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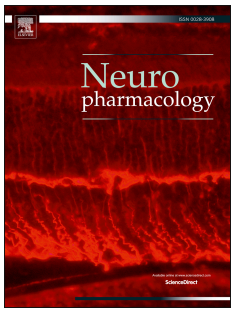

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PII: S0028-3908(17)30175-2

DOI: 10.1016/j.neuropharm.2017.04.025

Reference: NP 6681

To appear in: *Neuropharmacology*

Received Date: 19 November 2016

Revised Date: 10 April 2017

Accepted Date: 14 April 2017

Please cite this article as: Villa, R.F., Ferrari, F., Bagini, L., Gorini, A., Brunello, N., Tascedda, F., Mitochondrial energy metabolism of rat hippocampus after treatment with the antidepressants desipramine and fluoxetine, *Neuropharmacology* (2017), doi: 10.1016/j.neuropharm.2017.04.025.

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ACCEPTED MANUSCRIPT

Mitochondrial energy metabolism of rat hippocampus after treatment with the antidepressants desipramine and fluoxetine

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Abstract

Alterations in mitochondrial functions have been hypothesized to participate in the pathogenesis of depression, because brain bioenergetic abnormalities have been detected in depressed patients by neuroimaging *in vivo* studies. However, this hypothesis is not clearly demonstrated in experimental studies: some suggest that antidepressants are inhibitors of mitochondrial metabolism, while others observe the opposite.

In this study, the effects of 21-day treatment with desipramine (15 mg/kg) and fluoxetine (10 mg/kg) were examined on the energy metabolism of rat hippocampus, evaluating the catalytic activity of regulatory enzymes of mitochondrial energy-yielding metabolic pathways. Because of the micro-heterogeneity of brain mitochondria, we have distinguished between (a) non-synaptic mitochondria (FM) of neuronal perikaryon (post-synaptic compartment) and (b) intra-synaptic light (LM) and heavy (HM) mitochondria (pre-synaptic compartment).

Desipramine and fluoxetine changed the catalytic activity of specific enzymes in the different types of mitochondria: (a) in FM, both drugs enhanced cytochrome oxidase and glutamate dehydrogenase, (b) in LM, the overall bioenergetics was unaffected and (c) in HM only desipramine increased malate dehydrogenase and decreased the activities of Electron Transport Chain Complexes.

These results integrate the pharmacodynamic features of desipramine and fluoxetine at subcellular level, overcoming the previous conflicting data about the effects of antidepressants on brain energy metabolism, mainly referred to whole brain homogenates or to bulk of cerebral mitochondria. With the differentiation in non-synaptic and intra-synaptic mitochondria, this study demonstrates that desipramine and fluoxetine lead to adjustments in the mitochondrial bioenergetics respect to the energy requirements of pre- and post-synaptic compartments.

Keywords

Desipramine; Fluoxetine; Mitochondria; Functional Proteomics; Brain Energy Metabolism

Chemical Compounds:

Desipramine (PubChem CID: 2995); Fluoxetine Hydrochloride (PubChem CID: 62857).

Abbreviations

5-HT: 5-hydroxytryptamine; AD: Antidepressant Drugs; CBF: Cerebral Blood Flow; CCRS: NADH-cytochrome *c* Reductase Rotenone-Sensitive; CCRT: NADH-cytochrome *c* Reductase as Total; CMR_{glu}: Cerebral Metabolic Rate for Glucose; CNS: Central Nervous System; COX: Cytochrome Oxidase; CS: Citrate Synthase; ETC: Electron Transport Chain; FM: Non-synaptic Mitochondria; FST: Forced Swimming Test; GlDH: Glutamate Dehydrogenase; HM: Intra-synaptic Heavy Mitochondria; LM: Intra-synaptic Light Mitochondria; MDH: Malate Dehydrogenase; NE: Norepinephrine; NGF: Nerve Growth Factor; OGI: Oxygen-Glucose Index; PET: Positron Emission Tomography; rCBF: Regional Cerebral Blood Flow; SA: Specific Activity; SD: Sprague-Dawley; SDH: Succinate Dehydrogenase; SSRI: Selective Serotonin Reuptake Inhibitor; TCA: Tricyclic Antidepressant.

1. Introduction

The historically leading theory for the pathogenesis of depression is the biogenic amine hypothesis, that was suggested in 1960s because of the decreased concentrations of norepinephrine (NE) and 5-hydroxytriptamine (5-HT) observed in the brains of depressed patients (Crossland, 1963; Kety, 1963). The current main pharmacological therapies are still based on this hypothesis, as reviewed by Ferrari and Villa (2017).

However, depressive disorders are heterogeneous diseases and therapy of depression is not devoid of concerns, including: (i) the time-lag between the acute pharmacological effect, *i.e.* the increase of neurotransmitter brain concentrations, and the therapeutic efficacy, respectively occurring within hours and after weeks of treatment, and (ii) the presence of treatment-resistant depression, accounting for 30-40% of clinical cases. Therefore, new hypotheses and therapeutic strategies are needed.

In this context, many studies observed that mood disorders are associated with alterations in the intracellular signal transduction pathways originating from the activation of NE or 5-HT receptors (Brunello and Tascedda, 2003; Duman et al., 1997; Lenox et al., 1998; Manji et al., 1995, 1996; Perez et al., 2000; Popoli et al., 2000; Racagni et al., 1992). Consequently, the original biogenic amine hypothesis has been updated to include changes in down-regulation and desensitization of pre- and post-synaptic NE and 5-HT receptors (Hamon and Blier, 2013). Moreover, because many molecules of ATP are needed for the activation of the intracellular signaling pathways triggered by the binding of neurotransmitters to their receptors, an increasing interest has been developed about the

bioenergetic alterations of the cerebral tissue in mood disorders (reviewed by Moretti et al., 2003). This has lead to formulate the mitochondrial pathogenetic hypothesis of depression (Adzic et al., 2016; Ferrari and Villa, 2016; Wang and Dwivedi, 2016).

