THYROID HORMONE DEIODINASES RESPONSE IN BRAIN OF SPONTANEAUSLY HYPERTENSIVE RATS AFTER HYPOTENSIVE EFFECTS INDUCED BY MANDIBULAR EXTENSION

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ABSTRACT

Purpose: The deiodinases activate or inactivate the thyroid hormones (TH) in virtually all tissues in both physiological and pathological conditions. The three deiodinases, DIO1, DIO2 and DIO3, have different catalytic functions and regulate TH tissue distribution. The aim of the present study was to evaluate the modulation of gene expression of the deiodinases and TH transporters and protein levels of DIO1 in parietal and frontal areas of cerebral cortex of spontaneously hypertensive rats (SHRs), after two successive mandibular extension (ME).

Methods ME was performed on anesthetized rats by a dilatator appropriately designed and Real Time PCR and Western Blotting techniques were employed for gene expression and protein level study.

Results mean blood pressure (MBP) significantly decreased in 2ME-treated rats when compared to sham-operated rats ($p<0.001$) and this decrease lasted for the entire observation period. In gene expression analysis, in 2ME-treated rats we did not observe any significant variation of DIO1 and DIO3 with respect to the sham-operated rats. Differently, DIO2 gene expression significantly increased in frontal area of 2ME-treated rats, with respect to sham-operated rats ($p<0.01$). Furthermore, in parietal area, protein levels of DIO1 in 2ME-treated rats were significantly higher than in sham-operated rats ($p<0.01$). Moreover MCT8 and OATP1C1 both resulted significantly higher ($p<0.05$ and $p<0.001$) in sham frontal cortex.

Conclusion In summary, our data on SHRs, while confirming the hypotensive effect of two MEs, show that the treatment also solicits the three deiodinases production in the cerebral cortex.

Key words deiodinases, TH-transporters, spontaneously hypertensive rats (SHR), cerebral cortex, gene expression, protein levels

INTRODUCTION

Thyroid hormones (TH) regulate several processes in virtually all body tissues [1]. In physiological conditions, both TH (thyroxine, T4, and the biologically active triiodothyronine, T3) have stable concentrations in the circulation and their regulatory activities at the target sites are possible especially in virtue of local regulatory mechanisms of the TH signaling [2]. Circulating TH levels depend on the regulation of hypothalamic-pituitary-thyroid axis and the hormonal production by the thyroid gland [3]. Moreover, in most cases, TH action in tissues tends to correlate with circulating levels of the hormones. However, many studies showed the crucial importance of other factors in the regulation of TH dynamics at cellular level [3-5]. The principal key factor in the local control is based on the activities of the three TH deiodinases (DIO1, DIO2 and DIO3), the enzymes involved in the activation and inactivation of TH [6, 7]. Once inside the cell, T4 can be activated into T3 via DIO2 pathway, therefore T3 in the cell derived from both plasma and intracellular DIO2-mediated conversion. On the contrary, DIO3 is the inactivating enzyme acting at plasma membrane to decrease local TH concentrations. The role of DIO1 is less intuitive and more intriguing than the other enzymes, since it can have both activation and inactivation functions and it is believed that its main homeostatic role is to subtract T4 to DIO2 pathway, ensuring a higher protection against the excessive production of T4 [8]. In addition to deiodinases interplay, the other important regulatory aspect in local TH homeostasis is TH transporter mediation for hormonal passage across cellular membranes (9). Main TH transporters are the organic anion-transporting polypeptide 1c1 (OATP1C1) and monocarboxylate transporter 8 (MCT8). Data in the literature show that the two transporters are regulated in a similar way in the diseased brain (10). In particular, they are fundamental for TH cellular influx and efflux through the blood-brain and blood-cerebrospinal fluid barrier. More specifically, MCT8 regulates T3 uptake in the brain whereas both MCT8 and OATP1C1 regulate T4 uptake (11). These aspects are particularly interesting in the central nervous system where the TH availability needs a fine regulation in both developing and adult brain, to permit a correct growth and functioning of neural system. In

particular, TH are important regulators of cerebral vasculature and the pathological alteration of TH levels is strictly associated to marked changes in vascular and microvascular brain resistance [12]. Changes in the cerebrovascular morphology are known to be often associated with hypertensive states: microvascular pathology is a well-known consequence of hypertension [13] and one of the first sign of vascular pathology in hypertension is an increase of the arteriolar resistance due to remodeling of small arteries [14]. In addition, hypertension is also associated with thyroid disorders, since thyroid dysfunction, due to hypothyroidism and especially to hyperthyroidism, can increase hypertension risk [15].

Finally, the simultaneous presence (comorbidity) of hypertension and hyperthyroidism is known to compromise the ability of myocardium to respond to the hyperthyroidism-induced increase in metabolic needs, with important clinical consequences [16]. To our knowledge, no studies have specifically investigated the direct involvement of TH deiodinases in hypertension. To start filling this gap, in the present study, we have evaluated deiodinases activation in spontaneously hypertensive rats (SHR) which represent the more suitable animal model to study hypertension. Rats underwent a double mandibular extension (ME) treatment, in order to induce a hypotensive response throughout the trigeminal nerve stimulation [17, 18]. In general, the facial area presents many autonomic reflexes having trigeminal fibers as afferent branches. Brain parietal cortex is the site where principal trigeminal afferent fibers project, whereas frontal area is less involved in trigeminal nerve activity. In light of these considerations, gene expression and protein levels of TH deiodinases and gene expression of TH transporters were evaluated in the parietal and frontal cortical regions of SHR brain.

