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Differential regulation of catecholamine synthesis and transport in rat adrenal medulla by fluoxetine treatment

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ABSTRACT

We have recently shown that chronic fluoxetine treatment acted significantly increasing plasma norepinephrine and epinephrine concentrations both in control and chronically stressed adult male rats. However, possible effects of fluoxetine on catecholamine synthesis and re-uptake in adrenal medulla have been largely unknown. In the present study the effects of chronic fluoxetine treatment on tyrosine hydroxylase, a rate-limiting enzyme in catecholamine synthesis, as well as a norepinephrine transporter and vesicular monoamine transporter 2 gene expressions in adrenal medulla of animals exposed to chronic unpredictable mild stress (CUMS) for 4 weeks, were investigated. Gene expression analyses were performed using a real-time quantitative reverse transcription-PCR. Chronically stressed animals had increased tyrosine hydroxylase mRNA levels and decreased expression of both transporters. Fluoxetine increased tyrosine hydroxylase and decreased norepinephrine transporter gene expression in both unstressed and CUMS rats. These findings suggest that chronic fluoxetine treatment increased plasma catecholamine levels by affecting opposing changes in catecholamine synthesis and uptake.

Key words: adrenal medulla, antidepressant, gene expression, norepinephrine transporter, tyrosine hydroxylase.

INTRODUCTION

The syndrome of major depression, particularly the melancholic type, potentially reflects dysregulation of the stress response (Chrousos and Gold 1998). The body responds to stress by activating the sympathoadrenal system and secreting epinephrine (E) and norepinephrine (NE) in the "fight-or-flight" response (Cannon 1929). Elevated levels of catecholamines were also detected in patients with signs of depression (Hamer et al. 2007). Increased release of catecholamines during stress could result from the changes in catecholamine synthesis, storage and/or release in adrenal medulla. Tyrosine hydroxylase (TH) is a rate-limiting enzyme in catecholamine biosynthetic process. Catecholamines are stored in secretory granules of adrenal chromaffin cells and released by regulated exocytosis. Two membrane carriers are involved in their subsequent reuptake and storage. The antidepressant- and cocaine-sensitive Na^+, Cl^--dependent norepinephrine transporter (NET) functions at the plasma membrane, mediates both the clearance of catecholamines and their recovery into the vesicles for subsequent re-use. Immunocytochemistry of rat adrenal tissue showed that NET staining was co-localized with TH in

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medullary chromaffin cells (Kippenberger et al. 1999). The uptake of monoamines from the cytoplasm into secretory granules is mediated by vesicular monoamine transporters (VMATs) and requires an ATPase-generated proton gradient (Erickson et al. 1996, Eiden 2000). Several studies indicate that VMATs play a critical role not only in sorting, storing and releasing of monoamines, but also in fine-tuning of neuronal and endocrine informational output (Henry et al. 1994, Eiden et al. 2004). Two isoforms of the vesicular monoamine transporter (VMAT1 and VMAT2) have been characterized in rodent tissue (Erickson et al. 1992). VMAT1 is widely expressed in all adrenal chromaffin cells, while VMAT2 is co-localized with TH enzyme (Tillinger et al. 2010). A subpopulation of chromogranin A -expressing chromaffin cells of the adrenal medulla also express VMAT2 (Weihe et al. 1994). Chromogranins are highly efficient systems directly involved in monoamine accumulation and in the exocytotic release of catecholamines (Borges et al. 2010).

Numerous authors observed the changes in the synthesis of catecholamines in the brain after fluoxetine treatment, but the data on the effect of this drug on catecholamine synthesis in the adrenal glands, are sparse. While administration of tricyclic antidepressants causes decrease (Zavosh et al. 1999) or in contrast, increase of NET mRNA (Szot et al. 1993), SSRI antidepressants including fluoxetine have been reported to express no effect on NET mRNA expression (Benmansour et al. 2004, Shinkai et al. 2005). Blardi et al. (2005) detected increased levels of catecholamines in depressed patients receiving fluoxetine (SSRI) for 40 days. We have also found that chronic fluoxetine treatment leads to a marked increase in the amount of plasma catecholamines both in control and stressed rats (Dronjak et al. 2007).

