

# Effects of Various Kynurenine Metabolites on Respiratory Parameters of Rat Brain, Liver and Heart Mitochondria

Halina Baran<sup>1,3,\*</sup>, Katrin Staniek<sup>2</sup>, Melanie Bertagnol-Spörr<sup>1,2</sup>, Martin Attam<sup>1,2</sup>, Carina Kronsteiner<sup>3</sup> and Berthold Kepplinger<sup>3</sup>

<sup>1</sup>Neurophysiology, Institute of Physiology, Department of Biomedical Sciences, University of Veterinary Medicine, Vienna. <sup>2</sup>Institute of Pharmacology and Toxicology, Department of Biomedical Sciences, University of Veterinary Medicine, Vienna. <sup>3</sup>Karl Landsteiner Research Institute for Neurochemistry, Neuropharmacology, Neurorehabilitation and Pain Treatment, Mauer-Amstetten, Austria.

**ABSTRACT:** Previously, we demonstrated that the endogenous glutamate receptor antagonist kynurenic acid dose-dependently and significantly affected rat heart mitochondria. Now we have investigated the effects of L-tryptophan, L-kynurenine, 3-hydroxykynurenine and kynurenic, anthranilic, 3-hydroxyanthranilic, xanthurenic and quinolinic acids on respiratory parameters (ie, state 2, state 3), respiratory control index (RC) and ADP/oxygen ratio in brain, liver and heart mitochondria of adult rats. Mitochondria were incubated with glutamate/malate (5 mM) or succinate (10 mM) and in the presence of L-tryptophan metabolites (1 mM) or in the absence, as control. Kynurenic and anthranilic acids significantly reduced RC values of heart mitochondria in the presence of glutamate/malate. Xanthurenic acid significantly reduced RC values of brain mitochondria in the presence of glutamate/malate. Furthermore, 3-hydroxykynurenine and 3-hydroxyanthranilic acid decreased RC values of brain, liver and heart mitochondria using glutamate/malate. In the presence of succinate, 3-hydroxykynurenine and 3-hydroxyanthranilic acid affected RC values of brain mitochondria, whereas in liver and heart mitochondria only 3-hydroxykynurenine lowered RC values significantly. Furthermore, lowered ADP/oxygen ratios were observed in brain mitochondria in the presence of succinate with 3-hydroxykynurenine and 3-hydroxyanthranilic acid, and to a lesser extent with glutamate/malate. In addition, 3-hydroxyanthranilic acid significantly lowered the ADP/oxygen ratio in heart mitochondria exposed to glutamate/malate, while in the liver mitochondria only a mild reduction was found. Tests of the influence of L-tryptophan and its metabolites on complex I in liver mitochondria showed that only 3-hydroxykynurenine, 3-hydroxyanthranilic acid and L-kynurenine led to a significant acceleration of NADH-driven complex I activities. The data indicate that L-tryptophan metabolites had different effects on brain, liver and heart mitochondria. Alterations of L-tryptophan metabolism might have an impact on the bioenergetic activities of brain, liver and/or heart mitochondria and might be involved in the development of clinical symptoms such as cardiomyopathy, hepatopathy and dementia.

**KEYWORDS:** respiratory parameters of mitochondria, brain, heart, liver, kynurenine metabolites, xanthurenic acid, misfoldome, dementia

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**CORRESPONDENCE:** halina.baran@neuro-lab.eu

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## Introduction

L-tryptophan (L-TRP) degradation along the kynurenine pathway has a biologically notable function for the central nervous system (CNS) as well as for the periphery.<sup>1–4</sup> It is linked with the synthesis of neuroreactive metabolites with various properties – kynurenic acid (KYNA) with inhibitory activities,<sup>3</sup> quinolinic acid (QUIN) with excitatory,<sup>3</sup> and 3-hydroxykynurenine (3-OH-KYN) with neurotoxic properties.<sup>5–7</sup> KYNA is well known as an antagonist of ionotropic excitatory amino acid (EAA) receptors and of nicotinic cholinergic subtype  $\alpha 7$  receptors.<sup>3,8</sup> L-kynurenine (L-KYN), as a major intermediate in the oxidative metabolism of L-TRP,<sup>1–4</sup> is actively accumulated in different tissues (brain, liver, heart and others) and taken up by various cells (astrocytes, neurons, macrophages) including their mitochondria.<sup>9,10</sup> It can be converted to KYNA, 3-OH-KYN or anthranilic acid (ANA) in

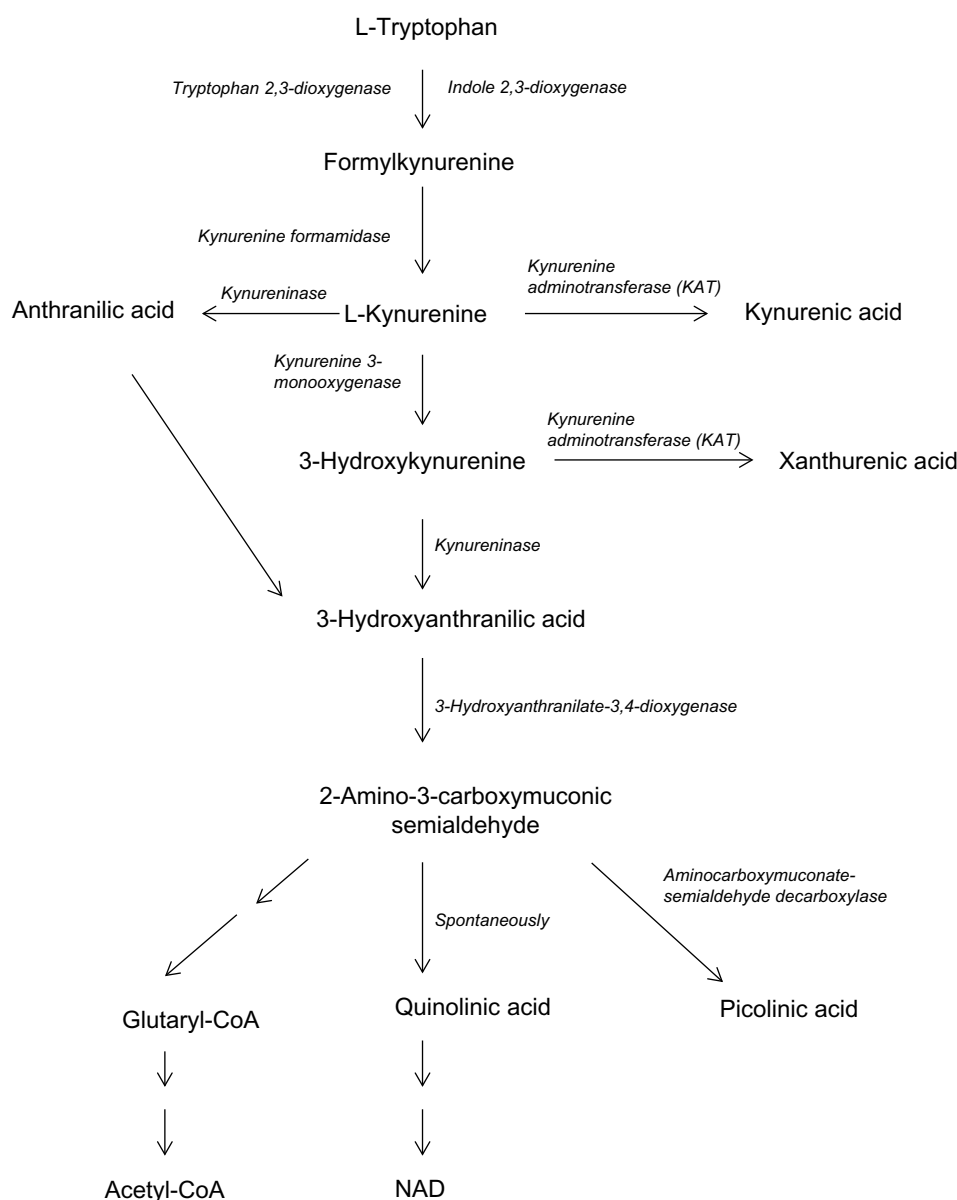
the brain, liver or heart.<sup>1–4</sup> The enzymes responsible for their formation, kynurenine aminotransferases, kynurenine-3-monooxygenase or kynureninase, are present in mitochondria and/or in the cytosol.<sup>10–14</sup>

