	− 3.90 ± 0.96	6	Partial	5–750	57,532
Haloperidol	467 ± 230	0.106 ± 0.241	− 1.14 ± 0.23	6	Partial	5–750	74,840
Risperidone	326 ± 23	0.740 ± 0.022	− 6.69 ± 2.50	3	Partial	5–700	52,244
Zotepine	107 ± 26	0.047 ± 0.190	− 1.84 ± 0.53	6	Full	5–250	17,147
Ziprasidone	86 ± 41	0.280 ± 0.138	− 0.92 ± 0.34	3	Partial	5–600	13,782
Aripiprazole	32.0 ± 2.8	0.369 ± 0.036	− 2.54 ± 0.44	3	Partial	0.6–150	5128
Clozapine	650 ± 24	− 0.001 ± 0.053	− 3.28 ± 0.35	3	Full	5–750	104,167
Olanzapine	419 ± 214	0.878 ± 0.069	2.16 ± 0.76	7	Partial, very little	0.5–500	67,147
Quetiapine	491 ± 18	− 0.015 ± 0.027	− 1.83 ± 0.10	3	Full	5–750	78,686

The AP/antimycin ratio represents the ratio of IC_{50} values determined for the antipsychotic (AP) and for antimycin A. The IC_{50} value of 0.00624 μmol/L for antimycin A was used from our previous study (Fišar et al. 2017). Values are means ± standard error

IC_{50} half maximal inhibitory concentration, N number of measurement, Residual residual activity at high drug concentration

[a] Inhibitory parameters of chlorpromazine were used from our previous study (Hroudová and Fišar 2012)

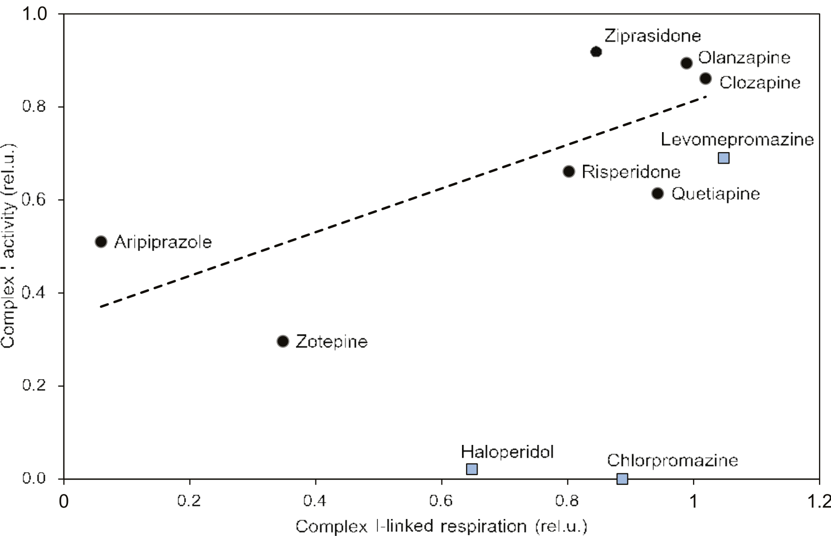


Fig. 7 Correlation between relative complex I activity and complex I-linked respiration in isolated pig brain mitochondria after the addition of antipsychotics at a final concentration of 50 μmol/L to the sample. The dotted line shows the correlation for atypical antipsychotics ($r = 0.753$, $p = 0.051$, $N = 7$)

mitochondrial respiration is only partially inhibited by chlorpromazine and at higher concentrations (Tables 2 and 3) (Hroudová and Fišar 2012). This can explain why the mitochondrial toxicity of chlorpromazine is not as high as one would expect from its effects on individual respiratory chain complexes. In contrast, zotepine exhibited inhibitory effects on individual ECT complexes and at the same time full inhibition of complex I- and complex II-linked respiration. There appears to be no simple correlation between the effects of drugs on individual ETC complexes and complex mitochondrial functions such as oxygen consumption rate or ATP production. We assume that this is due to the different drug-mitochondria interaction and different drug-induced compensatory or neuroprotective mechanisms (Robertson et al. 2019; Scatena et al. 2007), which can reduce the mitochondrial toxicity of drugs.

Antipsychotics and citrate synthase activity

CS is an enzyme localized in the mitochondrial matrix; it holds a regulatory function within cell energy metabolism. We did not find any significant AP-induced changes in CS activity (Fig. 4). In another study, aripiprazole enhanced the activity of CS, while clozapine and haloperidol did not affect CS activity in PC12 cells (Ota et al. 2012). Acute treatment with olanzapine inhibited CS activity in the cerebellum and prefrontal cortex, while the acute administration of olanzapine increased CS activity in the prefrontal cortex, hippocampus, and striatum in rats (Agostinho et al. 2011). Our in vitro study shows that the effects of APs on CS activity are small and are therefore probably not involved in the therapeutic or side effects of APs.

Possible mitochondria-related side effects of antipsychotics

Our observation that there is no significant correlation between the therapeutic concentration of APs and AP-induced inhibition of mitochondrial enzymes supports the hypothesis that the direct mitochondrial effects of APs are associated with their adverse rather than therapeutic effects.

Based on our data and already established facts about prolonged QTc intervals after treatment with APs (Leucht et al. 2013; Taylor 2003b), we noticed a relation between inhibition of complex I and prolongation of the QTc interval. AP-induced inhibition of complex I in our study (Fig. 1) may be associated with QTc inter-val prolongation (e.g., chlorpromazine, clozapine, haloperidol, risperidone, quetiapine, and ziprasidone) (Glassman and Bigger 2001; Haddad and Anderson 2002; Leucht et al. 2013; Spellmann et al. 2018; Taylor 2003a; Taylor 2003b; Vieweg 2003). In spite of this, ziprasidone, which causes the significant degree of drug-induced QTc interval prolongation, did not inhibit complex I activity, and aripiprazole, which effect did not differ from QTc prolongation with placebo (Leucht et al. 2013), inhibited complex I activity. Further basic research and consequent clinical trials are required to confirm that there is QTc interval prolongation as an adverse effect stemming from alterations in ETC enzyme activity.

Treatment with second-generation APs is accompanied with an increase in cardiovascular risk through insulin resistance, hypercholesterolemia, and accelerated weight gain. Altogether, these adverse effects might be called the metabolic syndrome. The metabolic side effects of APs could be connected with alterations in mitochondrial homeostasis, which

leads to an imbalance in the mitochondrial fusion/fission ratio and to an inefficient mitochondrial phenotype of muscle cells (Del Campo et al. 2018). Olanzapine induced a downregulation of genes involved in the mitochondrial enzymes of the ETC, as well as decreased enzyme activity, ATP synthesis, and oxygen consumption in blood cells of patients at elevated risk for metabolic syndrome (Scaini et al. 2018). The other effect of olanzapine was assessed on freshly isolated rat hepatocytes, and the results of the study showed cytotoxicity of olanzapine leads to hepatotoxicity, which are mediated by mitochondrial potential collapse and oxidative stress (Eftekhari et al. 2016). Autophagy of mitochondria in the SH-SY5Y neuronal cell line in humans was observed after olanzapine exposition. Thus, olanzapine induced mitochondrial damage related to ROS production and extensive mitochondrial depolarization, provoking the autophagic clearance of dysfunctional mitochondria (Vucicevic et al. 2014).