MATERIALS AND METHODS

Animals

Spontaneously hypertensive rats (SHR, 3-4 months, 250-300g) from Charles River (Calco, SO, Italy) were housed in polyethylene cages, under a 12/12 h light/dark cycle (light 8:00-20:00 h) at constant temperature (24±1°C) and humidity (60±5%) with free access to food and water.

Experiments were carried out in agreement with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Pisa and Ministry of Health (Permit Number: 157/2017-PR). All surgeries were performed under alpha-chloralose and urethane anesthesia in order to minimize suffering.

Surgery

Anesthesia, tracheotomy, intubation and mechanical ventilation were performed as previously described [16]. End-tidal CO_2 was continuously measured and the respirator was set to maintain end-tidal CO_2 between 4.5 and 5.0% to keep arterial blood gas tension within the normal range. MBP was measured by a catheter inserted in the left femoral artery.

The rats were secured to a heating stereotaxic frame to maintain the body temperature at 37.0 ± 0.5°C measured with a rectal probe.

At the end of the experiment, all rats were sacrificed by decapitation and samples of frontal and parietal cortex were rapidly removed and stored at -80°C until the use.

Mandibular extension (ME)

SHRs were divided in two groups: 2ME-treated and sham-operated control rats. 10-minutes ME was induced by a U-shaped dilatator appropriately designed for rats and already described previously [19, 20]. After 30 minutes of basal parameters acquisition, 2ME-treated rats underwent

a first ME (ME1) followed by 10 minutes of rest and then a second ME (ME2). Afterward, the rats were observed for further 240 minutes before their sacrifice. MBP and HR were constantly measured. Sham-operated SHRs underwent surgery without ME, and MBP and HR parameters were observed as long as 300 minutes, so to complete the recording at the same time as 2ME-treated rats. The experimental procedure on sham-operated and 2ME-treated rats are resumed in Figure 1 A and B respectively.

Mean blood pressure (MBP) and heart rate (HR) measurements

MBP was monitored with a transducer, model BLPR2 (World Precision Instruments, Sarasota, FL, USA) connected to an ad-hoc bridge amplifier (home-made) and recorded using LabView software (National Instruments S.R.L., Milan, Italy). MBP was measured every 60 sec automatically an averaged value was provided every 5 min. ECG recording (1 KHz sampling rate) was performed with a home-made differential amplifier for biomedical signals and data acquisition was obtained by LabView software. For statistical analysis and for graphical representations, we used values at intervals of 10 and 20 minutes. As baseline value, we considered the average of three recordings obtained at 10 minutes intervals immediately before ME1 and then we considered recordings at 10-minute interval for 30 minutes (corresponding to ME1, post ME1 and ME2 periods) and at 20 minutes interval for the subsequent observation period.

RNA extraction and reverse transcription

Total RNA was extracted from homogenized brain tissue (parietal and frontal cortex) with a miRNeasy Mini Kit (Qiagen, Milan, Italy), according to the manufacturer's instructions. The integrity of total RNA was detected by gel-electrophoresis and total RNA purity and concentration were evaluated spectrophotometrically (NanoDrop, Celbio, Milan, Italy). The RNA samples were stored at -80°C. A quantity of 1 μg of total RNA obtained from each sample was reverse

transcribed with iScript cDNA Synthesis Kit (Bio-Rad, Milan, Italy), according to the manufacturer's instructions.

Quantitative Real-Time PCR analysis of gene expression

Real-Time PCR reactions were carried out in a Bio-Rad 384-well CFX384 RT-PCR System, as already described previously [21]. Briefly, the reaction was performed 10 μl mixture, including 20 ng of template cDNA, 0.5 μM of each primer and Bio-Rad 2X iTaq Universal Sybr Green Supermix. Amplification protocol started with 95°C for 30 seconds, followed by 39 cycles at 95°C for 5 seconds and 60°C for 15 seconds. Each assay was performed in triplicates, with negative control. The CFX software technology was used to assess the stability of candidate reference genes selected for normalization. The average Ct values obtained from each triplicate was converted to a relative quantity and analyzed with CFX384 Manager algorithm. The analysis indicated the three most stably expressed housekeeping genes: HPRT-1: hypoxanthine phosphoribosyltransferase I; HMBS: Hydroxymethylbilane synthase; PAPBN-1: Polyadenylate-binding protein 1. Primer details are shown in Table 1. A standard curve for each target and housekeeping gene was evaluated to assess amplification efficiency and linearity.

Western Blotting

Cell lysate proteins (60 μg) were resolved by Bolt mini gel electrophoresis system (Life Technologies, Monza, Italy). Gels were blotted onto 0.2 mm PVC-membrane by iBlot Dry Blotting System (Life Technologies, Monza, Italy). Membranes were incubated with specific Antibody for DIO1 (sc-98392, Santa Cruz), and GAPDH (G9545, Sigma) and the appropriate secondary G-Immunoglobulin conjugated to horse radish peroxidase (IgG-HRP) was applied. Proteins were visualized with a chemiluminescence assay (Biorad, Milan, Italy) and the optical density (OD) of specific bands measured with Uvitec Alliance System (Eppendorf, Milan, Italy). The results were expressed as DIO1 OD, normalized to the reference protein GAPDH OD.