These results indicated the need to explore possible effects of fluoxetine on adrenal function using a chronic unpredictable mild stress (CUMS) paradigm in rats. The CUMS model was chosen because it induces many behavioral and biochemical characteristics that are similar to human depression, including anhedonia, reduced body weight and glucocorticoid hypersecretion (Willner et al. 1992, Papp et al. 1994, Dronjak et al. 2007). All these symptoms are reversed by chronic antidepressant treatment, indicating that this model has good predictive validity (Muscat et al. 1992). In order to examine whether the effects of fluoxetine on plasma catecholamine levels could be due to changes in their synthesis and/or release in adrenal medulla, the present study was undertaken to determine differences in TH, VMAT2 and NET gene expression in control and stressed rats after chronic fluoxetine treatment.

MATERIALS AND METHODS

Animals

Adult Wistar male rats weighing 280-320g at the onset of experiments and maintained in a temperature-controlled room (21±1.0°C) and 12h/12h light/dark cycle, were used. The care was taken to minimize the pain and discomfort of the animals according to the recommendations of the Ethical Committee of the "Vinca" Institute, Belgrade, which are in accordance with the Guide for Care and Use of Laboratory Animals of the National Institute of Health, Bethesda, MD, U.S.A. Fluoxetine (fluxilan®, Aeigis LTD, Cyprus) dissolved in sterile water and sonicated for approximately 10 min, was prepared *ex tempore*. Animals that were subjected to CUMS, according to method by Grippo et al. (2002), received daily injections of vehicle (sterile water) or fluoxetine (10 mg/kg) by i.p. route during 4 weeks. The fluoxetine dose was selected based upon the work of Reneric et al. (2002) and our previous study (Spasojevic et al. 2008). The control group was also receiving daily injections of vehicle. Upon completion of 4 weeks, the animals exposed to CUMS and controls were decapitated, the adrenal medulla rapidly dissected, frozen in liquid nitrogen and stored at − 70°C until analyzed.

RNA Isolation and cDNA Synthesis

Total RNAs were isolated using TRIZOL reagent (Invitrogen, CA, U.S.A.). Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Bead (AP, Biotech) and pd $(N)_6$ primer according to manufacturer's protocol.

Real-Time RT-PCR

TaqMan PCR assays were carried out using Assay-on-Demand Gene Expression Products (Applied Biosystems, USA) for TH (ID:Rn00562500-m1), NET (ID: Rn00580207_m1) and VMAT2 (ID: Rn00564688_m1). The reactions were performed in a 25 µl reaction mixture containing 1x TaqMan Universal Master Mix with AmpErase UNG, 1x Assay Mix (Applied Biosystems) and cDNA template (10 ng of RNA converted to cDNA). PCR reactions were performed in the ABI Prism 7000 Sequence Detection System at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. A reference, endogenous control, was included in each analysis to correct the differences in the inter-assay amplification efficiency and all transcripts were normalized to cyclophyline A (ID:Rn 00690933) expression. Quantification was done using the $2^{-\Delta\Delta Ct}$ method according to Livak and Schmittgen (2001).

Statistical Analysis

The results are reported as means ± S.E.M. Significance of the differences between the groups in gene expression levels of the examined catecholamine synthesising enzyme and transporters were estimated by Two-way ANOVA test, followed by the Tukey post hoc test. Statistical significance was accepted at $p<0.05$.

RESULTS

The exposure of rats to CUMS displayed low, although significant rise of 43% ($p<0.05$) in TH mRNA levels (Fig.1). Fluoxetine treatment also significantly increased TH mRNA expression both in control and stressed animals (by 43% and 34% respectively, $p<0.05$) in comparison to vehicle group.

