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Changes in hippocampal gene expression by 7-nitroindazole in rats submitted to forced swimming stress

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Nitric oxide (NO) is an atypical neurotransmitter that has been related to the pathophysiology of major depression disorder. Increased plasma NO levels have been reported in depressed and suicidal patients. Inhibition of neuronial nitric oxide synthase (nNOS), on the other hand, induces antidepressant effects in clinical and pre-clinical trials. The mechanisms responsible for the antidepressant-like effects of nNOS inhibitors, however, are not completely understood. In this study, genomic and proteomic analyses were used to investigate the effects of the preferential nNOS inhibitor 7-nitroindazole (7-NI) on changes in global gene and protein expression in the hippocampus of rats submitted to forced swimming test (FST). Chronic treatment (14 days, i.p.) with imipramine (15 mg/kg daily) or 7-NI (60 mg/kg daily) significantly reduced immobility in the FST. Saturation curves for Serial analysis of gene expression libraries showed that the hippocampus of animals submitted to FST presented a lower number of expressed genes compared to non-FST stressed groups. Imipramine, but not 7-NI, reverted this effect. GeneGo analyses revealed that genes related to oxidative phosphorylation, apoptosis and survival controlled by HTR1A signaling and cytoskeleton remodeling controlled by Rho GTPases were significantly changed by FST. 7-NI prevented this effect. In addition, 7-NI treatment changed the expression of genes related to transcription in the cAMP response element-binding pathway. Therefore, this study suggests that changes in oxidative stress and neuroplastic processes could be involved in the antidepressant-like effects induced by nNOS inhibition.

Keywords: Major depression, nitric oxide, neurogenesis, neuroplasticity, oxidative stress

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Nitric oxide (NO) is an atypical neurotransmitter involved in pathological processes related to major depression disorder (MDD) (Baranano *et al*. 2001). NO is synthesized from L-arginine by three different isoforms of NO synthase: neuronal (nNOS), inducible and endothelial NOS (eNOS) (Magarinos & Mcewen 1995). nNOS is widely but unevenly distributed in the mammalian brain, where it accounts for more than 90% of NO production (Abkevich *et al*. 2003; Joca *et al*. 2007).

The involvement of NO-mediated neurotransmission in depression is supported by several pieces of evidence. For example, a higher number of nNOS containing neurons in the hippocampus was described in post-mortem studies of depressive patients (Oliveira *et al*. 2008), and increased plasma NO levels have been reported in depressed patients with suicide attempts (Khovryakov *et al*. 2010; Kim *et al*. 2006). Moreover, systemic or intra-hippocampal treatment with 7-nitroindazole (7-NI), a preferential nNOS inhibitor, induces antidepressant-like effects in the forced swimming test (FST) (Joca & Guimaraes 2006; Yildiz *et al*. 2000). Furthermore, exposure of laboratory animals to uncontrollable or severe stressors, which can induce several physiological and behavioral changes that resemble human depression such as anedonia, memory impairments and excessive glucocorticoids (GC) secretion, induces nNOS expression in brain structures related to this disorders, including the hippocampus, amygdala and cortex (de Oliveira *et al*. 2000; Oliveira *et al*. 2008).

However, despite the evidence linking NO to biological processes associated with depression such as oxidative stress (Bergstrom *et al*. 2007; Packer *et al*. 2005), monoamine functions (Kiss & Vizi 2001; Yamamoto *et al*. 2001), S-nitrosilation signalin (Evans *et al*. 2002), and gene expression changes (Datson *et al*. 2001a), the molecular mechanisms responsible for the antidepressant-like effects of NOS inhibitors are still not completely understood. Therefore, this study was aimed at unveiling possible neuromolecular pathways

associated with antidepressant-like effects of nNOS inhibition. To achieve this goal, we measured total protein and gene expression in the hippocampus of rats treated chronically with a preferential nNOS inhibitor 7-NI (Alderton *et al*. 2001) or the standard antidepressant imipramine and submitted to the FST, an animal model that is widely used for detecting antidepressant-like effects (Nestler & Hyman 2010).

Methods

Animals

Male Wistar rats (200–220 g) were housed in pairs in a temperature-controlled room ($24 \pm 11^{\circ}C$) under standard laboratory conditions with free access to food and water (12 h light/12 h dark cycle). Procedures were conducted under approval by the Ethical Committee of FMRP-USP, in compliance with International laws and policies.

Drugs

Imipramine hydrochloride (15 mg/kg; Sigma Chemical, Saint Louis, Missouri, USA), 7-NI (60 mg/kg; Sigma Chemical, Saint Louis, MO, USA) or vehicle (DMSO:saline, 1:1; 2 ml/kg) was daily-injected i.p. for 14 days (1000 to 1400 h). The doses and treatment schedule were based on previous studies describing antidepressant-like effects of 7-NI (Yildiz *et al*. 2000). The five experimental groups ($n = 5–8$ animals/group) were naïve (NAI) animals not-submitted to FST stress, and treated with vehicle (VEH + NAI); naïve animals treated with 7-NI (7-NI + NAI); animals treated with vehicle and submitted to FST (VEH + FST); animals treated with 7-NI and submitted to FST (7-NI + FST); animals treated with imipramine and submitted to FST (IMI + FST).

On the day before the last injection, animals from the FST groups were submitted to the 15-min forced swimming pretest section (Porsolt *et al*. 1977). Twenty-three hours later they received the last injection and 1 h after were submitted to the forced swimming session where immobility time, meaning the period in which the animal remained immobile or with minimal movements necessary for floating, together with the latency for the first immobility episode, were recorded for 5 min. Two hours later the animals were sacrificed under deep anesthesia (urethane 25% 1 mg/kg) and hippocampus were dissected and stored at $-80^{\circ}C$ for later analyses.

Total RNA samples and Serial analysis of gene expression (SAGE) analysis

Total RNA was prepared from the hippocampus using a polytron homogenizer and TRIzol LS Reagent (Invitrogen Corporation; Carlsbad, CA, USA) according to manufacturer's instructions. A pool of three to four animal samples, resulting in 30 μg of total RNA, was used for the SAGE procedure.

