DDC expression is not regulated by NFAT5 (TonEBP) in dopaminergic neural cell lines

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ABSTRACT

The nuclear factor of activated T-cells 5 (NFAT5), also known as tonicity-responsive enhancer-binding protein (TonEBP), is a transcription factor that regulates osmoadaptive response in multiple tissues and is highly expressed in the developing central nervous system. A former study reported that NFAT5 activation through hypertonic stress increases the expression of the dopa decarboxylase enzyme (DDC), also known as aromatic-L-amino-acid decarboxylase (AADC), in human renal proximal tubule cells, leading to an increase of dopamine synthesis. In a previous study, we identified *NFAT5* as a candidate gene for cocaine dependence, a complex psychiatric disorder in which dopaminergic neurotransmission plays an important role. Therefore, under the hypothesis that NFAT5 may also affect dopamine levels in the nervous system through the regulation of *DDC* expression, we examined this regulation using two neural dopaminergic cell lines, SH-SY5Y and PC12.

The effect of NFAT5 on the expression of the neuronal isoform of *DDC* was evaluated by qRT-PCR. Upon hypertonic stress, NFAT5 was activated and accumulated into the nuclei and, subsequently, the expression of *NFAT5* and its known targets sodium/myo-inositol cotransporter 1 (*SMIT*) and sodium chloride/taurine cotransporter (*TAUT*) increased, as expected. However, the expression of *DDC* decreased. When silencing the expression of *NFAT5* with a specific shRNA we observed that the downregulation of *DDC* is independent from NFAT5 in both cell lines and is due to hypertonic stress.

In conclusion, NFAT5 does not regulate the expression of the neuronal isoform of *DDC* in neural dopaminergic cell lines and, consequently, it does not modulate dopamine synthesis through DDC.

Keywords: *NFAT5*; *DDC*; *TonEBP*; hypertonic stress; neural dopaminergic cell lines; SH-SY5Y; PC12.

1. Introduction

NFAT5 (TonEBP) is a transcription factor of the Rel family activated by hypertonicity that is ubiquitously expressed and is essential for the regulation of homeostasis under osmotic stress, and it has been especially related to immune system and inflammatory functions (Aramburu and López-Rodríguez, 2019; Ho, 2003; Lee et al., 2019). The activation of NFAT5, and its translocation to the nucleus, activates the expression of many genes, such as the osmolyte transporters *SMIT* (sodium/myoinositol cotransporter) and *TAUT* (sodium chloride/taurine cotransporter), and it also activates its own transcription (Aramburu et al., 2006; Halterman et al., 2012; Loyher et al., 2004). It has been reported that an acute hypertonic injection in rats increases the amount of NFAT5 protein in the nuclei of neuron cells (S Maallem et al., 2006; S. Maallem et al., 2006). However, NFAT5 is not only regulated by tonicity, and it can also be activated through other stimuli in hypertonic and isotonic tissues (Halterman et al., 2012). *NFAT5* is expressed in the adult brain, but it is also highly and specifically expressed in the developing brain at embryonic stages, suggesting its importance in embryogenesis and cellular homeostasis (Loyher et al., 2004; Yang et al., 2018). NFAT5 function in the brain varies among cell types. For example it protects neurons against ischemic damage, may participate in inflammation in microglia, regulates the expression of *AQP4* in astrocytes and could influence dopaminergic neurotransmission (Jeong et al., 2016; Mak et al., 2012; Yang et al., 2018; Yi et al., 2013).

In a previous study of our group, the *NFAT5* gene was found upregulated in a dopaminergic neuronal model after cocaine exposure, and it was also found to carry genetic risk variants predisposing to cocaine dependence (Fernàndez-Castillo et al., 2015). It has been suggested that NFAT5 may participate in modulating dopamine levels

in peripheral tissues through the direct regulation of DDC, an enzyme involved in dopamine synthesis (Hsin et al., 2011). The authors observed that the expression of *DDC* was upregulated by NFAT5 (TonEBP) in cells of the human renal proximal tubule (HK-2) and that this resulted in increased dopamine levels (Hsin et al., 2011). This connection between NFAT5 and dopamine is interesting, since addiction is a neuropsychiatric disorder in which this neurotransmitter (and also serotonin) plays an important role in mediating the effects of the drug on the reward system (Volkow et al., 2017).

DDC (also known as AADC, EC 4.1.1.28), the aromatic L-amino acid decarboxylase, catalyses the synthesis of dopamine and serotonin, which act as neurotransmitters and hormones in neural and endocrine tissues. Dopaminergic (and also serotonergic) neurotransmission in the brain plays a key role in several neuropsychiatric disorders, including addiction. The *DDC* gene has two tissue-specific isoforms that differ in their 5'UTR, one expressed in neuronal cell types and the other one in non-neuronal tissues (Ichinose et al., 1989).

Since NFAT5 has been reported to be a transcription factor for *DDC* in human renal proximal tubule cells (Hsin et al., 2011), it has been suggested that NFAT5 may also have a role in dopaminergic neurotransmission in brain (Yang et al., 2018). This would substantiate its possible contribution to cocaine addiction.

Therefore, the aim of the present study is to explore whether NFAT5 regulates the expression of the *DDC* neuronal isoform in dopaminergic neural cell lines, modulating in consequence the production of dopamine in the brain.

2. Material and methods

2.1.Cell culture and osmotic shock

PC12 cells from rat adrenal gland (ATCC) provided by Celltec UB were grown in RPMI medium supplemented with 10% horse serum, 5% fetal bovine serum, 1% penicillin/streptomycin and 1% glutamine (Life Technologies), and SH-SY5Y cells from human neuroblastoma (ATCC) were grown in DMEM:F12 (50:50) medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies), both at 37ºC in a humidified 5% CO_2 atmosphere incubator.

To activate and upregulate NFAT5 we used a hypertonic stress procedure based on previous studies performed in brain (with sucrose) (S Maallem et al., 2006; S. Maallem et al., 2006) or in neural cell lines (with NaCl) (Bitoun and Tappaz, 2000; Isaacks et al., 1994). Osmotic shock was performed at the same osmolality by adding a NaCl or sucrose solution to the medium up to 550 mOsm/Kg, examined by VAPRO 5520 osmometer (Wescor) to reach a hypertonic condition. Cells were exposed to the hypertonic medium for 6, 24, 36 or 48 hours before RNA isolation. A total of five replicates per condition were performed.

2.2.PCR and quantitative real-time PCR

RNA isolation was performed with the High Pure RNA Isolation Kit (Roche) and quantified with Nanodrop (NanoDrop Technologies). RNA was retrotranscribed to cDNA using the High-Capacity cDNA Reverse kit (Life Technologies) and RNase inhibitor (Applied Biosystems).

Since *DDC* has a neuronal and a non-neuronal isoform, we designed specific primers for the two isoforms to assess which ones are expressed in PC12 and SH-SY5Y by PCR. The primers used are indicated in Table S1. The expression of both isoforms was tested in PC12 and SH-SY5Y cells either not exposed to osmotic shock or exposed to 6h of hypertonic stress with NaCl up to 550mOsm/Kg. We used cDNA from rat liver and Hep3B cells as a positive control for the non-neuronal isoform. PCR products were resolved by electrophoresis on 2% agarose gels followed by staining with RedSafe (iNtRON Biotechnology).

