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ORIGINAL ARTICLE

The low affinity neurotensin receptor antagonist levocabastine impairs brain nitric oxide synthesis and mitochondrial function by independent mechanisms

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Abstract

Neurotensin is known to inhibit neuronal Na^+, K^+-ATPase, an effect that is rescued by nitric oxide (NO) synthase inhibition. However, whether the neurotensinergic and the nitrergic systems are independent pathways, or are mechanistically linked, remains unknown. Here, we addressed this issue and found that the administration of low affinity neurotensin receptor (NTS2) antagonist, levocabastine (50 μg/kg, i.p.) inhibited NO synthase (NOS) activity by 74 and 42% after 18 h in synaptosomal and mitochondrial fractions isolated from the Wistar rat cerebral cortex, respectively; these effects disappeared 36 h after levocabastine treatment. Intriguingly, whereas neuronal NOS protein abundance decreased (by 56%) in synaptosomes membranes, it was enhanced (by 86%) in mitochondria 18 h after levocabastine administration. Levocabastine enhanced the respiratory rate of synaptosomes in the presence of oligomycin, but it failed to alter the spare respiratory capacity; furthermore, the mitochondrial respiratory chain (MRC) complexes I–IV activities were severely diminished by levocabastine administration. The inhibition of NOS and MRC complexes activities were also observed after incubation of synaptosomes and mitochondria with levocabastine (1 μM) *in vitro*. These data indicate that the NTS2 antagonist levocabastine regulates NOS expression and activity at the synapse, suggesting an interrelationship between the neurotensinergic and the nitrergic systems. However, the bioenergetics effects of NTS2 activity inhibition are likely to be independent from the regulation of NO synthesis.

Keywords: cerebral cortex, levocabastine, mitochondria, neurotensin, nitric oxide synthase, synaptosomal membranes.

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Abbreviations used: DAF-2, 4,5-diaminofluorescein; DAF-2-DA, 4,5-diaminofluorescein diacetate; eNOS, endotelial nitric oxide synthase; FAD, flavin adenine dinucleotide; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhy-drazone; FMN, flavin mononucleotide; GSNO, *S*-nitrosoglutathione (*S*-nitrosylating agent); HEPES, (4-(2-hydroxyethyl)-1(-piperazineethanesulfonic acid); iNOS, inducible nitric oxide synthase; L-NAME, Nω-nitro-L-arginine methyl ester hydrochloride; L-NNA, Nω-nitro-L-arginine; MAO, monoaminoxidase; MSH, manitol-sucrose HEPES; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NTS1 receptor, high affinity neurotensin receptor; NTS2 receptor, low affinity neurotensin receptor; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TEMED, tetramethylethylenediamine; VDAC, voltage-dependent anion channel.

Nitric oxide (NO) is an intercellular messenger implicated in numerous physiological functions. At nervous system level, NO can act as a neurotransmitter or neuromodulator. Its involvement in synaptic plasticity and memory formation has been postulated (Kemenes *et al.* 2002). NO differs from conventional neurotransmitters in several aspects because it does not interact with specific synaptic receptors, it is not concentrated in synaptic vesicles and consequently it is not released by exocytosis. NO is a powerful signaling molecule that readily diffuses through the cell membranes and is not stored in the producing cell. NO signaling ability is controlled at the level of its biosynthesis and by local availability (see Holz and Fisher 2012).

For two decades, it was thought that NO, a short-lived gaseous molecule, is able to diffuse freely across relatively long distances and to traverse major parts of the cell, if not multiple cell layers. However, NO has been proven to be extremely reactive: it reacts with other reactive oxygen species, heavy metals, as well as with cysteine and tyrosine residues present in proteins. It is now widely accepted that once NO is generated, it is very short-lived and diffuses only over a short distance. For these reasons, it has been of interest to explore the local production of NO and the localization of NO synthases in the proximity of their downstream targets (Govers and Oess 2004).

The formation of NO is catalyzed by a family of isoenzymes, the NO synthases: a neuronal isoform (nNOS) widely distributed throughout the central nervous system; an endothelial isoform, which predominates in vascular endothelial cells and an inducible form present in glia (Knowles and Moncada 1994). The activity of the later isoform is Ca^{2+} insensitive, whereas the activities of nNOS and endotelial nitric oxide synthase are Ca^{2+}/calmodulin dependent. In fact, one of the most studied mechanisms of nNOS activation at the central nervous system is the stimulation of NMDA receptor and the consequent calcium entry to the cell (Dawson and Dawson 1996). Also, NO formed by NMDA receptor activation has been shown to exert a stimulatory action on neurotransmitter release (Kuriyama and Ohkuma 1995). Interestingly, a role of NO on the regulation of mitochondrial respiratory chain has been postulated, mainly by acting as an inhibitor of cytochrome oxidase (Brown 2001).

Neurotensin is a tridecapeptide widely distributed in the nervous system, at both central and peripheral levels. It can behave as a neurotransmitter or neuromodulator, exerting diverse biological actions (Kitabgi and Nemeroff 1992). Neurotensin effects are mediated by its binding to a group of receptors (Carraway and Leeman 1973; Vincent *et al.* 1999). Two of them, termed high affinity neurotensin receptor (NTS1) and low affinity neurotensin receptor (NTS2), bind neurotensin with high and low affinity, respectively. They are seven transmembrane domain receptors which are coupled to G proteins. Another two neurotensin receptor types are mainly localized intracellularly and are termed NTS3/sortilin and nts4/SorLA (Dobner 2005).

Previous results from our laboratory indicate the ability of neurotensin to inhibit synaptosomal membrane Na^+, K^+-ATPase activity, an essential enzyme for the maintenance of ionic gradients. This effect involves NTS1 receptor and- at least partially NTS2 receptor (López Ordieres and Rodríguez de Lores Arnaiz 2000, 2001; Álvarez Juliá *et al.* 2013; Gutnisky *et al.* 2016). The inhibitory effect of neurotensin on Na^+, K^+-ATPase activity is impaired by neonatal treatment to rats with Nω-nitro-L-arginine methyl ester hydrochloride, which blocks the synthesis of NO (López Ordieres *et al.* 2011).

These findings gave interest to explore the relationship between NO syntheses and neurotensinergic system. In the search of a potential interaction between neurotensinergic and nitrergic systems, levocabastine, antagonist for NTS2 receptor (Schotte *et al.* 1980) was administered to rats. NOS activity and nNOS expression were evaluated in synaptosomal membranes and mitochondrial fractions isolated from cerebral cortex. Mitochondrial respiratory function was also evaluated.

Materials and methods

Animals and drugs

Forty-four male Wistar rats (RRID: RGD_737960) 30 days old, weighing 100 g, were used. Animals were from Harlan Laboratories, Indianapolis, USA, without genetic modifications. Rats were kept in the Animal Housing of the School of Pharmacy and Biochemistry (ID 13208537, HsdFfyb: WI). All studies described were conducted in accordance with the Guide for Care and Use of Laboratory Animals provided by the National Institutes of Health, USA and had the legal ethical accreditation from Ethics Committee for Laboratory Animal Handling (CICUAL) of the Faculty of Medicine, Universidad de Buenos Aires where the protocol was performed (Res. 2843). All efforts were made to minimize suffering and reduce the number of animals used.

Groups of six animals were located per cage. The assignment of the animals to each treatment (controls, levocabastine 30 min, 18 and 36 h) was performed by a simple randomization method based on the use of a random number table. This method provided unbiased evaluation of biochemical parameters. No blinding was performed.