SAGE was carried out using the I-SAGE™ Kit (Invitrogen Corporation) following the manufacturer's protocol. The tag frequency counting was obtained by SAGE™ analysis software, and genes identification was extracted from CGAP SAGE Genie (http://cgap.nci.nih.gov/SAGE). The SAGE methodology was chosen in this study due to its similar sensitivity compared to microarrays, with the advantage of being potentially able to detect changes in genes not present in the array (Feldker *et al*. 2003; Martins-De-Souza *et al*. 2010; Weinreb *et al*. 2007). Moreover, since this method is based on relative frequency, SAGE libraries from independent studies could be more easily compared (Czibere *et al*. 2011). Also, new genes can be identified by re-analyses of unpredicted tags using Gene Banks.

To validate SAGE libraries, five genes of interest were chosen for quantitative real time (qRT) PCR using cyber green of PCR master mix (Applied Biosystem, California, CA, USA) on the basis of previous reports showing expression changes after stress exposure (Hill & Gorzalka 2005; Holmes *et al*. 1995; Mclaughlin *et al*. 2007; Mongeau *et al*. 2010; Pandey *et al*. 2010; Thome *et al*. 2001). They included the cannabinoid receptor 1 (CNR1), synaptophysin (SYP), glutathione S-transferase (GST), neurotrophic tyrosine kinase receptor type 2 (NTRK2), and serotonin receptor type 2C ($5\text{-}HT_{2c}$) genes.

Protein sample and MS/MS mass spectrometry analysis

Total protein was isolated from the organic phase of RNA TRIzol of the same samples used for SAGE analysis, following manufacturer's instructions with modifications. The protein content was determined by Bradford (Bio Rad, Hercules, CA, USA) reagent method, and stored at $-80^{\circ}C$ for later use.

In each experimental group. three bi-dimensional gels were prepared using 1 mg of total protein per gel, from a three to four pooled animal samples. The isoeletric focalization was performed using 18 cm 3–10 pH gradient (Amersham, Uppsala, Sweden) in the Ettan IPGPhor 3 (GE Healthcare, San Francisco, CA, USA), and the second dimension was run at SDS Ettan DALT six (GE Healthcare).

Protein expression levels were estimated by average of relative spot volumes obtained from two gels that presented spot location reproducibility higher than 95% by Image Master Software (GE Healthcare, version 5.0). Protein spots differently expressed were excised from the gel for MS/MS spectrometry in MALDI-TOF. To identify the proteins, the tryptic hydroxyoate peptide fingerprint was analyzed using Mascot method and identification by National Center for Biotechnology Information (NCBI) protein database (http://prospector.ucsf.edu/csfhtml4.0/msfit.htm). Proteins with Mascot score (MS) identity levels $\geq$36 were taken to be reliable identified.

Biological network analysis using MetaCore™

Results from SAGE and 2-DE-peptide mass fingerprint were analyzed by MetaCore software (v.5.1 build 16271, GeneGo, St. Joseph, MI, USA). This gene enrichment analysis approach is one of the methods that can be used to extract meaningful biological information from large genomic data set (Subramanian *et al*. 2005), grouping genes that share common biological function (Nikolsky *et al*. 2005).

Statistical analysis

The FST results were compared by one-way ANOVA followed by Duncan *post hoc* test. The sensitivity of the SAGE genome expression, reflecting the extent of the detected genes, was performed by calculating the ratio between new tags and total sequenced tags identified in each sequencing round. The saturation curves were compared by ANOVA. Hierarchical clustering, using GeneCluster 2.0 (Cambridge, MA, USA) was performed in order to evaluate the level of similarity-divergence among the SAGE libraries. Genes differentially expressed in the SAGE libraries were identified by confidence interval test (SAGEic) as previously described (Abkevich *et al*. 2003). The pairs of SAGE libraries and 2D protein spot volumes were compared as follow: VEH + NAI group against VEH + FST; VEH + FST group against 7NI + FST, and VEH + FST group against IMI + FST. The libraries VEH + NAI and VEH + 7NI were not compared by this method because the hierarchical analysis showed high similarity between these groups. The general level of gene activity was estimated by Kruskal–Wallis test for averages of fold change between the groups mentioned above. The qRT results were compared by one-way ANOVA, followed by Duncan *post hoc* test. Spearman correlation test was also used to evaluate the relationship between mean values of gene expression levels obtained by qRT and SAGE. Proteins spots were considered to be differently expressed when the relative volume between one spot and its homolog in another treated group was higher than two times and the *t*test resulted in a value of $P < 0.05$. The statistical method used by MetaCore software is based on gene ontological categorization to identify processes regulated by genes or proteins differentially expressed. The *P* values represent the statistical relevance of the ontological matches calculated as the probability of a match occurring by chance, given the size of the database. Lower *P* values indicated that more genes belong to a same pathway. The *P* value was calculated as previously described (Pan *et al*. 2010). Only ontological pathways with $P < 0.05$ were considered in this study.

Results

Behavioral effects of imipramine and 7-NI

Imipramine and 7-NI induced an antidepressant-like effect in the FST (Yildiz *et al*. 2000), significantly decreasing immobility time ($F_{2,18} = 15.83$, $P = 0.0001$) and increasing the latency for the first immobility episode ($F_{2,18} = 9{,}66$ $P = 0.0014$, Fig. 1a).

SAGE analyses

A total of 61 272; 62 031; 60 471; 61 390 and 60 754 gene tags were sequenced, with 26 406; 26 256; 19 270; 19 072 and 24 936 genes mapped and quantified in the hippocampus of VEH + NAI, VEH + 7-NI, VEH + FST, VEH + 7-NI and VEH + IMI groups, respectively. Analysis of the saturation curves showed that all SAGE libraries fitted into a hyperbolic curve with saturation on the abscissa ($R^2 < 0.99$, Fig. 1b), suggesting a high covering of hippocampal transcriptome using the SAGE method, as previously reported (Anisimov 2008). ANOVAs of SAGE libraries curves revealed statistical differences between the saturations curves ($F_{(4,34)} = 23.01$; $P < 0.0001$). These analyses suggest that SAGE libraries of VEH + NAI, 7-NI + NAI and IMI + FST presented a higher variety of gene expressed, compared to libraries VEH + FST and 7-NI + FST.