There is substantial evidence that in the rat brain, KYNA synthesis increases significantly under pathological conditions.<sup>3,15,16</sup> Increased KYNA formation has also been found in the brain of human patients suffering from CNS diseases such as Huntington's chorea,<sup>17</sup> Alzheimer's disease,<sup>18</sup> or Down's syndrome<sup>19</sup> and also in patients infected with HIV-1<sup>20–22</sup> and stroke patients.<sup>23</sup> Importantly, this augmentation of KYNA is in line with a marked increase of activity of the kynurenine aminotransferases, a group of mitochondrial enzymes.<sup>18,21,22</sup> Therefore, alterations of L-TRP metabolism, such as changes of enzyme activities and/or substrate availability, may influence mitochondrial function in cells and may contribute

to impairment of organs in the periphery and in the CNS. Metabolic disturbance accompanied by oxidative stress and dysfunction of mitochondria has been suggested in various neurodegenerative disorders.<sup>24–26</sup> In most cases, measurement of mitochondrial respiratory parameters, mitochondrial respiratory control or the ability of mitochondria to produce ATP allowed us to measure mitochondrial function and dysfunction in different systems.<sup>27</sup> Previously, we demonstrated that within L-TRP metabolites of the kynurenine pathway, KYNA, ANA, 3-OH-KYN and 3-hydroxyanthranilic acid (3-OH-ANA) influenced dose-dependently (between 125  $\mu$ M and 5 mM) respiratory parameters of rat heart mitochondria.<sup>28,29</sup> This suggested that alteration of L-KYN metabolism might influence heart cell function and cardiovascular processes.<sup>30</sup> Furthermore, our previous data also suggested that complex I of heart mitochondria is more sensitive to the KYNA

action.<sup>28,29</sup> Importantly, a marked and long-lasting increase of L-TRP and L-TRP metabolites in the blood has been reported in stroke patients, even up to 6 months after stroke.<sup>23</sup> Higher KYNA and/or ANA levels might impair heart function, likely causing congestive cardiac dysfunction.<sup>31,32</sup> Furthermore, it seems that high KYNA levels might significantly affect glutamatergic and nicotine cholinergic neurotransmission, causing impairment of memory or cognition.<sup>3,4,8,18,22</sup>

Therefore, the aim of the present study was to investigate if L-TRP and L-TRP metabolites via the kynurenine pathway (Fig. 1) such as L-KYN, KYNA, ANA, 3-OH-KYN, 3-OH-ANA, XAN or QUIN might affect brain and liver mitochondrial function of 3 month old rats, as we have observed them to affect heart mitochondria. Furthermore, the influence of L-TRP metabolites on enzymatic activities of complex I was studied in liver mitochondria.



**Figure 1.** Tryptophan metabolism along kynurenine pathway.

## Materials and Methods

**Chemicals.** ANA, 3-OH-ANA, L-KYN, 3-OH-KYN, KYNA, L-TRP, QUIN, xanthurenic acid (XAN), L-malic acid, rotenone, NADH (disodium salt), decylubiquinone (2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone) and fatty acid-free bovine serum albumin (BSA, fraction V) came from Sigma-Aldrich. Triethanolamine-HCl, KCN, succinic acid and L-glutamic acid were purchased from Fluka. ADP (potassium salt) was obtained from Boehringer. Other chemicals were from Merck.

**Animals.** Male, 3 month old, Sprague-Dawley rats (Him:OFA/SPF) were obtained from the breeding facility of the Medical University of Vienna and housed under standard laboratory conditions at a 12 h light/12 h dark cycle with free access to conventional animal food and water. For this study, 24 rats weighing  $350 \pm 31$  g were used. Food was withdrawn overnight before animals were killed for organ harvesting (one rat per experimental day) by cervical dislocation followed by decapitation. The study was discussed and approved by the ethics and animal welfare committee of the University of Veterinary Medicine, Vienna, in accordance with good scientific practice guidelines and national legislation, and was carried out according to Austrian ethical regulations.

**Isolation of mitochondria.** Immediately after killing each rat, the heart and liver were quickly removed. In parallel, the brain was rapidly extracted from the skull and removed from the brainstem, cerebellum and olfactory bulb. The isolated organs were plunged into ice-cold isolation buffer containing sucrose (300 mM for heart and brain; 250 mM for liver), 20 mM triethanolamine and 1 mM ethylenediaminetetraacetic acid (pH 7.4, 4 °C). All isolation steps were performed at 2–4 °C. The tissues were minced with scissors and subsequently homogenised by an electrically driven Potter-Elvehjem homogeniser. Mitochondria were separated by differential centrifugation. Tissue homogenates were centrifuged for 10 min at  $571 \times g$ . The supernatants were filtered through cheesecloth and centrifuged for 10 min at  $7410 \times g$  to sediment the mitochondria. The resulting pellets were manually re-suspended and centrifuged for 10 min at  $7410 \times g$ . This latter step was repeated once more for heart and brain mitochondria and twice for liver mitochondria. Final mitochondrial pellets were carefully re-suspended in respective isolation buffer giving protein concentrations of approximately 30–40 mg/mL. Protein concentrations were measured with the biuret method using BSA for the calibration curve. Errors due to interfering pigments and turbidity caused by lipids were eliminated by the addition of KCN.<sup>33</sup>

**Measurement of mitochondrial respiratory parameters.** Mitochondrial oxygen consumption rates were measured in an air-saturated incubation buffer (pH 7.4, 25 °C) consisting of 300 mM sucrose, 20 mM triethanolamine and 1 mM diethylenetriaminepentaacetic acid (DETAPAC) supplemented with 1 mg/mL BSA (for heart mitochondria: 0.5 mg/mL

BSA) within a DW1 oxygen electrode chamber connected to a CB1-D3 control unit (Hansatech Instruments).

State 2 respiration was stimulated in the presence of 4 mM potassium phosphate, either by 5 mM glutamate/5 mM malate (substrates for mitochondrial complex I) or 10 mM succinate (substrate for mitochondrial complex II) in the presence of 2 µg/mL rotenone to prevent electron transfer from complex I. Transition to state 3 respiration and thereby ATP synthesis was initiated by the addition of 250 µM ADP. The respiratory control (RC) value was calculated as the ratio of oxygen consumption rates during state 3 and state 2. The ADP/oxygen (ADP/O) ratio was calculated from the total amount of ADP added and the amount of oxygen consumed during state 3 respiration.

**Treatment of isolated mitochondria with tryptophan (L-TRP) and its metabolites.** Aliquot stock solutions of L-TRP and L-TRP metabolites (50 or 125 mM) were prepared while adding necessary amounts of KOH. Aliquots were frozen in liquid nitrogen (3-OH-ANA, 3-OH-KYN, XAN) or at -20 °C (ANA, L-KYN, KYNA, QUIN, L-TRP). During experiments, stock solutions were stored at 4 °C. In addition, 3-OH-ANA, 3-OH-KYN and XAN were protected from light.

Diluted mitochondrial suspensions were preincubated in the BSA-containing incubation buffer at 25 °C in an oxygen electrode chamber with L-TRP or L-TRP metabolites (1 mM final concentration) for 3 min before state 2 respiration was induced. Control mitochondria were preincubated for 3 min with 1 mM KCl (as a vehicle solution/ionic equivalent for L-TRP metabolites) under otherwise identical conditions.

Respiratory parameters of glutamate/malate and succinate-respiring brain, liver and heart mitochondria isolated from the same rat were measured on the same day. Each day two compounds, either L-TRP or L-TRP metabolites, and the respective controls were studied. These experiments were repeated with six mitochondrial suspensions prepared from six animals per treatment group and organ.

**Determination of NADH oxidase, NADH-ferricyanide and NADH-decylubiquinone oxidoreductase activities.** Due to a limited amount of brain and heart mitochondrial suspension, measurement of enzyme activity of complex I was performed only with liver mitochondria. For all enzymatic measurements, aliquots of rat liver mitochondria (10 mg protein per mL) were frozen at -20 °C (at least overnight), thawed at room temperature and stored at 4 °C during experiments. Enzyme activities were measured in an incubation buffer consisting of 300 mM sucrose, 20 mM triethanolamine and 1 mM DETAPAC (pH 7.4, 25 °C).

**NADH oxidase.** NADH oxidase activity was measured using a DW1 oxygen electrode. Mitochondria were suspended in the air-saturated incubation buffer and supplemented with tryptophan metabolites. After 1 min, 1 mM NADH was added and NADH oxidase activity was registered from oxygen consumption.