The expression levels of *NFAT5* and *DDC* were assessed by quantitative PCR (qRT-PCR), as well as those of the *SMIT* and *TAUT* genes, known NFAT5 targets. QRT-PCR experiments were performed using LightCycler® 480 SYBR Green I Master reagent (Roche) with the LightCycler® 480II system (Roche), and data were analyzed with the LightCycler® 480 Software, Version 1.5. For normalization, *GAPDH* and *PGK1* expression was used as a reference, which were stable across conditions. The primers used for this study are listed in Table S1.

2.3.Immunocytochemistry

PC12 or SH-SY5Y cells were stained during 15 min with Wheat germ agglutinin Alexa Fluor® 488 conjugate (Life Technologies) at 1:2000 in cold medium to stain the cellular membrane, fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.1M glycine and 0.5% Tween solution. After blocking non-specific binding with 10% normal donkey serum (blocking solution, BS) for 1h, we stained the samples during 1 hour at 37ºC with the anti-NFAT5 rabbit polyclonal primary antibody (Abcam) at 1:100 dilution in BS, 45min at 37ºC with the donkey anti-rabbit IgG-Cy3 secondary antibody (Jackson

ImmunoResearch) at 1:400 in BS and finally, 10 minutes at RT with DAPI (Invitrogen) at 1:10,000 diluted in BS. Images were acquired with a Leica SP2 AOBS Confocal Microscope (Leica Microsystems) with 63×/1.32–0.6 oil immersion objective and they were analyzed using ImageJ (Schindelin et al., 2012).

2.4.*NFAT5* silencing, lentiviral production and infection

To silence *NFAT5* expression, we used an shRNA against *NFAT5* (shNFAT5 5'-GGTCAAACGACGAGATTGTGA-3') previously used by others (Drews-Elger et al., 2009). The shRNA was inserted into a psi-LVRU6P plasmid that includes the U6 promoter and a puromycin selection cassette (Genecopoeia). A scrambled shRNA was produced in the same plasmid backbone (Genecopoeia,). We also used Tet-O-FUW-EGFP plasmid (Addgene) that contains the EGFP cassette under a tetracycline promoter and FUW-M2rtTA (Addgene) to quantify infection efficiency.

Third generation lentivirus particles were produced essentially as previously described (Ding and Kilpatrick, 2013). Briefly, the lentiviral expression vector (shRNAs or EGFP vectors) and three helper plasmids (pMD2.G, pMDLg/pRRE and pRSV-Rev (Addgene)) were cotransfected into HEK293T plated in T175 flasks using calcium phosphate transfection method, considering a 10:3:4:2 plasmid ratio respectively with a total amount of 142 µg. The medium was replaced 24h after transfection and viral supernatants were collected 24-30h later and filtered through a 0.45 µm filter. Finally, supernatants were ultracentrifuged and resuspended in 100 µl of DMEM medium.

PC12 cells or SH-SY5Y cells were plated in six-wells plates and infected with 0.8 µl of concentrated lentiviral particles in fresh medium with polybrene (6.5 µg/mL) (Sigma). Viral medium was removed after 24 hours and replaced with fresh medium or fresh

medium containing doxycycline (0.5 µg/mL) (Merck) for genes under tetracycline promoter. Twenty-four hours later, the osmotic shock was performed during 6, 24, 36 or 48h with NaCl as described above. A total of five replicates per condition were performed.

2.5.Statistical analysis

The data in all figures were presented as mean ± SD. The analyses of the effect of NFAT5 activation under hypertonic stress on *SMIT*, *TAUT* and *DDC* expression were performed with one-way ANOVA. Gaussian Generalized Linear Models were used to investigate the possible effect of *NFAT5* on the *SMIT*, *TAUT* and *DDC* expression in the experiments silencing *NFAT5*. In all the analyses, when necessary for normality assumption, *log* transformation was made. All computations were performed in R version 3.4.1 (R Core Team, 2014) and a value of $P< 0.05$ was considered to be statistically significant.

3. Results

The aim of the present study is to explore whether NFAT5, previously shown to modulate dopamine levels in renal tubule cells through activation of the DDC enzyme, also regulates the expression of the *DDC* neuronal isoform in two dopaminergic neural cell lines (PC12 and SH-SY5Y cells), and therefore regulates dopamine levels in the brain. To reach our objective, we activated NFAT5 by inducing hypertonic stress with salt or sucrose up to 550mOsm/Kg during 6, 24, 36 or 48h, as previously used to activate NFAT5 in neural cell lines (Bitoun and Tappaz, 2000; Isaacks et al., 1994).

First, we inspected which *DDC* isoforms were expressed in PC12 and SH-SY5Y and we determined that both cell lines express only the neuronal isoform in both conditions, with or without hypertonic stress (Fig. S1).

When activation of NFAT5 was induced under hypertonic stress (550 mOsm/kg) with salt (NaCl) we observed an expected significant upregulation of *NFAT5* expression, both in PC12 and in SH-SY5Y cells (Fig. 1). Similar results were observed when hypertonic stress was induced with sucrose at the same osmolality (Fig. S2). Immunocytochemistry demonstrated that the NFAT5 protein was translocated into the nuclei and therefore activated upon hypertonic stress with salt in both cell lines (Fig. 2). The genes *SMIT* and *TAUT*, known NFAT5 targets, were significantly upregulated under hypertonic stress with salt as expected, and the expression of *NFAT5* also increased significantly, which confirms the actual activation of the transcription factor NFAT5 in both cell lines (Fig. 1). However, the expression of *DDC* decreased significantly in PC12 and in SH-SY5Y (Fig. 1), contrarily to the effect reported in renal proximal tubule cells (Hsin et al., 2011). Similar results were observed when both cell lines were exposed to hypertonic

stress with sucrose (Fig. S2). So, for subsequent experiments we only induced hypertonic stress with salt, more used in neural cell culture studies (Bitoun and Tappaz, 2000; Isaacks et al., 1994).

In order to confirm that NFAT5 does not regulate the expression of *DDC* in these cell lines, we silenced *NFAT5* using a specific shRNA that inhibited its expression about 50% (Figs. 3 and 4). Hypertonic stress increased the expression (through NFAT5) of the targets of *NFAT5*-activated transcriptional activity *SMIT* and *TAUT*. When *NFAT5* was silenced, the transcription levels of both genes decreased compared to the control in the two cell lines, indicating an actual downregulation of *NFAT5* activity by the shRNA (Figs. 3 and 4). In contrast, the combination of *NFAT5* inhibition and hypertonic stress resulted in a reduction of *DDC* expression similar to that observed when *NFAT5* is not silenced (using a control shRNA) (Figs. 3 and 4). We observed a 70-80% decrease of *DDC* expression upon 24h of hypertonic shock in PC12 (Fig. 3) and 60-80% upon 6h of hypertonic shock in SH-SY5Y (Fig. 4). These results suggest that, in contrast to *SMIT* and *TAUT*, the expression of the neuronal isoform of *DDC* is regulated through a mechanism that is independent from NFAT5 in PC12 and in SH-SH5Y cells.

