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LMN diet could benefit the cognitive reserve enhancing catecholaminergic and cholinergic neurotransmission, hence reducing the Alzheimer's Disease risk.

Catecholaminergic and cholinergic systems of mouse brain are modulated by LMN diet, rich in theobromine, polyphenols and polyunsaturated fatty acids.

Laura Fernández-Fernández[1#], Gerard Esteban[1#], Mercedes Giralt[2], Tony Valente[1,3], Irene Bolea[1], Montse Solé[1], Ping Sun[1], Susana Benítez[1], José Ramón Morelló[4], Jordi Reguant[4], Bartolomé Ramírez[4], Juan Hidalgo[2], Mercedes Unzeta[1]

[1]*Institute of Neurosciences, and Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain.*
[2]*Institute of Neurosciences, and Department of Cellular Biology, Physiology and Immunology, Faculty of Biosciences, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain.*
[3]*Biomedical Research Institute of Barcelona (IIBB), Spanish National Research Council (CSIC), Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Department of Brain Ischemia and Neurodegeneration, Barcelona, Spain*
[4]*La Morella Nuts SA, Reus, Tarragona, Spain.*

[#]Both authors contributed equally.

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*Corresponding author: Institute of Neurosciences and Departament de Bioquímica i Biologia Molecular; Edifici M, Campus UAB, Bellaterra (Barcelona), 08193. Telephone: +34 93 581 1624 Fax: +34 93 581 3861. E-mail: Mercedes.unzeta@uab.es

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Abstract

The possible modulatory effect of the functional LMN diet, rich in theobromine, polyphenols and polyunsaturated fatty acids, on the catecholaminergic and cholinergic neurotransmission, affected in cognition decline during aging has been studied. 129S1/SvlmJ mice were fed for 10, 20, 30 and 40 days with either LMN or control diets. The enzymes involved in catecholaminergic and cholinergic metabolism were determined by both immunohistological and Western Blot analysis. Noradrenalin, dopamine and other metabolites, were quantified by HPLC analysis. Theobromine, present in cocoa, the main LMN diet component, was analysed in parallel using SH-SY5Y and PC12 cell lines. An enhanced modulatory effect on both cholinergic and catecholaminergic transmissions was observed after 20 days fed mice. Similar effect was observed with Theobromine, besides its antioxidant capacity inducing SOD-1 and GPx expression. The enhancing effect of the LMN diet and Theobromine on the levels of acetylcholine-related enzymes, dopamine and specially noradrenalin confirms the beneficial role of this diet on the "cognitive reserve" and hence a possible reducing effect on cognitive decline underlying aging and Alzheimer´s disease.

Introduction

Aging is a highly complex process that can affect multiple organs and induce changes that will disturb the correct functioning of the organism over time, leading to different pathologies [1]. In this concern, neurological disorders such as Alzheimer´s disease (AD), are deeply related to the aging process [2,3] and research in this area would become promising regarding the implications for both public health and social policies.

AD is a progressive neurodegenerative disorder characterized by memory loss and cognitive impairment. AD is a multifactorial disease in which oxidative stress, mitochondrial dysfunction, inflammation, metal dyshomeostasis, accumulation of miss-folded proteins, deficit of cholinergic transmission or apoptosis, among others, play an important role [4,5].

AD histological hallmarks such as the deposition of β-amyloid protein or the neurofibrillary tangles formation have been widely reported [6,7]. However, the correlation between the biological measurement of the pathology markers that are shown in brain at middle age and the real loss of cognitive function that appears at advanced life stages has not yet been found. This gap between symptoms and pathology has been explained by the "cognitive reserve" theory, in which a set of variables such as education, training, intelligence or mental stimulation allow the brain to adapt or to mask the pathology, maintaining the cognition in spite of the neuronal loss. It has been hypothesised that these key elements of "cognitive reserve" normalize the otherwise declining noradrenergic system during aging, and therefore the optimization of noradrenergic activity may reduce the risk of AD [8].

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The complexity of AD is corroborated by the fact that currently no drug is able to prevent this neurodegenerative process. To date, only five drugs have been approved by the Food and Drug Administration to enhance cholinergic transmission related to cognitive deficits associated to AD [9], leaving a vast majority of other potential AD targets nearly unaffected by current therapies.

Diet has been extensively reported to play an important role in cognition [10] and at present, there is mounting evidence that certain components of diet intake, especially those exhibiting antioxidant properties, could be beneficial for preventing and delaying neurodegenerative disorders such as AD [11,12]. Fish, vitamins and methionine-rich proteins have been identified to confer protection against AD [13].

In this regard, polyphenolic compounds present in dry fruits, nuts and almonds, wine, tea, berry fruits, cocoa and fish oils, have shown anti-aging and neuroprotective effects [14]. All of them are potent antioxidant agents that could be useful as a nutritional approach against the oxidative stress and inflammation associated to AD [15]. Therefore, diet supplementation may offer an alternative or supplementary therapy to the use of acetylcholinesterase inhibitors.

It has been previously reported that LMN cream intake, **[Patent ref WO2007063158 A2]**, based on cocoa, hazelnuts, polyphenols, vegetable oils rich in polyunsaturated fatty acids and flours rich in soluble fiber, is able to reduce the cardiovascular risk factors that are underlying AD [16]. Moreover, the LMN diet has been described as an inductor of neurogenesis in the adult mouse brain by promoting the proliferation and differentiation of neuronal cells in both the olfactory bulb and the hippocampus, being the latest one of the most highly affected brain regions in AD [17].

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Besides, the LMN diet is able to decrease the behavioural deterioration caused by aging in both wild type and in Tg2576 mice and to diminish the Aβ plaque formation. LMN also reduces the Aβ (1-40 and 1-42) plasma levels in adult mice [18]. Taken all these results into account, the increasing importance of polyphenols as human dietary supplements playing a potential role in ameliorating the cognitive impairment during aging and neurological disorders is corroborated.