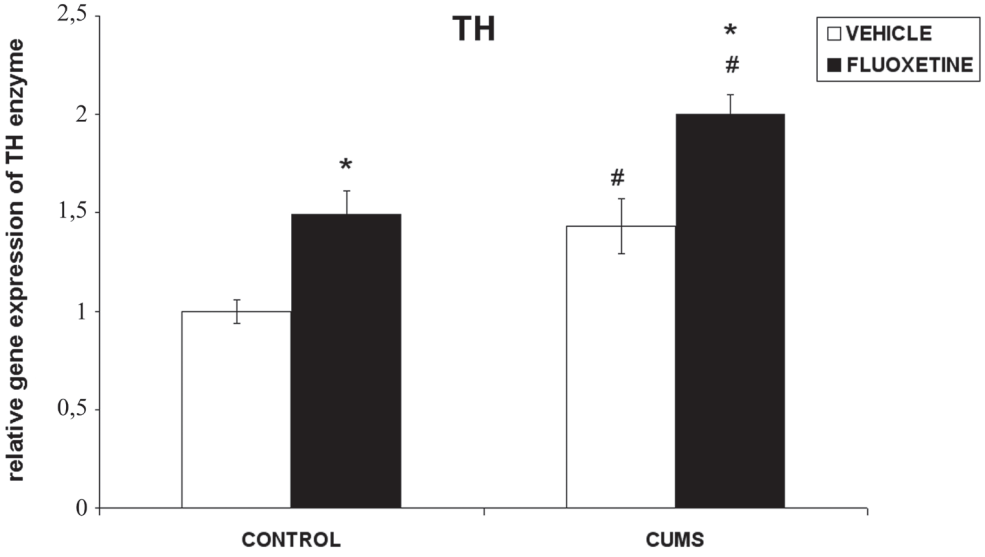

Figure 1 - Effect of chronic treatment with fluoxetine on tyrosine hydroxylase (TH) mRNA levels in adrenal medulla of rats exposed to CUMS for 4 weeks. The values are means ±S.E.M. of 6-8 rats. Statistical significance: # $p< 0.05$ CUMS *vs.* unstressed control; * $p<0.05$ placebo *vs.* fluoxetine (Tukey test). The final result was expressed as fold change relative to the calibrator and normalized to cyclophyline A.

Analysis of the data also displayed a significant chronic stress-related change ($F_{(1,16)}$ = 18.98, p<0.001) in VMAT2 mRNA levels. VMAT2 mRNA expression in medulla was decreased by 41% (p<0.01) after exposure of animals to CUMS, while fluoxetine expressed no effect (Fig. 2).

The results presented in Fig. 3. indicate a major influence of CUMS ($F_{(1,16)}$ = 17.94, p<0.001) on NET mRNA levels in the medulla. Post-hoc analysis demonstrated a significant decrease in stressed rats (by 41%, p < 0.05) in comparison to the controls. Long-term administration of the antidepressant altered NET mRNA content ($F_{(1,16)}$ = 20.72, p < 0.001) (Fig. 3). Fluoxetine significantly diminished NET mRNA levels both in control and stressed rats (by 43%, p<0.01 and 50%, p<0.05, respectively).

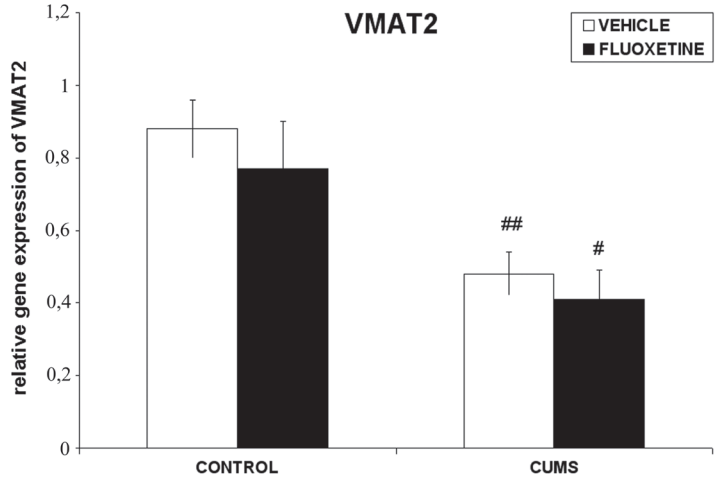

Figure 2 - Effect of chronic treatment with fluoxetine on vesicular monoamine transporter 2 (VMAT2) mRNA levels in adrenal medulla of rats exposed to CUMS for 4 weeks. The values are means ±S.E.M. of 6-8 rats. Statistical significance: # p< 0.05; ## p<0.01 CUMS *vs.* unstressed control (Tukey test). The final result was expressed as fold change relative to the calibrator and normalized to cyclophyline A.