Then, we assessed the effect of NFAT5 on the expression of these three genes using a generalized linear model. As reported previously (Aramburu et al., 2006; Halterman et al., 2012; Loyher et al., 2004), NFAT5 showed a significant effect on *SMIT* and TAUT expression in PC12 (P = 2.23E-04 and P = 6.05E-04, respectively) and SH-SY5Y (P = 1.34E-03 and P = 4.62E-02, respectively), both increasing their expression when *NFAT5* is upregulated, reinforcing their value as targets of *NFAT5*-regulated transcriptional activity (Figs. 3 and 4 and Table S2). On the other hand, *NFAT5* had no significant effect on the expression of *DDC* in PC12 (P = 0.13) nor in SH-SY5Y (P = 0.48) (Figs. 3

and 4 and Table S2). Furthermore, we observed that the expression of *DDC* decreased significantly by the effect of the osmotic shock upon 24h in PC12 (P< 2E-16), and at all time points in SH-SY5Y (2E-16 <P< 1.27E-08) (Figs. 3 and 4 and Table S2).

4. Discussion

The present study suggests that NFAT5 is not involved in dopamine neurotransmission in the nervous system (a hypothesis that had previously been formulated (Fernàndez-Castillo et al., 2015; Yang et al., 2018)), at least through the regulation of the expression of *DDC.* Our work in dopaminergic neural-like cells shows NFAT5 does not regulate the neuronal isoform of *DDC,* contrarily to what had been reported in renal tubular cells, where hypertonicity activates NFAT5, which in turn upregulates the non-neuronal isoform of *DDC,* increasing the levels of dopamine (Hsin et al., 2011). Our group previously described that NFAT5 was upregulated by cocaine and that genetic risk variants in this gene were associated with cocaine dependence, a complex psychiatric disorder in which dopaminergic neurotransmission plays an important role (Volkow et al., 2017). We therefore hypothesized that NFAT5 could also regulate *DDC* in the brain, activating its expression and leading to an increase of dopamine, a neurotransmitter involved in reward and motivation, important for addiction, but also related to other neuropsychiatric disorders. However, rather than observing an upregulation of *DDC,* we detected a decrease of *DDC e*xpression after the activation of NFAT5 through hypertonic stress in dopaminergic neural-like cell lines. Subsequently, we demonstrated that this decrease in the expression of the neuronal isoform of *DDC w*as due to hypertonic stress and independent from NFAT5 regulation.

Our results indicate that NFAT5 was expressed and translocated into the cell nuclei, where it becomes an active transcription factor, as shown by immunocytochemistry and by the increased expression of two known target genes and itself (Figs. 1 and 2), consistent with previous studies (Aramburu et al., 2006; Halterman et al., 2012; Loyher et al., 2004). But NFAT5 does not seem to regulate *DDC* expression in dopaminergic

neural cell lines, in contrast to the results reported by Hsin et al. in renal proximal tubule cells (Hsin et al., 2011). Thus, NFAT5 regulates the expression of the non-neuronal isoform of *DDC* but not that of the neuronal isoform. However, we cannot discard that NFAT5 may play a role in the regulation of dopamine levels in the brain not through DDC. We also show that the neuronal isoform of *DDC* is downregulated by osmotic shock in the two investigated neural cell lines. This observation goes in the opposite direction to what is seen in renal tubule cells, where dopamine has a role in regulating natriuresis. One possible explanation may be that the neuronal isoform of *DDC* is not involved in the osmoadaptive response mediated by dopamine and *DDC* would be downregulated as part of the transcription repression generated by a cell adaptation process (Burg et al., 2007) in neural cell lines.

There are some strengths and limitations in our study that should be discussed. Strengths: (i) Comparable results have been obtained for both dopaminergic neural cell lines studied (PC12 and SH-SY5Y), (ii) Expression changes are maintained up to 48 hours, (iii) Similar expression changes were obtained when hypertonic stress was induced with sucrose or NaCl at the same osmolality, (iv) We confirmed that both cell lines (PC12 and SH-SY5Y) express only the neuronal isoform of the *DDC*. Limitations: (i) The mechanism by which hypertonic stress modulates the expression of the neuronal isoform of *DDC* in PC12 and in SH-SH5Y cells has not been studied, (ii) NFAT5 may be involved in dopamine levels regulation through another mechanism not considered in the present study, (iii) Results obtained in these two neural cell lines may differ from mechanisms in the brain.

To sum up, we observed that the expression of the neuronal isoform of DDC is independent from NFAT5 regulation in two dopaminergic neural cell lines (PC12 and SH-SY5Y) upon hypertonic stress.

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FIGURE LEGENDS

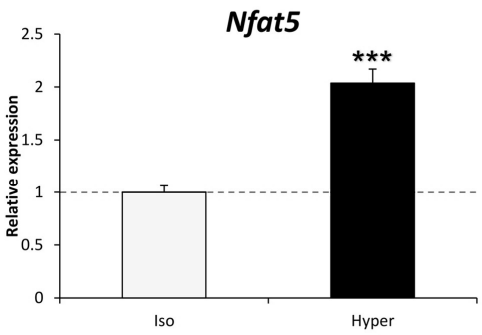
Fig. 1. **Gene expression response to hypertonic stress.** Cells were exposed to an osmotic shock with salt (NaCl) at 550 mOsm/kg during 6h. A) PC12 cell line B) SH-SY5Y cell line. Mean and SD is shown. *** $P< 0.001$ when compared to isotonic condition.Expression levels were normalized using *GAPDH* expression. Similar results were obtained when normalizing with *PGK1*.

Fig. 2. Immunocytochemistry images showing the translocation of NFAT5 (in red) from the cytoplasm to the nucleus of the cells after a hypertonic stress. Cells were exposed to an osmotic shock with salt (NaCl) at 550 mOsm/kg during 6h or 24h. NFAT5 (TonEBP) was visualized using an antibody targeting its C-terminal segment (red), DAPI (blue) was used to stain the nuclei and WGA (green) was used to stain the membrane. Scale bar: 10 μm.

Fig. 3. **Gene expression response to hypertonic stress in PC12 cells.** Cells were exposed to an osmotic shock at 550mOsm/kg during a 6, 24, 36 or 48h treatment with salt (NaCl), with or without *NFAT5* silencing. Two different shRNA were used, one against *Nfat5* (shNFAT5) and a scrambled shRNA as a control (shC). Mean and SD is shown. Expression levels were normalized using *Gapdh* expression.

Fig. 4. **Gene expression response to hypertonic stress in SH-SY5Y cells.** Cells were exposed to an osmotic shock at 550mOsm/kg during a 6, 24, 36 or 48h treatment with salt (NaCl), with or without *NFAT5* silencing. Two different shRNA were used, one against *NFAT5* (shNFAT5) and a scrambled shRNA as a control (shC). Mean and SD is shown. Expression levels were normalized using *GAPDH* expression.