The objective of the present work was to evaluate the possible modulatory effect of the LMN diet on both cholinergic and catecholaminergic systems in 129S1/SvlmJ mice fed for 10, 20, 30 and 40 days by immunodetection and high-performance liquid chromatography (HPLC) approaches.

Results

AChE and ChAT immunohistochemical analysis in striatum of LMN fed mice.

The effect of the LMN diet on AChE and ChAT as the main enzymes involved in Acetylcholine (ACh) metabolism has been studied since ACh is a depleted neurotransmitter in AD. Figure 1 shows a representative immunohistochemistry images for AChE and ChAT in basal ganglia (striatum) of 4-month old 129S1/SvImJ mice fed with the LMN diet for 10, 20, 30 and 40 days. The LMN diet induced a decrease of the AChE levels immunostaining in striatum at early times, which was significantly different at 20 days feeding. However, AChE levels were reestablished at longer times. In contrast, ChAT levels showed an opposite pattern, being increased at 20 days and returning to normal values afterwards.

Immunohistochemical quantification of TH expression in basal ganglia of LMN fed mice.

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The effect of the diet on the TH expression, as a key enzyme in the synthetic pathways of catecholamines DA and NA was analysed. TH expression was determined in the catecholaminergic circuits of both striatum and substantia nigra as shown in figure 2. The corresponding quantification of the immunofluorescence signal showed a significant increase of TH expression in the catecholaminergic fibers of the striatum at 20 and 30 days feeding. In substantia nigra, a progressive increase of the TH expression was observed in the neurons and fibers of catecholaminergic circuits. It was significant at 30 and 40 days feeding. The different period of time observed on the expression of TH in striatum and substantia nigra could be explained by the fact that the different components of the LMN diet are probably not processed by the gastro-intestinal track with the same efficacy and furthermore, they can cross the blood-brain barrier with different accessibility and hence to get the selected cerebral locations at different rate [19].

LMN cream modulates COMT expression in hippocampus

The expression of Catechol-O-Methyl transferase (COMT) was analysed in the hippocampus of 129S1/Svlmj mice fed with the LMN diet for 10, 20, 30 and 40 days. This brain region is involved in memory and learning processes and it is severely affected in AD. As shown in figure 3, the highest COMT expression was observed at 20 days while progressively decreased at longer times.

HPLC analysis of the catecholaminergic neurotransmitters DA and NA in striatum.

The levels of DA, NA, their metabolites DOPAC and HVA, and 5HIAA were determined by HPLC in basal nuclei of 4-month old mice fed with the LMN diet for 10,

20, 30 or 40 days. Figure 4 shows an increase of the levels of DA and NA increased at 20 days feeding. In case of their metabolites DOPAC and HVA, the same trend was observed. However, no effect on the levels of the serotonin metabolite 5-HIAA was detected.

Theobromine, the main component of in LMN cream shows similar antioxidant effects on SH-SY5Y cells.

The antioxidant effect of theobromine (TBr), present in cocoa, the main component of LMN cream, was also analysed and compared to that of LMN cream. Figure 5a shows a similar protective effect of both LMN cream and TBr on SH-SY5Y cells lesioned with 150μM H_2O_2 for 24h. Protection was observed in all doses being the highest effect detected with 10μg/ml LMN cream. An increase on the protein levels of the antioxidant enzymes Superoxide Dismutase-1 (SOD-1) (figure 5b) and Glutathione Peroxidase (GPx) (figure 5c) was observed after TBr and LMN cream treatment without H_2O_2 addition.

In order to corroborate this antioxidant effect, Nrf2 activation, the transcription factor that activates the gene expression of these antioxidant enzymes, was determined after LMN or TBr treatments without the addition of H_2O_2. Figure 5d shows representative images for Nrf2 immunofluorescence analysis in LMN or TBr-treated SH-SY5Y cells for 24h. These results clearly show a translocation of Nrf2 to the nucleus as a response to LMN and TBr treatments.

Theobromine as modulator of catecholaminergic metabolism in undifferentiated PC12 cells.

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Undifferentiated PC12 cells, derived from rat pheochromocytoma adrenal medulla, were treated with TBr (1-100μM) for 24h and catecholamines levels were subsequently quantified by HPLC-ED analysis. Broadly, levels of NA, L-DOPA, DA and DOPAC increased after treatments with all concentrations of TBr used (Table 1). However, no changes were observed in 3-MT or HVA levels.

Significant increase in the levels of NA (60.6%), DOPAC (65.3%) and DA (78.5%) were only detected at 10μM TBr versus non-treated cells. These results are in agreement with those previously determined in mice fed with LMN for 20 days.

Discussion

The main component of the LMN cream is cocoa that contains a large amount of polyphenols, natural substances that are present in plants, coloured fruits and vegetables as well as in olive oil, tea and red wine. This wide family of natural products contains flavonoids, the largest group of polyphenols including subclasses of flavones, isoflavones, flavonols, or flavans, among others. Because of their antioxidant properties, some of them are able to promote physiological benefits especially regarding the cognitive function and memory impairment [20]. The most abundant polyphenol present in green tea, the EGCG, also present in the LMN cream, has been reported to possess beneficial effects on cancer and cardiovascular function, with anti-inflammatory and antioxidant properties [21]. Furthermore, the importance of long-chain polyunsaturated fatty acids in neural development and neurodegeneration has been widely reported [22], such as omega-3, present in dry fruits like hazelnuts, one of the LMN cream components.

According to the cholinergic hypothesis of AD, this neurological disorder is characterized by a loss of cholinergic neurons present in nucleus basalis, responsible for learning and memory functions.