Exclusion criteria were based on the animal's good state of welfare. In this sense, animals were monitored to ensure that they were healthy, comfortable, well nourished, safe, able to express innate behavior, and not suffering from unpleasant states such as pain, fear, and distress. In our study, no animals were excluded.

As part of our study design, sample size was determined following Poisson regression. In addition, sample size was calculated using a power of 0.8 and $p < 0.05$. 4,5-diaminofluorescein diacetate (DAF-2-DA) was purchased from Calbiochem, San Diego, CA, USA. Catalase, superoxide dismutase, cytochrome *c*, dithiothreitol, horseradish peroxidase, L-arginine, NADH, NADPH, oxyhemoglobin, succinate, sucrose, sodium dodecyl sulfate, and Folin reagent, were

purchased from Sigma Chemical Co., St. Louis, MO, USA. Neuronal nitric oxide synthase (nNOS) rabbit antibody (RRID AB_630936), β-actin (RRID: AB_626632), α-tubuline (RRID: AB_628408), goat voltage-dependent anion channel antibody (VDAC, RRID: AB_793935), and enhanced chemiluminescence reagent were purchased from Santa Cruz Biotecnology Inc., Santa Cruz, CA, USA. Bisacrilamide, Laemmli buffer and tetramethylethylenediamine were purchased from Bio Rad Laboratories Inc., Research Foundation, OK, USA. Enhanced chemiluminescence substrate (ECL) Western blotting detection reagent and Hyperfilm ECL were from GE Healthcare, Little Chalfont, UK. Levocabastine clorhydrate and loratadine were kindly provided, respectively, by Janssen-Cilag Farmacéutica, S.A. and by Dr. Mario Loss, Bagó Laboratory, Argentina. All other reagents were analytical grade.

Administration of levocabastine

Levocabastine dissolved in saline solution was administered i.p. to male Wistar rats at a single dose of 50 μg/kg (Tyler *et al.* 1998). To serve as controls, rats were administered with saline solution (vehicle).

Preparation of crude mitochondrial fractions

Crude mitochondrial fractions from cerebral cortex of rats injected with levocabastine or vehicle were obtained by differential centrifugation. For each experimental point, cerebral cortices from two rats were pooled, homogenized at 10% (w/v) in cold 0.32 M sucrose (neutralized to pH 7.0 with 0.2 M Tris base) using a Teflon glass homogenizer of the Potter-Elvehjem type. The homogenates were centrifuged at 900 *g* for 10 min and the supernatants were separated. Pellets were resuspended in the homogenizing medium and centrifuged again at 900 *g* for 10 min to separate the sediments (nuclear fractions) from the supernatants. Supernatants from both centrifugations were pooled and centrifuged at 11 500 *g* for 20 min. Final supernatants were discarded and the pellets, which contains intact synaptosomes and free mitochondria, were resuspended in mannitol sucrose HEPES buffer (0.23 M mannitol, 0.07 M sucrose, and 5 mM HEPES, pH 7.4). The whole procedure for rat treatment and subcellular fractionation was replicated at least three times. Immediately after preparation, these fractions were employed for the assays of NO detection and oxygen consumption.

Preparation of purified synaptosomal membrane and mitochondrial fractions

Synaptosomal membrane and mitochondrial fractions from cerebral cortex were isolated by differential and sucrose gradient centrifugation as previously described (Rodríguez de Lores Arnaiz *et al.* 1967). For each experimental point, cerebral cortices from three rats were pooled, homogenized at 10% (w/v) in cold 0.32 M sucrose (neutralized to pH 7.0 with 0.2 M Tris base) using a Teflon glass homogenizer of the Potter-Elvehjem type. The homogenate was subjected to differential centrifugation to separate nuclear, mitochondrial, microsomal, and soluble fractions. The crude mitochondrial pellet was resuspended in redistilled water (pH 7.0 with 0.2 M Tris base) for the osmotic shock. A pellet containing mitochondria, synaptosomal membranes, and myelin was separated by centrifugation at 20 000 *g* for 30 min, then resuspended in 0.32 M sucrose, layered on top of a gradient containing 0.8; 0.9; 1.0, and 1.2 M sucrose and centrifuged at 50 000 *g* for 2 h in a SW 28 rotor of an L8 Beckman ultracentrifuge. The fraction at the level of 1.0 M sucrose was collected, diluted with 0.16 M sucrose and spun down at 100 000 *g* for 30 min to obtain the synaptosomal membrane fraction. The resulting pellet from the gradient was collected for mitochondrial assays. Synaptosomal membrane and mitochondrial fractions were stored at −70°C and, prior to enzyme assay, resuspended by brief homogenization in redistilled water and 0.16 M sucrose, respectively. The whole procedure was replicated at least three times.

NO detection by flow cytometry

Nitric oxide was detected in freshly prepared crude mitochondrial fractions by a cytometric method based on the use of potentiometric probe DAF-2-DA (López-Figueroa *et al.* 2000). This probe (DAF-2-DA) is deacetylated by intracellular esterases to DAF-2, which reacts with NO to yield the highly fluorescent triazolofluorescein. Assays were carried out as follows: crude mitochondrial fractions (25 μg protein/mL) were incubated at 37°C for 30 min in mannitol sucrose HEPES buffer supplemented with 5 mM malate, 5 mM glutamate, 2 mM phosphate, 1 mM $MgCl_2$ and 1.3 mM $CaCl_2$, in the presence of 10 μM DAF-2-DA (λexc = 495 nm, λem = 515 nm). Samples were protected from light until acquired by the FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). Autofluorescence was evaluated in samples without the probe. In addition, 5 mM *S*-nitrosoglutathione (*S*-nitrosylating agent) (GSNO) and 1 mM L-NNA were added as positive and negative controls, respectively. The effect of increasing concentrations of KCl (5–60 mM) was also tested. DAF-2 fluorescence was analyzed using the median value of the distribution of fluorescence events from each treatment. A common marker (M1) was fixed on control median value representing approximately 50% of the fluorescent events. Histogram differences in DAF-2 fluorescence were quantified as the number of events which drop under the median value of the relative fluorescence distribution corresponding to M1. A lower number of DAF-2 fluorescence events under M1 would reflect increased NO levels. Quantification of results was shown as bar graphs in which data were expressed as the percentage of control DAF-2 fluorescence.

Nitric oxide synthase activity

Nitric oxide production by NOS was measured in brain cortex synaptosomal membrane and mitochondrial fractions (0.1–0.5 mg protein/mL) using a double-beam dual-wavelength spectrophotometer, following the oxidation of oxyhemoglobin to methemoglobin at 577–591 nm ($\varepsilon_{577-591}$ = 11.2 per mM/cm) (Lores-Arnaiz *et al.* 1999; Boveris *et al.* 2002) at 37°C. The reaction medium contained 50 mM phosphate buffer (7.4 for synaptosomal membranes and pH 5.8 for mitochondria), 50 μM L-arginine, 100 μM NADPH, 10 μM dithiothreitol, 1 mM $CaCl_2$ and 25 μM oxyhemoglobin (expressed per heme group). To avoid the presence of $O_2^{\bullet -}$ and H_2O_2, 4 μM Cu-Zn superoxide dismutase and 0.1 μM catalase were also added to the reaction medium. When indicated, samples of the subcellular fractions were pre-incubated for 1 min with 1 μM levocabastine or loratadine before the addition of the corresponding reaction media. Results were expressed as nmol NO produced per milligram protein per minute.