Control experiments were performed in the absence of L-TRP or its metabolites.

**NADH-decylubiquinone and NADH-ferricyanide oxidoreductase.** NADH-decylubiquinone and NADH-ferricyanide oxidoreductase activities were determined with a U3300 spectrophotometer (Hitachi) set at 2 nm slit width in 1 mL quartz cuvettes under magnetic stirring. Mitochondria were suspended in the incubation buffer and blocked with 1 mM KCN to avoid electron flow via cytochrome oxidase onto oxygen.

**NADH-decylubiquinone oxidoreductase.** In the case of NADH-decylubiquinone oxidoreductase assays, KCN-inhibited mitochondria were incubated with 150  $\mu$ M NADH followed by the addition of L-TRP or its metabolites. After 1 min of incubation decylubiquinone (100  $\mu$ M) was added and the NADH-decylubiquinone oxidoreductase activity was monitored for 3 min at 340 nm as the oxidation of NADH.

Activities were calculated using an extinction coefficient of 6.22  $\text{mM}^{-1} \text{cm}^{-1}$  for NADH.<sup>34</sup>

**NADH-ferricyanide oxidoreductase.** Mitochondria were supplemented with 0.5 mM  $\text{K}_3[\text{Fe}(\text{CN})_6]$  followed by the addition of tryptophan or its metabolites. After 1 min of incubation, NADH (0.5 mM final concentration) was added and the NADH-ferricyanide oxidoreductase activity was monitored for 3 min at 420 nm as the reduction of  $\text{K}_3[\text{Fe}(\text{CN})_6]$ . Activities were calculated using an extinction coefficient of 1  $\text{mM}^{-1} \text{cm}^{-1}$  for ferricyanide.<sup>34</sup> The effect of 3-OH-ANA and 3-OH-KYN on NADH-ferricyanide oxidoreductase activity could not be determined due to direct interaction of ferricyanide with these tryptophan metabolites.

**Data analyses.** All measurements were performed in duplicate; respective averaged values were used for further statistical analysis. Data are mean  $\pm$  S.E.M of 6 separate experiments. Differences in respiratory parameters of control mitochondria and mitochondria treated with L-TRP or L-TRP metabolites were analyzed by a Student's *t*-test and by one-way ANOVA, followed by post-hoc two-tailed Dunnett's multiple comparison test. Differences in enzyme activities of control and treated mitochondria were analyzed by unpaired two-tailed Student's *t*-test. The level for statistical significance was  $P < 0.05$ . Asterisks indicate significant differences in comparison to control mitochondria: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

## Results

**Respiratory parameters of control brain, liver and heart mitochondria.** With regard to investigated control brain, liver and heart mitochondria, we observed that heart mitochondria exert the highest oxygen consumption rates (state 2:  $22.0 \pm 0.7 \text{ nmol O} \times \text{min}^{-1} \times \text{mg}^{-1}$ , state 3:  $138.1 \pm 5.3 \text{ nmol O} \times \text{min}^{-1} \times \text{mg}^{-1}$ ), followed by liver mitochondria (state 2:  $13.2 \pm 0.5 \text{ nmol O} \times \text{min}^{-1} \times \text{mg}^{-1}$ , state 3:  $54.5 \pm 1.8 \text{ nmol O} \times \text{min}^{-1} \times \text{mg}^{-1}$ ) and brain mitochondria (state 2:  $4.9 \pm 0.2 \text{ nmol O} \times \text{min}^{-1} \times \text{mg}^{-1}$ , state 3:  $20.7 \pm 1.1 \text{ nmol O} \times \text{min}^{-1} \times \text{mg}^{-1}$ ) using glutamate/malate as substrates. Succinate-respiring

mitochondria showed higher oxygen consumption rates in comparison to glutamate/malate-supplied mitochondria. However, the highest respiratory activities, as in the glutamate/malate data, were found in heart mitochondria (state 2:  $85.7 \pm 2.4 \text{ nmol O} \times \text{min}^{-1} \times \text{mg}^{-1}$ , state 3:  $269.4 \pm 6.7 \text{ nmol O} \times \text{min}^{-1} \times \text{mg}^{-1}$ ), followed by liver mitochondria (state 2:  $26.4 \pm 1.0 \text{ nmol O} \times \text{min}^{-1} \times \text{mg}^{-1}$ , state 3:  $101.0 \pm 2.8 \text{ nmol O} \times \text{min}^{-1} \times \text{mg}^{-1}$ ) and brain mitochondria (state 2:  $7.7 \pm 0.4 \text{ nmol O} \times \text{min}^{-1} \times \text{mg}^{-1}$ , state 3:  $32.2 \pm 1.3 \text{ nmol O} \times \text{min}^{-1} \times \text{mg}^{-1}$ ). The highest RC values were detected in glutamate/malate-respiring heart mitochondria ( $6.3 \pm 0.2$ ), while all other RC values were in the range of 3.2–4.2. Data represent means  $\pm$  S.E.M. of 24 mitochondrial preparations per organ. Control values of respiratory parameters of brain or heart mitochondria are in the line with previously published data.<sup>28,29,35</sup>

**Influence of tryptophan and its metabolites on respiratory parameters of brain mitochondria.** The most pronounced effects in brain mitochondria were detected in samples treated with 3-OH-ANA and 3-OH-KYN, which led to decreased RC values and ADP/O ratios in comparison to controls independently of whether mitochondria were supplemented with glutamate/malate (3-OH-ANA: 69.2% of CO,  $P < 0.001$  for RC; 3-OH-KYN: 89.3% of CO,  $P < 0.05$  for RC, 91.2% of CO,  $P < 0.01$  for ADP/O; Table 1) or succinate (3-OH-ANA: 77.7% of CO,  $P < 0.001$  for RC, 85.7% of CO,  $P < 0.01$  for ADP/O; 3-OH-KYN: 74.0% of CO,  $P < 0.001$  for RC and 79.1% of CO,  $P < 0.001$  for ADP/O; Table 2) as respiratory substrates. Decreased RC values resulted mainly from increased oxygen consumption rates during state 2 respiration (with glutamate/malate: 3-OH-ANA: 134.9% of CO,  $P = 0.126$ ; with succinate: 3-OH-KYN: 128.1% of CO; 3-OH-ANA: 127.1% of CO,  $P = 0.190$ ), but these effects were not statistically significant.

While pre-treatment of brain mitochondria with XAN significantly reduced RC values in mitochondria respiring glutamate/malate (88.8% of CO,  $P < 0.05$ ; Table 1), a moderate but not significant decrease of RC value was seen in the presence of ANA (83.9% of CO,  $P = 0.13$ ). ANA and XAN lowered State 3, but not significantly (83.9% of CO,  $P = 0.30$ ; 91.4% of CO,  $P = 0.65$ , respectively). No changes in respiratory parameters were found by XAN in succinate-consuming brain mitochondria (Table 2).

Brain mitochondria preincubated with 1 mM L-TRP, L-KYN, KYNA, ANA or QUIN, respectively, did not show any significant differences in comparison to control mitochondria, whether with glutamate/malate (one-way ANOVA, Table 1) or with succinate (one-way ANOVA, Table 2) as respiratory substrates.