Statistical analysis

All values are expressed as mean±SEM. One-way ANOVA with Bonferroni's post hoc test was used for multiple comparisons and a $p<0.05$ was considered statistically significant (StatView 5.0.1). For MBP and HR statistic, repeated-measure one-way ANOVA was run with the Holm Sidak test for post-hoc comparisons and analyses were done with the Sigma Stat package, 3.5 version (Jandel Corporation San Mateo, CA).

RESULTS

Mean Arterial Blood Pressure (MBP) and Heart Rate (HR)

Sham-operated SHRs did not show any significant change in MBP for the entire observation period of 300 minutes (Figure 2A). Conversely, 2ME-treated rats displayed a significant decrease in MBP ($p<0.001$ *vs.* sham-operated SHRs). MBP started to diminish immediately after ME2, declined by about 15 mmHg (from 189±1.5 mmHg to 174±1.8 mmHg) and attained a nadir at 161 mmHg 20 minutes after ME2, with an overall decrease after two MEs of about 30 mmHg. Thereafter, MBP remained stably low for the subsequent observation period of 220 minutes. For HR, no significant difference was measured in the observation period, either in sham-operated or 2ME-treated rats (Figure 2B).

RNA expression in Parietal and Frontal Cortex

DIO1, DIO2 and DIO3

In 2ME-treated rats, we did not observe any significant variation of DIO1 gene expression with respect to the sham-operated SHRs, both in parietal and frontal regions of cerebral cortex (Figure 3A). Furthermore, DIO2 gene expression was unchanged between sham-operated and 2M-treated rats in the parietal region, whereas in frontal area of 2ME-treatd rats it was significantly higher with respect to correspondent sham-operated ($p<0.01$, Figure 3B). While no difference in DIO2

gene expression was found between sham-operated groups in the two regions studied, a significant difference was observed between the two 2ME-treated groups ($p<0.001$, Figure 3B). In both parietal and frontal regions, no difference was found between DIO3 gene expression between sham-operated and 2ME-treatd rats ($p=0.18$, parietal area and $p=0.09$, frontal area, Figure 3C).

MCT8 and OATP1C1

In parietal region of 2ME-treated rats, we did not observe any significant difference in MCT8 and OATP1C1 gene expression with respect to sham-operated SHRs, however, in frontal area, a significantly higher expression of MCT8 and OATP1C1 was described in sham-operated SHRs with respect to the 2ME-treated rats ($p<0.05$, Figure 4A and $p<0.01$, Figure 4B, respectively). Furthermore, sham-operated levels of expression for MCT8 and OATP1C1 were significantly higher in frontal than parietal region ($p<0.05$, Figure 4A and Figure 4B, respectively, data not shown).

Protein Levels

Parietal and Frontal Cortex

In parietal area, protein levels of DIO1 in 2ME-treated rats were significantly higher than in sham-operated rats ($p<0.01$) and with respect to frontal region of both sham-operated ($p<0.01$) and 2ME-treated ($p<0.001$) rats (Figure 5A).

DISCUSSION

The relevance of TH action in brain development and functions has been established [22]. Studies on rats have shown that TH regulate a large number of genes in the CNS [23]. However, TH activity at cellular level greatly depends on critical regulating factors such as transporters and metabolizing enzymes, which in some cases determine a relevant discrepancy between TH circulating and tissue levels [24]. In the present study, we evaluated the variation of TH

transporters and deiodinases in the frontal and parietal cortex of SHRs, considered a reliable rat model of hypertension, in order to individuate and characterize a possible link between hypertension and TH specific regulation in different areas of the brain in this pathologic condition. In particular, we studied how the ME treatment, known to be associated to a hypotensive response both at systemic and local level [25], may affect the regulation of the deiodinase expression in brain tissue and, consequently, TH metabolism at frontal and parietal levels in the brain cortex.

Our results confirm previous observations in SHR [17, 18] showing a marked decrease in MBP and HR induced after two MEs and suggest, for the first time, a possible involvement of deiodinases in the compensative response to a hypotensive stimulus (ME) in rat brain cortex. So far, data available on deiodinases expression in the rodent brain are limited to observations in hyper- or hypo-thyroid conditions or in association with the brain maturation [15, 26, 27].

Both hyper- and hypothyroidism conditions have been associated to hypertensive state, however, the mechanisms involved are only partially described and clinical data on this topic are often controversial [15]. Furthermore, another aspect still scarcely investigated, regards the fact that mutations or polymorphisms occurring in several genes belonging to the genetic pattern of hypothalamic-pituitary-thyroid axis may affect different organs, promoting various diseases, including hypertension. As an example, the polymorphism of DIO2 gene, threonine 92 to alanine (Thr92Ala), is a determinant of hypertension susceptibility [28]. It is well established that the excess of TH leads to metabolic and hemodynamic changes, increases metabolic rate, cardiac preload and ventricular contractility and decreases systemic vascular resistance, causing an augmented of cardiac output and hypertension [29]. Given these assumptions, we hypothesize a possible link between the reduction of hypertension by ME and the deiodinases involvement in brain cortex of SHRs. Over the time, deiodinase role in TH homeostasis has been investigated in several pathological contexts and in different tissues [30]. Deiodinase availability in tissues is considered a crucial factor determining TH global levels and effects. The three enzymes owe their functional role mainly to their cellular distribution, in fact DIO1 and DIO3 are in the plasma

