

# Non-Nucleoside Reverse Transcriptase Inhibitors Efavirenz and Nevirapine Inhibit Cytochrome C Oxidase in Mouse Brain Regions

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**Abstract** The highly active antiretroviral therapy completely changed the clinical evolution of HIV infection, reducing the morbidity and mortality among human immunodeficiency virus (HIV)-1 infected patients. Therefore, in the present study we evaluated the effect of chronic efavirenz (EFV) and nevirapine (NVP) administration on mitochondrial respiratory chain complexes activities (I, II, II–III, IV) in different brain regions of mice. Mice were orally administered via gavage with EFV 10 mg/kg, NVP 3.3 mg/kg or vehicle (controls) once a day for 36 days. We observed that the complex IV activity was inhibited by both EFV and NVP in cerebral cortex, striatum and hippocampus of mice, but not in cerebellum, as compared to control group. In contrast, chronic EFV and NVP administration did not alter complexes I, II and II–III. We speculated that brain energy metabolism dysfunction could be involved in the CNS-related adverse effects.

**Keywords** Antiretroviral drugs · Efavirenz · Nevirapine · Mitochondrial respiratory chain · Energy metabolism

## Introduction

The development of the highly active antiretroviral therapy (HAART) completely changed the clinical evolution of HIV infection, reducing the morbidity and mortality among human immunodeficiency virus (HIV)-1 infected patients. Although HAART is successful in controlling, at least partially, acquired immune deficiency syndrome (AIDS), the concern with the long-term adverse effects induced by this therapy has been increasingly emphasized, including cognitive impairment and neurodegeneration [1].

The use of efavirenz (EFV) and nevirapine (NVP), two non-nucleoside reverse transcriptase inhibitors (NNRTIs) widely used in initial therapy for HIV infection, has been limited because of their propensity to cause adverse effects. In this scenario, side effects of EFV treatment are mainly associated with central nervous system [2], and neurological symptoms may affect up to one-half of patients during initial stage of treatment. It has been proposed that plasma and intracellular EFV concentrations and CYP2B6 genotype are predictors of early neuropsychological disturbances provoked by this drug [2, 3]. Similarly to EFV, NVP is also able to cross the blood–brain barrier, enter the central nervous system [4, 5], and cause some adverse effects [6]. However, NVP toxicity is primarily associated with skin and liver, which may range from mild to severe, and rarely result in life-threatening liver failure or toxic epidermal necrolysis. Recently, skin rash was shown to be immune-mediated in rats treated with NVP, which was prevented by partial depletion of CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T cells [7]. At the present, very little is known

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regarding the toxicity of these drugs and the exact mechanisms responsible for CNS-related side effects associated with EFV and NVP remain unknown. Therefore, in the present study we evaluated the effect of chronic EFV and NVP administration on mitochondrial respiratory chain complexes activities (I, II, II–III, IV) in different brain regions of mice.

## Experimental Procedure

### Animals

Male CF-1 mice (age 60–90 days; weight 30–40 g) were obtained from the State Foundation for Health Science Research (FEPPS-RS, Porto Alegre, Brazil), and maintained in the animal facility of Universidade do Extremo Sul Catarinense (UNESC) for at least 2 weeks before drug treatment. The animals were housed in plastic cages, eight per cage, under 12 h light/dark cycle (lights on 7:00 A.M.) at constant room temperature of  $23 \pm 1^\circ\text{C}$ ; with water and food ad libitum. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care, with the approval of local Ethics Committee.

### Drugs and Treatment

Two NNRTIs were used in this study: efavirenz (EFV-Patheon Inc., Mississauga, Ontario, Canada) and nevirapine (NVP-Boehringer Ingelheim Roxane, Inc. Columbus, Ohio, USA). Mice were orally administered via gavage with EFV 10 mg/kg, NVP 3.3 mg/kg or vehicle in a volume of 1 mL/kg, once a day for 36 days [8]. EFV and NVP were dissolved in distilled water slightly warmed with 1% Tween 80. The control group received a solution consisting of distilled water with 1% Tween 80. Drug solutions were daily prepared before the administration. The animals were killed by decapitation 3 h after the last administration of the drugs. The brain was immediately removed and cerebral cortex, striatum, hippocampus and cerebellum were isolated.