A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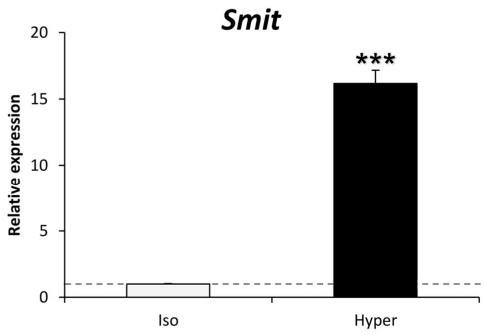
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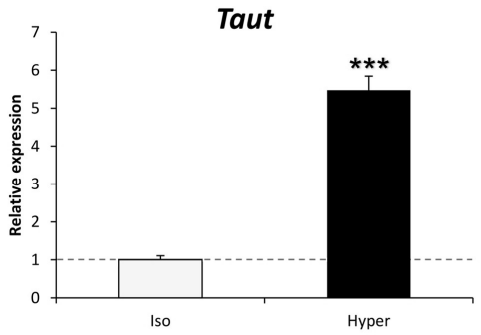
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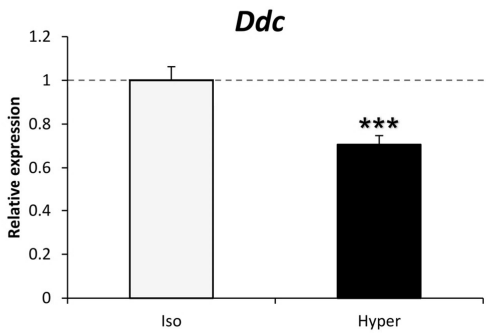
Ddc
Relative expression
1.2
1
0.8
0.6
0.4
0.2
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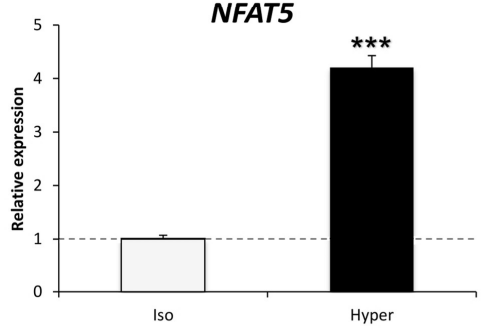
Hyper

B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Relative expression
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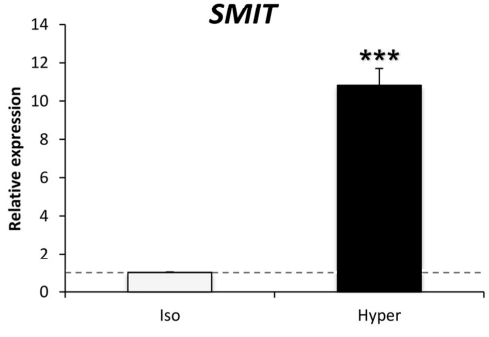
4
3
2
1
0
***
Iso
Hyper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Relative expression
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12
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8
6
4
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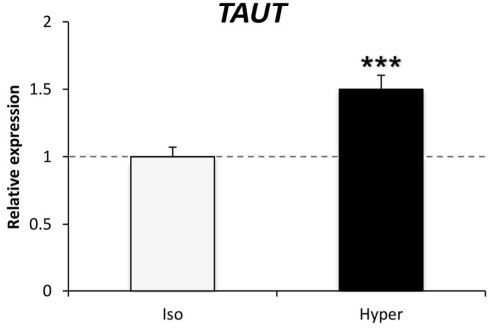
0
***
Iso
Hyper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Relative expression
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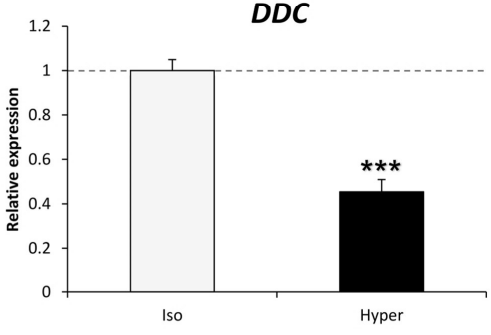
1
0.5
0
***
Iso
Hyper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Relative expression
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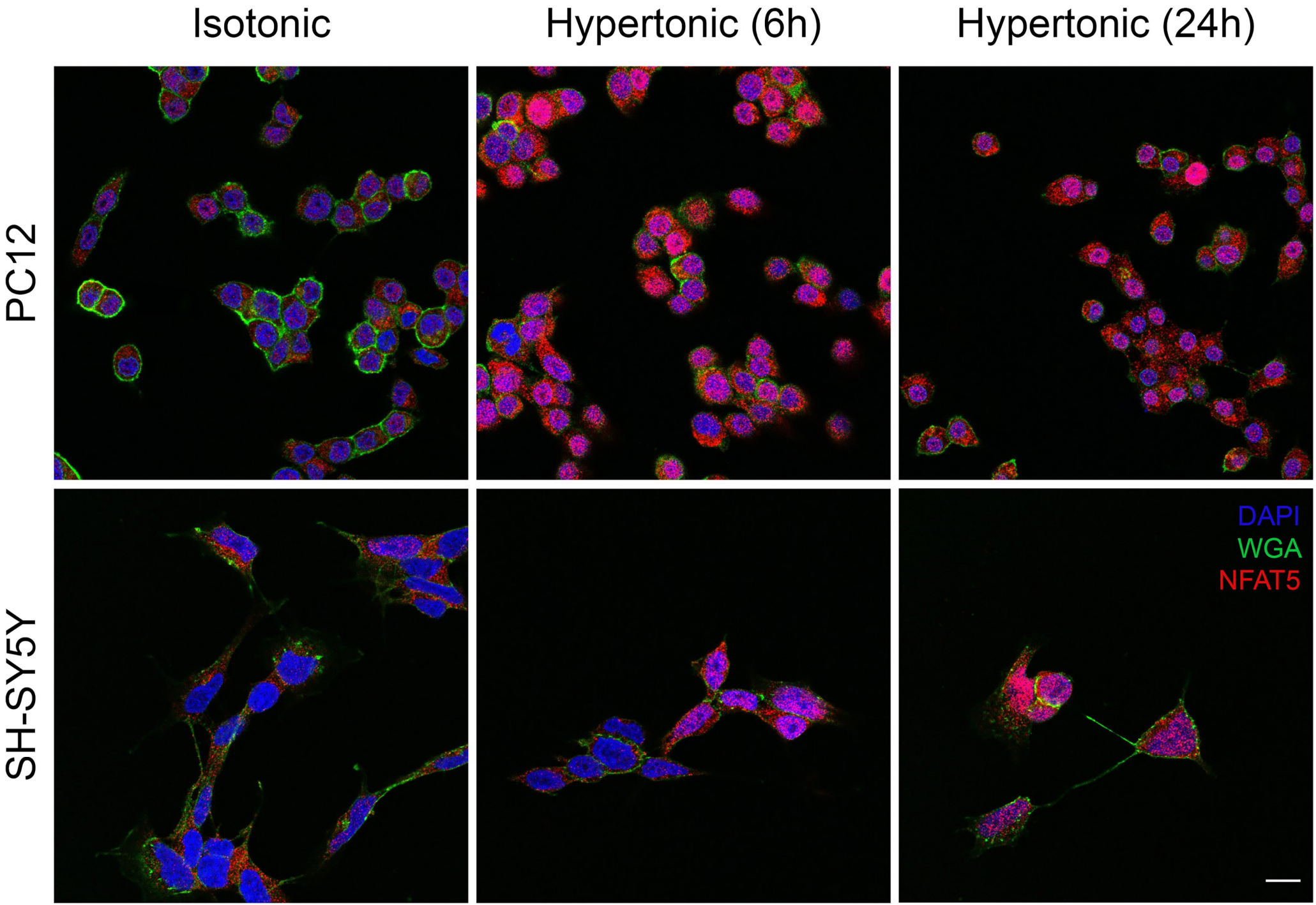
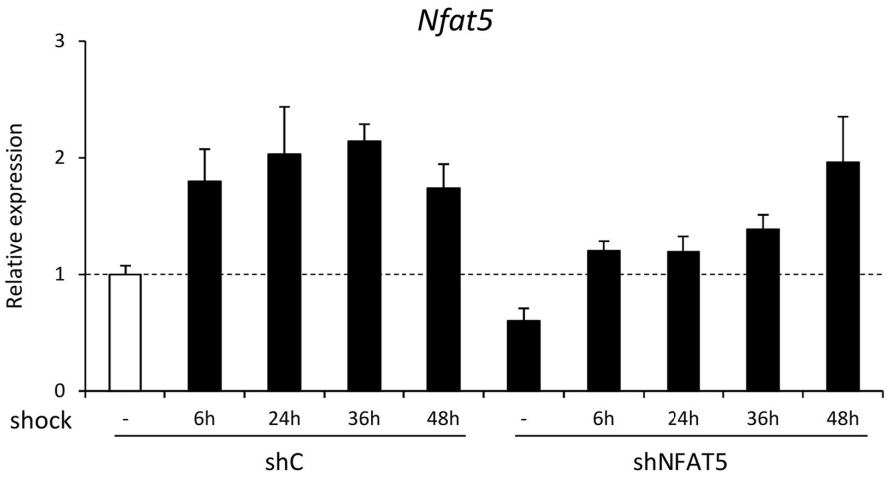
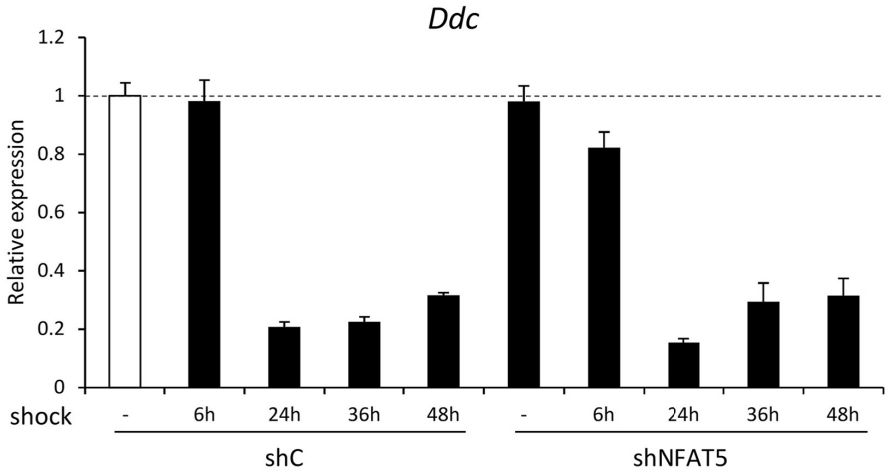
0.8
0.6
0.4
0.2
0
***
Iso
Hyper