membrane whereas DIO2 in the endoplasmic reticulum [31], assuming a more strategical position in the regulation of protein daily synthesis of T3. Differently, DIO1 is more involved in the production of circulating T3, which would explain also its fast equilibrating property between intra- and extra- cellular compartments (30 minutes of DIO1 *vs.* about 8 hours of DIO2) [31]. Furthermore, DIO1 seems to have a relevant scavenger activity, which permits the recovering of iodine for the recycling in *de novo* synthesis of TH [32]. Among the three deiodinases, DIO3 is the one more investigated in the brain, especially in relation to its role both in the developing and adult brain in limiting the availability and actions of TH [33]. In particular, DIO3 maintains a high activity in several regions also of the adult brain, included the cerebral cortex, protecting neural processes from untimely T3 action [34]. Previous data from the literature showed that inflammation associated to many diseased conditions particularly affects DIO2 expression in the rodent brain (9). Furthermore, in the absence of MCT8 and OATP1C1, was observed a dramatic reduction of T4 and T3 with respect to wild type values in brain tissue and vessels, thus confirming the crucial role of these two transporters in TH effects in target cells (11). It has been hypothesized that the two transporters have synergistic functions and common regulatory mechanisms that in large part need to be still defined. In future studies, further investigation at protein levels will better clarify the reciprocal role of transporters and deiodinases in TH intracellular metabolism of SHRs undergoing 2ME-treatment.

A novel aspect emerging in the present study, regards the observed DIO1 gene expression in the cortical brain of SHRs, which, in our knowledge, has not been described so far. Usually, DIO1 is considered involved in the activation of circulating TH throughout the conversion of T4 to T3 mainly in the thyroid, but also in liver and kidney and, typically, its presence is not evaluated (or considered irrelevant) in other tissues. In both parietal and frontal brain cortex of SHRs we did not observe any difference in DIO1 gene expression (Figure 3A), however, a significantly higher level ($p<0.01$) of DIO1 protein in the parietal region after ME treatment was observed (Figure 4A). Differently, DIO2 and DIO3 gene expression varied in the two cortical regions in response to ME:

in the frontal area of 2ME-treated SHRs, DIO2 was significantly higher than in sham-operated SHRs ($p<0.01$, Figure 3B), whereas DIO3 expression, did not significantly modify when compared to its control (Figure 3C). Notably, DIO2 levels of gene expression in sham-operated SHRs are comparable in the two brain regions studied whereas, after 2ME, they were significantly lower in parietal with respect to frontal region ($p<0.001$), suggesting a different involvement of DIO2 in the two compartments after 2ME. Unfortunately, no data are at this moment available on DIO2 and DIO3 protein levels and we hope to be able to get more information in the future. Taken as a whole, gene expression and protein data on deiodinases in the brain cortex showed the three deiodinases behavior after 2ME treatment: hypotensive stimulus, in fact, induced a major presence of 5'-monodeiodinases (DIO1 and DIO2) in parietal and frontal cortex, respectively. The observation that the two deiodinases promptly respond (within the 270 minutes of the study) to the ME hypotensive stimulus strongly suggests its involvement in MBP regulation. In future studies, it will be useful to introduce new observational time points immediately after ME to evaluate gene expression and protein levels of the three deiodinases. Furthermore, the analysis of enzyme activity will be diriment on the role of the different deiodinases. Based on our results, we can hypothesize that at brain cortex level, in association with a ME hypotensive treatment, a higher synthesis of T3 is needed and that the first responding enzyme is DIO1 in the parietal cortex, followed by DIO2 activation in the frontal region.

Conflict of interest

The authors declare that they have no conflict of interest

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LEGENDS OF FIGURES

Figure 1: Experimental procedure on sham-operated (A) and 2ME-treated (B) spontaneously hyperternsive rats (SHRs).

Figure 2 As an example, are represented the time courses in SHRs of (A) mean blood pressure (MBP, N.3) and (B) heart rate (HR) in sham-operated (N.2) and 2 ME-treated (N.3) SHRs. ME1 and ME2 indicate the timing of mandibular extension treatments. In (B), heart rates in sham-operated and 2ME-treated SHRs were represented on two different y-axes for major clarity. Values are reported as mean±SEM. *p<0.01, **p<0.001 *vs.* baseline values.

Figure 3: Expression levels of DIO1 (A), DIO2 (B) and DIO3 (C) genes in the parietal and frontal cortex of SHR. Data are expressed as mean±SEM. p<0.05 is considered significant. SHAM: Sham operated rats (N.6); ME: 2 Mandibular Extension-treated rats (N.9).

Figure 4: Expression levels of MCT8 (A) and OATP1C1 (B) genes in the parietal and frontal cortex of SHRs. Data are expressed as mean±SEM. p<0.05 is considered significant. SHAM: Sham operated rats (N.4); ME: 2 Mandibular Extension-treated rats (N.6).

Figure 5: Protein levels of DIO1 in the parietal and frontal cortex of SHRs. (A) Histograms representing the Optical Density (OD) of target DIO1 normalized to the OD of the reference GAPDH. (B) Examples of DIO1 (28KDa) and GAPDH (37KDa) blots. Data are expressed as mean±SEM. p<0.05 is considered significant. SHAM: Sham operated rats (N.4); ME: 2 Mandibular Extension-treated rats (N.4).