### Tissue and Homogenate Preparation

The brain structures were homogenized (1:10, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 IU/ml heparin). The homogenates were centrifuged at  $800\times g$  for 10 min and the supernatants were kept at  $-70^\circ\text{C}$  until the determination of the enzyme

activities. This preparation contains a suspension of mixed and preserved organelles. The period between homogenate preparation and enzyme activity analysis was always less than 5 days. Protein content was determined by the method described by Lowry and colleagues [9] using bovine serum albumin as standard.

### Mitochondrial Respiratory Chain Enzymes Activities

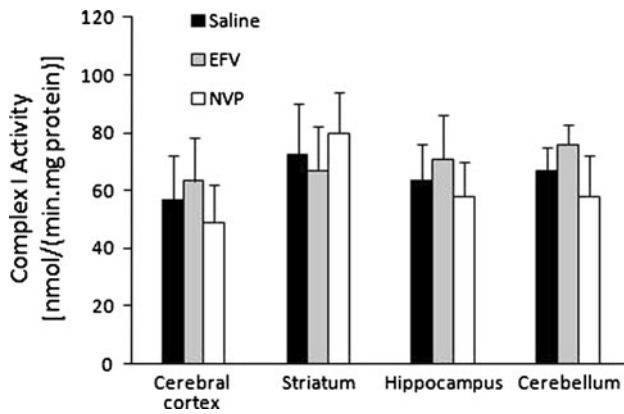
At the day of the assays, the samples were freeze-dried and thawed three times to disrupt inner mitochondrial membrane, therefore fully exposing the catalytic site of the enzymes to the substrates to achieve maximal activities. NADH dehydrogenase (complex I) activity was evaluated by assessing the rate of NADH-dependent ferricyanide reduction at 420 nm [10]. The succinate: DCIP oxidoreductase (complex II) and succinate: cytochrome *c* oxidoreductase (complex II–III) activities were determined according to the method described by Fischer and colleagues [11]. Complex II activity was measured by following the decrease in absorbance due to the reduction of 2,6-DCIP at 600 nm. Complex II–III activity was measured by following cytochrome *c* reduction from succinate at 550 nm. The activity of cytochrome *c* oxidase (complex IV) was assayed according to the method described by Rustin and colleagues [12] by following the decrease in absorbance due to the oxidation of previously reduced cytochrome *c* at 550 nm. Cytochrome *c* was reduced with the addition of sodium borohydride. The activities of the mitochondrial respiratory chain complexes were expressed as  $\text{nmol min}^{-1} \text{mg protein}^{-1}$ . In some experiments, specific inhibitors of the respiratory chain complexes were also added to the incubation medium. Residual activity of the different respiratory chain complexes in the presence of specific inhibitors was always less than 10%, as compared to controls.

### Statistical Analysis

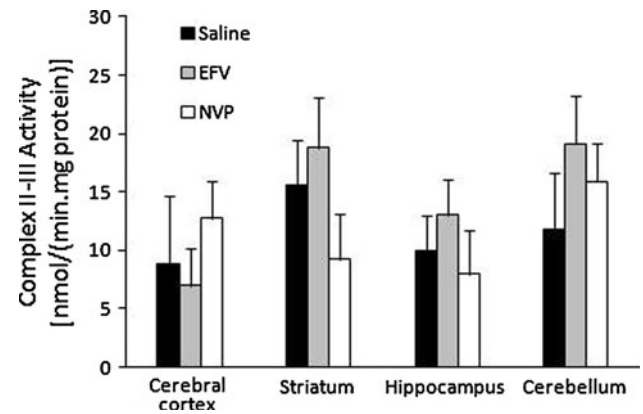
Data are expressed as mean  $\pm$  standard deviation and were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's test when *F* was significant. All analyses were performed using the Statistical Package for the Social Science (SPSS) software.