Isotonic
Hypertonic (6h)
Hypertonic (24h)
PC12
SH-SY5Y
DAPI
WGA
NFAT5


Nfat5
Relative expression
3
2
1
0
shock
-
6h
24h
36h
48h
-
6h
24h
36h
48h
shC
shNFAT5


Ddc
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
shock
-
6h
24h
36h
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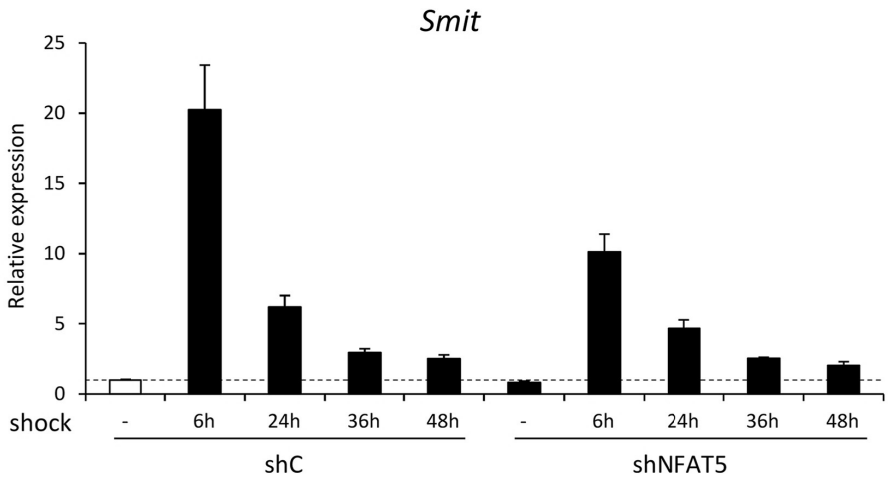
-
6h
24h
36h
48h
shC
shNFAT5