At present, proteomic studies performed on after death brains of depressed patients indicate that mood disorders share about 21% of modified proteins, being these proteins mostly related to deregulation of energy metabolism pathways (Saia-Cereda et al., 2016). In addition, neuroimaging *in vivo* studies on human depressed patients reported some modifications of brain energy metabolism, *i.e.* changes in Cerebral Blood Flow (CBF) and Cerebral Metabolic Rate of glucose (CMR_{glu}) (Drevets, 1999, 2000; Price and Drevets, 2012; Stoll et al., 2000). In particular, in the complex pattern of the detected abnormalities, the following neuroimaging findings may be summarized: (i) the thalamus and amygdala are hypermetabolic, while (ii) the anterior cingulate cortex, prefrontal cortex and hippocampus are hypometabolic. As a consequence, the control exerted by the cerebral cortex and hippocampus towards the amygdala does not properly function in depression, and a long-lasting stress-activated *status* is likely established. The resulting persistent glucocorticoid release sustains this vicious cycle and may cause: (i) cortical and hippocampal atrophy and (ii) the monoamine depletion as an adapting response (Ferrari and Villa, 2016).

Therefore, the frontal cerebral cortex and hippocampus seem to be primarily involved in energy metabolism abnormalities in depression (Detka et al., 2015) and the aim of the present research was to evaluate the effects of sub-chronic 21-day pharmacological treatment with desipramine (a tricyclic antidepressant, TCA) and fluoxetine (a selective serotonin reuptake inhibitor, SSRI) on brain energy metabolism of rat hippocampus. The effects of these drugs on rat frontal cerebral cortex have been previously assessed in the same experimental settings (Villa et al., 2016).

The energy metabolism has been studied assaying the catalytic activities of regulatory enzymes of mitochondrial energy-yielding metabolic pathways (functional proteomics). In fact, enzyme activities are indicative of the cerebral tissue ability to respond efficiently (i) to pathological *noxae* and (ii) to pharmacological treatments (Ferrari et al., 2015; Villa et al., 1992, 2013a, 2013b). Moreover, (iii) enzyme activities may be the direct molecular targets of drugs (Moretti et al., 2011, 2015a, 2015b; Villa and Gorini, 1997; Villa et al., 2012a).

In this context, it is of interest the possibility of distinguishing brain mitochondria according to their *in vivo* localization in different sub-cellular neuronal compartments, in

the perspective of evaluating the effects of drugs diversifying between pre- and post-synaptic terminals. Therefore, because of this micro-heterogeneity of brain mitochondria (Villa and Gorini, 1991; Villa et al., 1989, 2012b, 2013a), this research was performed on: (i) non-synaptic mitochondria of neuronal perikaryon, *in vivo* located within the post-synaptic compartment, and (ii) intra-synaptic light and heavy mitochondria (two types), *in vivo* located in the pre-synaptic compartment.

This technology was previously proven to be useful when evaluating the effects of the antidepressants desipramine and fluoxetine in the frontal cerebral cortex, where non-synaptic and intra-synaptic mitochondria underwent different modifications (Villa et al., 2016): (a) cytochrome oxidase activity was increased in non-synaptic mitochondria, while (b) malate dehydrogenase, succinate dehydrogenase and glutamate-pyruvate transaminase activities were decreased in intra-synaptic ones. Therefore, it is of interest to evaluate the bioenergetic modifications induced by these antidepressants also in rat hippocampus, in the perspective of confirming the validity of the employed functional proteomic approach.

In fact, this sub-cellular study may overcome the conflicting data so far obtained about the action of antidepressants on mitochondrial energy metabolism, that has been previously evaluated only on pooled mitochondria. For example, Souza et al. (1994) observed that fluoxetine *in vivo* administration stimulated the rat liver mitochondrial state 4 respiration for α-ketoglutarate or succinate oxidations, and this uncoupling effect of oxidative phosphorylation was described also in rat brain mitochondria after the administration of TCAs and other psychotropic drugs (Abdel-Razaq et al., 2011; Byczkowski and Borysewicz, 1979; Ferreira et al., 2014; Fromenty et al., 1989; Weinbach et al., 1986).

On the other hand, imipramine increased the intra-mitochondrial content of cytochrome *b* and $c + c_1$ after 1 week of treatment and that of aa_3 cytochrome after 2 week of treatment (Katyare and Rajan, 1995). Also nortriptyline was identified as a strong inhibitor of mitochondrial permeability transition and likely for this reason is neuroprotective in *in vitro* and *in vivo* models of cerebral ischemia (Zhang et al., 2008). Recently, Filipović et al. (2017) showed that fluoxetine treatment increased the energy metabolism towards the citric acid cycle and oxidative phosphorylation in a mitochondrial proteome study. Therefore, by taking into account the micro-heterogeneity of cerebral mitochondria populations, it would be possible to cast new insights into the molecular mechanisms of action of antidepressant drugs.

2. Materials and Methods

2.1. Care of the animals and pharmacological treatment

The experiments were performed on male CD Sprague-Dawley (SD) rats (Charles-River). The animals were kept from birth under standard cycling and housing conditions (temperature: 22 ± 1°C; relative humidity 60 ± 3%; lighting cycle: 12 hours light and 12 hours darkness; low noise disturbances), fed with a standard diet in pellets with water *ad libitum*.

The selection of the animals for pharmacological treatment was established by Fisher and Yates permutation tables, and the rats were divided in three experimental lots: (a) control animals treated with saline physiological solution; (b) animals treated with desipramine (desmethylimipramine; 3-(10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepin-5-yl)-N-methylpropan-1-amine) at the dose of 15 mg/kg b.w. per day, by intraperitoneal injection; (c) animals treated with fluoxetine hydrochloride ((*RS*)-*N*-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy] propan-1-amine) at the dose of 10 mg/kg b.w. per day, by intraperitoneal injection. The pharmacological treatment was started from the 7th week of age and continued for 21 days, so to take into consideration the known time-lag between the pharmacological and therapeutic effect of these drugs.

At the end of treatments, at the 10th week of age, the animals were sacrificed under anesthesia by ether (Merck Darmstadt, Germany) at 09:00 a.m., after 24 hours from the last drug administration, and the brain was isolated at the University of Modena and Reggio Emilia.