Moreover, the existing gap between symptoms and pathology in AD has been explained by the concept of "cognitive reserve" in which both cognition and memory are maintained despite the progress of the pathology. Cognitive reserve has been hypothesized to help people to avoid greater brain pathology and it has been considered a preventive factor for dementia. Compensatory adjustments, such as an enhancement on the noradrenergic transmission, have been proposed to be involved [8]. Regarding nutritional aspects, some authors report that mediterranian diet does not support a beneficial effect on cognitive function, irrespective of educational level, which is the strongest indicator of cognitive reserve [23]. These results are in controversy with other studies concluding that the mediterranian diet is associated with lower risk of dementia [24]. In this line, other authors have provided support for the hypothesis that cognitive reserve moderates the relationship between brain structure and cognition at middle age well before the onset of dementia [25].

Neurotransmitters NA, DA and ACh are considered neuromodulators released by neurons, whose cell bodies are found in specific nuclei in the brainstem. The function of many brain regions can be influenced through their wide spread projections. Dopaminergic neurons innervate the striatum, pre-frontal cortex and hippocampus. Noradarenalin has multiple effects on cellular excitability, intracellular cascades or synaptic plasticity of its target neurons. It is also able to enhance/block excitatory responses to glutamate depending on its concentration. The effect of NA on synaptic plasticity in the hippocampus promotes long-term potentiation (LTP) in both dentate

gyrus through the action on β-receptors and in prefrontal cortex improving working memory in primates [26].

In this concern, the search of some natural products able to modulate cholinergic and noradrenergic systems could help delaying the loss of cognition. In this work, adult male 129S1/SvImJ mice were selected as a mid-age model in which a strengthening of this neurotransmission could have beneficial effects during aging.

Basal nuclei, containing striatum, were selected as a rich area in both cholinergic as well as catecholaminergic terminals. The expression of both ChAT and AChE, the two main enzymes involved in the ACh metabolism, was modulated after LMN diet intake. The increase of ChAT expression indicates a stimulation of ACh synthesis, a neurotransmitter that is diminished in AD. Furthermore, the expression of AChE, the enzyme responsible for ACh degradation at the synaptic cleft, was significantly decreased after LMN feeding. Taken together, these results strongly suggest that the LMN diet shows a beneficial modulatory effect on the stimulation of the cholinergic transmission.

The previous results were correlated with the increasing TH expression in the dopaminergic fibers of both striatum and substantia nigra. This is the key enzyme responsible for the regulation of the catecholamine synthesis pathway, which controls reward-induced neurotransmitter change in cognitive brain regions and learning processes [27]. Therefore, the LMN diet enhances dopaminergic system and consequently, catecholamines production. COMT is the enzyme responsible for dopamine metabolism rendering HVA after its catalytic action on DOPAC, a metabolite of DA generated by both aldehyde dehydrogenase and monoamine oxidases activities. The increased expression of COMT in hippocampus, the specific area related with memory and

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cognition, strongly confirms the stimulatory effect of the LMN diet on catecholamine metabolism. All these data obtained by immunohistological assays were corroborated by the metabolites quantification by HPLC analysis. Levels of NA, DA and their metabolites were also found increased in PC12 cells treated with TBr.

The antioxidant properties of the LMN cream previously reported [17,28] were also observed with TBr. This main component of cocoa is able to stimulate the Nrf2 activation, a transcription factor responsible for the expression of both SOD-1 and GPx by themself, in absence of any oxidant contaminant.

All these results suggest that TBr may be one of the main responsible for the modulatory effects on cathecolaminergic and cholinergic vias observed with LMN cream. The effects observed only in a short-time window could be explained by the activation of compensatory mechanisms that need to be further elucidated.

Only male mice were used in this study due to their homogeneity. However, since females also experience aging and show higher AD prevalence, further studies carried out in females would also be interesting in order to confirm these results.

Experimental

Animals and diets

Mice were bred under controlled temperature and light conditions and were fed with Control diet prior to the experiments. Animal care and experimental procedures were performed in accordance with European Community Council Directive 86/609/ECC. Four-month 129S1/SvImJ adult male mice were fed either with Control diet (Harlan global diet 2014, Mucedola SRL, Milano, Italy) or Control diet supplemented with the LMN cream (LMN diet, Harlan Global Diet 2014 containing 9.27% LMN cream,

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Mucedola SRL, Milano Italy) for 10, 20, 30 or 40 days. The LMN cream consists of a mix containing at least one ingredient from each of the following four groups: dry fruits, cocoa, vegetable oils rich in unsaponifiable lipids and flours rich in soluble fibers **[Patent ref WO2007063158 A2]**. After feeding periods, all mice were killed by decapitation and brains immediately removed. Right hemispheres were dissected into regions and frozen in liquid nitrogen at -80ºC for subsequent western blot and HPLC analyses. Left hemispheres were fixed in 4% paraformaldehyde (PFA) for 24 hours. Then, tissue was cryoprotected in 30% sucrose/PBS solution during 48 hours at 4ºC, frozen in dry ice and cut in sagittal sequential 30μm sections in a Leica cryostat. These sagittal slices were used for immunohistochemistry and histological techniques.

Cell lines and treatments

Human neuroblastoma SH-SY5Y cells were purchased and cultured as previously described [18]. PC12 cell line was purchased from the American Type Culture Collection (ATCC) and grown in DMEM media containing 7% FBS, 7% foetal horse serum (FHS), 1.14mM HEPES pH 6.8, 60U/ml penicillin and 60μg/ml streptomycin. Both cell lines were maintained at 37ºC in a saturating humidity atmosphere containing 5% CO_2.

For treatments, both SH-SY5Y and PC12 cells were seeded at a density of $2.5 \cdot 10^4$ cells/mL in full media onto collagen type I (BD Biosciences)-coated plates until 70-80% confluence was reached. Before treatments, full medium was replaced by 1% FBS-medium overnight except for HPLC analysis. Both theobromine (TBr) and LMN cream were dissolved in PBS at 37ºC and added to the media for desired times. Non-toxic TBr concentrations (1, 10 or 100μM) were used for 24h. At the end of each treatment, cells were collected in PBS, centrifuged at 3,000 x *g* for 5 minutes and the pellets resuspended in 100μL of homogenization solution (0.25M $HClO_4$, 100μM

sodium bisulfate and 250 µM EDTA). Samples were sonicated for 10 seconds on ice and lysates kept at -80ºC for at least 24h prior to the HPLC analysis.