Nitric oxide synthase expression

Synaptosomal membrane and mitochondrial fractions were processed for western blot. Sample fractions (80 μg protein) were

separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7.5%), blotted onto a nitrocellulose membrane (Bio-Rad, München, Germany) and probed primarily with rabbit polyclonal antibodies (dilution 1 : 500) for the neuronal nitric oxide synthase (nNOS), epitope corresponding to amino acids 2–300, mapping to the amino terminus of NOS I. Then, the nitrocellulose membrane was incubated with a secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (dilution 1 : 5000), followed by development of chemiluminescence with the ECL reagent for 2–4 min (Lores-Arnaiz *et al.* 2007). Optical densities for paired samples (treated and control) developed in the same gel were quantified. As loading controls, β-actin and α-tubuline were processed for synaptosomal membranes and VDAC was processed for mitochondria. Densitometric analysis of nNOS and control bands were evaluated through NIH Image J 1.47b software (NIH, Rockville, Maryland, USA).

Preliminary assays for western blot in synaptosomal membrane fractions carried out with β-actin or tubuline as loading controls showed that levocabastine treatment modified not only the expression of nNOS but also those of loading control proteins. For this reason, data for nNOS optical density in synaptosomal membrane fractions were expressed as the ratio between treated *versus* control values. To calculate the ratios, data were normalized by assigning a value of 1.00 to optical density for the control. The values ratio indicates how much a given protein is expressed in the treated in comparison with the control. Data for nNOS optical density in mitochondrial fractions were expressed as the ratio of nNOS/VDAC taking control value as 100%.

Oxygen consumption

A two-channel respirometer for high-resolution respirometry (Oroboros Oxygraph; Paar KG, Graz, Austria) was used.

Oxygen consumption in fresh crude mitochondrial fractions was assayed in different conditions, taking into account that this fraction contains synaptosomes plus mitochondria. To evaluate synaptosomal respiration, 15 mM D-glucose plus 10 mM pyruvate were employed as substrates, in a reaction medium containing 120 mM NaCl, 3.5 mM KCl, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO4, 0.4% (w/v) fatty acid-free bovine serum albumin and 10 mM HEPES, pH 7.4.

For mitochondrial respiration, 6 mM malate-6 mM glutamate or 7 mM succinate were used as substrates in a reaction medium consisting of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 4 mM $MgCl_2$, 5 mM phosphate and 0.2% bovine serum albumin**.** Incubation proceeded at 30°C to measure state 4 respiration, and 1 mM ADP was added to measure state 3 respiration. The respiratory ratio (state 3 respiration/state 4 respiration) was determined (Estabrook 1967).

Maximum respiration was achieved in the presence of 4 μM carbonyl cyanide-4-(trifluoromethoxy)phenylhy-drazone (FCCP). Respiration driving proton leak was determined after the addition of 5 μM oligomycin, an inhibitor of ATP synthase. Spare respiratory capacity was estimated as a percentage ratio between maximum and basal respiration (Choi *et al.* 2011). Results were expressed as ng at O/min mg protein**.**

Evaluation of mitochondrial respiratory complexes I–III, II–III and IV

Assays were carried out in purified mitochondrial fractions after three cycles of freeze-thawing. NADH-cytochrome *c* reductase activity (complex I–III) was measured in mitochondrial fractions by following spectrophotometrically the reduction in cytochrome *c* at 550 nm (ε = 19.6 per mM/cm) in a reaction medium containing 100 mM phosphate buffer (pH 7.4), 0.2 mM NADH, 0.1 mM cytochrome *c* and 0.5 mM KCN at 30°C. Succinate-cytochrome *c* reductase activity (complex II + III) was determined as indicated for complex I–III, except that NADH was substituted by 20 mM succinate. Cytochrome oxidase activity (complex IV) was assayed spectrophotometrically at 550 nm by following the rate of oxidation of 50 μM ferrocytochrome *c* (Yonetani 1967). Enzyme activities for complex I–III and complex II + III were expressed in nmol cytochrome *c* reduced per mg of protein per minute. Enzyme activity for complex IV was expressed as k'/mg protein.

MAO activity

This enzyme activity was measured by following spectrophotometrically the oxidation of kynuramine at 30°C, in a reaction medium containing 50 mM phosphate buffer, pH 7.4 and kinetics were followed at 360 nm (ε = 4.28 per mM/cm) (Weissbach *et al.* 1960). Results were expressed as relative activity, taking as 100% values recorded for the corresponding controls.

Protein measurement

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Expression of results and statistical analyses

Results are expressed as mean values ± SEM of *n* experiments. Data were evaluated for normality distribution by Kolmogórov–Smirnov test to follow a posterior parametric or non-parametric statistical testing. The analysis of the results was performed using unpaired Student's *t*-test or ANOVA followed by post hoc Tukey or Bonferroni tests to analyze if differences between groups were statistically significant. IBM SPSS Statistics 20.0 was used and a difference was considered to be statistically significant when $p < 0.05$.

Results

NO detection by flow cytometry

Flow cytometric analysis using DAF-2-DA was performed to determine if crude mitochondrial fractions, which contain both intact synaptosomes and free mitochondria, were able to produce detectable NO levels after vehicle or levocabastine treatments.

Dotblots of Forward Scatter- Height (FSC-H) *versus* Side Scatter-Height (SSC-H) indicating the gated mitochondrial population are shown in Fig. 1(a) and (f) for control- and levocabastine-treated animals, respectively.

Assays were carried out in several conditions. As expected, DAF-2 relative fluorescence intensity after pre-incubation of the sample with the NOS inhibitor L-NNA (Fig. 1c) was significantly lower *versus* control samples (Fig. 1b). A significant increase in DAF-2 fluorescence was observed after the addition of the NO donor GSNO (Fig 1d). The addition of 5–60 mM KCl produced a slight