**Influence of tryptophan and its metabolites on respiratory parameters of liver mitochondria.** L-TRP and most of its metabolites tested in this study were without significant impact on respiratory parameters of glutamate/malate-respiring (one-way ANOVA, Table 3) as well as succinate-respiring liver mitochondria (one-way ANOVA, Table 4). However,



**Table 1.** Respiratory parameters of glutamate/malate-respiring rat brain mitochondria (2 mg protein per mL) in the absence (Control) or in the presence of 1 mM L-tryptophan (L-TRP) or its metabolites L-kynurenine (L-KYN), kynurenic acid (KYNA), anthranilic acid (ANA), 3-hydroxykynurenine (3-OH-KYN), 3-hydroxyanthranilic acid (3-OH-ANA), xanthurenic acid (XAN) and quinolinic acid (QUIN), respectively.

| SUBSTANCES | STATE 2     | STATE 3    | RC             | ADP/O         |
|------------|-------------|------------|----------------|---------------|
| Control    | 4.97 ± 0.30 | 23.4 ± 1.3 | 4.76 ± 0.29    | 2.24 ± 0.15   |
| L-TRP      | 4.67 ± 0.30 | 22.7 ± 1.1 | 4.94 ± 0.11    | 2.21 ± 0.15   |
| QUIN       | 4.90 ± 0.36 | 22.8 ± 1.2 | 4.70 ± 0.15    | 2.29 ± 0.17   |
| ANOVA, F   | 0.231       | 0.089      | 0.403          | 0.071         |
| P          | 0.797       | 0.915      | 0.675          | 0.932         |
| Control    | 5.28 ± 0.37 | 21.7 ± 1.9 | 4.11 ± 0.12    | 2.49 ± 0.04   |
| L-KYN      | 5.34 ± 0.44 | 22.3 ± 1.8 | 4.19 ± 0.15    | 2.44 ± 0.04   |
| 3-OH-KYN   | 5.64 ± 0.55 | 20.6 ± 1.9 | 3.67 ± 0.11*   | 2.27 ± 0.05** |
| ANOVA, F   | 0.169       | 0.209      | 4.763          | 6.831         |
| P          | 0.846       | 0.814      | 0.025          | 0.0078        |
| Control    | 4.42 ± 0.47 | 15.6 ± 1.9 | 3.55 ± 0.27    | 2.23 ± 0.06   |
| KYNA       | 4.80 ± 0.30 | 16.3 ± 1.5 | 3.41 ± 0.25    | 2.21 ± 0.07   |
| ANA        | 4.37 ± 0.27 | 13.1 ± 1.3 | 2.98 ± 0.22    | 2.19 ± 0.05   |
| ANOVA, F   | 0.442       | 1.116      | 1.449          | 0.095         |
| P          | 0.651       | 0.353      | 0.266          | 0.910         |
| Control    | 4.84 ± 0.55 | 22.1 ± 2.8 | 4.54 ± 0.11    | 2.31 ± 0.07   |
| 3-OH-ANA   | 6.53 ± 0.85 | 20.3 ± 2.6 | 3.14 ± 0.12*** | 2.12 ± 0.08   |
| XAN        | 5.10 ± 0.83 | 20.2 ± 2.8 | 4.03 ± 0.16*   | 2.36 ± 0.09   |
| ANOVA, F   | 1.440       | 0.142      | 29.514         | 2.417         |
| P          | 0.268       | 0.869      | <0.001         | 0.123         |

**Notes:** States are given in nmol O × min<sup>-1</sup> × mg<sup>-1</sup>. Data are means ± S.E.M; n = 6; \*P < 0.05, \*\*P < 0.01; \*\*\*P < 0.001 in comparison to the respective control.

3-OH-KYN and 3-OH-ANA significantly reduced RC values of glutamate/malate-consuming liver mitochondria (3-OH-KYN: 82.4% of CO, *P* < 0.01; 3-OH-ANA: 84.8% of CO, *P* < 0.05) mainly due to enhanced state 2 oxygen consumption rates (3-OH-ANA: 116.7% of CO, *P* < 0.05; 3-OH-KYN: 117.6% of CO, *P* = 0.07, Table 3). In the presence of 3-OH-ANA, the ADP/O ratios were mildly, but significantly decreased in comparison to control mitochondria (91.2% of CO, *P* < 0.05, Table 3). KYNA and ANA lowered RC values, but not to a statistically significant degree (86.9% of CO, *P* = 0.068 and 86.9% of CO, *P* = 0.071, respectively), mainly due to a tendency of increased state 2 oxygen consumption (111.0% of CO, *P* = 0.23; 107.9% of CO, *P* = 0.36).

While the RC values of succinate-consuming liver mitochondria were significantly reduced by treatment with 3-OH-KYN (88.0% of CO, *P* < 0.05), with 3-OH-ANA only a tendency of decrease was observed (92.1% *P* = 0.11; Table 4).

**Influence of tryptophan and its metabolites on respiratory parameters of heart mitochondria.** 3-OH-ANA, 3-OH-KYN, ANA and KYNA, (1 mM final concentration) significantly reduced the RC values of glutamate/malate-respiring heart mitochondria (3-OH-ANA: 62.7% of CO, *P* < 0.001; 3-OH-KYN: 64.4% of CO, *P* < 0.001; ANA: 68.5% of CO, *P* < 0.001; KYNA: 79.6% of CO, *P* < 0.01; Table 5) due to a decreased oxygen consumption during state 3

(3-OH-KYN: 63.1% of CO, *P* < 0.01; ANA: 71.5% of CO, *P* < 0.001; 3-OH-ANA: 75.3% of CO, *P* < 0.01; Table 5) and/or an increased state 2 respiration (3-OH-ANA: 120.9% of CO, *P* < 0.01; KYNA: 118.7% of CO, *P* < 0.05, Table 5). A significantly lower ADP/O ratio, in comparison to the respective control mitochondria, was only observed in heart mitochondria treated with 3-OH-ANA (87.1% of CO, *P* < 0.01; Table 5).

By contrast, L-TRP, QUIN, L-KYN and XAN did not significantly influence the bioenergetic function of glutamate/malate-respiring heart mitochondria (one-way ANOVA, Table 5).

In contrast to heart mitochondria consuming glutamate/malate, respiratory parameters of heart mitochondria supplemented with succinate were marginally affected by L-TRP and its metabolites (one-way ANOVA, Table 6) suggesting a selective interference of tryptophan metabolites KYNA, ANA or 3-OH-ANA in the activity of mitochondrial complex I. Only 3-OH-KYN significantly reduced RC values (88.1% of CO, *P* < 0.01), and XAN mildly but significantly decreased state 3 respiration (92.5% of CO, *P* < 0.05) of succinate-consuming heart mitochondria (Table 6).

**Influence of tryptophan and its metabolites on enzyme activities of liver mitochondrial complex I.** Since the most pronounced effects on respiratory parameters were found in

**Table 2.** Respiratory parameters of rotenone-inhibited succinate-respiring rat brain mitochondria (2 mg protein per mL) in the absence (Control) or in the presence of 1 mM L-tryptophan (L-TRP) or its metabolites L-kynurenine (L-KYN), kynurenic acid (KYNA), anthranilic acid (ANA), 3-hydroxykynurenine (3-OH-KYN), 3-hydroxyanthranilic acid (3-OH-ANA), xanthurenic acid (XAN) and quinolinic acid (QUIN), respectively.

| SUBSTANCES | STATE 2      | STATE 3    | RC             | ADP/O          |
|------------|--------------|------------|----------------|----------------|
| Control    | 8.25 ± 0.38  | 33.7 ± 1.3 | 4.10 ± 0.11    | 1.56 ± 0.05    |
| L-TRP      | 8.24 ± 0.50  | 33.2 ± 1.3 | 4.08 ± 0.13    | 1.51 ± 0.04    |
| QUIN       | 8.22 ± 0.34  | 33.6 ± 1.4 | 4.09 ± 0.07    | 1.51 ± 0.06    |
| ANOVA, F   | 0.0014       | 0.030      | 0.017          | 0.305          |
| P          | 0.999        | 0.970      | 0.983          | 0.742          |
| Control    | 7.20 ± 0.81  | 33.3 ± 3.0 | 4.70 ± 0.12    | 1.39 ± 0.02    |
| L-KYN      | 7.44 ± 0.62  | 33.4 ± 2.1 | 4.54 ± 0.13    | 1.33 ± 0.01    |
| 3-OH-KYN   | 9.22 ± 0.83  | 31.9 ± 3.2 | 3.48 ± 0.10*** | 1.10 ± 0.02*** |
| ANOVA, F   | 2.123        | 0.090      | 32.786         | 56.241         |
| P          | 0.154        | 0.914      | <0.001         | <0.001         |
| Control    | 6.99 ± 0.36  | 28.6 ± 2.2 | 4.08 ± 0.22    | 1.35 ± 0.01    |
| KYNA       | 6.54 ± 0.41  | 27.2 ± 1.9 | 4.17 ± 0.23    | 1.31 ± 0.02    |
| ANA        | 6.57 ± 0.42  | 26.9 ± 2.0 | 4.13 ± 0.17    | 1.37 ± 0.02    |
| ANOVA, F   | 0.397        | 0.197      | 0.048          | 2.872          |
| P          | 0.679        | 0.823      | 0.953          | 0.088          |
| Control    | 8.38 ± 1.12  | 33.3 ± 3.7 | 4.04 ± 0.14    | 1.47 ± 0.04    |
| 3-OH-ANA   | 10.65 ± 1.16 | 33.4 ± 3.6 | 3.14 ± 0.08*** | 1.26 ± 0.03**  |
| XAN        | 8.36 ± 1.41  | 32.4 ± 3.7 | 4.04 ± 0.22    | 1.46 ± 0.05    |
| ANOVA, F   | 1.131        | 0.020      | 10.847         | 8.051          |
| P          | 0.349        | 0.980      | 0.0012         | 0.0042         |

**Notes:** States are given in nmol O × min<sup>-1</sup> × mg<sup>-1</sup>. Values represent means ± S.E.M; n = 6; \*\*P < 0.01; \*\*\*P < 0.001 in comparison to the respective control.

glutamate/malate-respiring mitochondria, it was of interest to research whether mitochondrial complex I was directly targeted by tryptophan metabolites.