Histochemistry and Inmunohistochemistry

The brain sections were permeabilized in PBS-0.3% Triton X-100 for 30 min and blocked in PBS-Triton X-100 containing 10% FBS, 0.2M glycine and 1% BSA for 10 minutes. Afterwards, slides were incubated with goat anti-Choline Acetyltransferase (ChAT, 1:200, Millipore AB144P) overnight at 4 ºC, washing with PBS-Triton X-100; and incubated with donkey anti-goat HRP (1:200; Thermo PA 1-28664) for 1 or 2 hours at room temperature. After washing, sections were incubated with the Streptavidin-HRP (1:1,250; Sigma, S5512) for one hour and developed with DAB/H_2O_2 (0.5mg/0.22µl/ml Sigma). Acetylcholinesterase (AChE) histochemistry was performed according to Geneser-Jensen [29].

Quantification of ChAT and AChE was carried out in striatum. Brain sections from 4 animals per group were used and pictures were taken at 2X and 10X magnifications using the software ACT-1 version 2.70 (Nikon corporation). They were examined with a Nikon Eclipse 90i microscope interfaced to a DXM 1200F camera. Three to five pictures per section were evaluated and quantified in 10X images using image analysis software ImageJ.

Immunofluorescence

For tyrosine hydroxylase (TH) immunohistofluorescence, the brain sections were rinsed in PBS, treated with 2% H_2O_2 in methanol for 15 minutes, rinsed in PBS-0.5% Triton X-100, blocked with 10% of normal goat serum in PBS-0.5% Triton X-100 and incubated overnight at 4°C with polyclonal rabbit anti-TH (1:1,000; Millipore). After

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rinsing in PBS-0.5% Triton X-100, sections were incubated for 2h at room temperature with goat anti-rabbit ALEXA 546 (1:500; Molecular Probes). Finally, the sections were counterstained with DAPI and after being washed in PBS, were mounted in Mowiol medium (Sigma-Aldrich). For quantification of TH immunofluorescence, photographs of four sequential histological sections per animal were acquired with a Zeiss Axio observer Z1 microscope (Carl Zeiss) and a digital camera (QImaging Retiga Exi, QImaging). Using ImageJ sotfware and Paxinos brain mouse atlas, the striatum and substantia nigra areas were delimited (striatum: lateral 1.32 to 2.04 mm; substantia nigra: lateral 0.84 to 1.68 mm) and relative TH immunofluorescence densitometry was obtained for each individual section, as well as for relative Alexa 546 immunofluorescence. The quantification of TH immunofluorescence was obtained by its normalization with Alexa 546 tissue background.

SH-SY5Y cells fixed in 4% PFA were incubated with the primary antibody against Nrf2 (1:100; Santa Cruz) diluted in PBS containing 0.2% gelatin, 0.1% Triton X-100, 20 mM glycine and 5% FBS overnight at 4ºC. Secondary anti-rabbit conjugated to Alexa Fluor® 594 (1:1,000; Invitrogen) and 0.5mg/ml Hoechst were then incubated for 1h at room temperature and cells were mounted in Fluorescent Mounting Medium (Dako). Preparations were observed under a Nikon Eclipse TE 2000-E inverted fluorescence microscope with a Hamamatsu C-4742-80-12AG camera and Metamorph® Imaging System software.

Western blot analysis

SH-SY5Y cells were lysed in RIPA buffer, centrifuged at 3,000 x g for 10 min at 4ºC and the supernatants kept at -80ºC until their use. Protein concentration was determined using Bradford reagent (Bio-Rad). Twenty-five µg of total protein extract were resolved

by SDS-PAGE according to standard protocols. Primary antibodies used were: Catechol-O-Methyl Transferase (COMT) (1:1,000; Santa Cruz), Superoxide Dismutase-1 (SOD-1) (1:1,000; Santa Cruz), Glutathione Peroxidase (GPx) (1:1,000, Abcam), β-Tubulin (1:50,000; Sigma) and β-actin (1:40,000; Sigma). Secondary antibodies used were horseradish peroxidase-conjugated goat anti-mouse IgG (1:2,000; Dako) or goat anti-rabbit IgG (1:1,000; BD Biosciences). Densitometry analyses were performed using Quantity One® software (Bio-Rad) following manufacturer's instructions.

Cell viability analysis

Cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay. MTT solution was added at a final concentration of 0.5 mg/ml for 45 min. DMSO was used to dissolve the formazan blue precipitate formed, which was quantified at 560-620nm in a microplate reader (Labsystems multiscan RC).

Determination of catecholamines levels by HPLC

Catecholamines levels were determined by HPLC coupled to an electrochemical detector. The mobile phase consisted of 0.1 M citric acid, 0.05 mM EDTA and 1.2 mM sodium octylsulphate (SOS) was adjusted at pH 2.75 with triethylamine (TEA). Acetonitrile was added to reach 10% (v/v). Elution was performed at a flow rate of 1 mL/minute. A Coulochem 5100A electrochemical detector (ESA) with a Model 5011 dual-electrode analytical cell with porous graphite electrodes was used. The potential of the electrodes 1 and 2 was set at 70.05V and + 0.4 V, respectively.

PC12 cell lysates were thawed and centrifuged at 12,000 x g for 10min at 4ºC. Next, 20μL of each sample supernatants were injected into the HPLC system for analysis.

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Catecholamines analysis was performed at room temperature (20 – 25ºC). The level of metabolites was expressed in pg of metabolite/mg of protein.