From the isolated brains, the hippocampus was carefully dissected according to Glowinsky and Iversen (1966) and immediately frozen in liquid nitrogen (< 20 sec) to subsequently perform the sub-fractionation techniques and the enzymatic analyses at University of Pavia.

Animal maintenance and research approval for this study has been given by *ad hoc* authorities and in accordance with the guidelines of the Italian Ministry of Health, which are in compliance with international laws and policies.

2.2. Preparation of the synaptosomal fraction and of non-synaptic "free" mitochondria

The synaptosomal fraction and non-synaptic mitochondria were prepared according to the method of Lai et al. (1977), as modified for analytical evaluations for single brain area of single animal by Villa and co-workers (Villa and Gorini, 1991; Villa et al., 1989).

At the time of experiment, the frontal cerebral cortex was thawed and immediately placed in the isolation medium consisting of 0.32 M sucrose (Merck), 1.0 mM EDTA-K^+ (Sigma-Aldrich Company St. Louis, MO, USA), 10 mM Tris-HCl, pH 7.4 (Merck). The homogenate was obtained by a Teflon-glass homogenizer (Braun S Homogenizer, Melsungen, Germany) by five up and down strokes of the pestle (total clearance 0.1 mm) rotating at 800 r.p.m., with electronic control of the speed. The homogenate was diluted (7-10% w/v) and the nuclear fraction was removed by centrifugation at 3.6 x 10^3 *g* x min (2.37 x 10^7 ω^2t) in a Beckman J2-21 Supercentrifuge (Palo Alto, CA, USA), rotor JA-17 (Galway, Ireland). The nuclear pellet was washed twice by resuspension in the isolation medium and centrifuged in the same conditions.

The three combined supernatants were centrifuged at 288 x 10^3 g x min (189.2 x 10^7 ω^2t) to yield the *crude* mitochondrial pellet. The *crude* mitochondrial fraction, containing synaptosomes, was resuspended by soft homogenization in IM and was applied on a discontinuous Ficoll-sucrose gradient (7.5%-12% w/w). Ficoll (Pharmacia Biotech, AB Uppsala, Sweden) was dissolved in stock solution consisting of 0.32 M sucrose, 50 μM EDTA-K^+, 10 mM Tris-HCl, pH 7.4. This gradient was centrifuged at 175.2 x 10^4 *g* x min (123.7 x 10^8 ω^2t) in the OTD-65B Sorvall Ultracentrifuge, rotor AH-650 (Newtown, CT, USA).

After centrifugation, the myelin fraction was sucked off and the synaptosomal fraction at the interface of the 7.5-12% Ficoll-sucrose interphase was collected by aspiration, then diluted three-fold with isolation medium and centrifuged at 288 x 10^3 *g* x min (189.2 x 10^7 ω^2t), rotor JA-17.

The pellet, consisting of the purified *free* mitochondrial fraction (FM) was resuspended in sucrose solution 0.32 M, pH 7.4, and pelleted at 162.4 x 10^3 *g* x min (106.7 x 10^7 ω^2t); the pellet was then resuspended by soft homogenization in a small volume of 0.32 M sucrose buffered solution (pH 7.4) for the assay of the catalytic activity of enzymes. Remarkably, validation of the employed isolation technique (Villa et al., 1989) using various biochemical parameters (Battino et al., 2002; Genova et al., 1997) confirmed that, the presence of glial mitochondria is minimal, and post-synaptic mitochondria are "enriched" mainly of mitochondria of neuronal origin.

2.3. Preparation of intra-synaptic light and heavy mitochondria

The synaptosomal pellet previously isolated from Ficoll-sucrose gradient, was lysed by resuspension in 6 mM Tris-HCl, pH 8.1, by soft homogenization (Villa et al., 1989).

After osmotic shock, the lysate was centrifuged at 399 x 10^3 *g* x min (262.1 x 10^7 ω^2t) and the pellet obtained from lysed synaptosomes was again resuspended and centrifuged at 192.6 x 10^3 *g* x min (126.5 x 10^7 ω^2t); at the end of this centrifugation, the pellet was resuspended in a medium consisting of 3% w/w Ficoll, 0.12 M mannitol (Merck), 30 mM sucrose, 25 μM EDTA-K^+, 5 mM Tris-HCl, pH 7.4. This suspension was layered on a Ficoll discontinuous gradient consisting of two layer 4.5% w/w Ficoll in 0.24 M mannitol, 60 mM sucrose, 50 μM EDTA-K^+, 10 mM Tris-HCl, pH 7.4 and, at the bottom, 6% w/w Ficoll in the same solution. This gradient was centrifuged at 280.2 x 10^3 *g* x min (197.3 x 10^7 ω^2t), rotor AH-650.

At the end of this centrifugation, the upper phase, the *light* intra-synaptic mitochondrial fraction (LM), was sucked off and pelleted at 166.5 x 10^3 *g* x min (109.4 x 10^7 ω^2t); the pellet of this centrifugation and that from the gradient, the *heavy* intra-synaptic mitochondrial fraction (HM), were separately resuspended in 0.32 M sucrose buffered solution (pH 7.4) and centrifuged at 162.4 x 10^3 *g* x min (106.7 x 10^7 ω^2t). The washed pellets were finally resuspended in the same washing solution for the assay of the catalytic activity of enzymes.

We have expressed the centrifugation conditions in ω^2t to indicate the total accumulated centrifugal force applied in every centrifugation.

2.4. Enzyme assayed on purified non-synaptic and intra-synaptic mitochondria

On the different sub-cellular mitochondrial fractions, the maximum rate (V_{max}) of the following enzyme activities was evaluated: citrate synthase (citrate oxaloacetate-lyase, EC 4.1.3.7) (Sugden and Newsholme, 1975), succinate dehydrogenase (succinate: oxidoreductase, EC 1.3.99.1) (Ackrell et al., 1978), that is also part of the Electron Transport Chain as Complex II, malate dehydrogenase (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37) (Ochoa, 1955) for Krebs' cycle; NADH-cytochrome *c* reductase (NADH-cytochrome *c* oxidoreductase, EC 1.6.99.3) (Nason and Vasington, 1963); cytochrome oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) (Smith, 1955; Smith et al., 1974; Wharton and Tzagoloff, 1967) for Electron Transport Chain (ETC); glutamate dehydrogenase (L-glutamate: NAD^+ oxidoreductase deaminating, EC 1.4.1.3) (Sugden and Newsholme, 1975) for glutamate and related amino acids metabolism, and the mobility of amino groups.