Experimental approach

The suitability of using (1) pig brain mitochondria as a biological model, (2) mitochondrial isolation method and experimental protocol of mitochondrial respiration measurement, and (3) concentration of APs used in in vitro experiments may be discussed. (1) Pig is a relatively uncommon species for pharmacological studies, but pig brain mitochondria are successfully used in measuring the effect of drugs on mitochondrial respiration (Fišar and Hroudová 2016; Fišar et al. 2016b; Hroudová and Fišar 2012). It seems that pig mitochondria are more similar to human mitochondria than rodent mitochondria (Hroudova and Fisar 2010). (2) Different laboratories differ in both the method of preparing the mitochondria (the origin of the animal, the tissue, and the purification) and the method of measuring the oxygen consumption. To compare the mitochondrial effects of drugs, the effects of a known inhibitor (e.g., rotenone for complex I-linked respiration and antimycin A for complex II-linked respiration) should always be measured using the same experimental protocols (Fišar et al. 2017). Therefore, we also calculated the IC_{50} ratios AP/rotenone (Table 2) or AP/antimycin A (Table 3). (3) The use of examined APs at high (50 μmol/L) concentration for in vitro experiments could be relevant in a case of intraneuronal accumulation of some APs in the brain, e.g., zotepine reaches brain levels of the unchanged drug 20 to 30 times higher than serum levels (Noda et al. 1979).

Possibilities of structure-activity relationship studies

Structures of mitochondrial complexes and APs can contribute significantly to understanding the mechanisms associated with the regulation of cellular energy by drugs such as APs. Quantitative structure-activity relationship (QSAR) studies are widely used to develop new drugs and their toxicological analysis (Jafari et al. 2012). However, the QSAR model has not been used to study the interactions of APs with mitochondria and respiratory chain complexes, respectively.

Complex I is the largest enzyme of the mitochondrial respiratory chain consisting of 45 subunits arranged in hydrophilic and membrane domains. It has a key role in ATP production, is a major source of ROS, and its dysfunctions have been associated with aging and neurodegenerative disorders (Schapira 1998). Crystal structure of bacterial complex I has been determined (Efremov and Sazanov 2011); however, the atomic structure of mammalian complex I with sufficient resolution has only recently been achieved using single particle cryo-electron microscopy (Fiedorczuk et al. 2016; Fiedorczuk and Sazanov 2018). The structures of complexes II (Iverson 2013; Sun et al. 2005), III (Hunte et al. 2000; Hunte et al. 2008; Iwata et al. 1998), and IV (Tsukihara et al. 1995) were determined earlier by X-ray crystallography. The complexes I, III, and IV may form supercomplexes (respirasomes) whose function is still under investigation (Letts and Sazanov 2017; Sousa et al. 2018).

It is known that chlorpromazine acts as a potent inhibitor of mitochondrial complex I. In our study, complex I activity was strongly inhibited by haloperidol, which is structurally different from chlorpromazine. In contrast, complex I activity was not inhibited by levomepromazine, which is similar to chlorpromazine. The different effects of these typical APs could be related to chlorine on chlorpromazine. Similarly, no similar inhibitory effects on complex I were observed in structurally similar atypical APs, except for similar effects of clozapine and olanzapine. There was no association between the structure of APs and their effect on the activity of complex II + III. From the inhibitory effects of the tested APs on the activity of complex IV, only the possible role of chlorine and tricyclic structure in the inhibition by chlorpromazine or zotepine can be concluded. The observation that chlorpromazine is a full complex I inhibitor, but only a partial inhibitor of complex I-linked respiration in intact mitochondria, suggests the possible role of the lipid part of the inner mitochondrial membrane in AP effects.

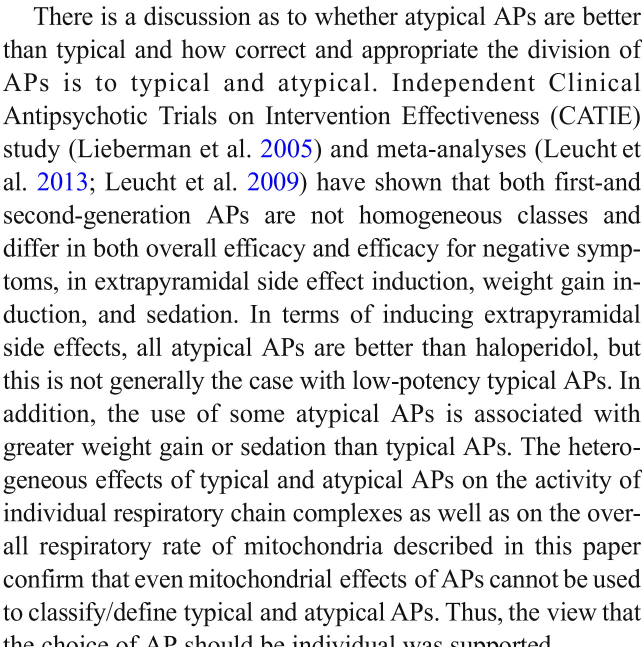
We did not observe the effect of certain molecular structures of the tested APs on their mitochondrial effects, assessed by their effect on the overall respiratory rate. In addition, some APs fully inhibit the measured mitochondrial function, some partially, and the biphasic course of complex II-linked respiration was even observed for clozapine effect (Figs. 5 and 6). We assume that this is due to the complexity

of the respiratory chain complex structures that consist of many subunits; it is known that dysfunction of some subunits is lethal for the function of the entire complex. Partial or full inhibition of mitochondrial respiration appears to be due to the cumulative effect of APs on the subunits of the individual complexes and possibly on the lipid part of the inner mitochondrial membrane. It can be hypothesized that while the therapeutic effects of APs are associated with their specific binding to neurotransmitter receptors and transporters, the mitochondrial side effects of APs are associated with AP-induced multiple allosteric regulation of various ETC components.

The structure-activity relationship of APs should be studied in terms of their effects on both individual mitochondrial complexes and supercomplexes. Obviously, recognizing the structural parts of the AP molecules responsible for their effect on mitochondrial functions would facilitate the development of new drugs. Although the possibilities and results of molecular modeling of AP interactions with mitochondrial complexes and supercomplexes are currently very uncertain and difficult to interpret, structure-activity relationship (SAR) studies on antipsychotics are essential to identify the potential modulatory sites not only on neurotransmitter receptors and transporters but also on mitochondrial proteins. We believe that in subsequent studies, attention should be paid to both SAR and the search for specific binding sites for APs in respiratory complexes using biochemical and biophysical methods.

Final notes

Schizophrenia frequently exhibits a neuroprogression and new APs should inhibit pathways of neurodegeneration, including mitochondrial dysfunction (Robertson et al. 2019). It is therefore necessary to study the mitochondrial effects of both existing and novel drugs. A comparison of the effects of antipsychotics on individual mitochondrial enzymes or enzyme complexes and on complex mitochondrial functions, such as oxygen consumption rate, has shown that complex mitochondrial functions may not fully reflect the disruption of individual components of ETC. It can be hypothesized that this is due both to the reversibility of AP-induced inhibition of ETC complexes and to the existence of feedbacks in the OXPHOS system leading to adaptive mitochondrial changes, similar to those described in vivo in platelets of patients with neurodegenerative disease (Fišar et al. 2016a; Fišar et al. 2019). Measurement of respiratory rate in mitochondria is much closer to the physiological situation and describes more suitably mitochondrial toxicity of drugs than measurement of inhibitory effects on individual ETC complexes. However, measuring the effects of drugs on individual complexes can help, at least in part, to clarify the drug-induced mitochondrial dysfunction mechanism.