Hierarchical clustering identified at least three distinct groups. The first one corresponded to animals submitted to stress by FST that received vehicle or 7-NI. The second one included non-FST stressed animals, treated or not with 7-NI, and the last one consisted of animals treated with imipramine (Fig. 1c).

To evaluate the pattern of hippocampal global gene expression induced by FST and drug treatment, we compared the SAGE libraries of VEH + FST group against VEH + NAI, 7-NI + FST and IMI + FST groups. Comparison between VEH + NAI and VEH + FST groups showed changes in the expression of 1469 genes. Among them, 1312 genes (89.3%) showed increased expression in the VEH + FST group against only 157 genes (10.7%) in the VEH + NAI. 7-NI and IMI, on the other hand, induced changes in the expression of 1201 and 1687 genes, respectively. 7-NI and IMI decreased the expression of 1078 (89.8%) and 1514 (89.8%) genes, respectively, compared to the VEH + FST group, whereas only 123 (10.2%) and 173 (10.3%) genes had their expression increased by these drugs (Table 1). Finally, stressed animals treated with vehicle (VEH + FST) presented a higher global gene expression compared to the other groups (Fig. 1d; Kruskal–Wallis test, $P < 0.0001$, followed by *post hoc* Dunn's test, $P < 0.05$).

To identify the biological processes preferentially modified by FST stress and detected by SAGE, the genes differently expressed between the VEH + NAI and VEH + FST groups were submitted to enrichment MetaCore analysis. The analysis indicated that a significant number of genes involved in oxidative phosphorylation, apoptosis signaled by 5-HT_{1A} receptor and cytoskeleton remodeling processes were statistically changed (Table 2).

Among the genes involved in oxidative phosphorylation, 25 were up-regulated while 4 were down-regulated by FST. The former included cytochrome-*c* oxidase polypeptide 6A1 mitochondrial, NADH dehydrogenase and ATP synthesis mitochondrial, which showed more than ninefold increase after FST compared to the control group (Table S1).

Genes of at least three pathways involving 5-HT_{1A} signaling were modified according to the GeneGO analyses. The first one is associated with activation of an alpha inhibitory G-protein (Gi), adenylate cyclase and regulatory protein PP2A, which modulates the BAX protein (Gi-ADC-PP2A-BAX). The second one involves AKT and ERK2-MAPK1. The last one is also related to AKT and NF-κB (Figure S1, Table S2). FST also modified the expression of several genes involved in cytoskeletal and neurophilament remodeling. FST (VEH + FST) stress induced more than 14-fold [0; 1.7]ci increase in the expression of glutamate receptor subunit GluR2 (Rn.91361) compared to VEH + NAI group (Table S3).

Comparison between vehicle and 7-NI treated groups submitted to FST stress performed by enrichment analysis showed that genes related to physiological processes associated to oxidative phosphorylation, cytoskeleton remodeling, cell adhesion and genes related with transcriptional factors responding to beta-adrenergic receptors and cAMP response element-binding (CREB) were significantly modified by 7-NI treatment (Table 2).

7-NI prevented the increase in the expression of several genes related to respiratory complex I, succinil dehydrogenase and ATPases. Moreover, 7-NI prevented the induction of subunits 6 and 8 of the cytochrome complex and increased the expression of eighth Cox subunit (Table S4). Similar effects were observed for cytoskeleton remodeling signaled by FKA and cell adhesion genes, with 7-NI preventing the up-regulation of genes such as $G_{(0)}$–protein, CDC42 and LIMK. This drug also increased the expression of the cytoskeleton genes beta-actin and actin (Table S5).

Regarding the physiological process related to CREB, 7-NI treatment prevented the increase in gene expression of calcium channel, Erk (MAPK1/3), c-Jun, c-Fos and AP1, and increased the expression of calmodulin 1 (Calm1) (Figure S2, Table S6).

The enrichment analysis of the genes differentially expressed in animals treated with vehicle against those treated with imipramine and submitted to FST showed that drug-induced gene expression modifications also occurred in biological pathways associated with oxidative phosphorylation, apoptosis/prolipheration signaled by 5-HT_{1A} receptor, cytoskeleton remodeling and transcriptional control (Table 2).

Imipramine prevented the increased expression of most genes related to oxidative phosphorylation (Table S7). It also prevented the induction of genes related to 5-HT_{1A} signaling promoted by FST stress. Within this pathway, imipramine inhibited the increased expression of NF-κB, STAT3 and caspase regulator factor PARP-1. The expression of PPA2 protein was also inhibited. In addition, imipramine treatment decreased the expression of the apoptosis-related genes c-Raf-1 and Map2K2. Moreover, it reduced the expression of G-protein potassium channel (Kcnj5), and induced the expression of ionotropic glutamate receptor alpha 3 (Gria3) when compared to VEH + FST group (Figure S3, Table S8).