Table 1. Reference and target genes: primer details

GENE	PRIMER 5'→3'	bp	GENE BANK N.
HPRT-1	F: CCCAGCGTCGTGATTAGTGATG R: TTCAGTCCTGTCCATAATCAGTCC	110	NM_012583
HMBS	F: TCTAGATGGCTCAGATAGCATGCA R: TGGACCATCTTCTTGCTGAACA	76	NM_013168
PAPBN-1	F: TATGGTGCGACAGCAGAAGA R: TATGCAAACCCTTTGGGATG	110	116697
MCT8	F: CCCAAGCAAGAGAGGCGCCC R: CGGTAGGTGCGCTGGCGAAA	98	NM_147216.1
OATP1C1	F: GGATCCCCAGTGGGTCGGGG R: ACCAGAAAGGCACGGCTGCA	82	NM_053441.1
DIO1	F: TCTATGTGATACAGGAAGGCAG R: CGGACTTCCTCAGGATTGT	76	BC083557.1
DIO2	F: AGACGCCTACAAACAGGTT R: TGCTTCAGGATTGGACACG	76	NM_031720.5
DIO3	F: GCCTACTTCGAGCGTCTCTATG R: CATAGCGTTCCAACCAAGTGCG	112	NM_001362.3

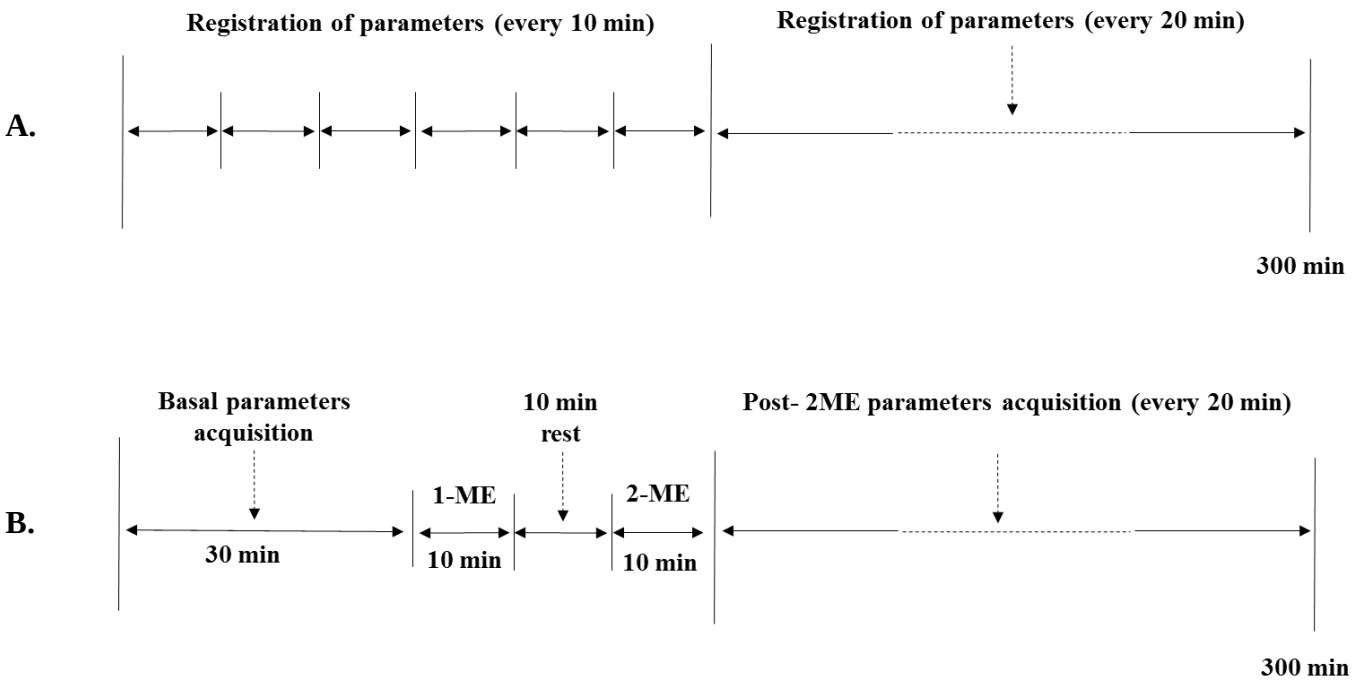
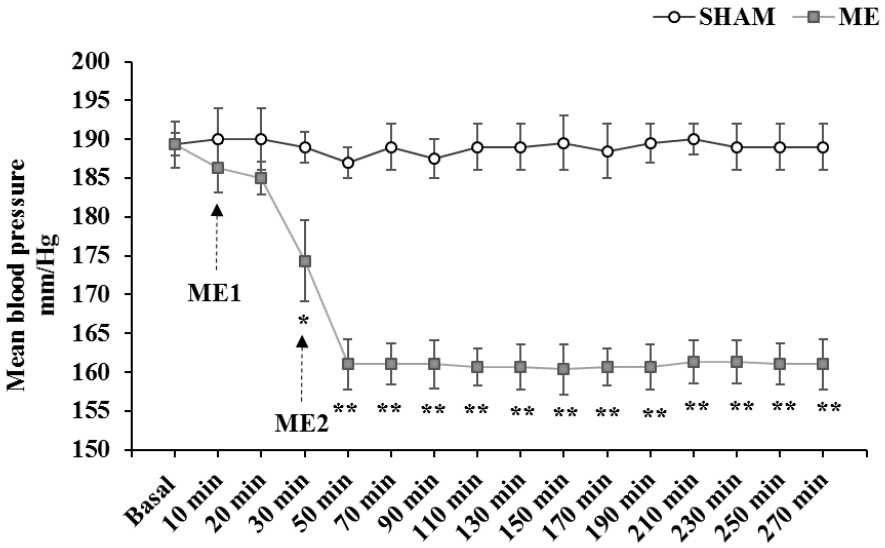