There is a discussion as to whether atypical APs are better than typical and how correct and appropriate the division of APs is to typical and atypical. Independent Clinical Antipsychotic Trials on Intervention Effectiveness (CATIE) study (Lieberman et al. 2005) and meta-analyses (Leucht et al. 2013; Leucht et al. 2009) have shown that both first-and second-generation APs are not homogeneous classes and differ in both overall efficacy and efficacy for negative symptoms, in extrapyramidal side effect induction, weight gain induction, and sedation. In terms of inducing extrapyramidal side effects, all atypical APs are better than haloperidol, but this is not generally the case with low-potency typical APs. In addition, the use of some atypical APs is associated with greater weight gain or sedation than typical APs. The heterogeneous effects of typical and atypical APs on the activity of individual respiratory chain complexes as well as on the overall respiratory rate of mitochondria described in this paper confirm that even mitochondrial effects of APs cannot be used to classify/define typical and atypical APs. Thus, the view that the choice of AP should be individual was supported.

Conclusions

From the obtained data, APs affected both ETC mitochondrial enzyme activities and mitochondrial respiration. The typical APs did not show greater potency in alteration of mitochondrial functions, except for complex I inhibition by chlorpromazine and haloperidol, compared to the atypical APs. AP-induced mitochondrial dysfunctions can be expected to be associated with adverse effects of APs related to neurodegenerative processes induced by bioenergetic disruption. The diverse but largely inhibitory effects of both typical and atypical APs on complex I- or II-linked mitochondrial respiration confirm that the direct effects of APs on cell energy are associated with adverse effects of APs rather than their therapeutic effects. However, it can be considered that the possible neuroprotective effects of some APs, as well as the adaptive processes induced by the inhibition of ETC complexes, may result in greater or lesser compensation of the undesirable mitochondrial toxicity of APs.

The precise molecular mechanism by which various APs affect mitochondrial enzymes must be still determined. In further clinical research, it must specified whether AP-induced mitochondrial dysfunction is causally related to clinically manifested effects, such as extrapyramidal syndromes, QTc interval prolongation, or metabolic syndrome. Due to the complex processes associated with mitochondrial activity, it is necessary to measure not only the effect of test substances on individual ETC complexes but also on the total respiration rate of the mitochondria. The experimental methods used in the present study can be applied to mitochondrial toxicity testing of newly developed APs.

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Author's contribution JH and ZF conceived and designed research. TC, YB, ML, and JH conducted experiments. TC, JH, and ZF analyzed data and wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

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REVIEW

Important Role of Mitochondria and the Effect of Mood Stabilizers on Mitochondrial Function

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Summary

Mitochondria primarily serve as source of cellular energy through the Krebs cycle and β-oxidation to generate substrates for oxidative phosphorylation. Redox reactions are used to transfer electrons through a gradient to their final acceptor, oxygen, and to pump hydrogen protons into the intermembrane space. Then, ATP synthase uses the electrochemical gradient to generate adenosine triphosphate (ATP). During these processes, reactive oxygen species (ROS) are generated. ROS are highly reactive molecules with important physiological functions in cellular signaling. Mitochondria play a crucial role in intracellular calcium homeostasis and serve as transient calcium stores. High levels of both, ROS and free cytosolic calcium, can damage mitochondrial and cellular structures and trigger apoptosis. Impaired mitochondrial function has been described in many psychiatric diseases, including mood disorders, in terms of lowered mitochondrial membrane potential, suppressed ATP formation, imbalanced Ca^{2+} levels and increased ROS levels. In vitro models have indicated that mood stabilizers affect mitochondrial respiratory chain complexes, ROS production, ATP formation, Ca^{2+} buffering and the antioxidant system. Most studies support the hypothesis that mitochondrial dysfunction is a primary feature of mood disorders. The precise mechanism of action of mood stabilizers remains unknown, but new mitochondrial targets have been proposed for use as mood stabilizers and mitochondrial biomarkers in the evaluation of therapy effectiveness.

Key words

Mitochondria • Oxidative phosphorylation • Reactive oxygen species • Calcium • Mitochondrial signaling • Mood stabilizers

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Introduction

Mitochondria as cellular organelles

The mitochondrion is one of the largest organelles in human cells, occupying approximately 25 % of the cytoplasmic volume. Evolutionarily, they are presumed to be descendants of bacteria on the basis of the endosymbiotic hypothesis of mitochondria origin. This hypothesis may explain the origin of the two organelle membranes border the two mitochondrial compartments: the intermembrane space and the matrix. The outer mitochondrial membrane is smooth and permeable to solutes smaller than 5 kDa. The inner mitochondrial membrane (IMM) is mostly impermeable and contains many transport channels. Its surface is enlarged by cristae, with a higher concentration of integral proteins, which enable adenosine triphosphate (ATP) formation (Lodish *et al.* 2013, Srivastava *et al.* 2018). In addition to ATP production, mitochondria have a role in Ca^{2+} homeostasis, production and disposal of reactive oxygen species (ROS), apoptosis regulation and thermoregulation (de Sousa *et al.* 2014).

Mitochondria are highly dynamic organelles that undergo frequent fusion (merging) and fission (dividing). Fission and fusion were likely developed to prevent the

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accumulation of mutated mitochondrial DNA. They are optimal tools for the isolating of damaged segments. Disrupted mitochondrial dynamics are often linked to genetic disorders, indicating that mitochondrial dynamics play important roles in mitochondrial physiology (Lodish *et al.* 2013).

Mitochondria as energy factories

The primary function of mitochondria is to use products of glycolysis, proteolysis, or lipolysis and through a series of a biochemical reactions leading to ATP formation (Andreazza and Nierenberg 2018). ATP is created *via* oxidative phosphorylation (OXPHOS) using the electron transport chain (ETC) as the machinery (Clay *et al.* 2011).

The pyruvate dehydrogenase complex, which converts pyruvate into acetyl-coenzyme A (acetyl-CoA), is able to control carbon entry from two main sources: carbohydrates and glucogenic amino acids. Fatty acids are metabolized in another way; first, they are converted into acyl-coenzyme A, attached to carnitine, and shuttled into the mitochondria. Second, acyl-CoA undergoes β-oxidation and results in the production of acetyl-CoA. Acetyl group from acetyl-CoA is oxidized into CO_2 concurrently with the production of substrates for OXPHOS: the reduced forms of high-energy carriers nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). The presence of oxygen is necessary to regenerate the oxidized forms of the electron carriers nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD^+) (Lodish *et al.* 2013, de Sousa *et al.* 2014).

Mitochondria function through a highly effective transport gradient in which electrons move from acceptors with less electronegativity to those with greater electronegativity at the same time that the redox reactions involving both, electron donors and acceptors are in progress. Simultaneously, through electron transport, H^+ ions are pumped into the intermembrane space (IMS), increasing the pH in the matrix, which increases its electronegativity, thereby creating proton motive force (PMF) (Divakaruni and Brand 2011, Lodish *et al.* 2013).

The ETC consists of 4 complexes with supramolecular organization, all of which have several prosthetic groups as heme or iron-sulfur clusters. Heme-based prosthetic groups are bound to cytochromes and are able to delocalize accepted electrons due to their porphyrin structure. Every cytochrome *c* (cyt *c*) molecule has a different reduction potential, and the electrons flow downhill. Iron-sulfur clusters disperse accepted electrons among iron atoms, and their true charge is approximately 2^+-3^+. Complexes I, III and IV are responsible for pumping H^+ into the IMS (Lodish *et al.* 2013, Ohnishi *et al.* 2018).