## Results

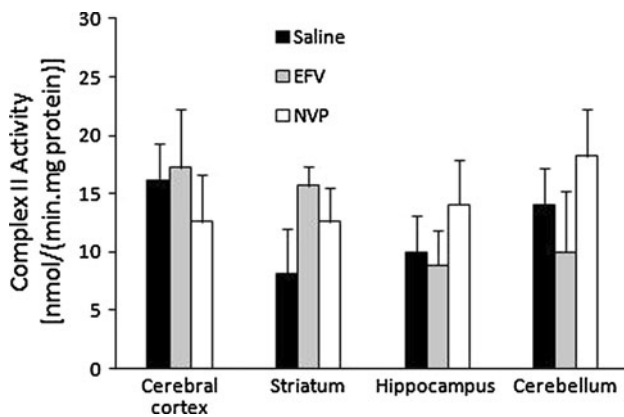
We evaluated the effect of chronic administration of anti-retroviral drugs, namely EFV and NVP, on the activities of the respiratory chain complexes I, II, II–III and IV in



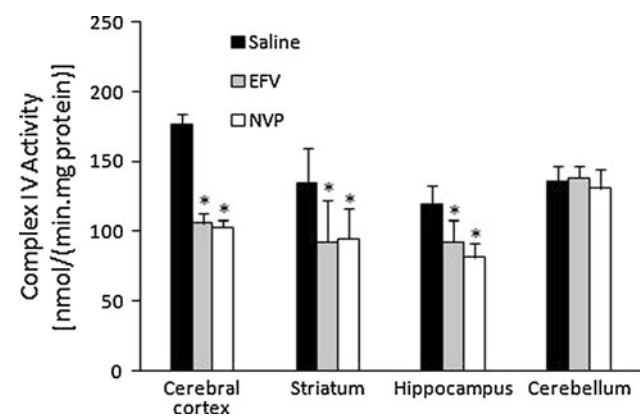
**Fig. 1** Effect of chronic efavirenz and nevirapine administration on complex I activity in cerebral cortex, striatum, hippocampus and cerebellum of mice. Data are expressed as means  $\pm$  SD for 6 independent experiments performed in duplicate. No differences were observed between groups



**Fig. 3** Effect of chronic efavirenz and nevirapine administration on complex II–III activity in cerebral cortex, striatum, hippocampus and cerebellum of mice. Data are expressed as means  $\pm$  SD for 6 independent experiments performed in duplicate. No differences were observed between groups



**Fig. 2** Effect of chronic efavirenz and nevirapine administration on complex II activity in cerebral cortex, striatum, hippocampus and cerebellum of mice. Data are expressed as means  $\pm$  SD for 6 independent experiments performed in duplicate. No differences were observed between groups



**Fig. 4** Effect of chronic efavirenz and nevirapine administration on complex IV activity in cerebral cortex, striatum, hippocampus and cerebellum of mice. Data are expressed as means  $\pm$  SD for 6 independent experiments performed in duplicate. \* $P < 0.01$  compared to control (Tukey's test)

different brain regions of mice; the animals received anti-retroviral drugs for 36 days.

It was first observed that chronic EFV and NVP administration did not alter enzyme activities of mitochondrial respiratory chain complex I, II and II–III in cortex, striatum, hippocampus and cerebellum of mice (Figs. 1, 2 and 3, respectively). In contrast, the complex IV activity was inhibited by both EFV and NVP in cerebral cortex (sal:  $176 \pm 33$ , EFV:  $107 \pm 21^*$ , NVP:  $102 \pm 22^*$ ,  $P < 0.05$ ,  $n = 6$ ), striatum (sal:  $146 \pm 22$ , EFV:  $94 \pm 31^*$ , NVP:  $96 \pm 21^*$ ,  $P < 0.05$ ,  $n = 6$ ) and hippocampus (sal:  $121 \pm 13$ , EFV:  $91 \pm 16^*$ , NVP:  $83 \pm 11^*$ ,  $P < 0.05$ ,  $n = 6$ ) of mice, as compared to control group (Fig. 4). Finally, complex IV activity was not altered in cerebellum of mice administered with EFV and NVP.