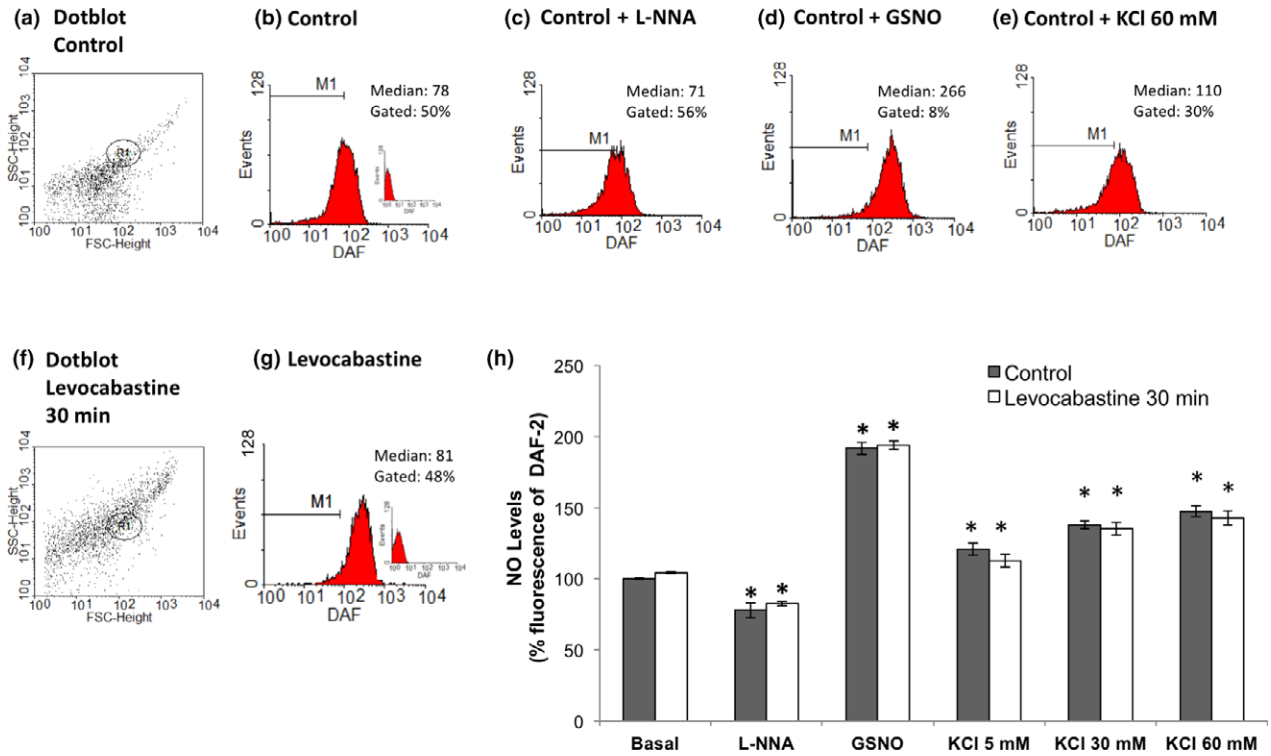

Fig. 1 Nitric oxide (NO) content in fresh mitochondria isolated from rat cerebral cortex following 4,5-diaminofluorescein (DAF-2) fluorescence. Brain cortex crude mitochondrial fractions were loaded with the probe DAF-2-DA and direct measurements of NO were obtained by flow cytometry. Typical dot-blot of FSC-H *versus* SSC-H indicating a gated mitochondrial population (R1) for control- or levocabastine-treated animals are shown in panels (a) and (f), respectively. Typical histograms of gated events *versus* relative fluorescence intensity (FL-1) are shown for control (b) or levocabastine-treated animals (g); samples without probe used for autofluorescence are presented as insets. Assays were carried out in the presence of 1 mM L-NNA, 5 mM *S*-nitrosoglutathione (*S*-nitrosylating agent) (GSNO) or 5–60 mM KCl for control- and levocabastine-treated animals. Typical histograms of DAF-2 fluorescence intensity of control samples after incubation with L-NNA, GSNO, and KCl are shown in (c–e), respectively. Bar graph quantification of the amount of DAF-2 relative fluorescence events is shown in (h). Fluorescence events were quantified as the number of events which drop under a common marker M_1 (fixed at the median value of the control histogram) taking control fluorescence events as 100%. Bars represent the mean ± SEM. The cytometric analysis was performed three times for each treatment ($n = 3$ corresponding to the number of total assays for each condition); ANOVA, Bonferroni's test $^*p < 0.05$, compared with basal conditions levels from the same group of animals.

concentration-dependent enhancement of NO production, as observed by an increase in DAF-2 fluorescence intensity (Fig. 1e and h). No differences were recorded in levocabastine *versus* vehicle-injected samples in any of the tested conditions (Fig. 1g and h).

Nitric oxide synthase activity

The activity of NOS was determined in synaptosomal membrane fractions after levocabastine administration. Enzyme activity from control rats injected with vehicle ranged between 0.9 and 1.1 nmol NO produced per mg protein per minute. NOS activity decreased, respectively, by 46 and 74% at 30 min and 18 h after levocabastine administration, but recovered control values at 36 h after drug treatment (Fig. 2).

These results gave interest to determine NOS activity in other fractions obtained 18 h after administration of levocabastine or vehicle. Taking NOS activity values recorded in control samples as 100%, activities for levocabastine-injected samples showed the following results: whole cerebral cortex homogenate, 48%; nuclear fraction, 125%; crude mitochondrial fraction, 100%; synaptosomal membrane fraction, 66%; microsomal fraction, 63%; and soluble fraction, 83% (data not shown).

In mitochondrial fractions, NOS activity was roughly 0.4 nmol NO produced per mg protein per minute. This value remained unaltered 30 min after levocabastine administration, decreased 42% at 18 h after the treatment and recovered control values at 36 h after drug administration (Fig. 3).

Absolute values for NOS activity in synaptosomal membrane fractions were roughly double than those recorded in mitochondrial fractions. The response to levocabastine administration in synaptosomal membrane fractions were

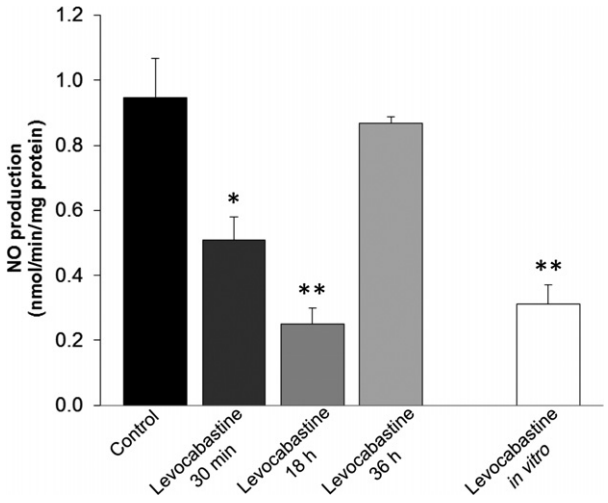

Fig. 2 Nitric oxide synthase (NOS) activity in synaptosomal membranes after levocabastine administration. Rats received i.p. a single dose of levocabastine or vehicle. Thirty minutes, 18 and 36 h later animals were decapitated, tissues harvested and subjected to subcellular fractionation to obtain synaptosomal membrane and mitochondrial fractions. Samples were processed for the assay of NO production. When indicated, enzyme assays were carried out in the presence of 1 μM levocabastine. Results are expressed as specific enzyme activity. For each experimental point, cerebral cortices from three rats were pooled. The whole procedure for rat treatment and subcellular fractionation was replicated three times and NOS activity assays were performed in triplicate ($n = 9$, corresponds to the number of total assays for each condition). Data are mean values ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ with respect to the control, by one-way ANOVA followed by Tukey's multiple comparison test.