Frozen samples from basal nuclei were homogenized in RIPA buffer. After that, samples were split into two parts and diluted (1:1) in homogenization solution containing 2,000 pg/ml of 3,4-dihydroxybenzylamine (DHBA), as internal standard for catecholamine determination and 4,000 pg/ml of NW-5-met-5HT as internal standard for serotonin determination. After homogenization, samples were sonicated and centrifuged (15,000 x g, 10 min, 4ºC) and supernatants were injected into the HPLC system for analysis of 3,4-dihydroxyphenylacetic acid (DOPAC), noradrenalin (NA), homovanillic acid (HVA), dopamine (DA), serotonin (5-HT) and serotonin metabolite 5-hydroxyindoleacetic acid (5HIAA). DHBA was used as internal standard.

Statistics

Graphs were preformed and data were analysed using the GraphPad Prism 4.02 software. Values of $p<0.05$ were considered to be statistically significant.

Conclusions

At present, there are few reports on the beneficial effect of nutrients on the stimulation of cholinergic and catecholaminergic transmission [30]. In this work, we report by first time the enhancement of cholinergic and cathecolaminergic transmissions, both highly impaired in neurodegenerative disorders such as AD, by some natural nutrients present in the LMN cream. These results will give an insight into the possible contribution of the LMN cream in the "cognitive reserve" [8] and its beneficial effect on the cognitive decline related to aging and neurological disorders.

Acknowledgements

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Figure Legends

Fig. 1 Immunohistochemical analysis of AChE and ChAT in striatum of 129S1/SvlmJ mice fed with LMN diet. a Representative images for acetylcholinesterase (AChE, 2X) and **b** for choline acetyltransferase (ChAT, 10X) immunohistochemistry in basal nuclei of 4-month old 129S1/SvImJ mice fed with the Control or the LMN diet for 10 (n=4), 20 (n=4), 30 (n=4) or 40 (n=4) days. Graphs at the bottom show the IHC quantifications carried out in 10X images. Data are related to each representative control. *$p < 0.05$ vs its own control (C), by Student t-test; [#]$p < 0.05$, [##]$p < 0.01$ by one-way ANOVA and the addition of Newman Keuls multiple comparison test.

Fig. 2 Quantification of TH expression in nucleus basalis of LMN fed mice. Representative images for tyrosine hydroxylase (TH) immunofluorescence in striatum and substantia nigra of 4-month old 129S1/SvlmJ mice fed with the LMN diet for 10 (n=4), 20 (n=4), 30 (n=4) or 40 (n=4) days. Graphs show the quantification for each brain area. *$p < 0.05$, ***$p < 0.001$ vs Control diet fed animals; [#]$p < 0.05$, [##]$p < 0.01$, [###]$p < 0.001$ vs LMN 20D in striatum or vs LMN 30D in substantia nigra, by one-way ANOVA and the addition of Tukey's multiple comparison test.

Fig. 3 Quantification of COMT in hippocampus from LMN fed mice. Representative western blots for cathecol-o-methyltransferase (COMT) in hippocampi from 4-month old mice fed with the Control or the LMN diet for 10 (n=3), 20 (n=4), 30 (n=4) or 40 (n=4) days. Graph shows the data corresponding to the western blot densitometric analysis. *$p < 0.05$ vs its respective control by Student t-test. [#]$p < 0.05$ by one-way ANOVA and the addition of Newman Keuls multiple comparison test.

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Fig. 4 HPLC analysis of catecholaminergic neurotransmitters: DA and NA in striatum. Quantification of noradrenaline (NA), dopamine (DA), the metabolites DOPAC and HVA and 5-HIAA in basal nuclei of 4-month old mice fed with the LMN diet for 10 (n=3), 20 (n=4), 30 (n=4) or 40 (n=4) days. Data are related to each respective control. [#]$p<0.05$ by one-way ANOVA and the addition of Bonferroni's multiple comparison test.

Fig. 5 Antioxidant effects of the LMN cream and its main component, theobromine, in SH-SY5Y cells. a SH-SY5Y cells were pretreated for 24h with the LMN cream or with theobromine (TBr) and then lesioned with 150μM hydrogen peroxide (H_2O_2) for 1h. Cell viability was determined by the MTT method. Data are the mean ± S.E.M. of three independent experiments. *$p<0.05$ vs H_2O_2 by one-way ANOVA and the addition of Newman-Keuls multiple comparison test. **b,c** Effect of the LMN diet (0.1μg/ml) or TBr (0.001-1μM) on the expression of Superoxide Dismutase-1 (SOD-1, **b**) or Glutathione Peroxidase (GPx, **c**) in 72h-treated SH-SY5Y. Tubulin (Tub) or β-actin (Act) were used as loading controls. Graphs represent the western blot quantification of three independent experiments. ***$p<0.001$ by one-way ANOVA and the addition of Tukey's multiple comparison test (TBr in SOD-1 analysis) or by Student *t*-test (LMN treatment in GPx analysis). **d** Representative images for Nrf2 immunofluorescence analysis in LMN or TBr-treated SH-SY5Y cells for 24h. Red staining represents Nrf2 whereas nuclei are stained in blue (DAPI).

Table 1 Modulation of the levels of catecholaminergic neurotransmitters in PC12 cell lysates treated with theobromine. PC12 cells were treated for 24h with theobromine (TBr) (1-100μM). The levels of the catecholamines were quantified by

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HPLC analysis. Values are expressed as the mean of the percentage increase from at least four independent experiments; *$p<0.05$ vs NT.