The protein concentration of the tested sub-fractions was determined using crystalline bovine serum albumin (Merck) as standard, according to Lowry et al. (1951).

2.5. Enzyme activity calculation and statistical analysis

Enzyme activities were measured by graphic recordings for 3 min in double beam recorder Spectrophotometer (Perkin-Elmer 554) and were expressed as specific activities [S.A.: μmoles of substrate transformed) x min^{-1} x (mg of protein)$^{-1}$].

Because all the obtained data are homoscedastic and fit in the Gauss' normal distribution, according to this experimental design, at first the homogeneity of variance was checked by Bartlett's test, and afterward the two-way homoscedastic ANOVA parametric test was used to evaluate the comparisons of each enzyme activity between: (i) the different mitochondrial populations; (ii) controls and desipramine-treated animals; (iii) controls and fluoxetine-treated ones. In addition, *post hoc* tests according to Tukey and Dunnett were used to compare the differences between individual groups, controlling statistical evaluation.

3. Results

3.1. Mitochondrial energy metabolism in control animals

In control animals (Figure 1), non-synaptic and intra-synaptic mitochondria show different levels of energy-linked enzyme activities, according to the observation that the metabolic features of these mitochondria are reflected by enzyme kinetics. This was reported also by previously published results about the effects of physiological aging, experimental physiopathology and pharmacological treatments on the same biochemical parameters (Villa et al., 2012a, 2012b, 2013a, 2016).

All Krebs' cycle enzyme activities (Figure 1A) are different in FM when compared to LM and HM (symbol: §). Citrate synthase activity was lower in intra-synaptic light and heavy mitochondria by about 61% and 56%, respectively. Succinate dehydrogenase was lower by 24% in LM and by 56% in HM *versus* FM, and by about 42% in HM *versus* LM; likewise, malate dehydrogenase activity was lower in both types of intra-synaptic mitochondria, by about 55% in intra-synaptic light and by 65% in heavy ones, being also lower in HM *versus* LM by 21% (symbol: °).

As regards ETC enzymes (Figure 1B), NADH-cytochrome *c* reductase total activity was lower in both intra-synaptic light and heavy mitochondria respect to FM by about 46%, while a different behavior was observed for cytochrome oxidase, that was higher in

intra-synaptic mitochondria compared to FM by 44% in LM and by 28% in HM, being 23% lower in HM *versus* LM.

Finally, glutamate dehydrogenase (Figure 1B) did not show any difference between the different types of hippocampal mitochondria.

3.2. Effects of desipramine and fluoxetine on mitochondrial energy metabolism

The results about the effects of sub-chronic treatment with desipramine and fluoxetine on the mitochondrial energy-linked enzyme activities of non-synaptic and intra-synaptic light and heavy mitochondria from the hippocampus of 10 week-old rats are reported in Figure 2.

Pharmacological treatment with desipramine modified some enzyme activities in non-synaptic and intra-synaptic heavy mitochondria, not affecting enzyme activities of intra-synaptic light ones.

In non-synaptic mitochondria, the drug enhanced the activities of cytochrome oxidase and markedly that of glutamate dehydrogenase. In intra-synaptic heavy mitochondria, desipramine increased malate dehydrogenase activity and decreased total NADH-cytochrome c reductase and cytochrome oxidase activities. Thus, desipramine exerted its effects differently on hippocampal mitochondria, showing complex variations particularly in the intra-synaptic heavy sub-type.

Also fluoxetine sub-chronic treatment induced selective modifications in the different mitochondrial populations but, otherwise from desipramine, only the intra-synaptic heavy mitochondria were unaffected by drug treatment.

In particular, in non-synaptic mitochondria, fluoxetine increased succinate dehydrogenase and cytochrome oxidase activities, and showed clear effects on glutamate dehydrogenase, enhancing its activity. In intra-synaptic light mitochondria, fluoxetine induced only a decrease in succinate dehydrogenase activity.

4. Discussion

4.1. Metabolic micro-heterogeneity between non-synaptic and intra-synaptic light and heavy mitochondria in control animals

Differences in the V_{max} of enzymes were observed between non-synaptic and intra-synaptic mitochondria in metabolic steady-state conditions (Figure 1). This implies that enzyme kinetics reflect the metabolic attitude and catalytic metabolic profile of these

different cerebral mitochondria. Coherently, in a quantitative proteomic analysis by Stauch et al. (2014) synaptic and non-synaptic mitochondria from 10 month-old mice differed in the expression of 522 proteins involved in oxidative phosphorylation, mitochondrial fission/fusion, Calcium transport and mitochondrial DNA replication and maintenance.

Comparing non-synaptic *versus* intra-synaptic mitochondria (Figure 1), citrate synthase and malate dehydrogenase activities were both lower in the latter mitochondrial populations. Both these activities are biochemically connected in the Krebs' cycle, because MDH refurnishes oxaloacetate as the substrate for CS activity (Villa et al., 2013a). These results are similar to those obtained in rat frontal cerebral cortex (Villa et al., 2016). Therefore, because citrate synthase is one of the major point of metabolic control of Krebs' cycle (ΔG (kJ/mol) = negative; $\Delta G^{0'}$ (kJ/mol) = - 31.5; Villa et al., 2013a), these data suggest that the metabolic flux in this cycle is higher in the neuronal soma, *i.e.* in non-synaptic mitochondria, independently from the cerebral area (cerebral cortex *versus* hippocampus).

On the contrary, the synaptic compartment is characterized by a higher energetic potential (Villa et al., 2012b), requiring less energy production, as confirmed also by a recent ultra-structural imaging study (Chavan et al., 2015). This observation is coherent with the lower total NADH-cytochrome *c* reductase (the integrated activity of Complex I-III, Genova et al., 1997) and succinate dehydrogenase (Complex II) activities in intra-synaptic mitochondria, being these enzymes respectively the first and the second entry point of the reducing equivalents produced in the Krebs' cycle in mitochondrial ETC.