A.
Registration of parameters (every 10 min)
Registration of parameters (every 20 min)
300 min
B.
Basal parameters acquisition
30 min
1-ME
10 min
10 min rest
2-ME
10 min
Post- 2ME parameters acquisition (every 20 min)
300 min

Fig.1

A.


SHAM
ME
Mean blood pressure mm/Hg
200
195
190
185
180
175
170
165
160
155
150
ME1
ME2
*
**
Basal
10 min
20 min
30 min
50 min
70 min
90 min
110 min
130 min
150 min
170 min
190 min
210 min
230 min
250 min
270 min

B.

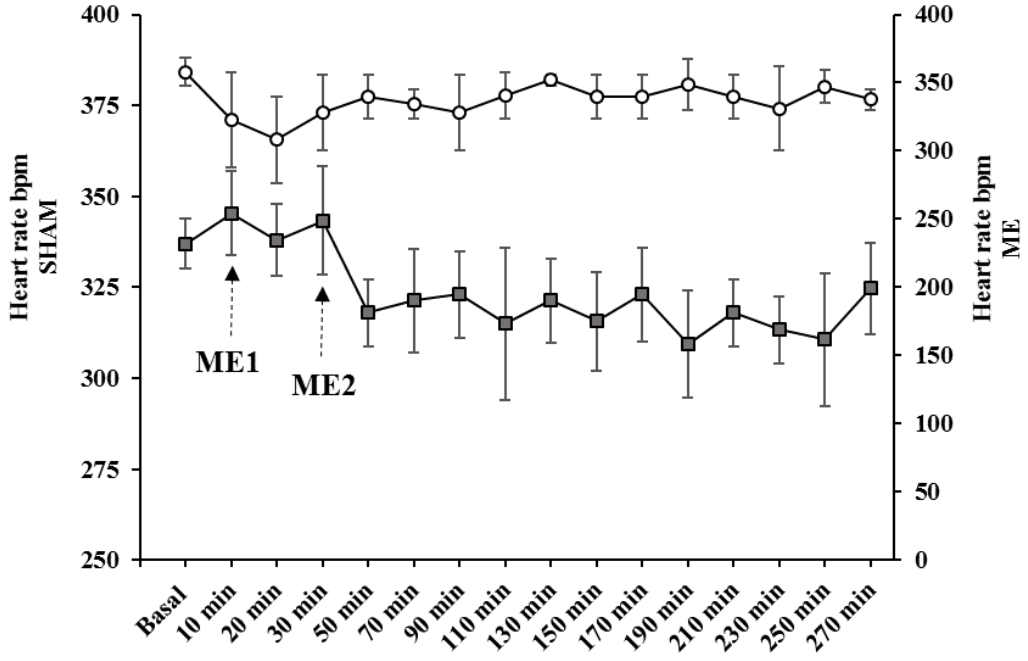
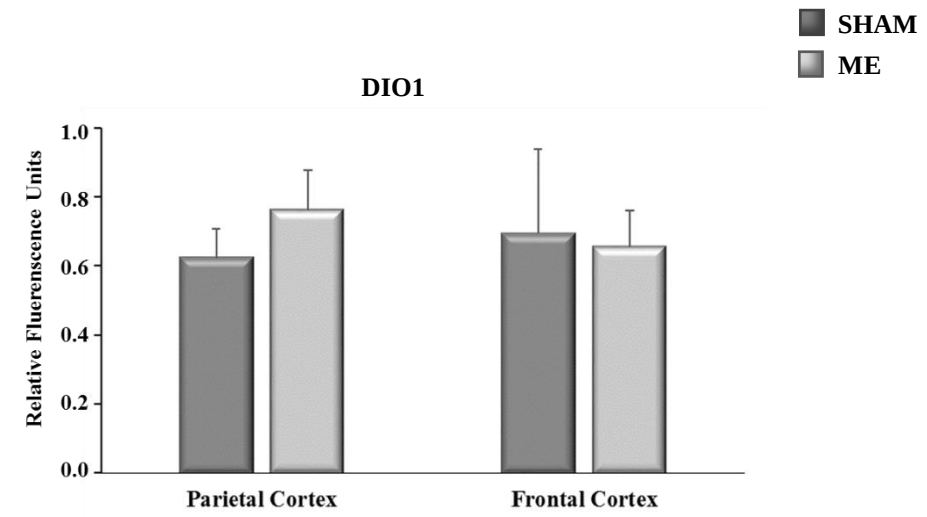
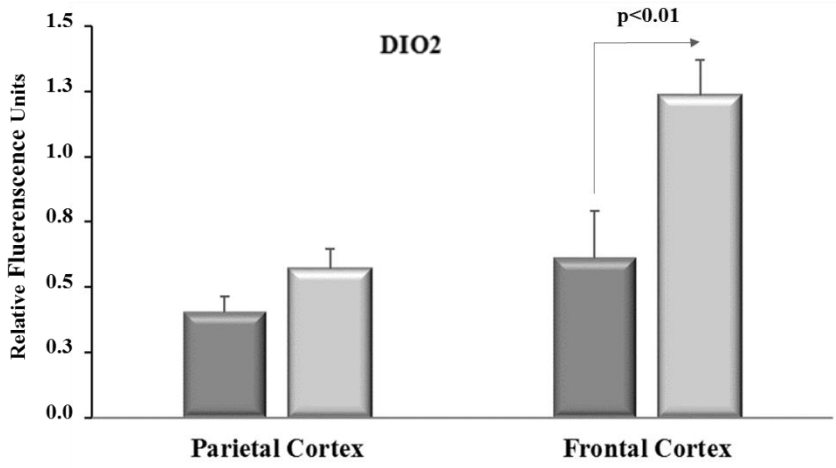