Smit
Relative expression
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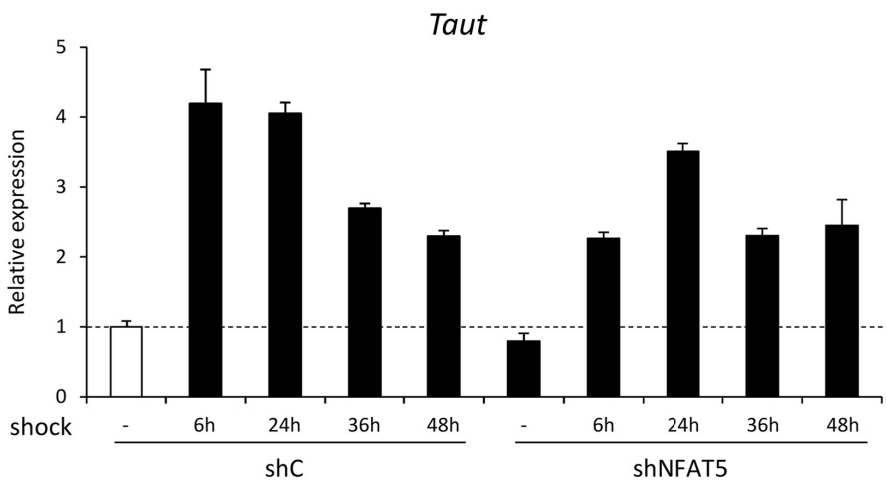
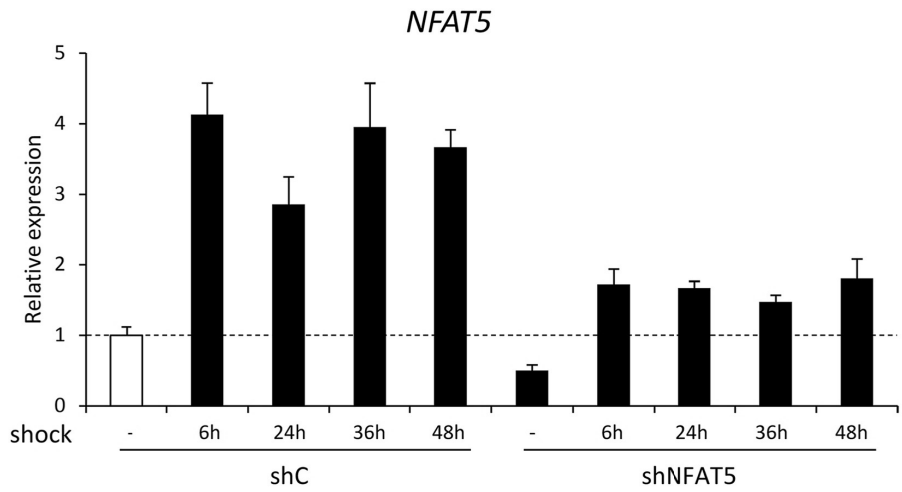
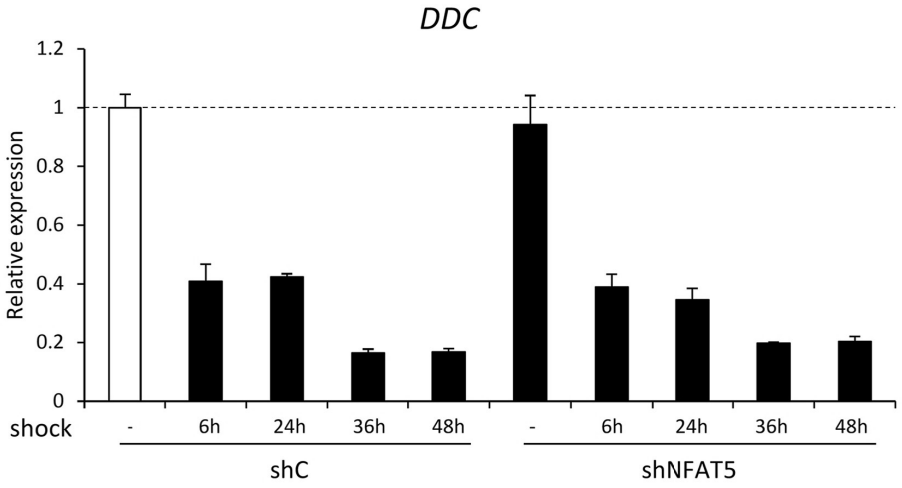
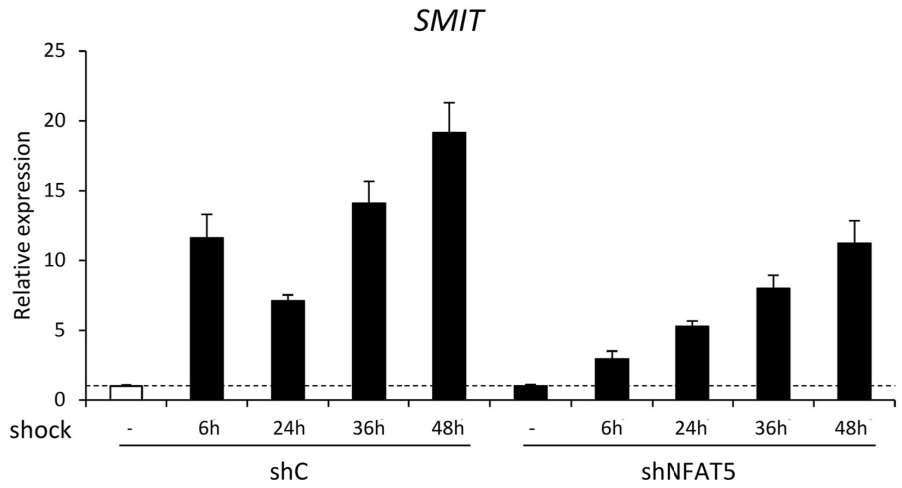
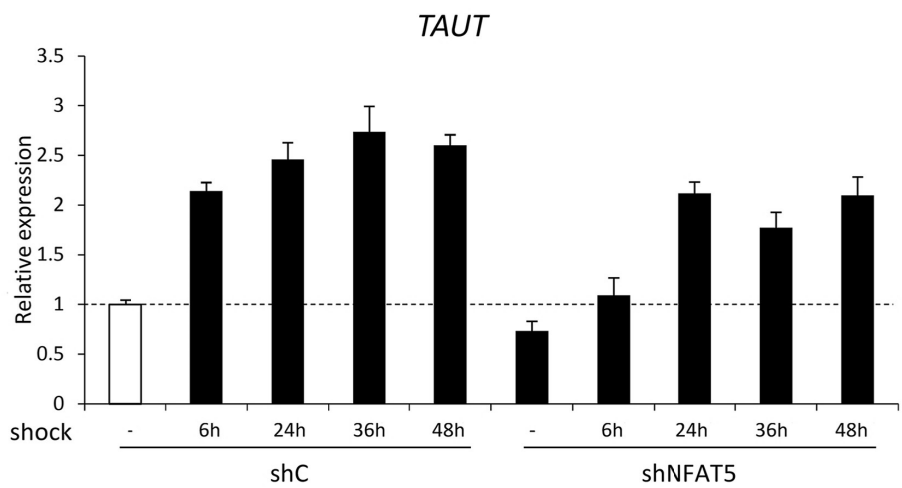
20
15
10
5
0
shock
-
6h
24h
36h
48h
-
6h
24h
36h
48h
shC
shNFAT5


Taut
Relative expression
5
4
3
2
1
0
shock
-
6h
24h
36h
48h
-
6h
24h
36h
48h
shC
shNFAT5


NFAT5
Relative expression
0
1
2
3
4
5
shock
-
6h
24h
36h
48h
-
6h
24h
36h
48h
shC
shNFAT5


DDC
Relative expression
0
0.2
0.4
0.6
0.8
1
1.2
shock
-
6h
24h
36h
48h
-
6h
24h
36h
48h
shC
shNFAT5


SMIT
Relative expression
0
5
10
15
20
25
shock
-
6h
24h
36h
48h
-
6h
24h
36h
48h
shC
shNFAT5


TAUT
Relative expression
0
0.5
1
1.5
2
2.5
3
3.5
shock
-
6h
24h
36h
48h
-
6h
24h
36h
48h
shC
shNFAT5

SUPPLEMENTARY MATERIAL

Table S1. Primers used for the *DDC* isoform analysis and for the real time quantitative PCR.