**NADH oxidase.** An indirect measure of complex I activity is the NADH oxidation by oxygen involving the whole mitochondrial electron transfer chain, which can be detected from oxygen consumption. NADH oxidase was monitored at saturating substrate concentration (1 mM NADH). Significant acceleration of NADH-driven oxygen consumption (Table 7) was observed in the presence of 3-OH-ANA (143.2% of CO, *P* < 0.001), followed by 3-OH-KYN (125.64% of CO, *P* < 0.001) and L-KYN (112.1%, *P* < 0.05), while KYNA and XAN were without significant influence on NADH oxidase activity.

**NADH-decylubiquinone oxidoreductase.** Among the tryptophan metabolites tested, only 3-OH-KYN significantly enhanced NADH-decylubiquinone oxidoreductase activity (130.15% of CO, *P* < 0.001; Table 8). The effect of 3-OH-ANA was mild but significant (107.23% of CO, *P* < 0.05, Table 8).

**NADH-ferricyanide oxidoreductase.** NADH-ferricyanide oxidoreductase was not significantly changed by L-TRP, ANA, KYNA or QUIN (Table 9). Treatment of liver mitochondria with L-KYN led to a moderate but significant increase of NADH-ferricyanide oxidoreductase (108.9% of CO, *P* < 0.05, Table 9).

## Discussion

With this investigation we demonstrated for the first time that respiratory parameters of brain or liver mitochondria were not affected by KYNA, independent of whether glutamate/malate or succinate was used as substrate for respiration. In contrast, in heart mitochondria KYNA significantly impaired RC values of glutamate/malate-respiring mitochondria and moderately lowered ADP/O ratio. The latter findings are in line with our previously published observations.<sup>28,29</sup> Furthermore, in heart mitochondria ANA was also found to lower selectively state 3 respiration and consequently RC values. This finding is also in line with our previously published data.<sup>28,29</sup>

In this study, the data indicate that enhanced KYNA synthesis due to distinct pathological conditions might lower ATP synthesis and cause impairment of heart mitochondria function, but not of the brain or liver mitochondria function. Previously we have demonstrated that in the asphyctic brain, KYNA levels are significantly increased,<sup>16</sup> and other investigators have demonstrated lowering of ATP synthesis in the brain during asphyxia.<sup>36</sup> These observations allowed us to suggest that lowering of ATP synthesis in the brain during asphyxia might be due to the effect of KYNA on brain mitochondria respiratory parameters, similarly to what we had observed in heart mitochondria.<sup>28,29</sup> However, the present data do not support this assumption, since respiratory parameters of brain



**Table 3.** Respiratory parameters of glutamate/malate-respiring rat liver mitochondria (1 mg protein per mL) in the absence (Control) or in the presence of 1 mM L-tryptophan (L-TRP) or its metabolites L-kynurenine (L-KYN), kynurenic acid (KYNA), anthranilic acid (ANA), 3-hydroxykynurenine (3-OH-KYN), 3-hydroxyanthranilic acid (3-OH-ANA), xanthurenic acid (XAN) and quinolinic acid (QUIN), respectively.

| SUBSTANCES | STATE 2     | STATE 3    | RC            | ADP/O        |
|------------|-------------|------------|---------------|--------------|
| Control    | 15.7 ± 1.4  | 63.6 ± 3.2 | 4.14 ± 0.21   | 2.27 ± 0.19  |
| L-TRP      | 15.7 ± 1.7  | 60.3 ± 4.3 | 3.94 ± 0.18   | 2.26 ± 0.19  |
| QUIN       | 14.5 ± 1.3  | 60.8 ± 4.3 | 4.23 ± 0.13   | 2.29 ± 0.18  |
| ANOVA, F   | 0.212       | 0.207      | 0.725         | 0.0058       |
| P          | 0.811       | 0.815      | 0.500         | 0.994        |
| Control    | 13.1 ± 0.7  | 52.1 ± 2.7 | 3.98 ± 0.16   | 2.57 ± 0.07  |
| L-KYN      | 13.3 ± 0.7  | 52.6 ± 3.2 | 3.96 ± 0.14   | 2.60 ± 0.05  |
| 3-OH-KYN   | 15.4 ± 0.9  | 50.5 ± 3.9 | 3.28 ± 0.15** | 2.53 ± 0.06  |
| ANOVA, F   | 2.601       | 0.106      | 7.114         | 0.397        |
| P          | 0.107       | 0.900      | 0.0067        | 0.679        |
| Control    | 12.7 ± 0.8  | 53.3 ± 3.6 | 4.22 ± 0.20   | 2.51 ± 0.08  |
| KYNA       | 14.1 ± 0.7  | 51.6 ± 3.6 | 3.67 ± 0.17   | 2.44 ± 0.10  |
| ANA        | 13.7 ± 0.6  | 50.3 ± 3.9 | 3.67 ± 0.18   | 2.42 ± 0.07  |
| ANOVA, F   | 0.957       | 0.157      | 2.877         | 0.369        |
| P          | 0.406       | 0.856      | 0.088         | 0.698        |
| Control    | 11.4 ± 0.5  | 49.0 ± 1.7 | 4.35 ± 0.18   | 2.51 ± 0.05  |
| 3-OH-ANA   | 13.3 ± 0.6* | 48.7 ± 2.5 | 3.69 ± 0.11*  | 2.29 ± 0.07* |
| XAN        | 11.2 ± 0.7  | 45.3 ± 2.5 | 4.07 ± 0.25   | 2.52 ± 0.11  |
| ANOVA, F   | 3.646       | 0.835      | 3.167         | 2.826        |
| P          | 0.051       | 0.453      | 0.071         | 0.091        |

**Notes:** States are given in nmol O × min<sup>-1</sup> × mg<sup>-1</sup>. Values represent means ± S.E.M; n = 6; \*P < 0.05, \*\*P < 0.01 in comparison to the respective control.

and liver mitochondria were not affected by KYNA, indicating profound differences between heart and brain or liver mitochondria. Importantly, ANA also did not significantly alter the respiratory parameters of brain or liver mitochondria, with glutamate/malate or with succinate. A moderate effect of ANA, namely lowering of RC, could be seen on brain mitochondria in the presence of glutamate/malate.

Thus, in contrast to glutamate/malate-consuming heart mitochondria, the complex I-selective effect of KYNA seen in heart mitochondria was not observed in liver or brain mitochondria. Although ANA is generally accepted as biologically inactive, it can form an anti-inflammatory complex with copper.<sup>37,38</sup> Interestingly, a neurodegenerative effect of ANA was shown in organotypic cultures of rat hippocampus *in vitro*,<sup>39</sup> but the mechanism of ANA-induced neurodegeneration has not been elicited yet. We cannot exclude that increased ANA levels might play a role indirectly in the impairment of mitochondria function, through the action of 3-OH-ANA synthesized from ANA.<sup>40</sup> Increased levels of ANA were observed in the periphery and CNS in various pathological conditions.<sup>23,31</sup>

Another metabolite of L-TRP, 3-OH-KYN, which has a role in toxic processes,<sup>5</sup> markedly affected the mitochondrial activities of all three investigated organs. RC values were reduced significantly by 3-OH-KYN in brain, liver and heart

mitochondria, independently of whether glutamate/malate or succinate was used. The ADP/O ratio was most affected by 3-OH-KYN in succinate-respiring brain mitochondria, followed by glutamate/malate-respiring brain mitochondria. Increase of 3-OH-KYN levels in the CNS has been documented in some disorders,<sup>41–43</sup> and can be involved in the impairment of the bioenergetic activity of brain mitochondria and brain function, respectively. Intracellular generation of reactive oxygen species (ROS), mainly superoxide radicals and H<sub>2</sub>O<sub>2</sub>, has been observed at low micromolar concentration of 3-OH-KYN, a process that strongly depends on 3-OH-KYN uptake ability, which is variable in different brain regions.<sup>44,45</sup> Our finding on lowering ADP/O ratio predominantly in brain mitochondria strongly evidences the neurotoxic activity of 3-OH-KYN. This mechanism could also be a possible cause of neurodegeneration, in addition to the ROS-generating activity of 3-OH-KYN.<sup>45</sup> On the other hand, 3-OH-KYN has been proposed as a peroxyl radical scavenger in inflammatory diseases<sup>44</sup> and under special conditions of cellular redox status can act as an anti-oxidant.<sup>46</sup> It seems that 3-OH-KYN has a role in anti-oxidant and pro-oxidant activities.