Heart rate bpm SHAM
400
375
350
325
300
275
250
Heart rate bpm ME
400
350
300
250
200
150
100
50
0
ME1
ME2
Basal
10 min
20 min
30 min
50 min
70 min
90 min
110 min
130 min
150 min
170 min
190 min
210 min
230 min
250 min
270 min

Fig.2

A.


SHAM
ME
DIO1
Relative Fluerenscence Units
1.0
0.8
0.6
0.4
0.2
0.0
Parietal Cortex
Frontal Cortex

B.


p<0.01
DIO2
Relative Fluerenscence Units
1.5
1.3
1.0
0.8
0.5
0.3
0.0
Parietal Cortex
Frontal Cortex

C.

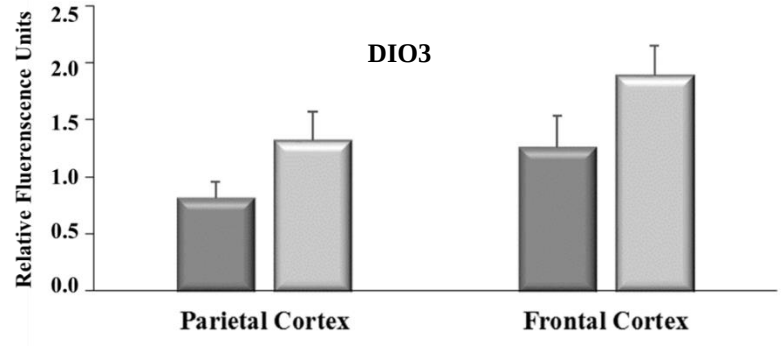
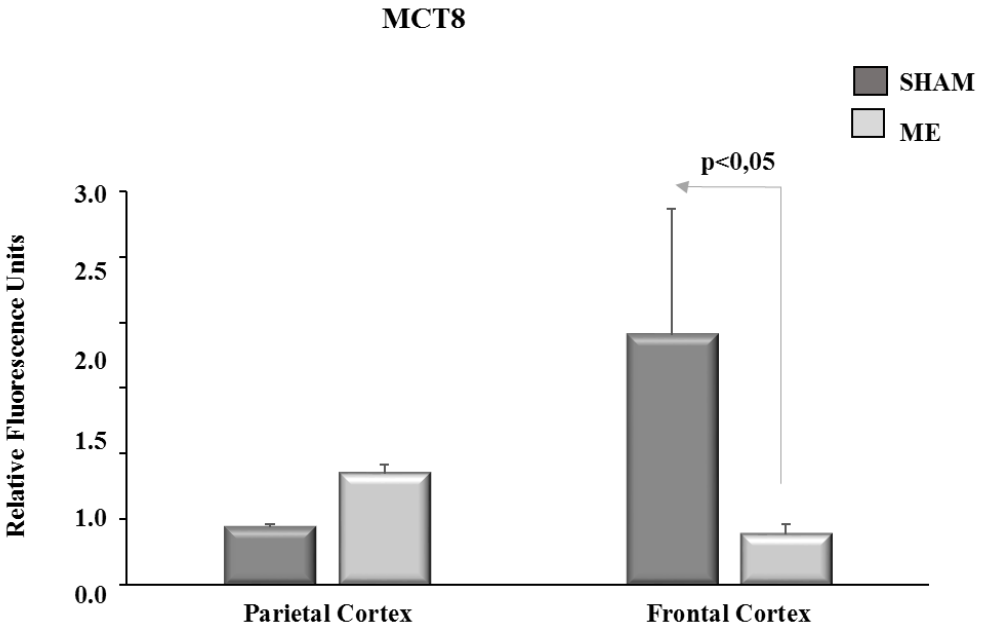

DIO3
Relative Fluerenscence Units
2.5
2.0
1.5
1.0
0.5
0.0
Parietal Cortex
Frontal Cortex

Fig. 3

A.


MCT8
SHAM
ME
p<0,05
Relative Fluorescence Units
3.0
2.5
2.0
1.5
1.0
0.0
Parietal Cortex
Frontal Cortex

B.