Some differences were observed also between intra-synaptic mitochondria themselves: malate dehydrogenase, succinate dehydrogenase and cytochrome oxidase (Complex IV of ETC) were higher in LM compared to HM. Therefore, considering that cytochrome oxidase activity is a marker of neuronal activation (Wong-Riley, 2012), light mitochondria are metabolically more active than heavy ones, which show an overall lower energy-yielding metabolic capacity as previously noted in the rat (Battino et al., 2002) and monkey brain (Battino et al., 1996). The different metabolic attitude of cerebral mitochondria is attributable to their *in vivo* localization in pre- and post-synaptic compartments, that display different energetic needs (Brand and Nicholls, 2011; Nicholls et al., 2015).

In previous studies, we focused on the proteomic characterization of these mitochondria, highlighting that HM showed many features of an aged mitochondrial population, *i.e.* (i) partial impairment of mitochondrial function due to the continuous

damage accumulating on membranes, and (ii) higher content of hydroperoxides (Battino et al., 2002; Villa and Gorini, 1991; Villa et al., 1989). As a consequence, we have hypothesized that the intra-synaptic heavy mitochondria are the aged light ones, damaged for the high metabolic energy requirements of synapses (Battino et al., 2002).

This hypothesis was confirmed by the observation that the oxygen-glucose index (OGI) declines after neuronal activation from 6 to 2.8 (Seifert et al., 2009). This mismatch may be explained by the absence of mitochondria in the post-synaptic density at the onset of stimulation, followed by return to the normal value thanks to the gradual accumulation of functional mitochondria (Li et al., 2004). According to the results obtained in the present study and in previously published papers (Battino et al., 2002; Villa and Gorini, 1991; Villa et al., 1989), the bioenergetic, biochemical and morphological features of intra-synaptic heavy mitochondria support this hypothesis, confirming the validity of the functional proteomic approach to evaluate the changes in energy metabolism at sub-cellular level.

Therefore, different maximal rates of enzyme activities are expressed in these various types of rat hippocampal mitochondria and these differences are connected to the energy requirements of the compartments in which these mitochondria are localized. These observations are fundamental from a pharmacological point of view, because mitochondrial enzyme activities may be differently involved in the molecular action of drugs in the pre- *versus* post-synaptic compartment, respectively affecting the energy metabolism in synaptic terminals and in neuronal peri-nuclear soma.

4.2. Effect of desipramine and fluoxetine on mitochondrial energy metabolism of rat hippocampus and comparison with the frontal cerebral cortex

Desipramine and fluoxetine modified some enzymatic activities of rat hippocampus in relation to the different types of mitochondria (Figure 2). In this Section, the effects of desipramine and fluoxetine pharmacological treatments on rat hippocampus will be discussed comparing these results with those obtained in the frontal cerebral cortex.

In particular, desipramine induced heterogeneous modifications. In non-synaptic mitochondria, the drug increased cytochrome oxidase (Complex IV) activity, the key enzyme of the mitochondrial ETC: this enzyme carries the electrons from cytochrome *c* to molecular oxygen in the final step of the respiratory chain. Cytochrome oxidase activity is mainly regulated by the concentrations of reduced cytochrome *c* (c^{2+}) and, because the availability of c^{2+} is at *equilibrium* with the oxidative phosphorylation (Wilson, 1994), the

activity of Complex IV is inversely proportional to ATP concentrations and thus to the energy state of the involved cerebral area. This means that higher cytochrome oxidase activity corresponds to lower ATP concentrations.

Moreover, desipramine stimulated glutamate dehydrogenase activity, the key enzyme linking the α-ketoglutarate formed in the Krebs' cycle to glutamate metabolism: by the inversion of glutamate dehydrogenase activity, glutamate formed from glutamine may enter in the Krebs' cycle as α-ketoglutarate. In fact, this enzyme operates at the intersection of the metabolisms of: (i) carbon units, (ii) glutamate and related amino acids, (iii) moiety of amino groups and (iv) ammonia detoxification (Villa et al., 2013a). Its metabolic relevance is suggested also by the considerations that: (i) the direction of glutamate dehydrogenase (from α-ketoglutarate to glutamate or *viceversa*) is regulated by ATP concentrations; (ii) the reaction has a positive ΔG [ΔG0' (kJ/mol) = + 30; Villa et al., 2013a] and (iii) the absolute values of specific activity are low.

Therefore, considering the effects of desipramine on these enzyme activities, this drug increases the metabolic efficiency of non-synaptic mitochondria, likely in response to the enhanced ATP utilization after neuronal activation of the post-synaptic compartment.

As regards intra-synaptic mitochondria, desipramine modified some enzyme activities only in the heavy sub-type, where malate dehydrogenase activity was slightly increased, while total NADH-cytochrome c reductase (integrated activity of Complex I-III) and cytochrome oxidase (Complex IV) were decreased. This implies a reduced metabolic flux through the ETC. Overall, desipramine exerts an inhibitory effect on the oxidative metabolism in intra-synaptic heavy mitochondria of hippocampus, located in pre-synaptic compartment.

Interestingly, in rat frontal cerebral cortex (Villa et al., 2016), desipramine induced different effects, being the similarity with hippocampus only the increased cytochrome oxidase activity in non-synaptic mitochondria (Table 1). Therefore, it is of interest to comparatively examine also the effect of fluoxetine treatment on the energy metabolism of hippocampal mitochondria, to evaluate if also this drug induces different effects in relation to the cerebral area and sub-cellular compartment.

Remarkably, on non-synaptic mitochondria, fluoxetine induced the same effect of desipramine, *i.e.* the stimulation of energy metabolism. In this case, together with the increased cytochrome oxidase and glutamate dehydrogenase activities, also succinate dehydrogenase activity (Complex II of ETC) was enhanced, indicating the stimulation of the second entry point into the ETC of reducing equivalents produced in the Krebs' cycle.

Despite the enhancement of oxidative metabolism in non-synaptic mitochondria, citrate synthase activity is not modified either by desipramine or fluoxetine, although this enzyme regulates the metabolic flux entry in Krebs' cycle. This result is in accordance with the study of Agostinho et al. (2011), where the sub-chronic treatment with fluoxetine (12.5 or 25 mg/kg; 28 days) did not modify this enzyme activity in male Wistar rats, while the acute treatment induced an increased in citrate synthase activity but only in the striatum.