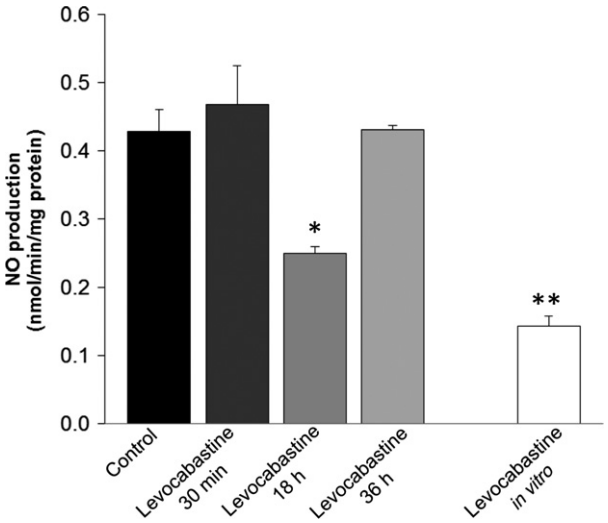

Fig. 3 Nitric oxide synthase (NOS) activity in mitochondrial fractions after levocabastine administration. Rats received i.p. a single dose of levocabastine or vehicle. Thirty minutes, 18 and 36 h later animals were decapitated, tissues harvested and subjected to subcellular fractionation to obtain synaptosomal membrane and mitochondrial fractions. Samples were processed for the assay of NO production. When indicated, enzyme assays were carried out in the presence of 1 μM levocabastine. Results are expressed as specific enzyme activity. For each experimental point, cerebral cortices from three rats were pooled. The whole procedure for rat treatment and subcellular fractionation was replicated three times and NOS activity assays were performed in triplicate ($n = 9$, corresponds to the number of total assays for each condition). Data are mean values ± SEM. $^{*}p < 0.05$, with respect to the control, by one-way ANOVA followed by Tukey multiple comparison test.

recorded earlier and proved greater than those observed in mitochondrial fractions. In both fractions, full recovery was observed at 36 h after drug administration (Figs 2 and 3).

It was of interest to test potential *in vitro* levocabastine effects. For this purpose, the drug was added to NOS incubation medium at 1 μM concentration, which is able to modify synaptosomal membranes Na^+, K^+-ATPase activity (López Ordieres and Rodríguez de Lores Arnaiz 2000). Such levocabastine concentration decreased 67% NOS activity in synaptosomal and mitochondrial fractions isolated from vehicle-injected animals (Figs 2 and 3).

Taking into account that levocabastine exerts antihistaminic properties, 1 μM loratadine – a well-known antiH1 receptor – was also tested, to observe a similar effect to that recorded with levocabastine. Loratadine was able to induce decreases in 79% and 58% in NOS activity from synaptosomal and mitochondrial fractions, respectively (data not shown).

Neuronal nitric oxide synthase protein expression

To explore whether NOS activity changes involved an alteration in enzyme expression, western blot assays were carried out. In each experiment, membrane samples isolated from tissue of levocabastine-injected rats were run in parallel with samples isolated from control tissue. Figures 4(a) and 5(a) show immunoblots from single representative experiments for cerebral cortex synaptosomal membranes and mitochondria. Figures 4(b) and 5(b) show quantitative data from 3 to 6 assays carried out with paired samples.

Levocabastine administration decreased the expression of tubuline (Fig. 4a) and β-actine (data not shown). Therefore, data for nNOS optical density of synaptosomal membrane fractions from control- and levocabastine-treated rats were expressed relativizing values to the control group (ratio: treated vs. control values). As shown in Fig 4(b), nNOS expression in synaptosomal membranes was decreased by 18 and 56% after 30 min and 18 h of levocabastine treatment, respectively.

An increasing trend in NOS expression was observed in purified mitochondria 30 min after levocabastine administration, whereas 86% enhancement in protein enzyme expression was observed 18 h after drug administration (Fig. 5).

Oxygen consumption in the crude mitochondrial fraction

Oxygen consumption in freshly prepared crude mitochondrial fractions was assayed in different conditions. Taking

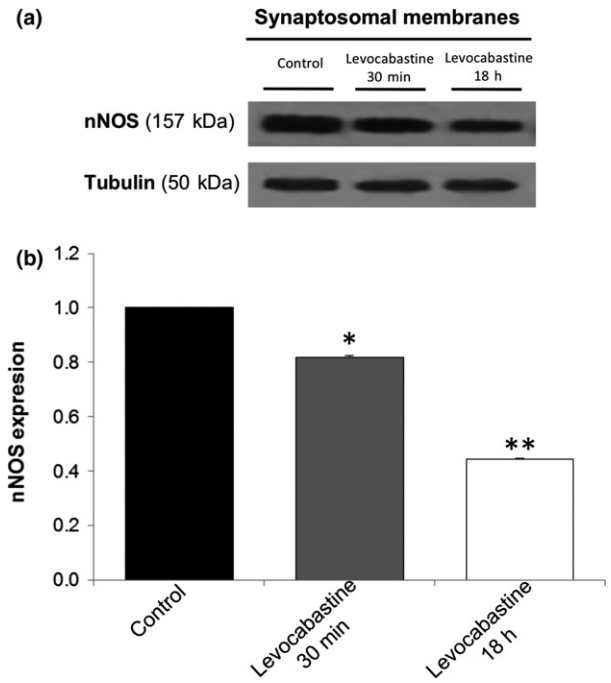

Fig. 4 Neuronal nitric oxide synthase (nNOS) protein expression in synaptosomal membranes after levocabastine administration. Western blots were carried out to analyze the expression of nNOS and tubuline in synaptosomal membranes isolated from rat cerebral cortex after i.p. administration of a single dose of levocabastine or vehicle. (a) Results shown are immunoblots from single representative experiments. (b) Results are expressed as the ratio between treated versus control optical density (mean ± SEM). For each experimental point, cerebral cortices from three rats were pooled. The whole procedure for rat treatment and subcellular fractionation was replicated three times and samples were processed for western blot assays. Each gel included 3–4 replicates per condition (n = 9–12, corresponds to the number of total data for each condition). Black bars, control values; dark gray bars, 30 min after levocabastine; light gray bars, 18 h after levocabastine. *$p < 0.05$, **$p < 0.01$ with respect to the control, by one-way ANOVA followed by Tukey multiple comparison test.

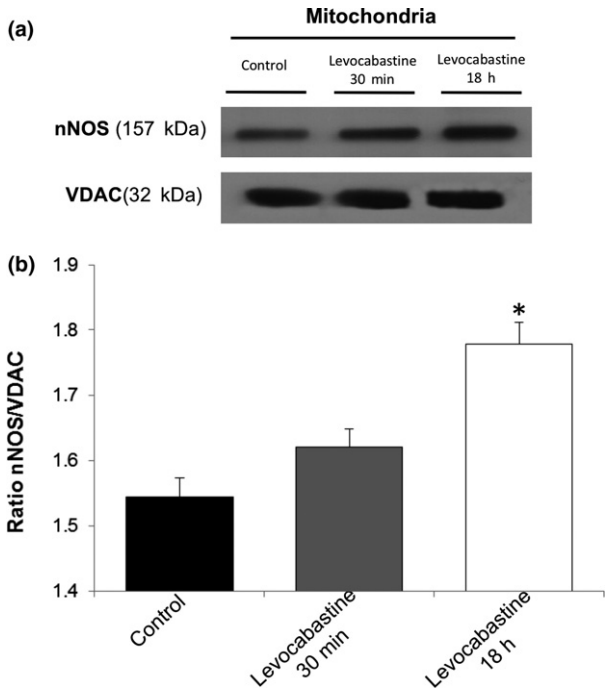

Fig. 5 Neuronal nitric oxide synthase (nNOS) protein expression in mitochondrial membranes after levocabastine administration. Western blots were carried out to analyze the expression of nNOS and voltage-dependent anion channel (VDAC) in mitochondrial fractions isolated from rat cerebral cortex after i.p. administration of a single dose of levocabastine or vehicle. (a) Results shown are immunoblots from single representative experiments. (b) Results are expressed as the ratio between nNOS and VDAC optical density (mean ± SEM). For each experimental point, cerebral cortices from three rats were pooled. The whole procedure for rat treatment and subcellular fractionation was replicated three times and samples were processed for western blot assays. Each gel included 3–4 replicates per condition (n = 9–12, corresponds to the number of total data for each condition). *$p < 0.05$, with respect to the control, by one-way ANOVA followed by Tukey multiple comparison test.

into account that this fraction contains mitochondria plus synaptosomes, appropriate media for each organelle were employed.