Complex I, NADH-CoQ oxidoreductase, is a large, multi-subunit enzyme with a noncovalently bound flavin mononucleotide (Ohnishi *et al.* 2018). Complex I serves as an entry point into the ETC and a key component for the respiration. The high-energy oxygen carrier NADH transfers two electrons to complex I, which catalyzes the regeneration of the reduced NADH and electrons are transferred to flavin mononucleotides and, then, further through the iron-sulfur cluster to oxidized coenzyme Q10 (CoQ10), also known as ubiquinone, which is reduced to ubiquinol (Hirst 2009). These redox reactions are exergonic, and every transported electron loses some potential in the process. The released energy is used for actively pumping four H^+ across the IMM (Fig. 1) (Lodish *et al.* 2013, Ohnishi *et al.* 2018). This reaction is completely reversible, and through PMF, the electrons can be transferred backward, while a massive amount of superoxide anion is produced in a process known as reverse electron transfer (Dubouchaud *et al.* 2018). Complex I is easily inhibited by psychopharmaca; therefore, it is an interesting target for further research (Hroudova and Fisar 2010).

$$NADH + CoQ + 6H^+_{in} \rightarrow NAD^+ + H^+_{in} + CoQH_2 + 4H^+_{out}$$

Complex II, succinate dehydrogenase, is a four subunit lipoprotein with a large soluble domain and a smaller integral-membrane domain (Hroudová and Fišar 2013, Iverson 2013). It is the smallest respiratory chain complex and is fully encoded by nuclear DNA (Grimm 2013). Complex II partly consists of covalently bound flavin adenine dinucleotide, several redox active centers, iron-sulfur clusters and quinone binding sites (Cecchini 2013). Succinate dehydrogenase enables the side entry of high energy carriers into the ECT, specifically reduced $FADH_2$, which is formed in the Krebs cycle during the oxidation of succinate to fumarate, which occurs in the soluble domain of complex II (Grimm 2013, Iverson 2013). Complex II has a role in OXPHOS by participating in the reversible oxidoreduction of the quinol and quinone (Grimm 2013). Succinate dehydrogenase is directly involved in the Krebs cycle: two electrons are released during succinate-fumarate oxidation are transferred *via* NAD^+ through

iron-sulfur cluster to CoQ10 (ubiquinone), reducing it to ubiquinol (Fig. 1) (Lodish *et al.* 2013).

$$\text{Succ} + \text{CoQ} \rightarrow \text{Fum} + \text{CoQH}_2$$

First, a partially reduced semiquinone radical is formed, which is immediately stabilized after the second electron is transferred. This mechanism most likely serves prevents excessive electron leakage (Grimm 2013). All reactions catalyzed *via* succinate dehydrogenase are reversible, although they are more efficient in their preferred catalytic direction. However, this enzyme reversibility may link complex II with some metabolic diseases (Cecchini 2013). CoQ10 is the main transporter of electrons from complex I and II to complex III (Rodriguez-Hernandez *et al.* 2009). Energy released during this step is not sufficient to pump H^+ into the IMS because part of the energy is absorbed during the reduction of CoQ10; thus, the PMF is not generated by this reaction (Lodish *et al.* 2013). Recent studies have suggested that the subunits of complex II might have tumor-suppressor gene function. Complex II can be then involved in apoptosis, thereby acting as a pro-apoptotic sensor and transducer of apoptotic signals (Grimm 2013).

Complex III, CoQ : cyt *c* oxidoreductase, is a transmembrane enzyme complex consists of two centers, Q_i facing the matrix and Q_o oriented toward to IMS, and three main subunits: cyt c_1, the Rieske center and cyt *b* (Iwata *et al.* 1998, Hroudová and Fišar 2013). Complex III catalyzes the oxidation of one molecule of reduced coenzyme Q ($CoQH_2$) and the reduction of two molecules of cyt *c.* It pumps 4 H^+ into the IMS and absorbs 2 H^+ from the matrix in a two-step process called the Q cycle (Speijer 2018).

$$\text{CoQH}_2 + 2\text{cyt } c^{3+} + 2\text{H}^+ \rightarrow \text{CoQ} + 4\text{H}^+ + 2\text{cyt } c^{2+}$$

One molecule of $CoQH_2$ is oxidized at the Q_o center, two protons are released into the IMS, two electrons are released and CoQ dissociates from complex III. However, each cyt *c* is can carry only one electron, which is the reason that the Q cycle is two-step reaction. The first electron travels *via* the high-potential chain through the Rieske center (iron-sulfur clusters) and cyt c_1 and finally reduces cyt *c*. The second electron travels *via* a low-potential chain (Q_i center) through cyt b_L and b_H to partially reduce CoQ and create a stable semiquinone radical. The second step of the Q cycle is exactly the same as that of a new $CoQH_2$ binding to complex III, and the second electron completes the reduction of semiquinone. Complex III consumes 2 H^+ protons from the matrix, and the reduced $CoQH_2$ is recycled (Fig. 1). Altogether, a normally operating complex III both produces and consumes $CoQH_2$ (Lodish *et al.* 2013, Speijer 2018).

Complex IV, cytochrome *c* oxidase, consists of 13 subunits, of which only three form the catalytic core of the complex. Complex IV enables the terminal reduction of oxygen to H_2O (Hroudová and Fišar 2013, Lodish *et al.* 2013).

$$4\text{cyt } c^{2+} + 8\text{H}^+_{\text{in}} + \text{O}_2 \rightarrow 4\text{cyt } c^{3+} + 2\text{H}_2\text{O} + 4\text{H}^+_{\text{out}}$$

Reduction proceeds partially when 4 molecules of reduced cyt *c* bind sequentially to complex IV and transfer the electron from its heme first to the Cu_a^{2+} ions, then to the heme in cyt *a*, next to the Cu_b^{2+} ions and finally to the heme of cyt a_3. All together, these results create the oxygen reduction center, creating four molecules of H_2O (Fig. 1). During this process, another four H^+ are translocated (2 per every ½ of O_2) across the membrane (Lodish *et al.* 2013). The important regulator of this complex is the protein kinase A/cyclic adenosine monophosphate (PKA/cAMP) signaling pathway, which stimulates the phosphorylation of subunit IV and increases the activity of complex IV (Bouchez and Devin 2019).

Complex V, ATP synthase, is the fifth and the last enzyme of the OXPHOS process and contains two functional domains: F_O and F_1. F_O is a domain with three types of integral membrane proteins – *a*, *b* and *c*. Protein *a* serves as an ion channel and is rigidly connected to *b* and protein *c* forms a "*c* ring". F_1 is a hydrophilic catalytic domain containing five polypeptides with the composition $\alpha_3\beta_3\gamma\delta\varepsilon$ that are strongly bound to the F_O at the surface of the membrane. F_1 contains the catalytic machinery for both, ATP synthesis and hydrolysis. The domains are connected *via* two stalks: subunit γ of the F_1, which is firmly bound to the rotating cavity of the F_O "*c* ring", and subunit *b* of F_O, which serves as a stator and prevents the $\alpha_3\beta_3$ hexamer situated on the top of subunit γ a from rotating (Fig. 1) (Jonckheere *et al.* 2011, Lodish *et al.* 2013, Neupane *et al.* 2019). Complex V exists as both a monomer and a dimer supercomplex, which optimizes cristae shape and proton flow (Barca *et al.* 2018). ATP synthase is the smallest biological nanomotor and is responsible for ATP creation, which it generates using a proton gradient with membrane potential as a battery.