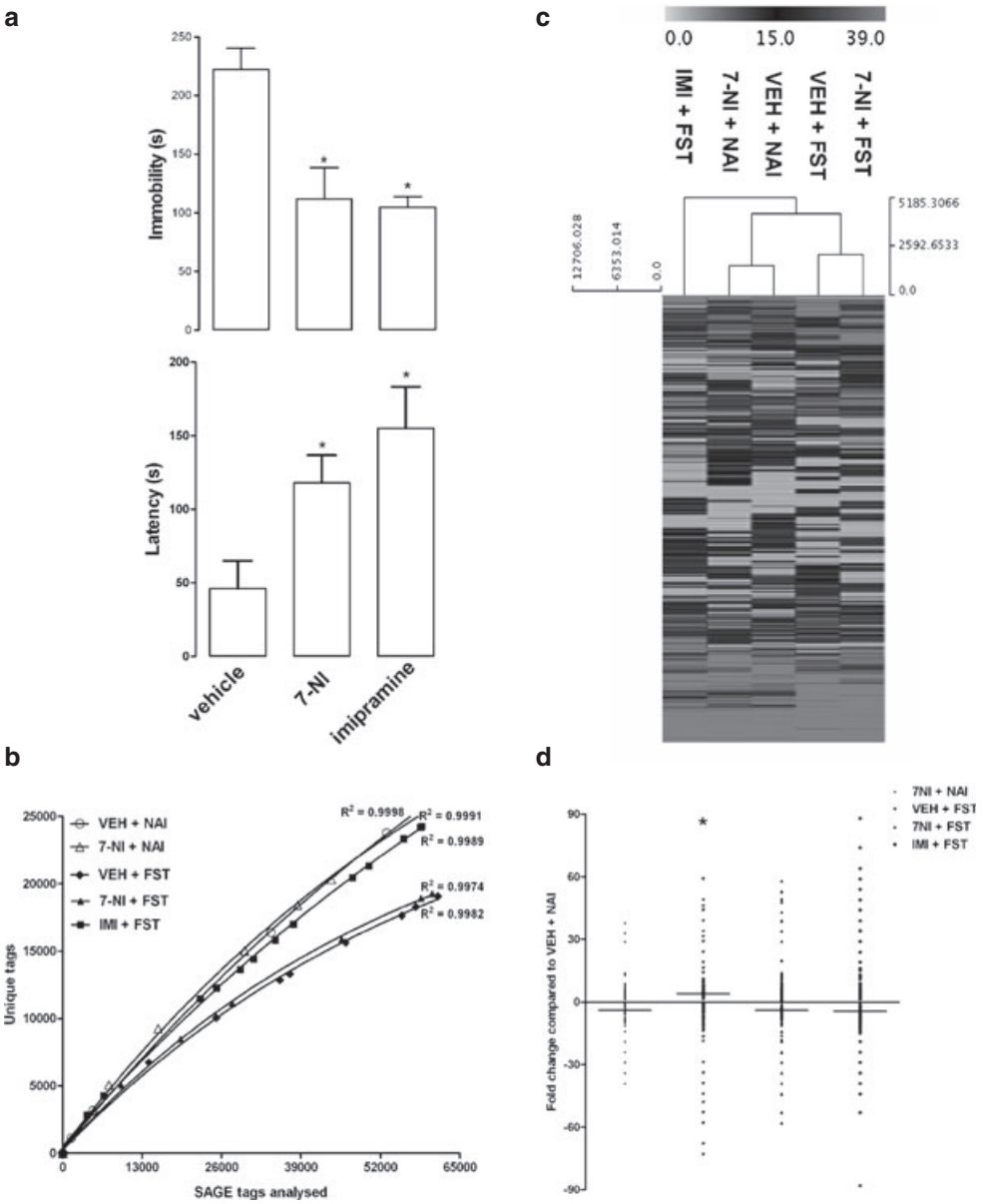

Figure 1: Effect of 14 days chronic treatment with 7-NI, imipramine or vehicle on FST and hippocampal pattern of gene expression. (a) Total immobility time and latency to the first immobility episode of rats ($n = 5–8$/group) submitted to the FST. The bars represent means ± SEM. $^*P < 0.05$ compared to vehicle (ANOVA). (b) Serial analyses of gene expression (SAGE) tags saturation curve ($n = 3–4$/group). The curves represent the relation of new sequenced tags (unique tags) by the total sequenced tags at each library (SAGE tags analyzed). R^2 = fitting hyperbolic curve. (c) Hierarchical clustering of SAGE libraries. (d) Differential gene expression in fold changes for SAGE libraries of the groups 7-NI + NAI, VEH + FST, 7NI + FST e FST + IMI compared to the group VEH + NAI. VEH + NAI = animals treated with vehicle (saline:DMSO, 1:1, 2 ml/kg for 14 days), 7-NI + NAI = animals treated with 7-NI (60 mg/kg by 14 days), VEH + FST = animals treated with vehicle (saline:DMSO, 1:1, 2 ml/kg for 14 days) and submitted to FST, 7-NI + FST = animals treated with 7-NI (60 mg/kg for 14 day) and submitted to FST, IMI + FST = animals treated with imipramine (15 mg/kg for 14 days) and submitted to FST.

Similar to 7-NI, cytoskeleton-related genes were also significantly changed in animals treated with imipramine. This drug inhibited their expression although, for at last three of them, the precursor of activator of tissue plasminogen (PLAT), cytoskeleton actin (Actg1) and myosin heavy head type II (MyHC), the expression was enhanced. Different from 7-NI, imipramine changed the expression of genes related to DNA methylation such as a family of protein 14-3-3

Table 1: Number of genes and proteins with expression changes into each comparison set

Experimental groups	Total genes evaluated		Total protein spots evaluated	
VEH + NAI	26 406		340	
7-NI + NAI	26 256		296	
VEH + FST	19 270		616	
7-NI + FST	19 072		446	
IMI + FST	24 936		496	
Compared groups	N genes (%)	Total	N protein (%)	Total
VEH + NAI X VEH + FST	↑ 157 (10.68)	1469	↑ 33 (50.77)	65
	↑ 1312 (89.32)		↑ 32 (49.23)	
VEH + FST X 7-NI + FST	↑ 123 (10.24)	1201	↑ 10 (9.34)	107
	↓ 1078 (89.76)		↓ 97 (90.66)	
VEH + FST X IMI + FST	↑ 173 (10.25)	1687	↑ 43 (47.26)	91
	↓ 1514 (89.75)		↓ 48 (52.74)	

(over 60-fold increase) and class II histone deacetylases and histone H3 (14 and 19-fold decrease, respectively). Chronic imipramine treatment also decreased the expression of G-beta/gamma protein compared to animals treated with vehicle (Table S9).

The enrichment analysis for gene function was also performed for the non-stressed animals comparing vehicle- vs. 7-NI treated animals. The MetaCore database indicated only major effects in cytoskeleton remodeling and cell adhesion gene. There were no significant changes in oxidative phosphorylation, cell signaling mediated by CREB, pathways signaled by 5-HT_{1A} or beta-adrenergic receptor related genes (Table 2).

qRT PCR analysis

The SAGE libraries were validated using an independent method. qRT was performed for genes that showed changes in the SAGE analysis. They included genes related to oxidative stress (GST), neuronal plasticity (CNR1, SYN) and serotonin-mediated neurotransmission (5-HT_{2C}) (Figure S4). Spearman test showed positive correlation ($r = 0.609$, $P < 0.001$) between gene expression analyses by SAGE and qRT, suggesting a reproducibility of SAGE method (Fig. 2).