In a medium to evaluate synaptosomal oxygen consumption, basal respiration values from levocabastine-treated rats were similar to those obtained in control animals. The presence of 5 μM oligomycin decreased 50% basal respiration in control samples, whereas it produced no effect on levocabastine samples. As a result, respiration rate in the presence of oligomycin achieved a value 147% higher in levocabastine samples than that recorded in control samples. As compared with data in the presence of oligomycin, the further addition of the uncoupler FCCP to control samples induced a 104% increase in respiration, whereas it failed to modify this value in levocabastine samples (Table 1).

Spare respiratory capacity (%) from control rats was 116.1 ± 3.6 and was not modified by levocabastine treatment, being 109.0 ± 3.8 ($n = 4$).

Assay of oxygen consumption in a medium to evaluate mitochondrial respiration showed that respiratory controls with substrates malate-glutamate and succinate were 2.05 and 2.75 for control samples (vehicle-injected), but 1.40 and 1.80 for levocabastine samples, both, respectively (mean values from two experiments). These findings indicated that levocabastine administration induced decreases of 32 and 53% in respiratory controls when malate-glutamate and succinate were used as substrates, respectively. Values for the respiratory controls for vehicle-injected samples with 1 μM levocabastine added *in vitro* were 1.20 and 1.60, measured with substrate malate-glutamate and succinate, respectively, indicating roughly 55% decrease by the presence of the drug.

Table 1 Respiration rate parameters of brain cortex crude mitochondrial fractions from control and 30-min-treated levocabastine animals

Oxygen consumption (ng at O/min.mg protein)	Control	Levocabastine 30 min
Basal respiration	10.0 ± 1.5	12.3 ± 1.8
+ Oligomycin	5.7 ± 0.8*	14.1 ± 1.6***
+ FCCP	11.6 ± 1.6	13.4 ± 2.0

Basal respiration was registered in the presence of substrates 15 mM D-glucose plus 10 mM pyruvate. Respiration driving proton leak was measured in the presence of 5 μM oligomycin. Maximum respiration was achieved by the addition of 4 μM FCCP. For each experimental point, cerebral cortices from two rats were pooled. The whole procedure for rat treatment and subcellular fractionation was replicated three times and oxygen consumption assays were performed in triplicate (n = 9, corresponds to the number of total assays for each condition). Data are mean values ± SEM.

*p < 0.05, compared with basal respiration.

***p < 0.001, compared with the same condition from control animals.

Activity of respiratory complexes

Respiratory complex activities were measured in cerebral cortex mitochondria. Enzymatic activities of 47, 40, and 27 nmol per mg protein per min were recorded for NADH-cytochrome *c* reductase (Complex I–III), succinate-cytochrome *c* reductase (Complex II–III) and cytochrome oxidase (Complex IV), respectively. The activity of mitochondrial respiratory complexes 30 min after levocabastine administration was decreased by 60%, 63%, and 42% for Complex I–III, II–III, and IV, respectively. After 18 h of levocabastine treatment, decreases in the activity of respiratory complexes were 83%, 82%, and 74% for Complex I–III, Complex II–III, and Complex IV, respectively (Table 2).

In vitro addition of 1 μM levocabastine decreased roughly 75–90% the activity of all three mitochondrial enzymatic complexes (Table 2). Similarly, significant decreases were observed after *in vitro* incubation of control mitochondrial fractions with 1 μM loratadine (data not shown).

MAO activity

To estimate mitochondrial enrichment after isolation procedure, values recorded for monoaminoxidase (MAO) activity from control animals in brain cortex whole homogenates were compared with those obtained in control purified mitochondrial fractions. Specific MAO activity resulted to be roughly 3-fold higher in mitochondrial fractions *versus* whole homogenates (data not shown).

MAO activity in purified mitochondrial control samples was 12.5 ± 0.6 nmoles oxidized substrate per mg protein per minute (mean values ± SEM, n = 4). Enzyme activity was, respectively, 25% and 60% lower at 30 min and 18 h after levocabastine treatment. Enzyme activity at 36 h after levocabastine administration failed to differ *versus* control values (Fig. 6).

Table 2 Effect of levocabastine on mitochondrial enzymatic complex activity

	Enzymatic complex activity		
Condition	Complex I–III (nmol/min mg protein)	Complex II–III (nmol/min mg protein)	Complex IV (k'/mg protein)
Control	46.7 ± 4.3	39.2 ± 2.6	27.6 ± 5.2
Levocabastine i.p., 30 min	18.8 ± 1.5*	14.6 ± 0.9*	15.9 ± 1.7*
Levocabastine i.p., 18 h	7.9 ± 0.7*,***	6.9 ± 0.5*,***	7.2 ± 1.3*,***
Levocabastine *in vitro*	5.6 ± 0.4*	3.7 ± 0.6*	6.8 ± 0.8*

Purified mitochondrial fractions were obtained from rat cerebral cortex at 30 min and 18 h after i.p. administration of levocabastine or the vehicle. For each experimental point, cerebral cortices of three rats were processed and the activity of enzymatic complexes was assayed. The whole procedure for rat treatment and subcellular fractionation was replicated three times and activity of enzymatic complexes assays were performed in triplicate (n = 9, corresponds to the number of total assays for each condition). Data are mean values ± SEM.

In addition, samples from vehicle-injected animals were incubated with 1 μM levocabastine for *in vitro* assays.

*p < 0.001 versus Control.

***p < 0.001 versus Levocabastine i.p., 30 min.

Discussion

Herein NOS activity and expression were evaluated in rat cerebral cortex after the administration of levocabastine, an antagonist for neurotensinergic NTS2 receptor. Decreases in NOS activity were recorded in synaptosomal membranes, a result which was accompanied with a reduction in enzyme protein expression. Mitochondria functionality was likewise impaired by the treatment.

Flow cytometry methods have been extensively used to detect and measure intracellular NOS-induced NO production in cellular systems such as isolated cardiomyocytes (Strijdom *et al.* 2004). The DAF-2/DA system is employed for the detection of low levels of NO (2–5 nM) produced by the constitutive forms of NOS under basal conditions (López-Figueroa *et al.* 2000). This method was applied herein to detect NO generation in fresh crude cerebral cortex mitochondrial fractions, known to contain mitochondria and intact synaptosomes (Rodríguez de Lores Arnaiz and Pellegrino de Iraldi 1985). No differences between the fractions obtained 30 min after administration of levocabastine or vehicle were found in NO generation in any of the conditions tested. Fractions tested were functionally active because NO generation decreased in the presence of NOS inhibitor L-NNA and it enhanced with the NO donor GSNO and in a depolarizing condition (with KCl). NOS activation can be induced by the depolarizing effect of KCl after elevation of