As regard the intra-synaptic mitochondria of hippocampus, no appreciable modifications were detected, apart a slight decrease of succinate dehydrogenase in the light mitochondria by fluoxetine.

Therefore, the observed modifications in energy metabolism are clearly noteworthy in relation to the pharmacodynamic features of the studied antidepressants: overall, this functional proteomic approach allows to study the metabolic changes occurring in the neuronal compartments and structures where energy metabolism is carried out *in vivo*, overcoming the contradictory experimental data so far obtained about AD effects on brain energy metabolism (see Introduction). The conflicting results reported in literature may be due to a variety of factors, such as: (i) biases in experimental study design, *e.g.* using whole brain homogenates and bulk of cerebral mitochondria; (ii) not taking into account the metabolic macro-heterogeneity of cerebral areas that has been observed through neuroimaging *in vivo* studies on the brain of depressed patients (Drevets, 2000); (iii) the lack of *in vivo* analyses at sub-cellular level, not considering the possible differential effects of ADs on pre-synaptic nerve endings and on post-synaptic terminals.

In fact, as summarized in Table 1, desipramine and fluoxetine showed a common pattern of modifications of the energy-linked enzyme activities respect to the sub-cellular compartments: (i) in non-synaptic mitochondria of frontal cerebral cortex and hippocampus, the energy metabolism is *stimulated* by ADs; (ii) in light intra-synaptic mitochondria, energy production pathways are *inhibited* in the frontal cerebral cortex and *unchanged* in the hippocampus; (iii) in heavy intra-synaptic mitochondria, the drugs induced heterogeneous modifications in these brain areas. This latter observation likely occurs because of the biochemical-metabolic characteristics of these mitochondria and because of their different bioenergetic responsiveness linked to the assumption that they may be the damaged intra-synaptic light mitochondria (Battino et al., 2002).

From a pharmacological point of view, the differences observed in the pre-synaptic compartment after AD treatment between the frontal cerebral cortex and hippocampus may be due to: (i) the expression of different sub-types of NE (Day et al., 1997; Scheinin et al.,

1994) and 5-HT receptors (Kaufman et al., 2015; Marinova et al., 2015) and (ii) to the different intracellular transduction pathways activated by these receptors. In fact, in a metabolomic study on the C57BL/6N mouse model of chronic unpredictable mild stress (CMS), Zhao and collaborators (2015) recently observed a disparity between fluoxetine and imipramine (of which desipramine is an active metabolite) groups: the phosphoinositide pathway involving myo-inositol is likely to be one of the downstream pathways affected by fluoxetine, while lysine and oleic acid may be predictive markers of imipramine treatment efficacy.

This hypothesis deserves further studies, also because it could account also for the different outcomes in active behaviors exerted after desipramine and fluoxetine administration in the forced swimming test (FST). In fact, even if both desipramine and fluoxetine have been proven to be effective in various behavioral tests (Brenes and Fornaguera, 2011; Brunello et al., 1985; Cuomo et al., 1983; Rygula et al., 2006; Willner et al., 1987), in the FST, SSRIs reduce immobility and increase swimming without affecting climbing, while NE reuptake inhibitors reduce immobility and increase climbing without altering swimming (Armario et al., 1988; Cryan et al., 2005; Detke et al., 1995; Page et al., 1999). The different effects of ADs on these behavioral tests may be the consequence of neurochemical changes related to the maintenance of mood rather than of cognitive alterations (Armario et al., 1988).

In any case, the present results provide new insights about desipramine and fluoxetine pharmacodynamics. In fact, the primary mechanism of action of desipramine is to inhibit NE and 5-HT reuptake systems in the pre-synaptic terminal, *i.e*. NET and SERT, while fluoxetine selectively inhibits only the 5-HT one (SERT). Upon administration of these drugs, NE and 5-HT concentrations increase in synaptic cleft and, afterwards: (i) the intra-cellular signaling pathways are activated after the binding of NE and 5-HT to their post-synaptic receptors, increasing energy production requirements in post-synaptic compartment; (ii) NE and 5-HT neurotransmitter concentrations decrease in pre-synaptic compartment, because of the inhibitory action on NET and SERT reuptake systems. Consequently, monoamine pre-synaptic regulatory receptors are stimulated, activating feedback inhibitory intracellular signaling pathways, thus reducing the need of energy production in this compartment.

In this context, it should be pointed out that mitochondria accumulate at sites where ATP consumption is higher (Hroudová et al., 2013) and Chen et al. (2013) observed a significantly increased number of mitochondria after treatment with imipramine in the

hippocampal CA_1 region of Flinders rats, that display a genetic susceptibility to depressive behavior.

Finally, our results may be discussed also respect to the rapid pharmacological effects of ADs on NE and 5-HT concentrations in the synaptic cleft *versus* the delayed therapeutic efficacy of these drugs: the observed metabolic modifications in the post-synaptic compartment may account for the time necessary to revert the down-regulation of monoamine receptors induced by the persistent lack of stimulation by their endogenous ligands in depressive state. In fact: (i) the synthesis of a protein of *n* amino acids requires the consumption of *n* - 1 molecules of ATP, just to activate each amino acid (Moretti et al., 2003), and (ii) the *de novo* synthesis of receptors is energy and time-consuming, explaining the time-lag between the pharmacological and therapeutic actions of these drugs.

In particular, this study demonstrated that desipramine and fluoxetine *in vivo* treatments lead to adjustments in the neuronal energetic state, in relation to the energy requirements of pre- and post-synaptic compartments after drug administrations. This conclusion is in accordance: (i) with the recent observation that inorganic phosphate correlates positively with the severity of depression in gray matter (Harper et al., 2016) and (ii) with the statement of Renshaw et al. (2001), according to which, studying neuroimaging abnormalities in human patients, the agents that increase the cerebral ATP availability may have antidepressant properties.