Proteomic analysis

The analysis of gels by Image Master Software allowed us to identify a different number of protein spots in each five experimental groups (VEH + NAI: 340; 7-NI + NAI: 296; VEH + FST: 616; 7-NI + FST: 446; IMP + FST: 496, Fig. 3). The increase in protein spots in the stressed group and their decrease by drug treatment is similar to the global gene expression changes found by SAGE analysis. To identify the proteins that had their expression modified by FST stress, the gels of animals treated with VEH + NAI were compared

Table 2: Biological processes enriched by genes differentially expressed between experimental groups

GeneGo pathway	N genes	*P*
VEH + FST X VEH + NAI		
Oxidative phosphorylation	29	2.68^{-11}
Apoptosis and survival controlled by HTR1A signaling	20	2.60^{-8}
Cytoskeleton remodeling controlled by Rho GTPases	11	3.45^{-7}
Cytoskeleton remodeling on neurophilaments	11	7.90^{-7}
VEH + FST X 7-NI + FST		
Oxidative phosphorylation	22	2.28^{-08}
Beta-adrenergic receptors transactivation	10	4.01^{-07}
Cell adhesion	17	2.05^{-06}
Cytoskeleton remodeling FAK signaling	12	3.97^{-06}
Transcription induced by CREB pathway	9	1.24^{-05}
VEH + FST X IMI + FST		
Oxidative phosphorylation	36	2.28^{-8}
Apoptosis and survival signaling by HTR_{1A}	18	1.28^{-7}
Cytoskeleton remodeling	22	3.78^{-6}
Transcription control by heterochromatin protein 1 family	11	5.25^{-6}
Neurophysiological process signaling by neuronal HTR_{1A}	14	6.02^{-6}
VEH + NAI X 7-NI + NAI		
Cytoskeleton remodeling	41	4.63^{-14}
Cytoskeleton remodeling associated with TGF and WNT	41	1.30^{-12}
Cell adhesion	36	7.37^{-11}
Cytoskeleton remodeling modulated by PKA	20	1.52^{-09}
Cytoskeleton remodeling modulated by integrin outside signaling	20	1.15^{-07}

to those of VEH + FST animals. From a total of 65 protein spots, 32 showed increased expressions, while 33 were down-regulated in VEH + FST group when compared to VEH + NAI. Among them, 28 proteins were sequenced by MALDI-TOF method (Table S10).

When the proteins of VEH + FST rats were compared to 7-NI + FST animals it was found that 10 protein spots had their expression enhanced and 97 decreased at least twofold by the drug (Table 1). From this total, 28 proteins were sequenced and characterized by MS/MS spectrometry (Table S11). A similar comparison with imipramine-treated animals showed enhanced expression of 43 protein spots and reduction of 48 (Table 1). From this total, 54 protein proteins were sequenced and characterized by MS/MS spectrometry (Table S12).

Finally, the comparison of 7-NI + NAI and VEH + NAI groups showed that drug treatment alone was able to increase or decrease the expression of only 12 or 15 proteins, respectively. Thus, because of the low number of proteins differently expressed between these groups (7-NI + NAI and VEH + NAI), a trend also observed in hierarchical clustering performed in SAGE libraries, they were not sequenced.

The enrichment analysis by MetaCore method for screening proteins differently expressed between the groups VEH + NAI and VEH + FST indicated that, among the functional

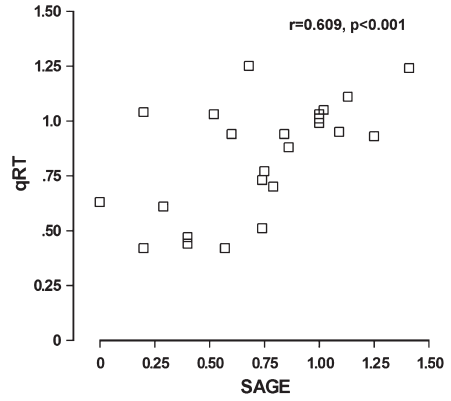

Figure 2: Spearman correlation to SAGE and qRT data. Each dot represents the mRNA expression level to cannabinoid receptor 1 (CNR1), synaptophysin (SYP), glutathione S-transferase (GST), neurotrophic tyrosine kinase receptor type 2 (NTRK2) and serotonin receptor type 2C (5-HT_{2c}) expression evaluated by SAGE and qRT methods.

pathways significantly represented by these genes, there were the oxidative phosphorylation ($P = 4.59^{-3}$), oxidative stress ($P = 3.72^{-2}$), and prevention of apoptosis induced by reactive oxygen species (ROS) ($P = 3.97^{-2}$).

In stressed rats, 7-NI significantly changed proteins related to apoptosis and/or proliferation associated to BAD ($P = 2.79^{-3}$), pathways driven by CREB ($P = 2.93^{-3}$) and cytoskeleton regulation and rearrangement ($P = 9.19^{-4}$). Imipramine, on the other hand, modified proteins associated to GABA-A signaling ($P = 2.75^{-5}$), mitochondrial tricarboxylic acid chain (TCA) ($P = 5.78^{-3}$), responses to oxidative stress and hypoxia ($P = 3.28^{-4}$), process linked to actin cytoskeleton ($P = 2.19^{-2}$) and catalytic process of hydrogen peroxide ($P = 2.87^{-7}$).

Discussion

The preferential nNOS inhibitor 7-NI decreased immobility time in the FST, thus confirming its reported antidepressant-like effect (Joca & Guimaraes 2006; Yildiz *et al*. 2000). The primary mechanisms of this effect are not fully understood, although 7-NI shares some properties with imipramine such as the facilitation of neurogenesis and hippocampal neuroplasticity (Brown 2010; Lu *et al*. 1999). A genomic and proteomic analysis was employed in this study to further investigate these mechanisms. Although the SAGE strategy has a sensitivity similar to other genomic strategies such as microarray (Weinreb *et al*. 2007), it is considered to be an open approach, capable of detecting and quantifying unpredictable genes expressed at different biological samples (Martins-De-Souza *et al*. 2010). In addition to SAGE, proteomic analysis was also employed as a source of additional information to detect changes in protein expression levels.

Molecular analyses showed that forced swimming stress changed the pattern of gene and protein expression in about 4% to 5%. Hierarchical clustering analyses dividing the animals into two main groups, stressed and non-stressed, agreed with this finding, indicating a main effect of stress on gene expression pattern.