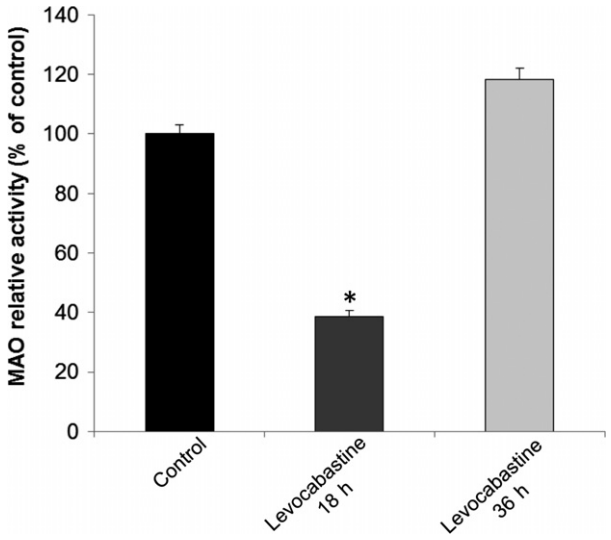

Fig. 6 Monoaminoxidase (MAO) activity in purified mitochondrial fractions after levocabastine administration. Enzyme activity was assayed in mitochondria isolated from rat cerebral cortex 18 and 36 h after i.p. administration of a single dose of levocabastine or vehicle. Results are expressed as enzyme activity taken control values as 100%. For each experimental point, cerebral cortices from three rats were pooled. The whole procedure for rat treatment and subcellular fractionation was replicated three times and MAO activity assays were performed in triplicate (n = 9, corresponds to the number of total assays for each condition). Data are mean values ± SEM. *$p < 0.0001$, significantly different from control.

intracellular Ca^{2+} concentration (Oka *et al.* 1999). NO generation after incubation with KCl was concentration-dependent in both control and levocabastine samples, an effect most likely attributable to the presence of intact synaptosomes in the fraction, because they behave as miniature cells (Rodríguez de Lores Arnaiz and De Robertis 1972).

Owing to the fact that NO levels were not modified by levocabastine treatment in these crude mitochondrial fractions; it was of interest to continue the study assaying NO production in purified synaptosomal membrane and mitochondrial fractions.

Absolute values for NOS activity in rat cerebral cortex subcellular fractions were in the range 0.5–1.1 nmol NO produced per mg protein per minute, in agreement with data already reported for mouse brain fractions (Czerniczyniec *et al.* 2006). Results presented here indicated that NO synthesis is greatly diminished by administration of NTS2 receptor antagonist levocabastine. NOS activity decreases in synaptosomal membrane and mitochondrial fractions were reversible in time.

Several variables influence the amount of NO production in cells, including the availability of substrates L-arginine, NADPH and tetrahydrobiopterin, and cofactors flavin adenine dinucleotide and flavin mononucleotide. NOS activity changes recorded are hardly attributable to differences in those substance concentrations because enzyme assays were carried out in optimal conditions for substrates and cofactors.

It is known that other variables may influence NO production. They include protein-protein interactions such as NOS dimerization, and the association with specific proteins, including PSD-95, caveolins, and calmodulin. Direct interactions of a variety of proteins bearing PDZ domains with the PDZ domain of nNOS have been shown to influence the subcellular distribution and/or activity of the enzyme in brain and muscle (Kone 2000; Fulton *et al.* 2001). The differences recorded after levocabastine treatment do not seem to be because of an alteration of organelle distribution during tissue subcellular fractionation procedure because the amount of protein recovered in each fraction failed to differ between control and levocabastine samples. However, changes in nNOS protein expression after administration of levocabastine here observed could be related to an effect on protein synthesis or degradation, or else owing to interaction with specific proteins. In fact, nNOS is recruited to membranes via protein–protein interactions, as it is the case with PSD-95, known to be targeted to the post-synaptic membrane (Imamura *et al.* 2002). As mentioned before, this interaction involves the unique N-terminal PDZ domain which is absent from the other NOS isoforms (Govers and Oess 2004). When the nerve endings are subjected to an osmotic shock, the post-synaptic membrane remained firmly attached to the pre-synaptic membrane (Rodríguez de Lores Arnaiz and Pellegrino de Iraldi 1985). To explore whether levocabastine treatment was able to modify PSD-95 likewise, western blot assays were carried out, to observe that this protein dramatically decreased by the treatment (data not shown). It is tempting to suggest that NOS decrease in synaptosomal membranes may involve the disassembling between NOS and PSD-95.

NOS activity was inhibited by *in vitro* addition of levocabastine or loratadine (an antagonist for histamine receptor) during the enzyme assay. As far as we know, the potential effect of levocabastine on histamine H_1 receptor has not been described. Ligand binding to H_1 receptor is linked to the activation of several intracellular messengers (Leurs *et al.* 2012), such as the stimulation of cGMP synthesis (Prell and Green 1986). NO can likewise activate soluble guanyl cyclase, enhancing the concentration of cGMP, which in turn may activate specific protein kinases (see Holz and Fisher 2012; Pittinger *et al.* 2012). Therefore, there might be a convergence of mechanisms for levocabastine and loratadine on decreasing NOS activity.

The subcellular distribution of the NOS isoenzymes is important because the intracellular location of NOS is critical for the coupling of extracellular signals to efficient NO production (Sessa *et al.* 1993; Sakoda *et al.* 1995). The subcellular localization of nNOS by electron microscopic

immunocytochemistry has been studied in the brain of salmon (Holmqvist and Ekström 1997), the superficial gray layer of the rat superior colliculus (Batista *et al.* 2001) and the retina of turtle (Cao and Eldred 2001). Taken jointly, results indicate that NOS enzyme is always attached to cellular structures such as the external membrane of mitochondria, endoplasmic reticulum, pre- and post-synaptic membranes, ribosomes, microtubules, vesicle membranes, the inner part of the external membrane and it is also diffusely distributed in the cytosol (Rodrigo *et al.* 1997). Accordingly, western blot assays allow disclosing a protein of 157 kDa able to react with anti-nNOS (amino terminal) antibody in mouse brain submitochondrial membranes, synaptosomes, and cytosolic fractions (Czerniczyniec *et al.* 2006). As expected, NOS activity was detected in all the fractions tested herein.

In synaptosomal membranes, a slight and a marked decrease in nNOS protein expression were observed at 30 min and 18 h after levocabastine administration, respectively. It is of interest to recall that changes in nNOS protein expression have been observed in brain subcellular fractions in different experimental models, including apoptosis (Bustamante *et al.* 2002) and aging (Bustamante *et al.* 2008; Lores-Arnaiz and Bustamante 2011). On the other hand, conditions which modify synaptic plasticity are likewise accompanied by modification in nNOS protein expression (Lores-Arnaiz *et al.* 2006, 2007, 2010). The expression of nNOS protein in subcellular fractions is altered after administration of several drugs. To illustrate, western blot assays carried out in different brain areas, including cerebral cortex, hippocampus and striatum, disclose pharmacological regulation of this protein expression by administration of antipsychotic drugs haloperidol (Lores-Arnaiz *et al.* 1999) and chloropromazine (Lores Arnaiz *et al.* 2004).

The observed decrease in nNOS protein expression in synaptosomal membranes may explain the decrement registered in NOS activity after levocabastine treatment. Meanwhile, in mitochondrial fractions, nNOS protein expression was increased after 18 h of levocabastine treatment. To interpret these findings, it is worthwhile to recall that NO and its derivative peroxynitrite inhibit mitochondrial respiration by distinct mechanisms (Brown 1999). Therefore, it may occur that when NO production is decreased, a compensatory mechanism might be triggered leading to increased nNOS protein expression in mitochondria.