This battery is powered by decreased pH and a higher concentration of H^+ ions in the IMS and increased pH and a lower concentration of H^+ ions in the mitochondrial matrix. This situation is a result of H^+ pumping across the IMM during electron movement through the ETC. The electrochemical energy that is thus generated is translated to the PMF: H^+ ions return to the matrix through the *a* subunit of the F_O domain and power ATP synthesis. The binding-change mechanism is describes the rotation mechanism of ATP synthase during the synthesis of ATP (Neupane *et al.* 2019). All three β subunits are able to bind adenosine diphosphate and phosphate to create ATP during indirect coupling with the proton flow of the exoplasmic medium. The PMF is used for the rotation of the "*c* ring" with subunit γ to induce cyclical changes in the β subunits during the 360° turnover. There are three conformational states of subunit β: open, loose and tight, all of which have a different affinity for substrates and products. At least two protons must past through the membrane for synthesis of one molecule of ATP. Synthesis and hydrolysis of ATP depend only on the direction of the rotation (Lodish *et al.* 2013, Neupane *et al.* 2019). When the IMM is not sufficiently polarized, complex V acts as an ATP hydrolase and uses released energy from the hydrolyzed ATP to move the H^+ protons uphill and restore the Δp (Nesci *et al.* 2016). However, Liu *et al.* (2016) disagree with the conventional model that indicates reliance on the rotating "*c* ring". In their opinion, the model is defective because the number of c subunits (8-15) is indivisible by the number of rotations (3x120° = 360°). They propose an alternative rotary model, inspired by the mechanism of a retractable click ballpoint pen, in which the $\alpha_3\beta_3$ hexamer turns instead of the "*c* ring" (Liu *et al.* 2016). Complex V is able to undergo dimerization and oligomerization, which facilitates ATP synthesis and is beneficial for the cell (Jonckheere *et al.* 2012).

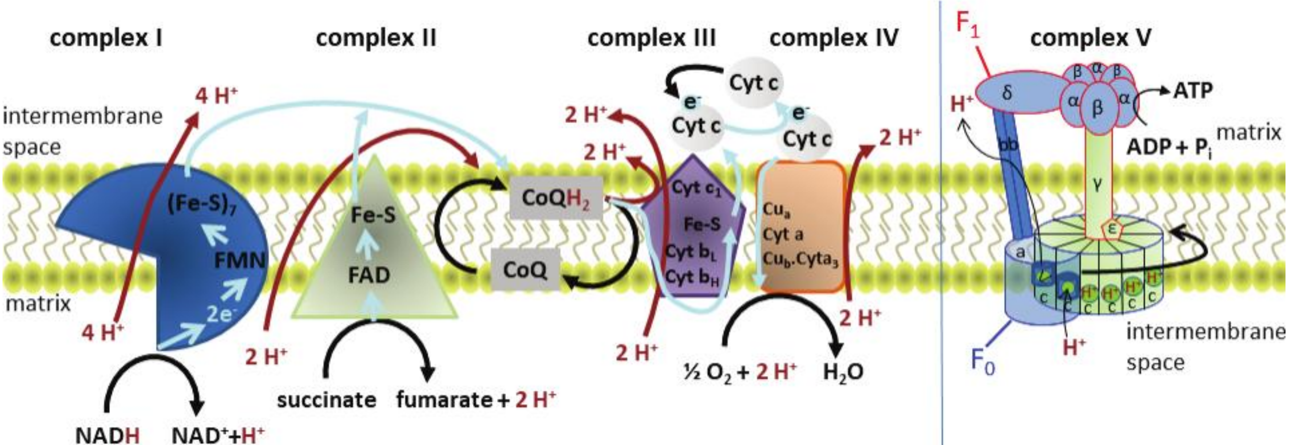

Fig. 1. The electron transport chain and structure of ATP synthase (adapted from Lodish et al. 2013). Electrons (blue arrows) from reduced nicotine adenine dinucleotide (NADH) move through **complex I** via flavin mononucleotide (FMN) and iron-sulfur clusters (Fe-S) and together with 2 protons, they bind oxidized coenzyme Q (CoQ) to form reduced coenzyme Q ($CoQH_2$). The electron flow enables 4 H^+ (red arrows) to be pumped into the intermembrane space. **Complex II** enables electron side entry and the electrons move from succinate through oxidized flavin adenine dinucleotide (FAD^+) and Fe-S form $CoQH_2$. Electrons flow from $CoQH_2$ to **complex III** and then via cytochrome c (Cyt c) to **complex IV**. In total, 10 H^+ per one NADH or 6 H^+ per one $FADH_2$ are translocated into the intermembrane space. **Complex V** is formed from the stator (blue) and rotor (green). The F_0 domain of ATP synthase consists of 3 **a** and 3 **b** subunits, and 10 **c** subunits, which form the c-ring. Subunit **a** contains the H^+ ion half-channel the mediates proton movement across the membrane. The subunits **α** and **β** of F_1 form a hexamer on the top of the **γ** subunit that is inserted into the c-ring.

Mitochondrial ROS production

ROS are highly unstable molecules which can damage different cellular structures, e.g. lipids, proteins and nucleic acids. The most common forms of ROS are superoxide anion, hydrogen peroxide and hydroxyl radical. The physiological concentration of ROS is important for maintaining homeostasis and transducing signals, whereas massive ROS production is connected with the induction of apoptosis, necrotic cell death, and pathophysiological consequences (Lanciano *et al.* 2013).

In mitochondria, ROS are byproducts of OXPHOS, which couple substrate with adenosine diphosphate phosphorylation. This coupling is not complete because some protons are able to return to the matrix *via* other pathways that enable them to bypass ATP synthase. This process is known as a basal proton

leak (Jastroch *et al.* 2010). Electron leakage occurs when electrons avoid some part of the ETC and partially reduce oxygen to create superoxide, which is superoxide anion in aqueous environments. Superoxide anion may be converted to hydrogen peroxide, catalyzed by superoxide dismutase, which is transformed into the most damaging product: hydroxyl radical (Jastroch *et al.* 2010, Lanciano *et al.* 2013). In OXPHOS superoxide is generated mostly by complex I and complex III. Complex I releases it into the matrix, and complex III releases it on both sides of the IMM (Hroudová and Fišar 2013). There are several ways in which ROS escape out of the mitochondrial matrix. Hydrogen peroxide is able to cross phospholipid barriers, but membrane pores such as mitochondrial permeability transition pore (mPTP), aquaporins and anion channels play crucial roles (Daiber *et al.* 2017).

There are also other sources of mitochondrial ROS, such as monoamine oxidase, catabolizes monoamines and produce hydrogen peroxide. Another mitochondrial enzyme, α-ketoglutarate dehydrogenase, produces H_2O_2, especially when the ratio of NADH and NAD^+ is high (Hroudová and Fišar 2013). Mitochondria can also stimulate neighboring mitochondria to release more ROS in a process called ROS-induced ROS release. ROS are able to activate inner membrane anion channels, the mPTP, and ATP-sensitive K^+ channels to disrupt mitochondrial membrane potential. High amounts of NADH and $FADH_2$ must be consumed to compensate for the activation of these channels, which causes even greater production of ROS (Bertero and Maack 2018). Interestingly, the production of ROS is lower when complex I and complex III assemble to form a supercomplex (Bolanos *et al.* 2016).