Alterations of L-TRP metabolism, eg, changes of enzyme activities and/or substrate availability, may influence mitochondrial function in cells and may contribute to impairment of organs in the periphery and in the CNS.<sup>24</sup> Markedly

**Table 4.** Respiratory parameters of rotenone-inhibited succinate-respiring rat liver mitochondria (0.5 mg protein per mL) in the absence (Control) or in the presence of 1 mM L-tryptophan (L-TRP) or its metabolites L-kynurenine (L-KYN), kynurenic acid (KYNA), anthranilic acid (ANA), 3-hydroxykynurenine (3-OH-KYN), 3-hydroxyanthranilic acid (3-OH-ANA), xanthurenic acid (XAN) and quinolinic acid (QUIN), respectively.

| SUBSTANCES | STATE 2    | STATE 3     | RC           | ADP/O       |
|------------|------------|-------------|--------------|-------------|
| Control    | 28.4 ± 2.4 | 99.1 ± 5.1  | 3.53 ± 0.11  | 1.61 ± 0.05 |
| L-TRP      | 29.6 ± 1.9 | 100.5 ± 4.6 | 3.42 ± 0.11  | 1.59 ± 0.03 |
| QUIN       | 27.8 ± 2.2 | 99.4 ± 4.7  | 3.62 ± 0.13  | 1.62 ± 0.04 |
| ANOVA, F   | 0.167      | 0.021       | 0.741        | 0.134       |
| P          | 0.848      | 0.979       | 0.494        | 0.875       |
| Control    | 26.4 ± 1.9 | 98.7 ± 5.6  | 3.76 ± 0.14  | 1.51 ± 0.03 |
| L-KYN      | 24.9 ± 1.8 | 93.2 ± 8.1  | 3.72 ± 0.09  | 1.55 ± 0.05 |
| 3-OH-KYN   | 29.0 ± 1.3 | 95.6 ± 3.9  | 3.31 ± 0.13* | 1.48 ± 0.06 |
| ANOVA, F   | 1.494      | 0.203       | 4.180        | 0.519       |
| P          | 0.256      | 0.819       | 0.036        | 0.605       |
| Control    | 23.1 ± 1.2 | 98.1 ± 4.6  | 4.30 ± 0.12  | 1.57 ± 0.05 |
| KYNA       | 22.7 ± 1.1 | 94.6 ± 3.0  | 4.20 ± 0.13  | 1.51 ± 0.03 |
| ANA        | 21.7 ± 1.0 | 89.5 ± 3.5  | 4.17 ± 0.19  | 1.53 ± 0.04 |
| ANOVA, F   | 0.464      | 1.302       | 0.198        | 0.545       |
| P          | 0.638      | 0.301       | 0.822        | 0.591       |
| Control    | 27.7 ± 2.0 | 108.3 ± 7.4 | 3.93 ± 0.06  | 1.65 ± 0.04 |
| 3-OH-ANA   | 29.0 ± 2.3 | 103.3 ± 5.4 | 3.62 ± 0.17  | 1.55 ± 0.04 |
| XAN        | 25.9 ± 2.0 | 102.3 ± 5.7 | 4.01 ± 0.19  | 1.62 ± 0.03 |
| ANOVA, F   | 0.524      | 0.260       | 1.890        | 2.190       |
| P          | 0.603      | 0.775       | 0.185        | 0.146       |

**Notes:** States are given in nmol O × min<sup>-1</sup> × mg<sup>-1</sup>. Values represent means ± S.E.M; n = 6; \*P < 0.05 in comparison to the respective control.

increased kynurenine aminotransferase activity in the brain has been reported in patients with bronchopneumonia or AIDS.<sup>22</sup> Another important factor is the aging process, since kynurenine aminotransferase activity is increased during the aging process in the rat brain and kidney, but not in the liver.<sup>47,48</sup> Increased KYNA levels were reported in aged rat brains too.<sup>48,49</sup> However, there are also data indicating reduction of L-TRP metabolism during the aging process.<sup>50</sup> Importantly, enhanced KYNA levels were also observed in human cerebrospinal fluid,<sup>51</sup> and accumulated data strongly suggest a correlation between increased KYNA levels and age-related neurodegenerative disorders.<sup>17–23</sup> Although the present study doesn't support the assumption of a correlation between neurodegeneration and the impairment of mitochondria caused by KYNA, it is important to note that an enhancement of kynurenine aminotransferase activities in the brain may increase the formation of XAN from 3-OH-KYN, as well. In the presence of XAN, the RC value of brain mitochondria was significantly lowered, since oxygen consumption during the passive state was slightly increased, whereas oxygen consumption during the active state was moderately lowered in the presence of glutamate/malate. Although the biological function of XAN is still obscure, accumulated data suggest its involvement in neurodegeneration. XAN possesses antioxidant activity, has been found to scavenge peroxyl radicals *in vitro*,<sup>44</sup> and was recently

shown to inhibit haem- and iron-induced lipid peroxidation mainly through chelation of haem and iron.<sup>52</sup> Notably, XAN was found to act as an apoptosis-inducing metabolite in vascular smooth muscle and lens epithelial cells.<sup>53,54</sup> Malina and Hess<sup>55</sup> demonstrated that accumulation of XAN in the human aortic smooth muscle cell causes proapoptotic conformation of the mitochondrial proteins (Bax, Bak, Bcl-xs and Bad). Furthermore, the authors stated that Bax-driven apoptosis may be important in the development of diabetes, atherosclerosis and neurological disorders. Interestingly, they also showed that in isolated rat liver mitochondrial fraction XAN leads to accumulation of Ca<sup>2+</sup>, while low micromolar concentrations of XAN increase oxygen consumption in the presence of succinate. Gobaille et al provided interesting data indicating a role for XAN in neurotransmission/neuromodulation.<sup>56</sup> XAN synthesis has been found to occur in the rat brain and is thought to be the main preventer of the accumulation of toxic 3-OH-KYN,<sup>56</sup> but XAN affects mitochondria as well. Importantly, XAN can modify the regulatory sequences of protein, leading to stable interactions between proteins and formation of a new pathological network, called *misfoldome*.<sup>57</sup> With respect to that, we can expect increased kynurenine aminotransferase activities, observed in the brain of Alzheimer's patients or HIV-1 infected patients or in patients with pneumonia, to enhance not only KYNA,<sup>18,22</sup> but probably





**Table 5.** Respiratory parameters of glutamate/malate-respiring rat heart mitochondria (0.5 mg protein per mL) in the absence (Control) or in the presence of 1 mM L-tryptophan (L-TRP) or its metabolites L-kynurenine (L-KYN), kynurenic acid (KYNA), anthranilic acid (ANA), 3-hydroxykynurenine (3-OH-KYN), 3-hydroxyanthranilic acid (3-OH-ANA), xanthurenic acid (XAN) and quinolinic acid (QUIN), respectively.