According to the results presented herein, it may be hypothesized that NOS activity in mitochondrial fractions could be because of contamination with synaptosomal membranes. However, the changes recorded in mitochondria were notably different or even opposite to those recorded in synaptosomal membrane fractions, ruling out the possibility that present results are exclusively because of the contamination of mitochondria with synaptosomal membranes.

Results in synaptosomal membrane fractions may be associated with the ability of levocabastine to antagonize NTS2 receptor. At variance, findings in mitochondrial fractions can hardly be attributed to NTS2 blockade because this receptor has not been detected in mitochondria so far. Therefore, causal involvement of NTS2 blockade in mitochondrial findings is untenable and may be interpreted as concomitant effects.

The possibility of a direct effect of the drug on several parameters at CNS level can be sustained by the fact that after systemic administration, levocabastine goes through the blood–brain barrier (Tyler *et al.* 1998). In line with this concept, it may be recalled that the treatment employed increases the activity of synaptosomal membrane Na^+, K^+-ATPase (Álvarez Juliá *et al.* 2013) as well as [^{3}H]-ouabain binding to brain membranes (Gutnisky *et al.* 2016), suggesting that the drug reaches the brain after systemic administration.

The extent of NOS inhibition achieved in synaptosomal membranes by 1 μM levocabastine (67%) is similar to that observed *ex vivo* 18 h after administration of levocabastine (74%). Therefore, after systemic administration levocabastine may well reach brain tissue at a concentration similar to that achieved when the drug was added *in vitro* to assay the enzyme activity. Some of the results may then be attributed to direct effects of the drug, such as the decrease in NOS activity in synaptosomal membranes, as well as the inhibition of MAO and mitochondrial complex activities. As a matter of fact, both the administration of levocabastine and the inclusion of this drug in the assay medium produced dramatic decreases in mitochondrial complex activities.

However, the notion of a direct effect should hardly be applicable to other results, such as those recorded for NOS activity and expression in mitochondrial fractions. Although levocabastine administration decreased NOS activity, nNOS expression became enhanced. Therefore, the *in vitro* results may indicate a direct effect, whereas the *ex vivo* results on mitochondrial nNOS protein expression cannot be fully explained at present but might be attributed to a compensatory mechanism.

Decreases in the activities of NOS, MAO and mitochondrial complexes recorded in assays carried out *ex vivo* and *in vitro* might be attributed to an alteration in mitochondrial number/level by levocabastine treatment. However, this hypothesis seems untenable for two reasons: a) *in vitro* assays carried out by levocabastine addition to control samples led to similar results than those recorded *ex vivo*, and b) no differences in the protein content for mitochondrial fractions separated by gradient centrifugation 18 h after levocabastine administration *versus* control were recorded (10.6 ± 0.9 and 11.4 ± 1.1 mg protein per g fresh tissue, mean values ± SEM, $n = 3$).

Additional assays showed that levocabastine administration also decreased the expression of actin and tubuline

protein. Changes in cytoskeleton proteins expression after levocabastine treatment could be because of direct effects of neurotensin receptor blockade. Alterations in actin cytoskeleton by neurotensin have been reported in human U373 glioblastoma cells, thus suggesting a modulating effect of this neuropeptide on tumor astrocyte migration into the brain parenchyma (Servotte *et al.* 2006). Most interesting, dysregulation of synaptic actin has been proposed as a convergent mechanism of mental disorders (Yan *et al.* 2016).

Decreases in NO synthesis have been associated with alterations in mitochondrial respiratory chain activity. For instance, deprenil, an inhibitor of MAO B, decreases NOS activity in mouse brain subcellular fractions both *ex vivo* and *in vitro* and, at the same time, enhances mitochondrial function (Czerniczyniec *et al.* 2006, 2007). At variance**,** levocabastine, which also inhibited MAO activity, decreased likewise the activity of NOS and diminished mitochondrial functionality both *ex vivo* and *in vitro*. This was evidenced by both a decreased mitochondrial respiratory control and impaired synaptosomal oxygen uptake, 30 min after levocabastine administration, and also by the marked inhibition found in mitochondrial enzymatic complexes of the respiratory chain, observed 30 min and 18 h after levocabastine treatment.

Regarding synaptosomal oxygen uptake determinations, the response to oligomycin and FCCP additions differed significantly between levocabastine and control samples. While oligomycin decreased basal respiration in control samples, it did not modify synaptosomal respiration rates 30 min after levocabastine treatment. A low respiratory rate in the presence of oligomycin indicates that the intrasynaptosomal mitochondria maintain a sufficiently high proton motive force to restrict electron transport. The higher respiratory rate in the presence of oligomycin observed after levocabastine treatment, as compared with control samples, suggests altered proton leakiness *in vivo* (Brand and Nicholls 2011).

The ability of neuronal mitochondria to respond to an increased cellular ATP demand with enhanced respiration is known as spare respiratory capacity (Nicholls 2008). Spare respiratory capacity has been proposed to be a primary factor involved in the survival of neurons under certain conditions such as the rotenone model of partial complex I deficiency (Yadava and Nicholls 2007) or after astrocytes treatment with exogenous glutamate (Yan *et al.* 2017). In our study, spare respiratory capacity was unaffected by levocabastine administration. Being spare respiratory capacity a critical factor in maintaining a reserve of ATP generating capacity (Choi *et al.* 2009), it seems that the ability of the synaptosome to adjust oxygen consumption in response to intracellular ATP demands was preserved after levocabastine treatment, even though proton leakiness seemed to be altered. Regarding to this point, it may be mentioned that during aging, spare respiratory capacity remains unaffected, whereas some parameters of mitochondrial function are altered (Lores-Arnaiz *et al.* 2016).

When mitochondrial respiratory rates were measured in the presence of the corresponding mitochondrial substrates malate-glutamate or succinate plus ADP, mitochondrial respiratory controls were markedly decreased after 30 min levocabastine treatment, thus showing severe mitochondrial dysfunction. The effect on mitochondrial respiratory rates was also observed after *in vitro* incubation of mitochondrial samples with levocabastine thus suggesting a direct effect of the drug on mitochondrial respiratory function.

As mentioned above, causal involvement of NTS2 blockade in levocabastine effects after its administration may explain the results obtained in synaptosomal membrane fractions but not those findings related to mitochondrial function. In addition, it may be concluded that levocabastine effects on NO metabolism and on mitochondrial respiratory chain, may only be interpreted as parallel actions rather than interrelated phenomena.

Regarding MAO activity in purified mitochondrial fractions after levocabastine administration decreases were recorded at 30 min and 18 h. This effect seemed to be reversible in time because enzyme values failed to differ from those in control samples at 36 h after drug administration.

In summary, a new central action induced by levocabastine administration was described herein. Results indicated that this NTS2 antagonist markedly modified NOS activity and expression at CNS synapsis, suggesting an interrelationship between the activities of neurotensinergic and nitrergic systems. Concomitantly, levocabastine both *ex vivo* and *in vitro* was able to modify several respiratory function parameters, including synaptosomal and mitochondrial oxygen uptake as well as mitochondrial respiratory complexes activity. The maintenance of normal spare respiratory capacity might contribute to providing an adequate neuronal bioenergetic state, necessary for the recovery in time for NOS and MAO activities after the treatment. Further studies will help to elucidate the possible mechanisms involved in the alterations of NO metabolism and mitochondrial function after NTS2 receptor blockade.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

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