Human		
Gene	**Forward (5' – 3')**	**Reverse (5' – 3')**
Non-neuronal *DDC*	AAGGAATTCGAATTTCCAGCAT	GCGGATCCTCAATGCCTTCCATGTAGTT
Neuronal *DDC*	TTCTGTGCCTCTTAACTGTCACTG	GCGGATCCTCAATGCCTTCCATGTAGTT
NFAT5	TATTTGATGCCGACAGTGCC	GCTCCTTTCCCTCACTTTTAAC
DDC	ACCACAACATGCTGCTCCTTTG	CATTCAGAAGGTGCCGGAACTC
SMIT	CACTCGCCGATCCTCCAG	ACTCTCCACAAGACCATCAGC
TAUT	CACCCAGGCTCTCTGAAATG	GACCAGCAGCACCAGGAG
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
PGK1	ACCGAATCACCGACCTCTCT	TCTCTCCAGCCACTAAGCCA
Rat		
Non-neuronal *Ddc*	TTGCAGAGCTGGACTGAGTG	ACACTGGACGTCCCTCAATG
Neuronal *Ddc*	GCAAGAGAGCGAATAGAGAGGA	ACACTGGACGTCCCTCAATG
Nfat5	CGAGTAAAGCCACACGGATTC	ATTCTGGCCTCAACATCAGC
Ddc	GTTGTCACCCTAGGAACCACATC	CGCCATTCAGAAGATACCGGAAC
Smit	GCTCATGCCAAAGGTTCTACTC	CTGCTTCCACACACTTGCATG
Taut	GCCTACCCAAAAGCTGTGACC	CCCGACGATAACCCTTCCTTAG
Gapdh	ATGGTGAAGGTCGGTGTGA	CTTGCCGTGGGTAGAGTCAT
Pgk1	GCAAAGACTGGCCAAGCTAC	GCCTCAGCATATTTCTTACTGCT

Table S2. Generalized linear model p-values and effects for the different factors that play a role in gene expression of SMIT, TAUT and DDC.

PC12				**SH-SY5Y**			
	Smit				*SMIT*		
	Effect	P value	Sig.		Effect	P value	Sig.
Intercept	-0.259	6.70E-04	***	**Intercept**	-0.194	4.97E-03	**
***Nfat5* expression**	0.258	2.23E-04	***	***NFAT5* expression**	0.188	1.34E-03	**
Shock 6h	2.792	< 2E-16	***	**Shock 6h**	1.866	1.28E-13	***
Shock 24h	1.552	< 2E-16	***	**Shock 24h**	1.620	< 2E-16	***
Shock 36h	0.785	1.76E-11	***	**Shock 36h**	2.095	3.78E-16	***
Shock 48h	0.732	5.95E-14	***	**Shock 48h**	2.454	< 2E-16	***
shNFAT5	-0.090	7.47E-02		**shNFAT5**	0.106	6.97E-02	
Shock 6h and shNFAT5	-0.446	2.51E-07	***	**Shock 6h and shNFAT5**	-1.041	6.46E-10	***
Shock 24h and shNFAT5	0.025	7.45E-01		**Shock 24h and shNFAT5**	-0.183	5.66E-02	
Shock 36h and shNFAT5	0.133	8.92E-02		**Shock 36h and shNFAT5**	-0.207	1.41E-01	
Shock 48h and shNFAT5	-0.195	2.40E-02	*	**Shock 48h and shNFAT5**	-0.294	1.29E-02	
	Taut				*TAUT*		
	Effect	P value	Sig.		Effect	P value	Sig.
Intercept	-0.207	1.43E-03	**	**Intercept**	-0.099	7.73E-02	
***Nfat5* expression**	0.204	6.05E-04	***	***NFAT5* expression**	0.094	4.62E-02	*
Shock 6h	1.269	< 2E-16	***	**Shock 6h**	0.472	3.24E-03	**
Shock 24h	1.192	< 2E-16	***	**Shock 24h**	0.729	2.17E-09	***
Shock 36h	0.762	3.57E-13	***	**Shock 36h**	0.731	6.75E-06	***
Shock 48h	0.684	3.23E-15	***	**Shock 48h**	0.711	2.34E-06	***
shNFAT5	-0.149	9.80E-04	***	**shNFAT5**	-0.266	1.08E-06	***
Shock 6h and shNFAT5	-0.340	2.65E-06	***	**Shock 6h and shNFAT5**	-0.192	9.57E-02	
Shock 24h and shNFAT5	0.176	1.24E-02	*	**Shock 24h and shNFAT5**	0.229	5.09E-03	**
Shock 36h and shNFAT5	0.145	3.24E-02	*	**Shock 36h and shNFAT5**	0.066	5.70E-01	
Shock 48h and shNFAT5	0.160	3.03E-02	*	**Shock 48h and shNFAT5**	0.223	2.30E-02	*
	Ddc				*DDC*		
	Effect	P value	Sig.		Effect	P value	Sig.
Intercept	0.952	< 2E-16	***	**Intercept**	-0.029	5.88E-01	
***Nfat5* expression**	0.048	1.34E-01		***NFAT5* expression**	0.032	4.76E-01	
Shock 6h	-0.057	1.17E-01		**Shock 6h**	-1.006	1.27E-08	***
Shock 24h	-0.842	< 2E-16	***	**Shock 24h**	-0.921	8.90E-13	***
Shock 36h	-0.829	< 2E-16	***	**Shock 36h**	-1.903	< 2E-16	***
Shock 48h	-0.720	< 2E-16	***	**Shock 48h**	-1.871	< 2E-16	***
shNFAT5	0.000	9.95E-01		**shNFAT5**	-0.051	2.70E-01	
Shock 6h and shNFAT5	-0.131	7.04E-04	***	**Shock 6h and shNFAT5**	0.084	4.46E-01	
Shock 24h and shNFAT5	-0.013	7.29E-01		**Shock 24h and shNFAT5**	-0.119	1.21E-01	
Shock 36h and shNFAT5	0.105	7.15E-03	**	**Shock 36h and shNFAT5**	0.316	6.67E-03	**
Shock 48h and shNFAT5	-0.011	7.94E-01		**Shock 48h and shNFAT5**	0.302	1.83E-03	**

Sig: Significancy; * P < 0.05; ** P < 0.01; *** P < 0.001.