## Discussion

Despite the effectiveness of therapy, adherence to medications during HAART has been impaired due to severe CNS-related adverse effects in patients. EFV and NVP are non-nucleoside analogue inhibitors of HIV-1 reverse transcriptase commonly used in HAART. There are some reports showing a variety of neuropsychiatric features including anxiety, hostility and depression seen in patients related to long-term EFV use and its high serum level. Although mainly associated to skin and liver injuries, NVP has also been demonstrated to provoke some undesirable effects in CNS, especially neuropsychiatric complications and headaches [3, 13]. In this context, we have recently reported a genuine anxiogenic-like effect to EFV, since it

reduced exploration to open arms of elevated plus-maze test without affecting spontaneous locomotion. Additionally, both drugs impaired recognition memory, while only the treatment with EFV impaired significantly aversive memory [8].

Therefore, there are direct clinical evidences that the NNRTIs are able to cross blood–brain barrier and to penetrate the CNS. Both EFV and NVP can be detected in the cerebrospinal fluid in concentrations effective to suppress HIV viral levels [14, 15], which may explain their proven CNS efficacy or the manifestations of CNS-related side effects. In fact, NVP presented a higher brain accumulation as compared to other antiretroviral drugs, including abacavir, amprenavir and ritonavir [16, 17], and reached CSF/plasma ratio ranging from 15 to 40% [4].

In the present study, we demonstrated that the mitochondrial respiratory chain is inhibited by chronic administration of EFV and NVP in cortex, striatum and hippocampus of mice, at the level of complex IV activity. As regards to the possible metabolic consequences of complex IV impairment to neural metabolism and function, it should be stressed that inhibition of cytochrome *c* oxidase activity at an extent similar to that found in the present study was previously reported in clinical conditions associated with a decrease of ATP synthesis and manifested by encephalopathy, as observed in various neurodegenerative disorders such as Alzheimer's disease, amyotrophic lateral sclerosis and Leigh disease [18–28]. Additionally, complex IV inhibition may also increase ROS generation in mitochondria, leading to defects in the mitochondrial genome, lipoperoxidation and protein oxidative damage [29, 30]. Since a correlation between the intensity of HIV infection and the degree of mitochondrial damage has already been suggested [31], a possible additive or synergistic effect exacerbating mitochondrial toxicity involving EFV/NVP and HIV infection should also be considered in HIV-infected patients during HAART regimen.

On the other hand, we found that complex IV activity was not altered in cerebellum of mice receiving EFV and NVP. At the present, we cannot explain the difference between the effects of these drugs in the different brain structures. However, it may be speculated that the distinct effects elicited by EFV and NVP in the various brain structures may possibly be attributed to distinct composition of cellular milieu, to tissue-specific isoforms of various nuclear-encoded subunits of the respiratory chain complexes [12, 32, 33] or alternatively, by a lower permeability of EFV and NVP into the cerebellum. In addition, since there is a short time interval between the last drug administration and tissue collection, it is also uncertain as to whether any effect on mitochondria is a pharmacological (or direct) effect of the drug.

In conclusion, to our knowledge the present study reported for the first time that EFV and NVP disrupt brain energy metabolism in mice by inhibiting cytochrome *c* oxidase in a structure-specific manner. Our data are in line with previous findings showing that chronic EFV and NVP administration impair creatine kinase activity in brain of mice [34]. It may therefore be speculated that brain energy metabolism dysfunction could be involved in the CNS-related adverse effects observed in HIV-infected patients during HAART therapy with EFV and NVP.

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