| SUBSTANCES | STATE 2      | STATE 3       | RC             | ADP/O         |
|------------|--------------|---------------|----------------|---------------|
| Control    | 22.4 ± 1.8   | 151 ± 11      | 6.81 ± 0.32    | 2.45 ± 0.09   |
| L-TRP      | 22.5 ± 2.0   | 142 ± 11      | 6.34 ± 0.29    | 2.49 ± 0.11   |
| QUIN       | 22.3 ± 2.2   | 143 ± 11      | 6.56 ± 0.38    | 2.47 ± 0.10   |
| ANOVA, F   | 0.0034       | 0.219         | 0.506          | 0.046         |
| P          | 0.997        | 0.806         | 0.613          | 0.955         |
| Control    | 23.7 ± 1.7   | 150 ± 13      | 6.32 ± 0.29    | 2.63 ± 0.07   |
| L-KYN      | 23.3 ± 1.5   | 145 ± 15      | 6.18 ± 0.31    | 2.63 ± 0.07   |
| 3-OH-KYN   | 23.2 ± 1.6   | 94.7 ± 8.6**  | 4.07 ± 0.21*** | 2.48 ± 0.07   |
| ANOVA, F   | 0.022        | 6.117         | 21.191         | 1.393         |
| P          | 0.978        | 0.011         | <0.001         | 0.279         |
| Control    | 20.9 ± 1.0   | 123.7 ± 5.5   | 5.97 ± 0.28    | 2.51 ± 0.04   |
| KYNA       | 24.8 ± 0.9*  | 117.4 ± 4.1   | 4.75 ± 0.15**  | 2.43 ± 0.05   |
| ANA        | 21.7 ± 0.8   | 88.6 ± 1.7*** | 4.09 ± 0.12*** | 2.51 ± 0.07   |
| ANOVA, F   | 5.418        | 20.955        | 24.161         | 0.798         |
| P          | 0.017        | <0.001        | <0.001         | 0.469         |
| Control    | 21.0 ± 0.6   | 127.8 ± 7.8   | 6.09 ± 0.29    | 2.64 ± 0.05   |
| 3-OH-ANA   | 25.4 ± 1.0** | 96.4 ± 3.8**  | 3.82 ± 0.12*** | 2.30 ± 0.08** |
| XAN        | 22.6 ± 0.6   | 126.8 ± 8.8   | 5.61 ± 0.31    | 2.56 ± 0.08   |
| ANOVA, F   | 9.341        | 6.213         | 21.843         | 6.530         |
| P          | 0.0023       | 0.011         | <0.001         | 0.0091        |

**Notes:** States are given in nmol O × min<sup>-1</sup> × mg<sup>-1</sup>. Values represent means ± S.E.M; n = 6; \*P < 0.05, \*\*P < 0.01 \*\*\*P < 0.001; in comparison to the respective control.

XAN, too. This could impair mitochondria function and play a role in the induction of a new pathological network and formation of plaques. It is believable that lowering of kynurenine aminotransferase activities by inhibitors might represent an excellent pharmacological and therapeutic approach for both processes; lowering of XAN might reduce plaque formation and lowering of KYNA might improve brain activities. Interestingly, Cerebrolysin, an anti-dementia drug, blocked kynurenine aminotransferase activities in the rat and human brain in an *in vitro* study,<sup>58</sup> and the drug is effective in preventing cognitive impairment in different experimental animal models.<sup>59,60</sup> Interestingly, in a transgenic model of Alzheimer's disease Cerebrolysin significantly ameliorated cerebrovascular amyloidosis.<sup>61</sup>

Similarly to 3-OH-KYN, 3-OH-ANA also significantly affected the bioenergetic activities of brain mitochondria with glutamate/malate and succinate as respiratory substrates, whereas liver and heart mitochondria were affected only in the presence of glutamate/malate, suggesting organ- and complex-dependent differences. This is in line with Quagliariello et al.<sup>62</sup>, who demonstrated an inhibition of oxygen uptake and oxidative phosphorylation induced by 1 mM 3-OH-ANA in rat liver and rat heart mitochondria respiring  $\alpha$ -oxoglutarate or other complex I-dependent substrates, while succinate-driven

complex II-dependent respiration was not inhibited by 3-OH-ANA. A further similarity to 3-OH-KYN is that 3-OH-ANA is a potent antioxidant<sup>63</sup> and a pro-oxidant<sup>46</sup> depending on its concentration, at least *in vitro*.<sup>45</sup> Both 3-OH-ANA and 3-OH-KYN, having an amino and a hydroxyl group in ortho-position in their aromatic rings, are good electron donors able to reduce cytochrome c as well as to autooxidize under aerobic conditions. Oxidation of these amino-phenols gives rise to quinone-imines and can concomitantly lead to ROS production.<sup>46,64</sup> Within investigated metabolites L-TRP, L-KYN and QUIN had no effect on the bioenergetic parameters of rat brain, liver or heart mitochondria independent of whether glutamate/malate or succinate were used. Lack of effect may be related to the chemical structure of these compounds. L-TRP contains a ring structure that stabilizes radicals,<sup>65</sup> and it was found to be the amino acid with the highest antiradical activities in the ferrous/cumene hydroperoxide system.<sup>66</sup> In similarity to L-TRP, L-KYN is also considered as a potential endogenous antioxidant.<sup>67–69</sup> Bordelon et al reported a significant reduction of RC values and state 3 respiration rates in brain mitochondria isolated 6 and 12 h after intrastriatal injection of QUIN, in comparison to saline-treated controls.<sup>70</sup> But this different outcome in the aforementioned *in vivo* model is the neurotoxic effect of QUIN in the striatum,

**Table 6.** Respiratory parameters of rotenone-inhibited succinate-respiring rat heart mitochondria (0.25 mg protein per mL) in the absence (Control) or in the presence of 1 mM L-tryptophan (L-TRP) or its metabolites L-kynurenine (L-KYN), kynurenic acid (KYNA), anthranilic acid (ANA), 3-hydroxykynurenine (3-OH-KYN), 3-hydroxyanthranilic acid (3-OH-ANA), xanthurenic acid (XAN) and quinolinic acid (QUIN), respectively.

| SUBSTANCES | STATE 2    | STATE 3      | RC            | ADP/O       |
|------------|------------|--------------|---------------|-------------|
| Control    | 81.3 ± 2.3 | 262 ± 10     | 3.25 ± 0.10   | 1.34 ± 0.04 |
| L-TRP      | 84.5 ± 3.1 | 268 ± 11     | 3.18 ± 0.07   | 1.34 ± 0.04 |
| QUIN       | 82.5 ± 2.9 | 254 ± 11     | 3.07 ± 0.05   | 1.30 ± 0.04 |
| ANOVA, F   | 0.342      | 0.476        | 1.301         | 0.404       |
| P          | 0.715      | 0.630        | 0.301         | 0.675       |
| Control    | 77.8 ± 6.2 | 269 ± 23     | 3.46 ± 0.08   | 1.31 ± 0.03 |
| L-KYN      | 80.9 ± 7.6 | 270 ± 26     | 3.33 ± 0.03   | 1.27 ± 0.03 |
| 3-OH-KYN   | 79.6 ± 7.3 | 242 ± 24     | 3.05 ± 0.05** | 1.23 ± 0.04 |
| ANOVA, F   | 0.048      | 0.400        | 13.050        | 1.673       |
| P          | 0.9538     | 0.677        | <0.001        | 0.221       |
| Control    | 88.0 ± 3.0 | 265 ± 10     | 3.03 ± 0.04   | 1.18 ± 0.04 |
| KYNA       | 95.2 ± 6.2 | 260 ± 11     | 2.78 ± 0.12   | 1.19 ± 0.02 |
| ANA        | 90.1 ± 6.7 | 262 ± 13     | 2.96 ± 0.12   | 1.21 ± 0.04 |
| ANOVA, F   | 0.446      | 0.046        | 1.702         | 0.235       |
| P          | 0.648      | 0.955        | 0.216         | 0.793       |
| Control    | 95.7 ± 4.1 | 281.4 ± 7.3  | 2.97 ± 0.11   | 1.21 ± 0.05 |
| 3-OH-ANA   | 96.0 ± 5.0 | 267.2 ± 4.2  | 2.84 ± 0.15   | 1.21 ± 0.05 |
| XAN        | 91.7 ± 4.1 | 260.4 ± 3.5* | 2.87 ± 0.13   | 1.23 ± 0.05 |
| ANOVA, F   | 0.299      | 4.148        | 0.311         | 0.054       |
| P          | 0.746      | 0.037        | 0.738         | 0.947       |

**Notes:** States are given in nmol O × min<sup>-1</sup> × mg<sup>-1</sup>. Values represent means ± S.E.M; n = 6; \*P < 0.05, \*\*P < 0.01 in comparison to the respective control.

involving increased Ca<sup>2+</sup> influx and accumulation of Ca<sup>2+</sup> in mitochondria leading to membrane potential collapse.