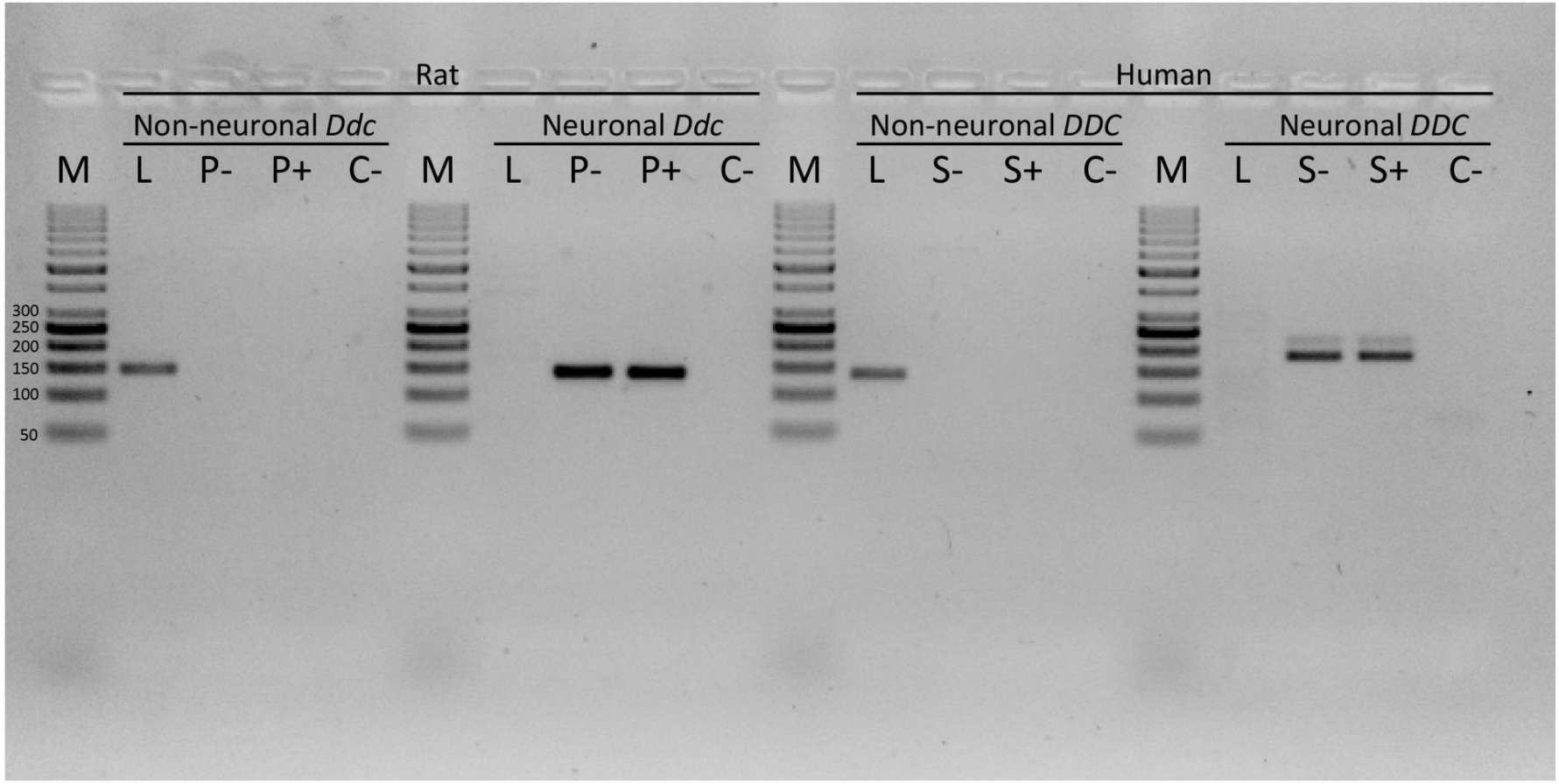


Figure S1. Analysis of the *DDC* isoforms expressed in rat and human. M: 50 bp marker; L: liver; P-: PC12 cells not exposed to osmotic shock; P+: PC12 cells exposed to 6h osmotic shock at 550mOsm/kg; S-: SH-SY5Y cells not exposed to osmotic shock; S+: SH-SY5Y cells exposed to 6h osmotic shock at 550mOsm/Kg; C-: negative control.

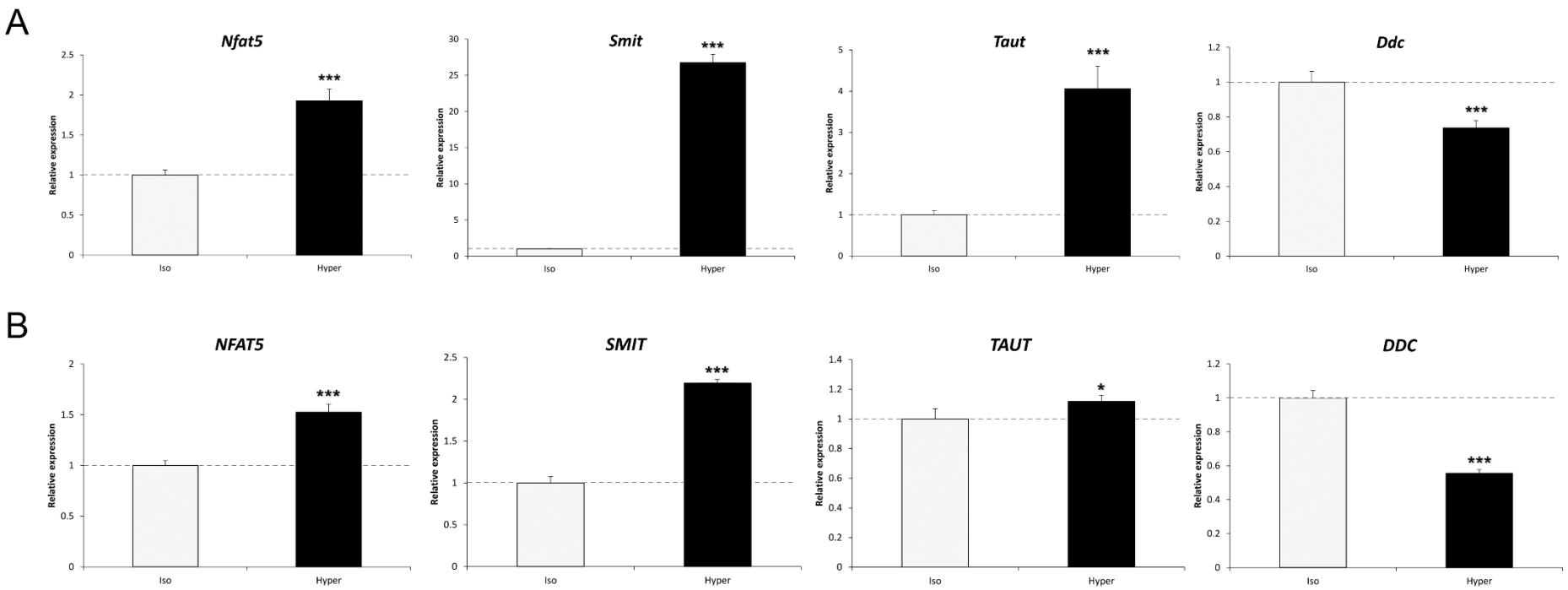


Figure S2. **Gene expression response to hypertonic stress with sucrose.** Cells were exposed to an osmotic shock at 550mOsm/kg during a 6 hours treatment with sucrose compared to non-treated controls. A) PC12 cell line B) SH-SY5Y cell line. Iso: cells exposed to an istotonic medium; Hyper: cells exposed to 6h osmotic shock at 550mOsm/Kg. Mean and SD is shown * $P < 0.05$, *** $P < 0.001$. Expression levels were normalized using *GAPDH* expression. Similar results were obtained when normalizing with *PGK1*.