4. Conclusions

The effects of desipramine and fluoxetine have been evaluated on different types of hippocampal mitochondria. The study has confirmed the validity of considering the changes of energy-linked enzyme activities (i) as representative of the AD-induced modifications in energy metabolism and (ii) as molecular targets of drug actions. In fact, respect to the *in vivo* localization in pre- or post-synaptic compartments, enzyme activities of non-synaptic and intra-synaptic light and heavy mitochondria underwent different modifications after desipramine and fluoxetine pharmacological treatments.

Remarkably, the results (i) strongly indicate that these modifications are in accordance with the acknowledged mechanism of action of these drugs and, (ii) thanks to the sub-cellular bioenergetic analysis, they overcome the conflicting data so far obtained in experimental studies about the effects of ADs on brain energy metabolism.

At this regard, the results encourage further studies evaluating AD effects on the same enzymatic systems in experimental animal models of mood disorders, also in accordance with recent evidence (Bansal and Kuhad, 2016; Ferrari and Villa, 2016; Głombik et al., 2016; Scaini et al., 2011), that propose mitochondrial energy metabolism as potential target for AD therapeutic strategies.

Author Disclosures

Role of Funding Source

This work was supported by Grants from Italian Ministry of University and Research (MIUR) and by a Grant of the "*Enrico ed Enrica Sovena Foundation*", Rome, Italy, for the Fellowship of Dr. Ferrari Ph.D., ACCP.

The Authors are grateful to Dr. Martina Vitelli for language editing of the article.

Conflict of Interest

The authors declare no conflict of interest.

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Figure Legend

Figure 1 – Specific Enzymatic Activities [expressed as (µmoles of substrate transformed) x min^{-1} x (mg of protein)$^{-1}$] of Citrate Synthase (CS), Succinate Dehydrogenase (SDH), Malate Dehydrogenase, value divided by 10 (MDH :10), Total NADH-cytochrome *c* Reductase (CCRT), Cytochrome Oxidase, value divided by 10 (COX :10) and Glutamate Dehydrogenase (GlDH) assayed on non-synaptic mitochondria (FM), intra-synaptic light (LM) and heavy (HM) mitochondria from hippocampus of 10 week-old male control animals. Results are the mean ± S.E.M. of n = 6-8. Statistical analysis by ANOVA and control-tests. Three levels of significance; 1 symbol: $P < 0.05$; 2 symbols: $P < 0.01$; 3 symbols: $P < 0.001$. Symbols of comparisons: § FM *versus* LM and HM; ° LM *versus* HM.

Figure 2 – Specific Enzymatic Activities [expressed as (µmoles of substrate transformed) x min^{-1} x (mg of protein)$^{-1}$] assayed on non-synaptic mitochondria (FM), intra-synaptic light (LM) and heavy" (HM) mitochondria from hippocampus of 10 week-old male control animals and treated with desipramine (15 mg/kg, i.p., 21 days) and fluoxetine (10 mg/kg, i.p., 21 days). Results are the mean ± S.E.M. of n = 6-8. Statistical analysis by ANOVA and control-tests. Three levels of significance; 1 symbol: $P < 0.05$; 2 symbols: $P < 0.01$; 3 symbols: $P < 0.001$. Symbols of comparisons: * controls *versus* treated animals.

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Table 1. Comparative scheme of qualitative effects of desipramine (Des) and fluoxetine (Flu) sub-chronic treatment on catalytic activities of indicated enzymes evaluated on non-synaptic mitochondria (FM) and intra-synaptic "light" (LM) and "heavy" (HM) mitochondria from frontal cerebral cortex and hippocampus of 10 week-old male Sprague-Dawley rats.

BRAIN AREA	**FRONTAL CEREBRAL CORTEX**					
MITOCHONDRIA	**FM**		**LM**		**HM**	
ENZYMES	**Des**	**Flu**	**Des**	**Flu**	**Des**	**Flu**
CS	=	=	=	=	↑↑	=
SDH	=	=	↓↓↓	↓↓↓	=	=
MDH	=	=	↓↓↓	↓↓↓	=	=
CCRT	=	=	=	=	=	=
CCRS	=	=	=	=	=	=
COX	↑↑↑	↑↑↑	=	=	↑↑	↑↑↑
GLDH (-Tx)	=	↑↑↑	n.d.	n.d.	n.d.	n.d.
GLDH (+Tx)	=	↑↑↑	n.d.	n.d.	n.d.	n.d.
GOT	=	=	=	=	=	=
GPT	=	=	↓↓↓	↓↓↓	=	↓

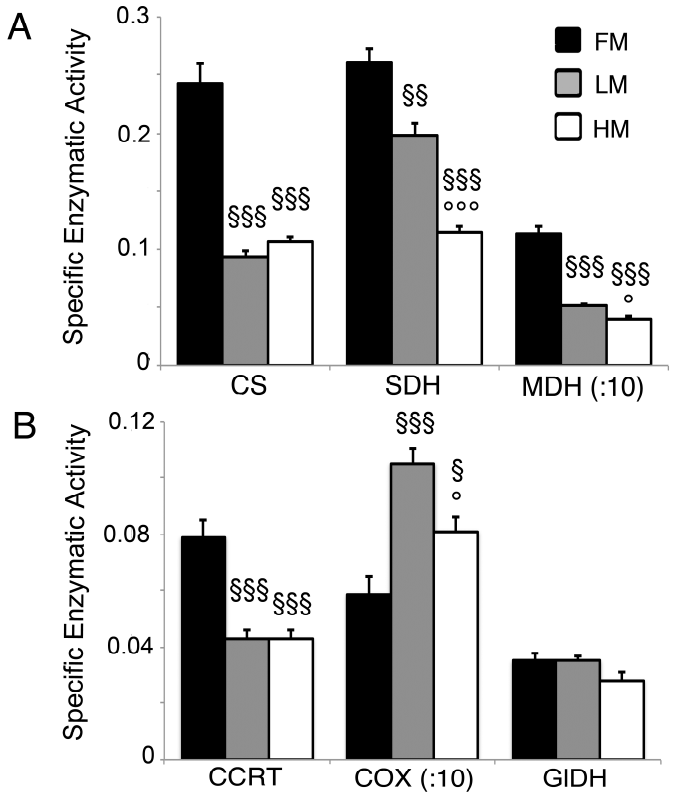
BRAIN AREA	**HIPPOCAMPUS**					
MITOCHONDRIA	**FM**		**LM**		**HM**	
ENZYMES	**Des**	**Flu**	**Des**	**Flu**	**Des**	**Flu**
CS	=	=	=	=	=	=
SDH	=	↑	=	↓	=	=
MDH	=	=	=	=	↑	=
CCRT	=	=	=	=	↓	=
COX	↑	↑↑↑	=	=	↓↓↓	=
GlDH	↑↑↑	↑↑↑	=	=	=	=