Moreover, in this study, forced swimming stress modified, for the most part, the expression of genes related to cellular respiratory chain, oxidative stress responses, apoptosis control and neuroplasticity. Also, imipramine and 7-NI were able to attenuate FST-induced changes in the expression of most of these genes. For example, 7-NI prevented the stress-induced increase in the expression of a number of genes related to respiratory complex I-III, succinate dehydrogenase and ATP synthesis.

Functional analyses of both SAGE and proteomic libraries indicated that the expression of genes associated with oxidative phosphorylation, oxidative stress and prevention of apoptosis induced by ROS are consistently modified by the FST. These findings corroborate other reports from the literature showing changes in the expression of genes enrolled in mitochondrial oxidative chain and metabolism control in suicidal patients with history of depression or rodents after treatment with high doses of corticoids or exposure to the learned helplessness model of depression (Datson *et al*. 2001b; Knapp & Klann 2002; Rivas-Arancibia *et al*. 2009). Depressive states have also been associated with an increase in oxidative damage in different brain sites, an effect that can be partially prevented by antidepressant drugs (Bilici *et al*. 2001; Eren *et al*. 2007). In addition, a recent study using microarray has also found significant effects on metabolic-related genes induced by different classes of antidepressant agents (Lee *et al*. 2010). Finally, anti-oxidant drugs induce antidepressant-like effects in the FST (Berk *et al*. 2008a; Ferreira *et al*. 2008).

Together, these results corroborate the metabolic/oxidative stress hypotheses of mood disorders (Andreazza *et al*. 2008; Berk *et al*. 2008b; Hess *et al*. 2005; Hroudova & Fisar 2010); Moreover, several pieces of evidence indicate that NO could be an important mediator linking stress exposure, metabolic/oxidative changes and behavioral consequences, including (1) increases of NO production by nNOS following stress-related NMDA activation by glutamate (Rivas-Arancibia *et al*. 2009), although other NOS isoforms could also be involved (Fu *et al*. 2010; Khovryakov *et al*. 2010); (2) increases in GC levels and neuronal energy demands (Lizasoain *et al*. 1996); (3) impairments of NADH formation due to inhibition of the respiratory chain by NO and ROS (Kamsler & Segal 2003; Wang *et al*. 2006); (4) joint effects of increased energy demands and inhibition of the respiratory chain, causing an imbalance between pro- and anti-oxidant mechanisms and increasing oxidative stress (Eren *et al*. 2007; Herken *et al*. 2007). As a final consequence, these mechanisms could lead to neuronal functional impairments (Meffert & Baltimore 2005), dendritic remodeling, apoptosis and impairment of hippocampal neurogenesis (Kaltschmidt *et al*. 2005).

In addition to oxidative stress, changes in genes and proteins related to apoptosis/survival processes mediated by NF-κB/BAX-cytochrome-*c*, cytoskeletal remodeling and neurophilament formation were also observed. The nuclear κB factor is a member of a transcriptional factor family

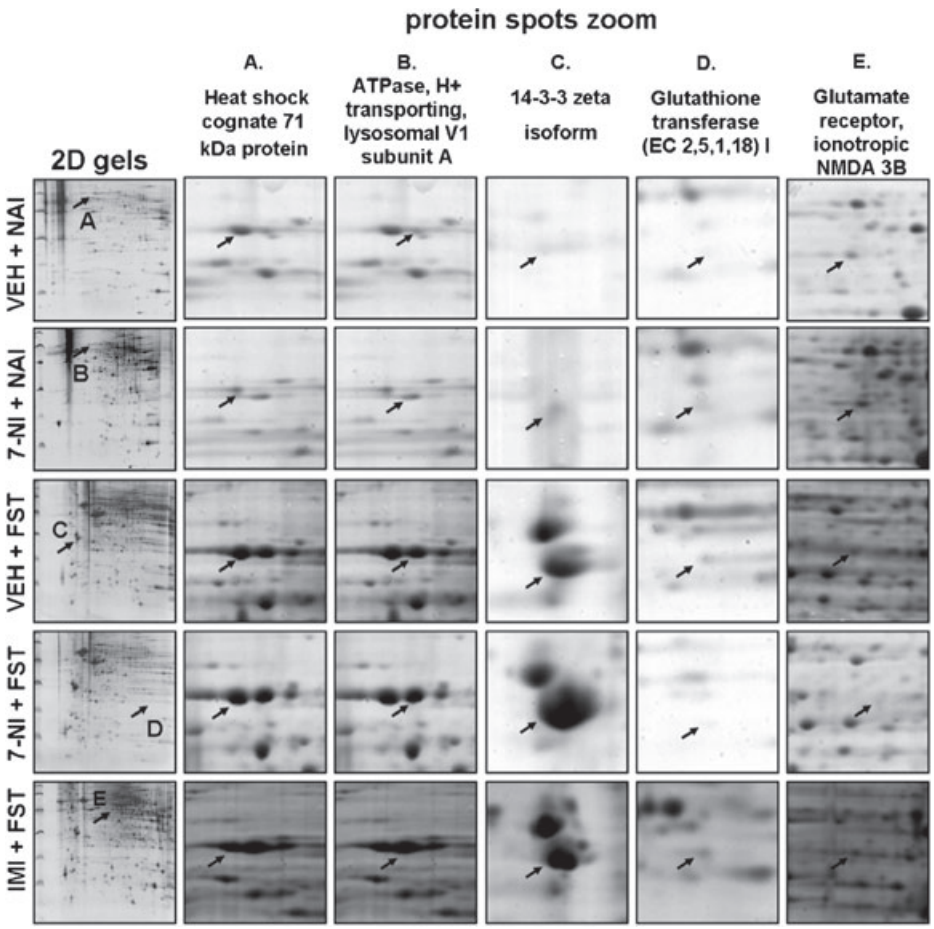

Figure 3: Bidirectional protein gels of proteomic analysis of VEH + NAI, 7-NI + NAI, VEH + FST, 7NI + FST and FST + IMI groups. The arrows indicate representative proteins differently expressed. VEH + NAI = animals treated with vehicle ($N = 4$; saline:DMSO, 1:1, 2 ml/kg for 14 days), 7-NI + NAI = animals treated with 7-NI ($N = 4$; 60 mg/kg by 14 days), VEH + FST = animals treated with vehicle ($N = 4$; saline:DMSO, 1:1, 2 ml/kg for 14 days) and submitted to FST, 7-NI + FST = animals treated with 7-NI ($N = 3$; 60 mg/kg for 14 days) and submitted to FST, IMI + FST = animals treated with imipramine (15 mg/kg for 14 days) and submitted to FST.