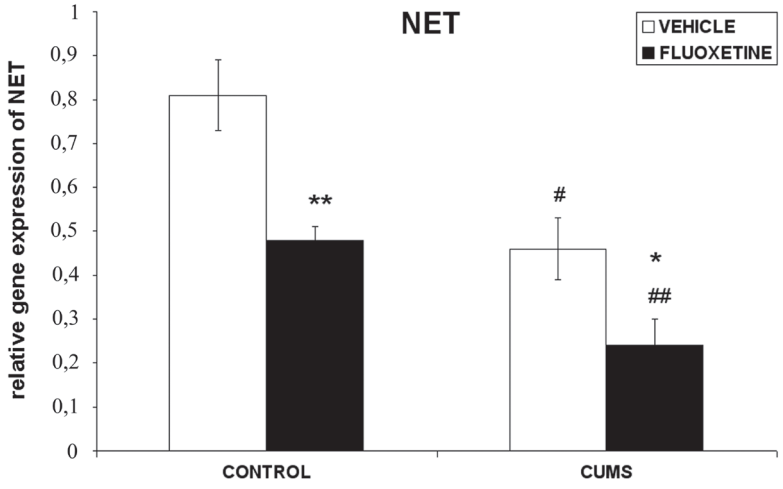

Figure 3 - Effect of chronic treatment with fluoxetine on norepinephrine transporter (NET) mRNA levels in adrenal medulla of rats exposed to CUMS for 4 weeks. The values are means ±S.E.M. of 6-8 rats. Statistical significance: # p< 0.05; ## p<0.01 CUMS *vs.* unstressed control; * p<0.05, ** p<0.01 placebo *vs.* fluoxetine (Tukey test). The final result was expressed as fold change relative to the calibrator and normalized to cyclophyline A.

DISCUSSION

In the present study, the effects of fluoxetine a widely used antidepressant on the levels of mRNAs encoding TH, a rate-limiting enzyme in catecholamine biosynthesis and two transporters NET and VMAT2 in adrenal medulla of CUMS rats, were examined. CUMS led to increased TH mRNA levels in the adrenal medulla. Similar to our results, Serova et al. (1998) had previously found elevated basal levels of TH mRNA in the adrenal medulla in Flinder's Sensitive Line (FSL), a genetic rat model of depression. Numerous authors have confirmed corticosterone regulation of TH mRNA (Kumai et al. 2000, Tank et al. 1986). Animals exposed to CUMS were shown to have elevated plasma corticosterone levels (Dronjak et al. 2007), which could be the cause of the observed changes in the TH gene expression. Among SSRIs, fluoxetine uniquely increased extracellular NE and serotonin (5-HT) levels (Pozzi et al. 1999, Bymaster et al. 2002). According to Brady et al. (1992) and Heydendael and Jacobson (2010) this antidepressant stimulates the synthesis of NE in the brain by increasing TH mRNA levels. Our results also demonstrated that repeated fluoxetine administration led to increased expression of TH mRNAs in the adrenal medulla. Thome et al. (2000) reported that chronic treatment of rats with a number of clinically effective antidepressants significantly increased cAMP-controlled gene transcription, as well as phosphorylation (and activation) of the transcription factor, a cAMP response element binding protein (CREB), in several brain regions. Fluoxetine was shown to increase cAMP levels (Edgar et al. 1999) and to promote CRE-dependent gene transcription in rat brain (Duman 2004). This transcription factor is essential for the proper TH gene regulation in the adrenal gland (Sabban et al. 2004). Besides this signaling pathway, Shinkai et al. (2007) reported that milinacipran, a serotonin norepinephrine reuptake inhibitor, activates TH through a p44/42 mitogen-activated protein kinase (MAPK)-dependent pathway in cultured bovine adrenal medullary cells.