ACCEPTED MANUSCRIPT

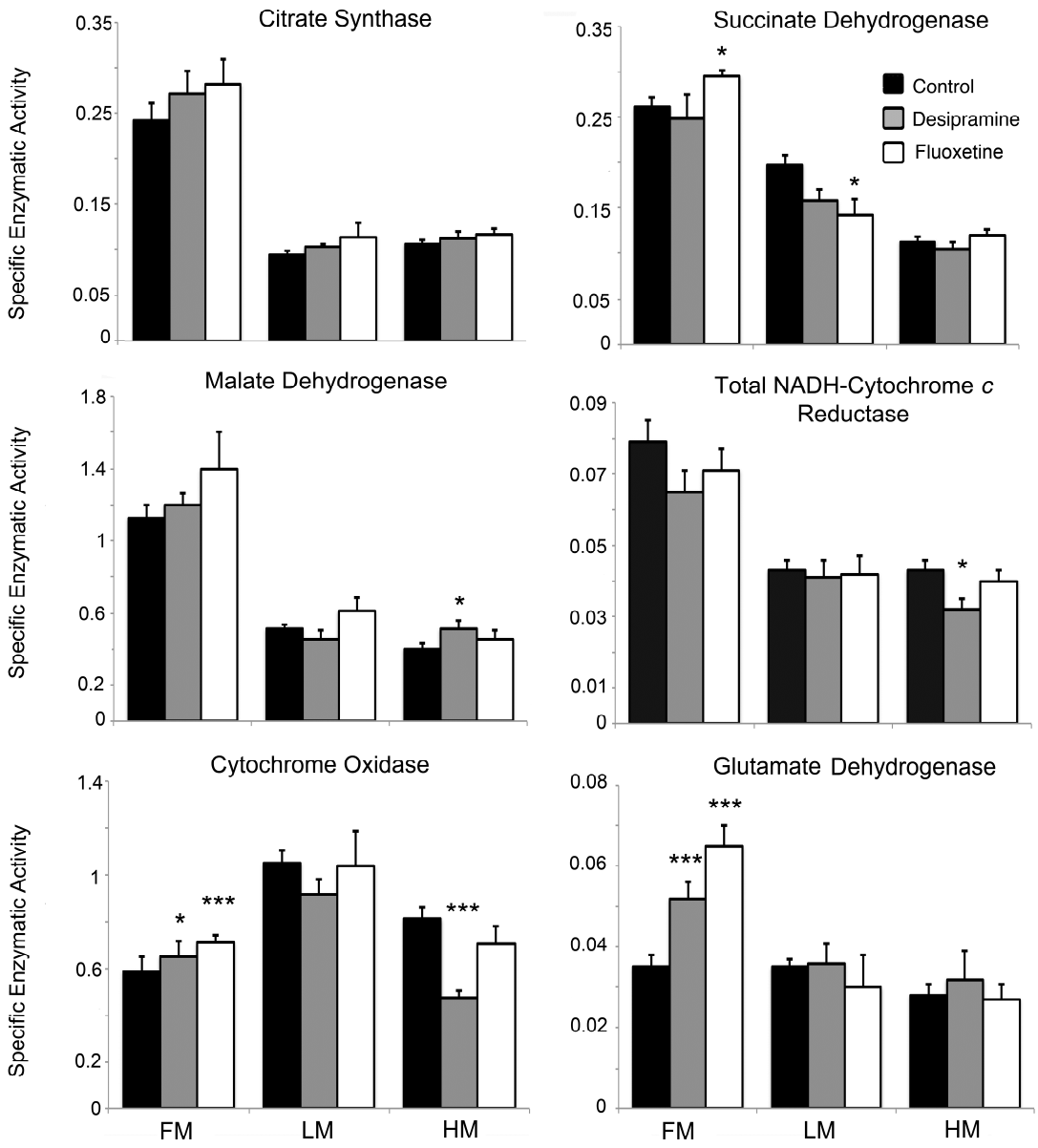
Significance of changes: (🡅) increased or (🡇) decreased enzyme activity in control *versus* treated animals. Statistical significances: 1 symbol: $P < 0.05$; 2 symbols: $P < 0.01$; 3 symbols: $P < 0.001$. n.d.: not determined; =: no detected changes.

Legend: Citrate Synthase (CS), Succinate Dehydrogenase (SDH), Malate Dehydrogenase (MDH), Total NADH-cytochrome *c* Reductase (CCRT), Rotenone-sensitive NADH-cytochrome *c* Reductase (CCRS), Cytochrome Oxidase (COX), Glutamate Dehydrogenase assayed without [GlDH (–Tx)] and with Triton X-100 [GlDH (+Tx)], Glutamate-oxaloacetate Transaminase (GOT), Glutamate-pyruvate Transaminase (GPT).

ACCEPTED MANUSCRIPT



ACCEPTED MANUSCRIPT


Citrate Synthase
Succinate Dehydrogenase
Malate Dehydrogenase
Total NADH-Cytochrome c Reductase
Cytochrome Oxidase
Glutamate Dehydrogenase
Specific Enzymatic Activity
Control
Desipramine
Fluoxetine
FM
LM
HM

ACCEPTED

ACCEPTED MANUSCRIPT

Highlights

- Desipramine and fluoxetine effects on rat hippocampal energetics were assessed.
- Mitochondrial energy metabolism was studied by Functional Proteomics.
- Non-synaptic somatic mitochondria and intra-synaptic ones were purified.
- Energy metabolism increased in somatic mitochondria, decreased in intra-synaptic.
- The drugs modified enzyme activities coherently with their pharmacodynamics.

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