Guillemin and Brew pointed out significant correlations between the kynurenine pathway and the neuropathogenesis of Alzheimer's disease, particularly the aspects of QUIN neurotoxicity and its roles in lipid peroxidation and the amplification of local inflammation.<sup>25</sup>

Based on mitochondrial respiratory parameters determined in the absence and presence of L-TRP and its metabolites, we suggested that complex I of the respiratory chain,

**Table 7.** NADH oxidase activity of freeze-thawed rat liver mitochondria (1 mg protein per mL) in the presence or absence of tryptophan metabolites, respectively, given in nmol O × min<sup>-1</sup> × mg<sup>-1</sup>.

| SUBSTANCES | 0 μM       | 1000 μM       |
|------------|------------|---------------|
| L-KYN      | 74.9 ± 3.0 | 84.0 ± 1.9*   |
| KYNA       | 77.5 ± 4.5 | 81.8 ± 2.0    |
| 3-OH-KYN   | 69.8 ± 1.5 | 87.7 ± 2.3*** |
| XAN        | 70.8 ± 1.0 | 73.4 ± 2.0    |
| 3-OH-ANA   | 40.5 ± 1.8 | 58.0 ± 1.3*** |

**Notes:** Data are means ± S.E.M; n = 4; \*P < 0.05, \*\*\*P < 0.001 in comparison to the respective control (0 μM).

NADH-ubiquinone oxidoreductase, could be the target involved in the action of several L-TRP metabolites. Studies on enzymatic activities of complex I revealed that complex I of liver mitochondria was more sensitive to 3-OH-KYN and 3-OH-ANA, but not to KYNA or ANA, which specifically affected heart mitochondria. Glutamate/malate-respiring

**Table 8.** NADH-decylubiquinone oxidoreductase activity of cyanide-inhibited freeze-thawed rat liver mitochondria (0.2 mg protein per mL) in the presence or absence of tryptophan or its metabolites, respectively, given in nmol NADH × min<sup>-1</sup> × mg<sup>-1</sup>.

| SUBSTANCES | 0 μM            | 125 μM             | 1000 μM         |
|------------|-----------------|--------------------|-----------------|
| L-TRP      | 41.7 ± 1.2 (12) | 41.9 ± 1.9 (8)     | 39.9 ± 1.2 (12) |
| L-KYN      | 41.7 ± 0.7 (8)  | 42.3 ± 0.9 (8)     | –               |
| KYNA       | 43.3 ± 1.4 (8)  | 42.8 ± 1.3 (8)     | –               |
| ANA        | 41.3 ± 1.5 (12) | 41.2 ± 1.4 (12)    | 40.5 ± 1.2 (12) |
| 3-OH-KYN   | 39.8 ± 1.2 (12) | 51.8 ± 1.2 (12)*** | –               |
| XAN        | 40.6 ± 1.1 (12) | 39.4 ± 1.3 (12)    | –               |
| 3-OH-ANA   | 40.1 ± 0.8 (12) | 43.0 ± 0.7 (12)*   | –               |
| QUIN       | 40.9 ± 1.4 (12) | 41.3 ± 1.2 (12)    | 39.8 ± 1.0 (12) |

**Notes:** Data are means ± S.E.M. (n); \*P < 0.05, \*\*\*P < 0.001 in comparison to the respective control (0 μM).

**Table 9.** NADH-ferricyanide oxidoreductase activity of cyanide-inhibited freeze-thawed rat liver mitochondria (0.02 mg protein per mL) in the presence or absence of tryptophan or its metabolites, respectively, given in  $\text{nmol K}_3[\text{Fe}(\text{CN})_6] \times \text{min}^{-1} \times \text{mg}^{-1}$ .

| SUBSTANCES | 0 $\mu\text{M}$ | 1000 $\mu\text{M}$ |
|------------|-----------------|--------------------|
| L-TRP      | 2212 $\pm$ 81   | 2210 $\pm$ 63      |
| L-KYN      | 2108 $\pm$ 53   | 2296 $\pm$ 49*     |
| KYNA       | 2304 $\pm$ 74   | 2303 $\pm$ 80      |
| ANA        | 2119 $\pm$ 19   | 2133 $\pm$ 17      |
| QUIN       | 2025 $\pm$ 50   | 2048 $\pm$ 47      |

**Notes:** Data are means  $\pm$  S.E.M; n = 13; \* $P < 0.05$  in comparison to the respective control (0  $\mu\text{M}$ ).

liver mitochondria treated with 3-OH-KYN or 3-OH-ANA showed significantly increased NADH-decylubiquinone oxidoreductase activities corresponding to the accelerated oxygen consumption observed during state 2. The activity of NADH oxidase involving electron transfer from complex I to complex IV was likewise enhanced by 3-OH-ANA and 3-OH-KYN. L-TRP and its metabolites, except 3-OH-KYN and 3-OH-ANA, influenced neither NADH-decylubiquinone oxidoreductase nor respiratory activities of liver mitochondria. Interestingly, NADH oxidase and NADH-ferricyanide oxidoreductase were moderately but significantly increased by L-KYN while oxygen consumption rates of glutamate/malate-respiring liver mitochondria were not affected by L-KYN. This difference might be due to a partial conversion of L-KYN to 3-OH-KYN by kynurenine 3-monooxygenase located in the outer mitochondrial membrane.<sup>50</sup> Nicotinamide nucleotide transhydrogenase, an enzyme of the inner mitochondrial membrane, could deliver the co-substrate NADPH required for this conversion, from NADH, which was present in saturating concentrations (1 mM) in NADH oxidase and NADH-ferricyanide oxidoreductase assays with freeze-thawed liver mitochondria.

The effect of L-TRP and its metabolites on respiratory parameters of brain, liver and heart mitochondria was tested at high concentration (1 mM). Therefore, one could argue that the physiological significance of these *in vitro* model experiments for the *in vivo* situation could be questionable. It is important to mention that submicromolar endogenous levels of L-TRP metabolite, as reported by our studies<sup>18,21–23,51</sup> and several others<sup>3,17,20,25,43</sup> do not take into account that concentration found in homogenate of whole tissue could be significantly lower than *in vivo* concentrations in compartments of their synthesis, eg, mitochondria.

The metabolism of L-TRP via kynurenine is over-activated under various pathological conditions as can be seen in the CNS or serum of HIV-1 infected patients,<sup>20–22</sup> stroke patients,<sup>23,31</sup> picorna virus encephalomyocarditis,<sup>71</sup> and particularly in neuroborreliosis (personal observation).

In addition, several metabolites might have synergistic effects contributing to the impairments of mitochondria and

to pathological processes.<sup>72</sup> Therefore, the combined effects of the neuroactive kynurenine pathway metabolites, ie 3-OH-KYN and ANA or 3-OH-KYN and QUIN etc, and also dose response, will be of particular significance. In our previous study, we tested only the combinatorial effect of KYNA and ANA on heart mitochondria,<sup>29</sup> but the effect of combinations of L-TRP metabolites on brain, liver or heart mitochondria needs to be the topic of further investigation.

In summary, our data revealed significant differences between respiratory parameters of brain, liver and heart mitochondria of adult male Sprague-Dawley rats regarding their response to L-TRP, L-KYN, KYNA, ANA, 3-OH-KYN, XAN, 3-OH-ANA or QUIN in the presence of glutamate/malate or succinate. Brain and liver mitochondria were less affected by kynurenines compared to heart mitochondria. Notably, differences in the effect of KYNA on heart mitochondria and the effect of XAN on brain mitochondria might be of particular significance, taking into consideration the aging process and inflammatory and/or degenerative processes.

## Abbreviation List

ANA: anthranilic acid  
ADP/O: adenosine diphosphate/oxygen ratio  
BSA: bovine serum albumin  
CNS: central nervous system  
CO: control  
DETAPAC: diethylenetriaminepentaacetic acid  
EAA: excitatory amino acid  
KYNA: kynurenic acid  
L-KYN: L-kynurenine  
L-TRP: L-tryptophan  
3-OH-ANA: 3-hydroxyanthranilic acid  
3-OH-KYN: 3-hydroxykynurenine  
XAN: xanthurenic acid  
QUIN: quinolinic acid  
RC: respiratory control  
ROS: reactive oxygen species

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## Author Contributions

Conceived and designed the experiments: HB. Analyzed the data: KS, MB-S, MA, HB, BK, CK. Wrote the first draft of the manuscript: HB. Contributed to the writing of the manuscript: KS, BK. Agree with manuscript results and conclusions: HB, KS, MB-S, MA, CK, BK. Jointly developed the structure and arguments for the paper: HB, KS, BK. Made critical revisions and approved final version: KS, BK, HB. All authors reviewed and approved of the final manuscript.



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