Under physiological conditions, ROS are neutralized by and antioxidant system consisting of different enzymes, such as superoxide dismutase, catalase, glutathione peroxidase and glutathione transferase. The superoxide is converted to hydrogen peroxide by superoxide dismutase and is then eliminated by glutathione peroxidase or peroxiredoxin. This system, together with other antioxidant molecules, such as coenzyme Q10, creatine and tocopherol, protects tissues from oxidative damage, but its efficiency might be decreased under pathophysiological conditions (Wang 2007, de Sousa *et al.* 2014, Bertero and Maack 2018).

Increased production of ROS occurs when the membrane potential is low, mitochondria are depolarized a response is mounted based on hypoxia or calcium signaling (Angelova and Abramov 2018). Overproduction of ROS or dysregulation in the antioxidant system damages lipids, proteins and nucleic acids and the structures of which they are part (Bolisetty and Jaimes 2013). Prolonged overproduction causes oxidative stress and induce cell death, which is associated with many pathologies, including neurodegeneration, obesity or diabetes (Mailloux and Harper 2012). Mitochondrial permeability is largely increased under inflammatory conditions, which enables even more ROS to escape to the cytosol (Daiber *et al.* 2017). Complex I generates a significant amount of superoxide anions during reverse electron transfer, during which electrons are transferred backwards, from quinone to NAD^+ (Dubouchaud *et al.* 2018). These superoxides are especially likely to be produced under hypoxic conditions. During hypoxia, electron flow stops, and the mitochondrial membrane potential drops. ATP is then taken up by mitochondria from the cytosol and hydrolyzed by ATP synthase, and the mitochondrial membrane potential is restored. However, the impaired electron flow leads to only partial oxygen reduction and contributes to increased ROS production (Bernardi *et al.* 2015). Complex II is considered to be an important regulator of the ROS production by complexes I and III and may be a major source of ROS only during heart failure (De Giusti *et al.* 2013).

The mitochondrial dysfunction and the increased ROS production are probably one of the main factors in brain aging processes. During the aging, the number of defective mitochondria that produce more ROS is increased and the antioxidant system is also less effective. Decreased OXPHOS capacity and mitochondrial DNA damage is also described (Hroudová and Fišar 2013, Cedikova *et al.* 2016). Because the brain is the greatest oxygen consumer in the body, brain cells are the most vulnerable to oxidative damage (Wang 2007). In addition, ROS and reactive nitrogen species are also considered key factors in cardiovascular and inflammatory diseases. Superoxide acts as a direct antagonist of nitric oxide, which is endogenous vasodilator. Their reaction produces peroxynitrite which leads to endothelial dysfunction of vessels (Daiber *et al.* 2017).

Mitochondrial ROS and signaling

The initial studies conducted in 1998 revealed that mitochondria release hydrogen peroxide under hypoxic conditions. Increased levels of hydrogen or peroxide activate hypoxia-inducible factor, which is important transcription factor for the metabolic

adaptation to low oxygen levels (Chandel *et al.* 1998). Currently, ROS are considered the secondary signaling messengers; that is, they change the redox state of the signal transduction proteins (Lenaz 2012). The signaling tasks of ROS in mitochondria are both short-term (redox signaling) and long-term (redox regulation of transcription). They influence several different systems: bridge energy metabolism to inflammatory responses *via* redox-sensitive molecules such as nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB); link bioenergetics, autophagy and circadian control; participate in phospholipase activation; associate with oxygen sensing in astrocytes and breathing regulation; and regulate cellular signaling in proliferating cells (Bolanos *et al.* 2016). A mild increase in ROS generation associated with the separation of the Nrf2 protein from Keap1 (marker for ubiquitin-dependent proteasomal degradation) and the translocation of Nrf2 to the nucleus and its binding with the antioxidant response element (ARE). The Nrf2-ARE pathway is a very important component of cellular antioxidant, stress adaptation and anti-inflammatory defense systems. Another oxidative stress-responding transcription factor is NFκB, which is activated by hydrogen peroxide. Although mild oxidative stress is increased upon activation of NFκB, higher concentrations of ROS oxidize and inactivate NFκB (Bolisetty and Jaimes 2013, Indo *et al.* 2017). ROS are markers during fuel sensing and use and also serve as important regulators of hypothalamic neuropeptide-Y and pro-opiomelanocortin neurons to regulate food intake and body weight (Gyengesi *et al.* 2012). ROS may also activate extracellular endopeptidases referred to as matrix metalloproteinases and influence cell proliferation and differentiation, transcriptional regulation, wound healing, tissue remodeling and other functions (Indo *et al.* 2017).

Mitochondria as Ca^{2+} regulators

Mitochondria play crucial roles in intracellular Ca^{2+} homeostasis by regulating Ca^{2+} uptake through the Ca^{2+} uniporter and Ca^{2+} efflux *via* Na^+/Ca^{2+} exchanger or mPTP, which is very important for synaptic plasticity and for the subsequent regulation of gene transcription (Ca^{2+} ions activate Ca^{2+}/calmodulin-dependent kinases) (Wang 2007, Srivastava *et al.* 2018). Mitochondrial Ca^{2+} also regulates ATP production, mitochondrial metabolism and cell death (Giorgi *et al.* 2018).

The outer mitochondrial membrane is permeable to Ca^{2+} through voltage-dependent anion channels, which enable Ca^{2+} to enter into the IMS. Mitochondria serve as high-capacity transient Ca^{2+} stores. There is a finite capacity for mitochondrial Ca^{2+}. In high Ca^{2+} levels, mitochondria maintain low Ca^{2+} forming calcium phosphate precipitate with inorganic phosphate (Feissner *et al.* 2009). Ca^{2+} cross the IMM *via* the Ca^{2+} uniporter (a selective Ca^{2+} channel) when the Ca^{2+} concentration falls below the submicromolar threshold (rising phase). This transport is possible due to the large negative potential across the IMM. Subsequently Ca^{2+} is released *via* a Na^+-dependent Na^+/Ca^{2+} exchanger or Na^+-independent H^+/Ca^{2+} exchanger (both inhibited by Bcl-2 proteins). This Ca^{2+} cycle is likely linked with the Ca^{2+} release channels of the endoplasmic reticulum (comprising inositol triphosphate and ryanodine receptors), which serves as a buffer for both intracellular is and extracellular Ca^{2+}, enabling mitochondria to participate in Ca^{2+} signal transduction. Disruptions in mitochondrial membrane potential reduce the ability of mitochondria to maintain Ca^{2+} homeostasis and result in cell damage. The mPTP connect the outer and inner mitochondrial membrane and is composed of an adenine nucleotide translocator, a voltage-dependent anion channel and the modulatory protein cyclophilin D (Brini 2003, Camello-Almaraz *et al.* 2006, Wang 2007, D'Orsi *et al.* 2017). Some studies have suggested that the mPTP is formed from ATP synthase dimers through conformational change, but the specific mechanism of its creation remains unknown (Bernardi *et al.* 2015). Studies have proposed two gating models of mPTP: one that is irreversible and leads to permanent pore opening and ultimately to cell death and one which the opening is reversible and transient (Hou *et al.* 2014). Massive mitochondrial Ca^{2+} concentration might accelerate metabolic enzymes and activate the mPTP opening (resulting in mitochondrial membranes becoming permeable for any molecule smaller than 1.5 kDa), which permits Ca^{2+} and proapoptotic molecules, such as apoptosis-inducing factor and cyt *c* (from complex III), to cross which consequently results in cell death (Camello-Almaraz *et al.* 2006, Wang 2007, D'Orsi *et al.* 2017). Not all mitochondria are equally susceptible to Ca^{2+}-induced mPTP opening. Drahota *et al.* (2012) proved that cardiac mitochondria of neonatal rats are more resistant to the Ca^{2+} overload than adult rats. This observation was only confirmed for the cardiac, not the liver mitochondria, probably because of the higher ischemic tolerance of the neonatal heart (Drahota *et al.* 2012). The mPTP opening is under control of the Bcl-2 family of proteins. This