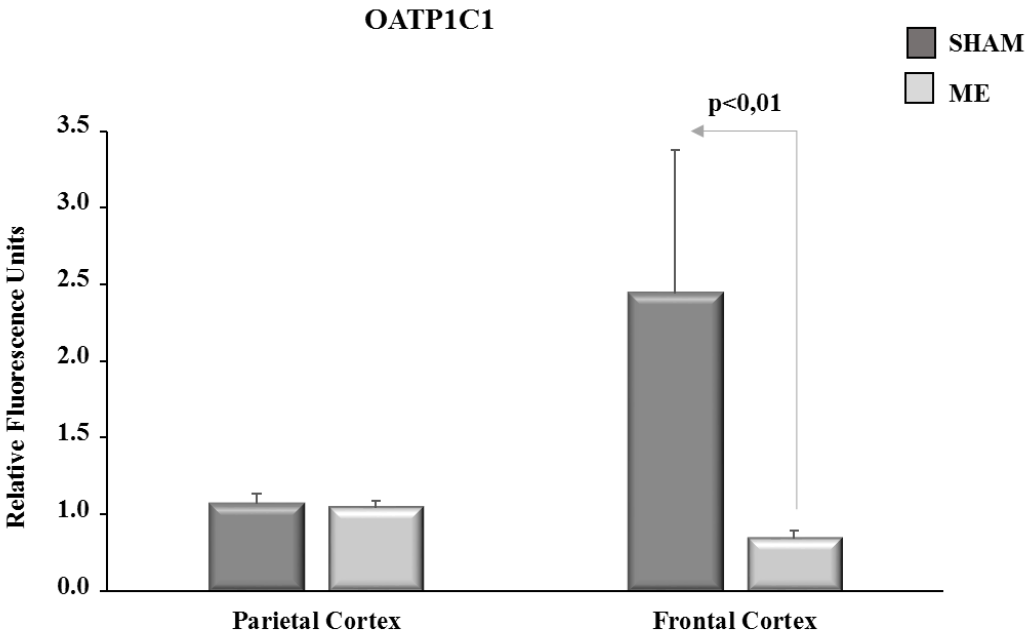
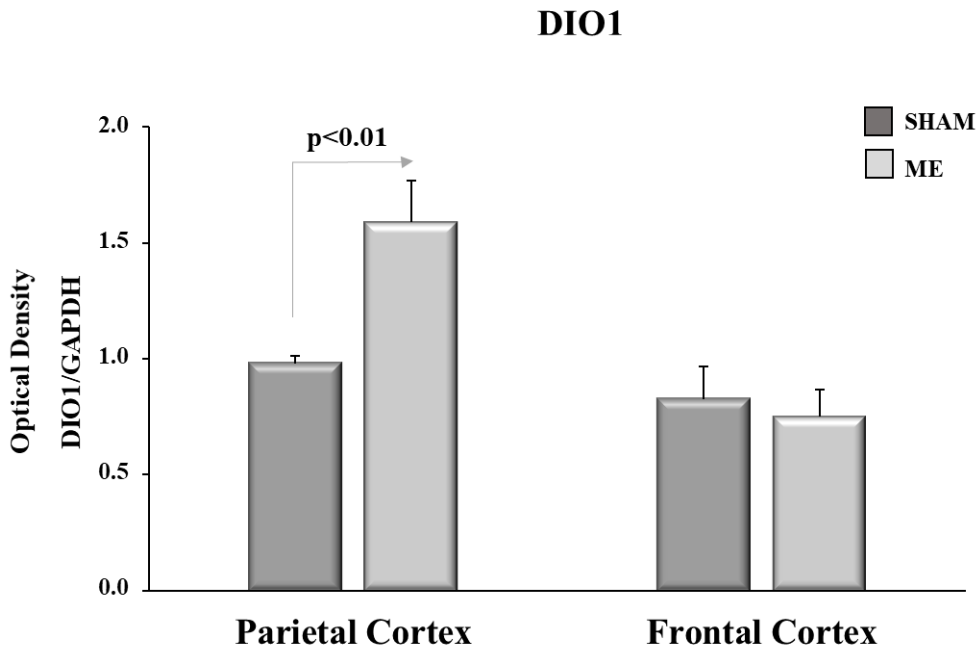

OATP1C1
SHAM
ME
p<0,01
Relative Fluorescence Units
3.5
3.0
2.5
2.0
1.5
1.0
0.0
Parietal Cortex
Frontal Cortex

Fig.4

A.


DIO1
SHAM
ME
p<0.01
Optical Density
DIO1/GAPDH
2.0
1.5
1.0
0.5
0.0
Parietal Cortex
Frontal Cortex

B.

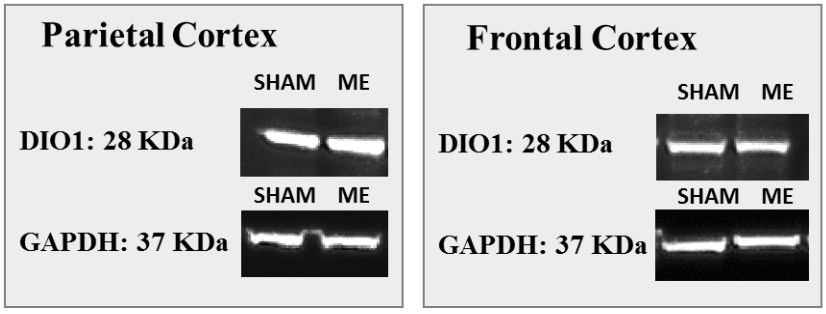

Parietal Cortex
SHAM ME
DIO1: 28 KDa
SHAM ME
GAPDH: 37 KDa
Frontal Cortex
SHAM ME
DIO1: 28 KDa
SHAM ME
GAPDH: 37 KDa

Fig. 5