a   AChE

b   ChAT

FIGURE 1

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TH

	Striatum	Substantia Nigra
CONTROL		
LMN 10D		
LMN 20D		
LMN 30D		
LMN 40D		

STRIATUM

SUBSTANTIA NIGRA

FIGURE 2

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FIGURE 3

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FIGURE 4

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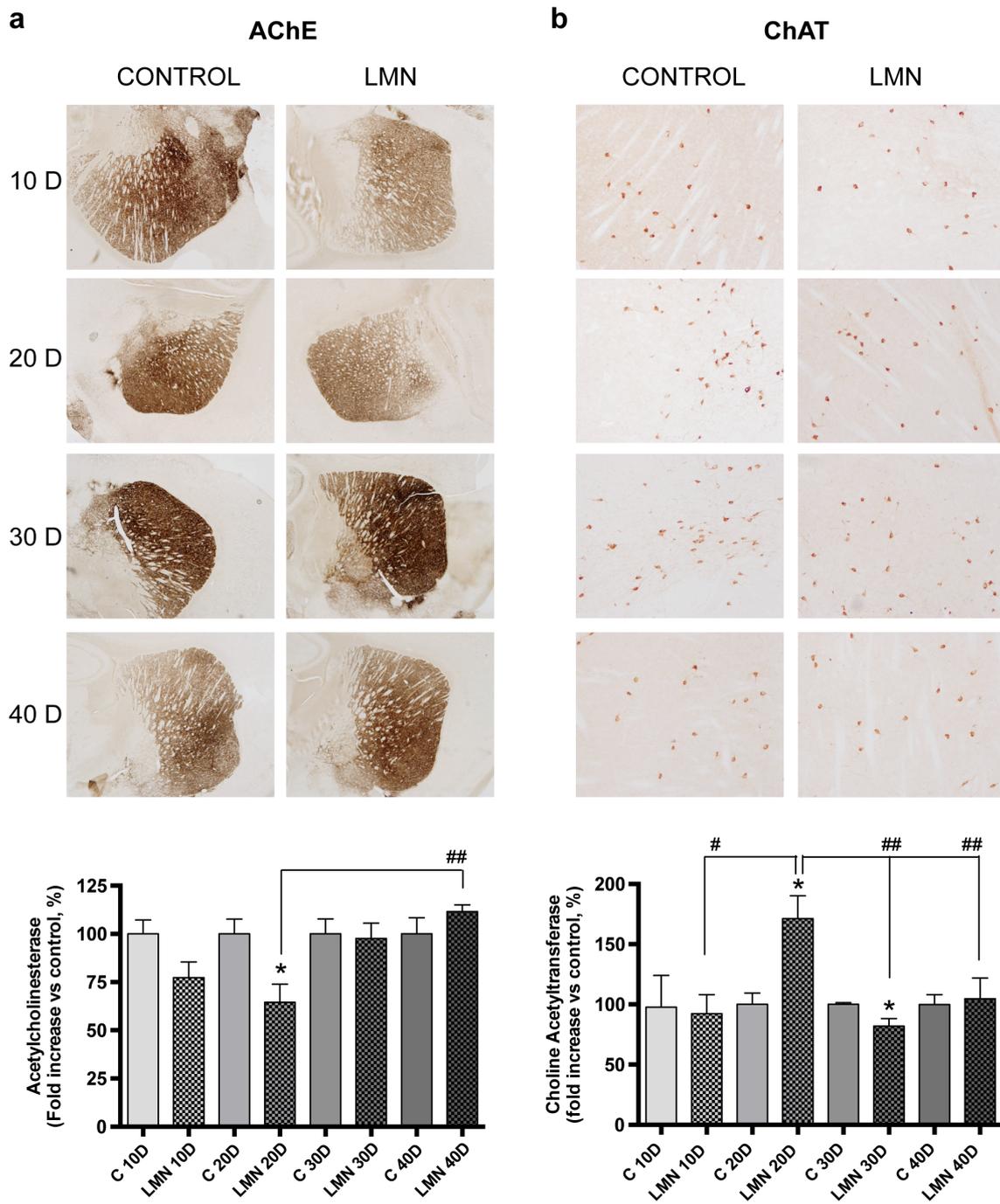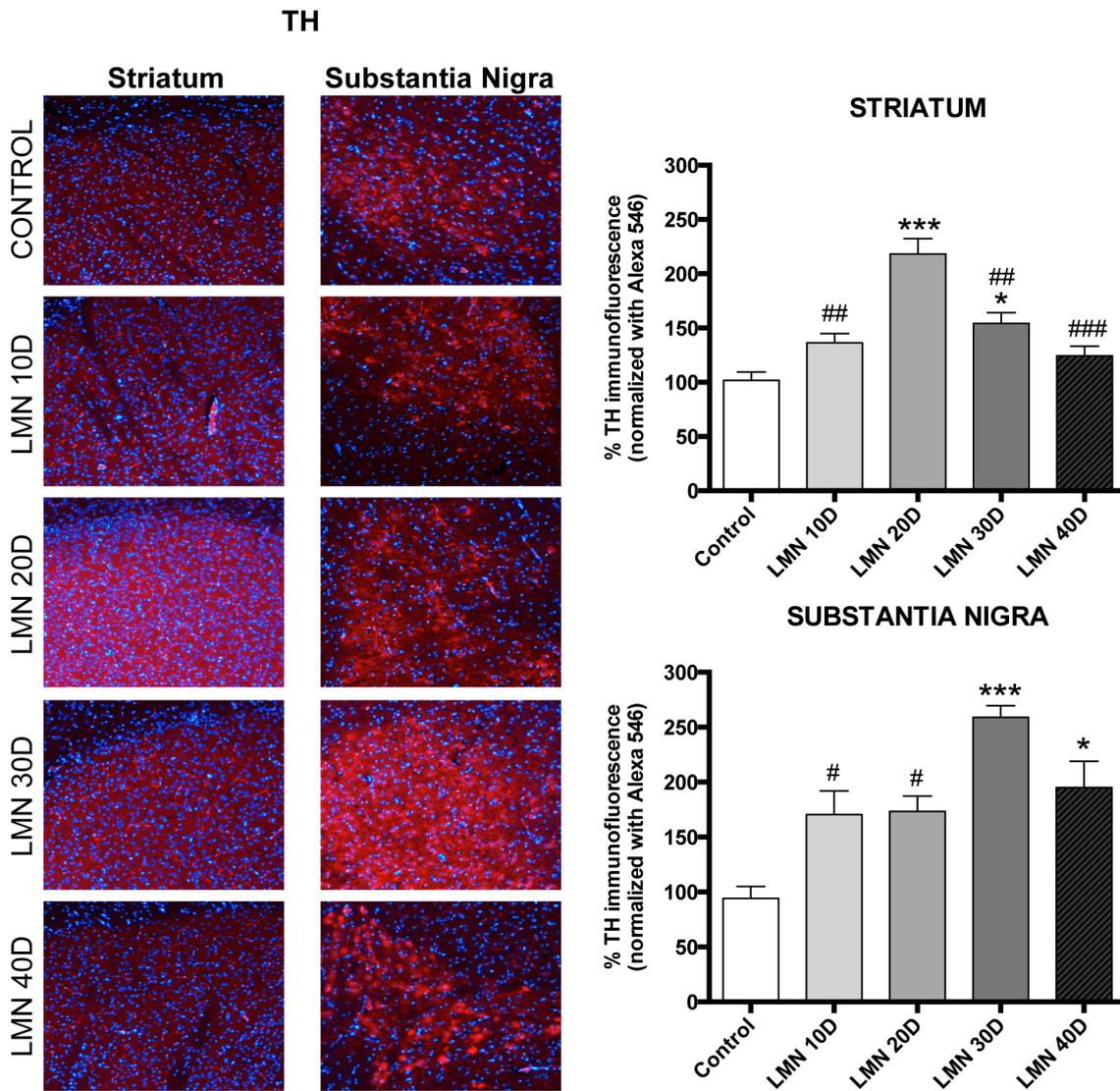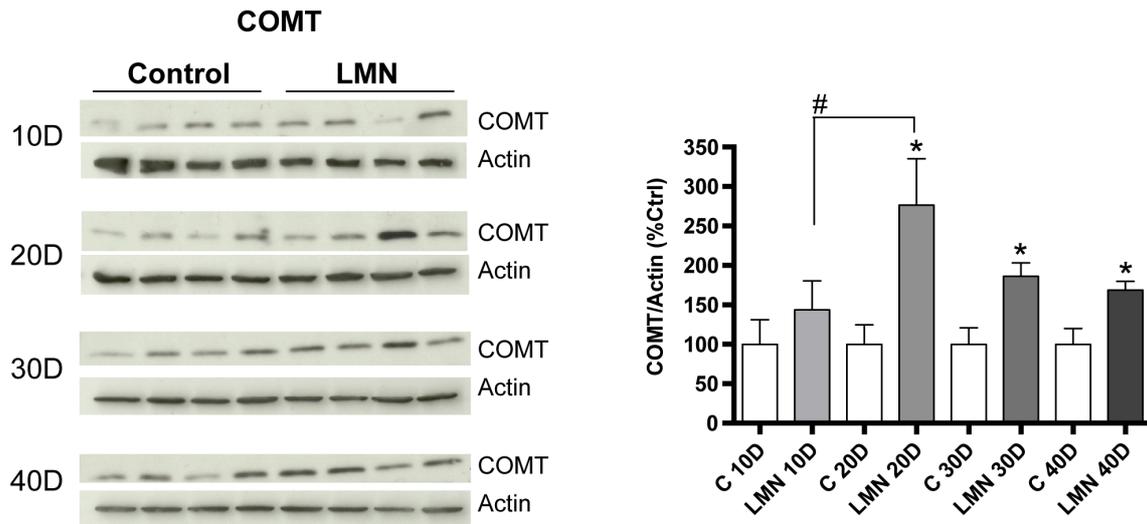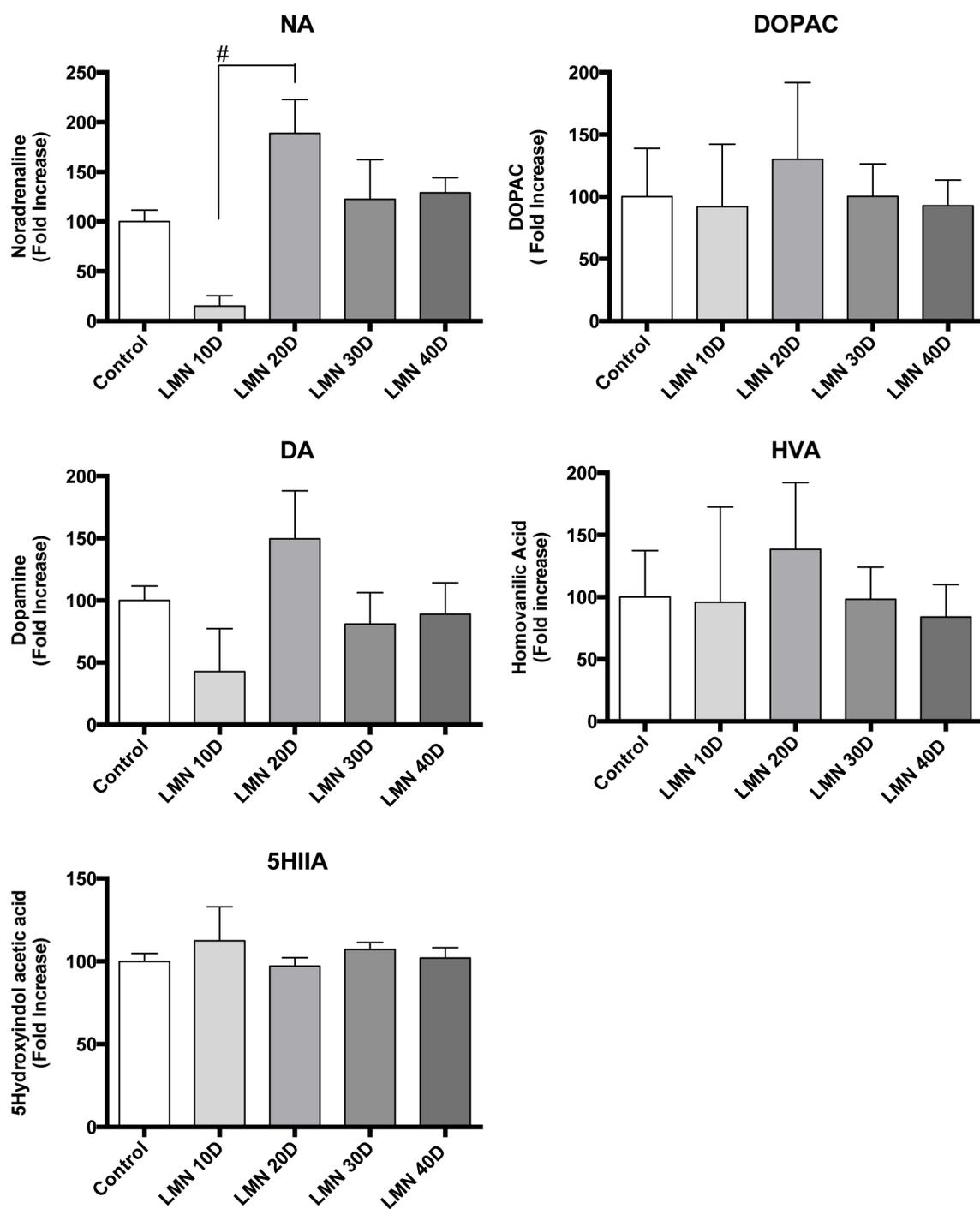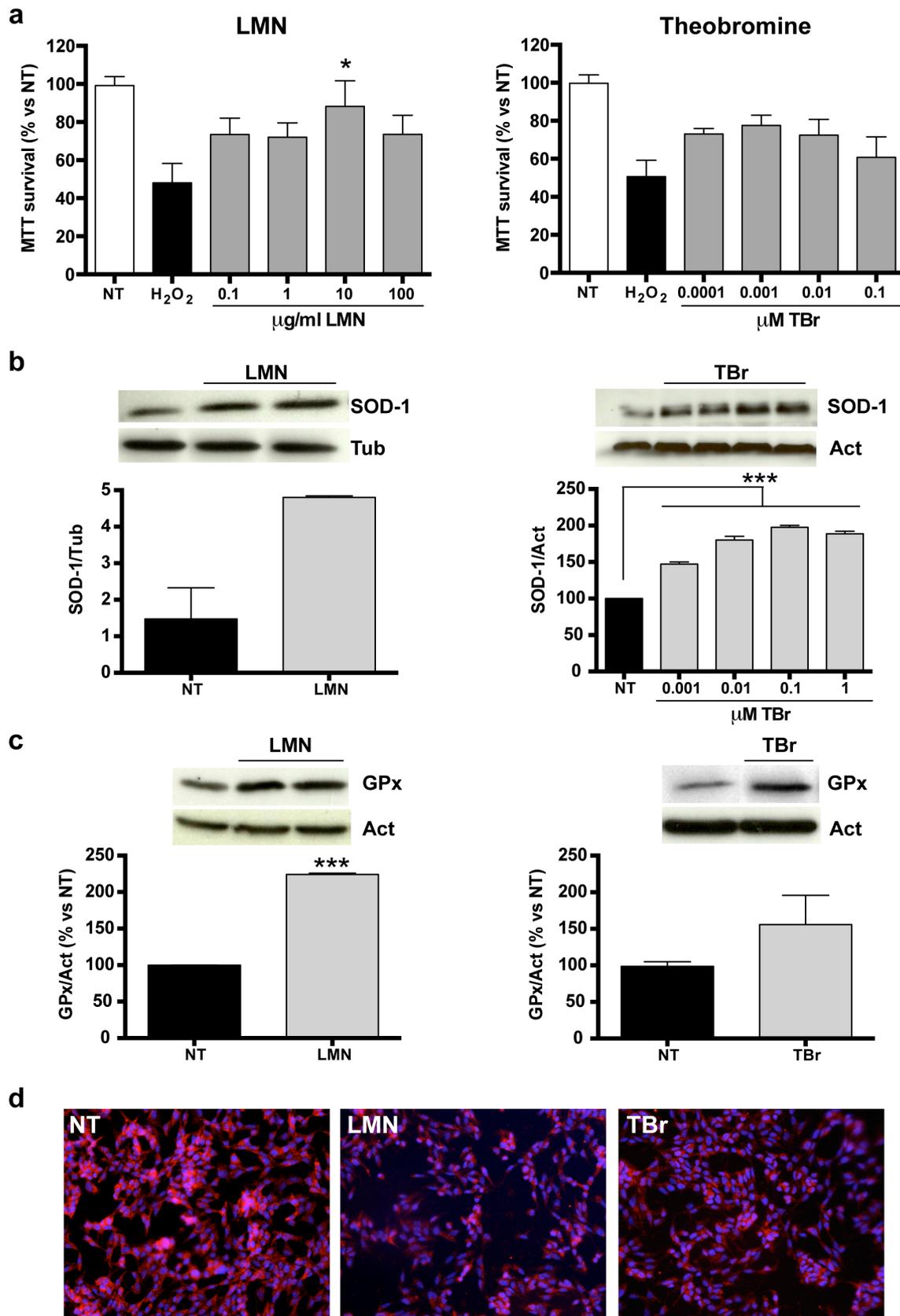
FIGURE 5

Table 1. Catecholaminergic neurotransmitters levels in pC12 cells treated with theobromine.

Metabolite	NT	[Theobromine](μM)		
		1	10	100
Noradrenalin	100.0± 6.6	151.8±15.5	160.6±22.8*	138.2±12.0
L-DOPA	100.0±14.3	131.1± 7.9	135.8±27.1	104.8± 9.7
DOPAC	100.0± 5.1	105.0±11.4	165.3±23.5*	120.6± 9.3
Dopamine	100.0± 4.3	142.6±21.5	178.5±35.0*	124.9±16.8
HVA	100.0± 4.4	83.1± 4.3	92.7± 5.9	96.2± 6.2
3-MT	100.0± 6.7	123.8±16.6	119.8±10.5	113.8±11.4

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