composed of five elements that has been related to apoptotic mechanisms (Wang *et al*. 1998). Moreover, the expression of genes enrolled in apoptotic processes such as STAT3, cytochrome-*c*, genes related to mitosis activated protein (MAPK) pathway, and several isoforms of Poly (ADP-ribose) polymerase and Ppp2r were also changed by FST stress. This latter gene controls the expression of IκB (IKK), a inhibitor of NF-κB activation (Chang *et al*. 2003; Shioda *et al*. 2009). 7-NI and imipramine revert the changes in these apoptosis-related genes, suggesting that the common proliferative and neuroprotective effects previously described to 7-NI and imipramine (Castren *et al*. 2007; Dranovsky & Hen 2006) could be associated to inhibition of NF-κB/Ppar activity (Sarnico *et al*. 2009; Veuger *et al*. 2009).

The MAPK pathway, composed by the protein kinase regulated by extracellular signaling (ERK1/2), the Jun-N-terminal kinase (JNK) and MAPK p38, is also closely involved in processes such as cell proliferation, differentiation and stress response (Sousa *et al*. 2000). The MAPK p38 and ERK pathways are usually associated with cell death and citoprotection, respectively (Liu *et al*. 2010). The increased expression of MAPK1-3 found in stressed animals would favor the balance for apoptotic signaling (Harvey 1964). In this study, only imipramine was able to prevent the changes in the MAPK pathway, indicating a distinct profile of gene expression changes compared to 7-NI.

The alteration of several isoforms of genes related to cytochrome-*c* in animals submitted to FST that were, also for the most part, prevented by 7-NI and imipramine, suggests the involvement of apoptotic-related genes associated to the balance of anti-apoptotic Bcl and pro-apoptotic BAX proteins (Chung *et al*. 2008; Yung *et al*. 2004). Corroborating this proposal, 7-NI has been shown to prevent NO-induced cell death mediated by p53- and Bax-dependent pathway (Martin *et al*. 2011). Therefore, it is possible that exposure to the FST results in an oxidative stress insult, producing

the engagement of pro- and anti-apoptotic factors, with antidepressive drugs changing the balance in favor of the latter.

Exposure to repeated restraint stress induces plastic changes in the hippocampus such as dendritic remodeling (O'kane *et al*. 2004). Although the stress procedure (forced swimming) used in the present work is much shorter than those reported to cause these changes, it did modify the expression of genes related to neurophilaments and cytoskeleton remodeling controlled by Rho GTPase. Rho belongs to a super protein family linked to GTPase named as Ras family (Rex *et al*. 2009), which also includes Ras and Ran proteins. They can be turned on–off by fast conformational shift when bound to GTP or GDP, being characterize as fast shift proteins (Sarandol *et al*. 2007). Through extracellular signaling, RhoA-GTPase regulates the function of LIM1 and 2 (LIMK1/2) proteins which, together Confilin, down-regulate the dynamic control of polymerization/depolarization of actin filaments (Chen *et al*. 2006; Kreis & Barnier 2009). The increased expression of several genes related to the RhoA/Rac/Cdc42 pathway in stressed animals (Cdc42, Cfl1, Pfn1, Cdc42), and its inhibition by 7-NI (Cdc42) and imipramine (Cdc42, Rho GDP) observed in this study, corroborates the proposal that neurophilament remodeling induced by stress stimuli could be related to the behavioral changes observed in depressive patients (Abkevich *et al*. 2003).

The LIM-Confilin system is also controlled by Cdc42, acting synergically in the dynamic control of actin filaments. The Rho-GTPase-LIM-Confilin complex seems to be involved in dendritic retraction, with arrest of actin filaments to lamelipody and filipody formation, while Cdc42 would be linked to prolongation of growing cones and dendritic ramification (Chen *et al*. 2006). Inhibition of GTPase-Rho-kinase increases LTP amplitude and interfere with synaptic plasticity (Park *et al*. 2007; Seo *et al*. 2006). Recent studies have shown that cytoskeletal components associated to a sub-family of Rho protein are important for LTP formation. The RhoA-ROCL-LIM2 signaling is proposed to promote the initial stages of LTP formation while the Rac1-LIMK1 pathway acts on its consolidation and maintenance (Rex *et al*. 2009).

The changes in several genes related to CREB induced by 7-NI and detected by both SAGE and proteomic analyses suggest that NO-mediated changes in the CREB pathway could be related to the antidepressant-like proprieties of NOS inhibitors. CREB is a nuclear transcriptional factor crucial for the expression of several genes that affect the function of entire neural circuits (Carlezon *et al*. 2005). Stress exposure reduces CREB activation and the expression of brain derived neurotrophic factor (BDNF) and its receptor tyrosine kinase B (TrkB), affecting the neurotrophic signaling pathway and reducing hippocampal cell proliferation. On the other hand, antidepressant treatment increases CREB phosphorylation and CREB, BDNF and trkB expression (Gass & Riva 2007), preventing plastic consequences of stress. It has been proposed that activation of BDNF-trkB-CREB pathway, with consequent alteration in hippocampal plasticity and neurogenesis, is a common key point to antidepressant effects (Castren *et al*. 2007; Shirayama *et al*. 2002). The increase in expression of CREB-related genes such as Calm1 and TrkB suggest that the antidepressant-like effects of imipramine could also be related to this pathway (Nibuya *et al*. 1996; Tardito *et al*. 2009).

Although sharing some common effects on gene expression, the present data suggest that imipramine and 7-NI effects also involve different mechanisms. For example, chronic treatment with 7-NI increased the expression of genes related to cell adhesion and cytoskeletal remodeling by FAK, while imipramine increased mainly genes linked to cytoskeletal remodeling and neurophysiological process signaled by the 5-HT1A receptor. Moreover, only imipramine changed the expression of genes related to DNA acetylation/deacetylation, a mechanism that has been associated with epigenetic mechanisms related to stress consequences (Carlezon *et al*. 2005; Tsankova *et al*. 2006).