family consists of anti-apoptotic proteins such as Bcl-2 and Bcl-xL, which maintain the mPTP in a close state, and pro-apoptotic proteins such as Bax and Bak, which induce mPTP opening and facilitate the transfer of Ca^{2+} from the ER. The results showed that ROS are able to increase Ca^{2+} concentration by internal release and disrupt Ca^{2+}-buffering system. It could be said that, in general, mitochondrial inhibitors such as rotenone, myxothiazol or antimycin A decrease Ca^{2+} oscillation and ROS production. Additionally, any Ca^{2+} mobilization in mitochondria is a signal for metabolic enzymes to increase productivity, which leads to greater production of ROS; thus, there is a direct connection between the metabolic state and ROS production (Camello-Almaraz *et al.* 2006, Wang 2007, D'Orsi *et al.* 2017). Because Ca^{2+} is part of apoptotic cascade initiation, it is not surprising that many diseases, especially those of neurodegenerative nature, have been associated with altered Ca^{2+} homeostasis and abnormal patterns of Ca^{2+} signals. Disturbances in Ca^{2+} signaling are very often connected with tissue dysfunction, and it has been proven, that early mitochondrial Ca^{2+} defects are involved in the pathogenesis of Huntington's disease (Duchen 2000, Brini 2003).

Mitochondrial dysfunction and psychiatric diseases

Mitochondria might play a crucial role in the pathogenesis of psychiatric disorders, such as depression, anxiety, schizophrenia and bipolar affective disorder (Hroudova and Fisar 2011). The impaired function of OXPHOS and decreased energy production could easily endanger the brain, very dependent on energy supplies. The brain represents around 3 % of body mass but use around 20 % of the oxygen and 25 % of the glucose. Alterations in mitochondrial functions could have the negative impact on the energy consumption of the brain, and increase the vulnerability to psychiatric disorders (Filiou and Sandi 2019).

In brief, wide range of mitochondrial dysfunctions was observed in patients with psychiatric diseases. Polymorphisms and mutations in mitochondrial and nuclear DNA leading to dysregulations of Ca^{2+}, the abnormal cellular energy and metabolism, the decreased pH and ATP formation, the impaired function of respiratory complexes and enzymes, and increased levels of ROS. The *post-mortem* examination of patients' brains revealed also anatomical and neuroanatomical abnormalities. More data could be found in the topic-related literature (Jou *et al.* 2009, Hroudova and Fisar 2011, Manji *et al.* 2012, Toker and Agam 2015).

Several studies suggest the possibility that damaged bioenergetics in circulating platelets might project impaired mitochondrial functions in metabolically active organs and help monitor the treatment in patients with Alzheimer´s disease or depression. In platelets of depressive patients, the decreased mitochondrial respiration and the drop in maximal capacity of ETC was described. In patients with Alzheimer´s disease, the decreased activity of the enzymes of citric acid cycle, the complex III and IV, the increased levels of nitric oxide which form peroxynitrite in reaction with superoxide, and the decreased maximal capacity of ETC was described (Fisar *et al.* 2019, Petrus *et al.* 2019). Fisar *et al.* (2019) observed reduced activity of the citrate synthase and a increased activity of the complex I in patients with Alzheimer´s disease, and increased activity of the complex II in patients with the Alzheimer´s disease and depression.

Effects of psychopharmaca on mitochondrial functions

Disrupted mitochondrial functions, manifesting as increased oxidative stress, decreased energy production and Ca^{2+} imbalance have been observed across the whole spectrum of psychiatric disorders (Quiroz *et al.* 2008, Srivastava *et al.* 2018). Therefore, how psychopharmaca affect mitochondrial functions is a very interesting phenomenon (Clay *et al.* 2011). Generally, changes due to mood stabilizers, such as decreased intracellular pH, globally upregulated or downregulated mitochondrial genes, increased expression of the antiapoptotic gene *BCL2* and increased Ca^{2+} storage capacity in the ER have been observed in the brains of patients. Several studies have hypothesized that mood stabilizers induce mitochondrial migration to synaptic terminals, which influences mitochondrial functions and neurotransmitter release and uptake (Scaini *et al.* 2016). This review is focused on drugs mainly or potentially used in bipolar affective disorder therapy.

Lithium

Lithium (Li) has protective functions in human cells. Itblocks tyrosine phosphorylation of the 2B subtype of the N-methyl-D-aspartate receptor and prevents massive Ca^{2+} influx and excitotoxicity. Li levels

in patients with bipolar affective disorder during depressive episodes and complex I activity are positively correlated. Study results also suggest that Li enhances mitochondrial respiration, decreases the proapoptotic enzyme glycogen synthase kinase-3 and inhibits the expression of proapoptotic genes Bax and p53 while simultaneously stimulating antiapoptotic Bcl-2 gene expression (Machado-Vieira *et al.* 2009, Cikankova *et al.* 2017). Interesting connection also exists between Li, Ca^{2+} and the mPTP. Li is able to augment the mitochondrial Ca^{2+} capacity, desensitize mitochondria against Ca^{2+}, inhibit calcium-mediated formation of mPTP to suppress membrane depolarization, swelling and the release of cyt *c* from the mitochondria of rat brain and liver and mitochondria isolated from heart tissue. It also prevents the release of Ca^{2+} from the ER by blocking the inositol monophosphatase pathway (Shalbuyeva *et al.* 2007). The same protective effect against Ca^{2+} overload was also observed in rat brain mitochondria. In homogenates of human prefrontal cortex cells, Li increased the activity of mitochondrial complexes I, II and III. Li also promoted the expression of the scavenger glutathione transferase (Clay *et al.* 2011). In the rat mania model, Li stimulated the activity of complex I in the prefrontal cortex (Valvassori *et al.* 2010). In *post-mortem* brain tissue, Li dose-dependently increases complex I activity (Maurer *et al.* 2009). Some studies have demonstrated the inhibition of ROS formation and increased glutathione levels after Li application in rat cerebral cells (Machado-Vieira *et al.* 2009). Li also had a cytoprotective effect. treatment inhibited aluminum-induced cyt *c* translocation, prevented Bcl-2 activity from decreasing and inhibited pro-caspase-3 cleavage in rabbits (Ghribi *al.* 2002). Li also attenuated rotenone-induced proapoptotic caspase-3 activation (King *et al.* 2001). In another rotenone-Li experiment, Li reduced complex I dysfunction and suppressed ATP production, cellular mortality and apoptosis, and DNA methylation and hydroxymethylation in rat neurons (Scola *et al.* 2014).