There are some limitations in this study: (1) Individual analysis of gene expression could have allowed for correlations between specific gene expression and behavior changes. However, most similar studies employing SAGE or microarray approaches have used pooled samples (Czibere *et al*. 2011; George *et al*. 2011; Guipponi *et al*. 2011). Moreover, the reliability of our SAGE approach was tested in individual samples by an independent method (qRT) (Figure S4), which found a positive correlation ($r = 0.609$, $P < 0.001$) between means of individual results obtained by qRT and SAGE data (Fig. 2); (2) Even if all groups were submitted to the same administration procedure, the stress of the i.p. injections could have also affected gene expression. It is doubtful, however, that the present approach (SAGE) would have been sensitive enough to detect such changes. Corroborating this idea, a recent study using microarray that compared several antidepressant agents administrated orally or by i.p. injection (1, 3 or 7 days) failed to detect any difference in gene expression due to the drug administration route (Lee *et al*. 2010); (3) Although no single drug administration group was included, the main objective of the study was to investigate gene expression changes induced by chronic treatment with the preferential inhibitor of neuronial nitric oxide synthase, 7-NI, in animals submitted to FST. Rats (different from mice) are usually not sensitive to single antidepressant injections in this test, but only to subchronic (three injections in 24 h) treatment. Although this treatment regime could have been used in this study, the technique employed might not be sensitive enough to detect small effects on gene expression. Moreover, several neurobiological changes that have been implicated in antidepressant-like actions such as increased hippocampal neurogenesis (Dranovsky & Hen 2006; Liu *et al*. 2006) and synaptic plasticity (Bachis *et al*. 2008; Dranovsky & Hen 2006) are usually detected after chronic, but not single drug administration.

In conclusion, despite these limitations, the present paper confirmed that inhibition of NO formation produces antidepressant-like effects in animals and suggests that this effect could involve changes in the expression of genes related to oxidative stress, neuroplastic and neurogenic processes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1: Genes differentially expressed between the VEH + FST and VEH + NAI groups associated to oxidative phosphorylation, according to GeneGo analyses.

Table S2: Genes differentially expressed between VEH + FST and VEH + NAI groups associated with apoptosis and survival controlled by 5-HTR1A signaling, according to GeneGo analyses.

Table S3: Genes differently expressed between the VEH + NAI and VEH + FST groups associated to regulation of action cytoskeleton by Rho GTPase and Cytoskeleton remodeling, according to GeneGo analyses.

Table S4: Genes differently expressed between the VEH + FST and 7-NI + FST groups associated to oxidative phosphorylation, according to GeneGo analyses.

Table S5: Genes differentially expressed between the VEH + FST and 7-NI + FST associated to cytoskeleton remodeling signaling by FAK and cell adhesion, according to GeneGo analyses.

Table S6: Genes differentially expressed between the groups VEH + FST and 7-NI + FST associated to transcriptional CREB pathway and pathway signaling by β-adrenergic receptor, according to GeneGo analyses.

Table S7: Genes differentially expressed between the groups VEH + FST and IMI + FST associated with oxidative phosphorylation, according to GeneGo analyses.

Table S8: Genes differentially expressed between the groups VEH + FST and IMI + FST associated to apoptosis and survival HTR1A and neurophysiological process 5HTR1A signaling in neuronal cells according to GeneGo analyses.

Table S9: Genes differentially expressed between the VEH + FST and IMI + FST groups associated to cytoskeleton remodeling and transcription control by heterochromatin protein 1 (HP1) family according to GeneGo analyses.

Table S10: Proteins differentially expressed between the VEH + NAI and VEH + FST groups.

Table S11: Proteins differentially expressed between the VEH + FST and 7NI + FST groups.

Table S12: Proteins differentially expressed between the VEH + FST and IMI + FST groups.

Figure S1: Biological pathways signaled by HTR1A and responsible for controlling cell apoptosis and survival, according to GeneGo bank. Columns indexed by number 1 indicate the fold change rate for the genes differently expressed between VEH + NAI and VEH + FST. Red columns indicate increase of expression, and blue columns indicate increase of expression compared to the group VEH + NAI.

Figure S2: Biological pathways associated to gene transcription regulated by the CREB pathway (A) and beta-adrenergic receptors transactivation pathway (B) according to GeneGo. Columns indexed by number 1 indicate the fold change rate for the genes differently expressed between VEH + FST and 7-NI + FST. Red columns indicate increase of expression, and blue columns indicate increase of expression compared to the group 7-NI + FST. Columns indexed by number 2 indicate the fold change rate for the genes differently expressed between VEH + NAI and VEH + FST. Red columns indicate increase of expression, and blue columns indicate increase of expression compared to the group VEH + NAI.

Figure S3: Biological pathways linked to apoptosis and survival signaled by HTR1A according to GeneGo. Red columns indicate increase of expression, and blue columns indicate increase of expression compared to the group IMI + FST. Columns indexed by number 2 indicate the fold change rate for the genes differently expressed between VEH + NAI and VEH + FST. Red columns indicate increase of expression, and blue columns indicate increase of expression compared to the group VEH + NAI.

Figure S4: Effect 7-nitroindazole, imipramine or vehicle on hippocampal gene expression at rats ($n = 4–8$/group) submitted to the forced swimming test. The black bars represent means ± SEM of fold change for qRT, $*P < 0.05$, Duncan test. VEH + NAI = animals treated with vehicle ($N = 4$; saline:DMSO, 1:1, 2 ml/kg, for 14 days), 7-NI + NAI = animals treated with 7-NI ($N = 4$; 60 mg/kg by 14 days), VEH + FST = animals treated with vehicle ($N = 4$; saline:DMSO, 1:1, 2 ml/kg for 14 days) and submitted to FST, 7-NI + FST = animals treated with 7-NI ($N = 3$; 60 mg/kg for 14 days) and submitted to FST, IMI + FST = animals treated with imipramine (15 mg/kg for 14 days) and submitted to FST.

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