However, not all the articles claim only a positive effect of Li on mitochondria and cells. Li lowered complex IV activity in mitochondria isolated from pig brain (Hroudova and Fisar 2010). Because of complex I activity stimulation, it came as no surprise that Li enhanced ROS formation such that mitochondrial structures were damaged. The results showed a decrease in antioxidants and a drop in mitochondrial membrane potential in rat hepatocytes after Li treatment (Eskandari *et al.* 2012). In mitochondria isolated from rat heart, Li concentrations from 75-1,000 μM inhibited complex II activity and ATP formation, enhanced ROS generation and the activity of proapoptotic caspase-3, caused swelling, and abrogated the mitochondrial membrane potential (Salimi *et al.* 2017). Similar to the action of VPA, the effect of Li likely depends on many factors.

Mood stabilizing anticonvulsants

Valproate (VPA) is often associated with the disruption of mitochondrial functions. It has been established, that VPA blocks the entry of long-chain fatty acid into mitochondria (and β-oxidation) by lowering cofactors such as carnitine and CoA. It also enhances ROS formation, mostly by inhibiting complex II activity and inducing mPTP opening. Higher ROS levels and enhanced mPTP formation lead to a decrease in membrane potential, swelling and release of the cyt *c* and apoptosis. In rat digitonin-permeabilized hepatocytes, VPA notably inhibited ATP synthesis and pyruvate oxidation when pyruvate was used as a substrate for respiratory reactions. This effect was not observed when succinate or glutamate/malate was used as a substrate gradient (Silva *et al.* 1997). These results presumed an interaction between VPA and pyruvate, which was confirmed by Aires *et al.* (2008). VPA and its metabolites inhibited pyruvate uptake in inverted submitochondrial vesicles, which impaired the mitochondria as described above (Aires *et al.* 2008). Inhibition of 2-oxoglutarate and glutamate-driven OXPHOS has been described in rat liver mitochondria (Luis *et al.* 2007). Structural Li abnormalities in the ETC proteins and VPA inhibit complex I and IV activity and decrease superoxide dismutase (Finsterer and Zarrouk Mahjoub 2012, *et* Cikankova *et al.* 2017, Finsterer and Scorza 2017, Costa *et al.* 2018). VPA decreased complex I and IV activity in mitochondria isolated from pig brain and facilitated mitochondrial cholesterol transport in Y1 adrenocortical cells (Hroudova and Fisar 2010, Brion *et al.* 2011).

In contrast, some studies confirmed the inhibition of cyt *c* release, increased Bcl-2 gene expression and reduced apoptosis *via* inhibition of the proapoptotic enzyme glycogen synthase kinase-3. Cell protection from glutamate-induced Ca^{2+} overload and excitotoxicity was also observed (Kazuno *et al.* 2008, Bachmann *et al.* 2009, Kato 2011, Cikankova *et al.* 2017). A similar mitochondria-stabilizing effect was observed in a rat mania model study. Rats pretreated with VPA, in addition to the same results found for LIT, showed lower amphetamine-induced citrate synthase and

succinate dehydrogenase inhibition (Correa *et al.* 2007, Feier *et al.* 2013). VPA also reversed ouabain-induced alterations to the Krebs cycle and mitochondrial enzymes and protected mitochondria from ouabain-induced lipid peroxidation and superoxide formation (Lopes-Borges *et al.* 2015, Valvassori *et al.* 2015). Chronic treatment with therapeutic doses of VPA and Li doubled Bcl-2 levels in the frontal cortex of rats (Quiroz *et al.* 2008). This gene expression was likely mediated *via* the inhibition of histone deacetylase because deacetylated histones inhibit transcription (Clay *et al.* 2011). The influence on Bcl-2 seems to be crucial for the beneficial effect of VPA on mitochondria (Bachmann *et al.* 2009).

The effect of VPA on mitochondrial functions likely depends on several factors, such as the specific type of cell population, presence of mitochondrial pathophysiology, cytotoxic stimulus and, of course, the concentration and duration of VPA treatment (Lai *et al.* 2006). It seems that VPA confers its cytoprotective effects mostly in the presence of another cytotoxic signal, whereas VPA alone tends to damage mitochondrial functions.

Carbamazepine decreases mitochondrial respiration, ATP production and membrane potential and inhibits the Ca^{2+}-induced swelling of rat liver cells (Cikankova *et al.* 2017, Finsterer and Scorza 2017).

Lamotrigine effectively inhibits the cytotoxic effect of rotenone and 1-methyl-4-phenypyridinium on PC12 cells, mostly by maintaining the mitochondrial membrane potential, preventing the opening of mPTP and increasing the levels of glutathione (Kim *et al.* 2007). The possible neuroprotective effect of lamotrigine may be mediated by respiratory complex I inhibition (Hroudova and Fisar 2011).

Conclusions

Mitochondria produce ATP supplies using substrates from proteins, lipids and carbohydrate metabolism. In addition to ATP synthesis, mitochondria participate on buffering of free Ca^{2+} concentration, maintaining of physiological levels of Ca^{2+}, and produce and regulate ROS, trigger intrinsic apoptotic pathway and participate on thermogenesis. These molecules serve as signaling molecules, are involved in the overproduction of ROS or the decreased activity of the antioxidant system damage cellular structure, leading to apoptosis or necrosis and are connected with aging and the pathophysiology of many diseases, including mood disorders.

Altered mitochondrial functions, e.g. suppressed ATP formation, reduced mitochondrial respiratory complex activity, overproduction of ROS or/and insufficient activity of the antioxidant system, and impaired Ca^{2+} balance have been observed in affected neurons in people with mood disorders and/or being treated with mood stabilizers. The dysfunctional mitochondrial hypothesis is one of current hypotheses attempts to explain the origin of mood disorders. Many studies have confirmed that mood stabilizers affect mitochondrial functions, even though the exact mechanism or localization of action is unknown. Both inhibition and stimulation of mitochondrial functions was both observed as a result of psychopharmaca treatment. Therefore, changes in mitochondrial parameters induced by mood-stabilizing drugs support the suggestion that mitochondrial dysfunction could be a primary feature of mood disorders. Further knowledge requires the investigation of other psychopharmaca used in bipolar disorder treatment, e.g. antidepressants, benzodiazepines and antipsychotics. The identification of the mechanism(s) of psychopharmaca action, new drug that target mitochondria, and measurable markers of therapy effectiveness is the focus of current biological psychiatry research.

Conflict of Interest

There is no conflict of interest.

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Abbreviations

ARE – antioxidant response element, ATP – adenosine triphosphate, CoA – coenzyme A, CoQ – oxidized coenzyme Q10, $CoQH_2$ – reduced coenzyme Q10, cyt *c* – cytochrome *c*, ETC – electron transport chain, FAD^+ – oxidized form of flavin adenine dinucleotide, $FADH_2$ – reduced form of flavin adenine dinucleotide, IMM – inner mitochondrial membrane, IMS – intermembrane space, Li – lithium, mPTP – mitochondrial permeability transition pore, NAD^+ – oxidized form of nicotinamide adenine dinucleotide, NADH – reduced form of nicotinamide adenine dinucleotide, NFκB – nuclear factor kappa-light-chain-enhancer of activated B cells, Nrf2 – nuclear factor erythroid 2-related factor 2, OXPHOS – oxidative phosphorylation, PMF – proton motive force, ROS – reactive oxygen species, VPA – valproate.

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Attachment 6

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