Our results show that VMAT2 and NET mRNA levels are reduced in rats exposed to CUMS. Tillinger et al. (2010) have recently observed that VMAT2 is up-regulated in chromaffin cells *in vivo* following immobilization stress. The differences of these and our results related to VMAT2 gene expression could be ascribed to the differences in the types of stress paradigms. On the other hand, our finding of reduced NET mRNA levels in medulla of chronically stressed rats agrees well with that of Cubells et al. (1995) who reported that reserpine-induced activation led to increased TH mRNA levels but at the same time to a significant decrease in NET mRNA levels both in locus coeruleus and adrenal medulla. Wakade et al. (1996) provided the evidence that glucocorticoids decreased NET mRNA and functional activity in rat chromaffin cells maintained in culture, primarily through the changes in activator protein-1 (AP-1) motif/binding protein interactions. Reduced expression of these transporters could result from increased demand for catecholamine secretion, because it would decrease the reuptake of NE and thus enhance NE release into the circulation. Finally, the observed opposite TH and NET gene expression regulation in the adrenals of CUMS rats would be anticipated to contribute to the maintenance of stress response.

Yasumoto et al. (2009) suggested that fluoxetine was able to deplete catecholamines by inhibiting the activity of VMAT2. However, our results did not indicate the impact of this drug on the gene expression of VMAT2 transporter. In the present experiments we detected a significant decrease in NET mRNA after chronic fluoxetine treatment. This is the first direct evidence clearly demonstrating the effect of fluoxetine on gene expression of NET in rat adrenal medulla. These results indicate that we should not exclude the possibility that the inhibition of NE uptake, contributes to the increased NE concentration in plasma, induced by fluoxetine. Rodent NE transporter promoter does not possess a cAMP response element, but it does have an

AP-1 site (Fritz et al. 1998) and immediate-early gene products that bind to this site (Fos and Jun) and can be induced by cAMP (Herdegen 1996). Direct treatment of bovine adrenal chromaffin cells *in vitro* with the agents that elevate or mimic intracellular cAMP was reported to reduce NE uptake (Bunn et al. 1992). Besides, nerve growth factor (NGF) and other neurotrophins were also present in developing and mature adrenal chromaffin cells (Suter-Crazzolara et al. 1996). After treatment of rat pheochromocytoma cell line PC12 with NGF, Ikeda et al. (2001) demonstrated decreased NET mRNA, but increased TH mRNA levels. On the other hand, chronic application of fluoxetine led to NFG enhancement in the frontal cortex (Hassanzadeh and Hassanzadeh 2009).

Amplifying catecholaminergic signaling, observed after fluoxetine treatment, could be associated with cardiovascular side effects. Rotondi et al. (2011) reported the clinical case in which duloxetine may have precipitated "tako-tsubo" cardiomyopathy by increasing plasma catecholamine concentration. Considering the fact that we have recently also recorded a significant increase of β_1-adrenoreceptor mRNA levels in the heart of CUMS rats treated with fluoxetine (Spasojevic et al. 2011), these findings should be taken into account when treating depressed patients with concomitant cardiac disease. Further studies are necessary to confirm our data and establish the functional significance of these results.

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RESUMO

Mostramos recentemente que o tratamento crônico com fluoxetina agiu de forma a aumentar significativamente as concentrações de noradrenalina e adrenalina no plasma tanto nos controles quanto em ratos machos adultos cronicamente estressados. No entanto, os possíveis efeitos da fluoxetina na síntese de catecolaminas e recaptação na medula adrenal são em grande parte desconhecidos. No presente estudo, os efeitos do tratamento crônico com fluoxetina sobre a tirosina hidroxilase, uma enzima limitante da velocidade de síntese de catecolaminas, bem como a expressão gênica de um transportador de norepinefrina e do Transporter 2 de monoamina vesicular na medula adrenal foram investigados em animais expostos ao estresse leve crônico imprevisível (CUMS) durante 4 semanas. Análises de expressão gênica foram realizadas utilizando transcrição reversa-PCR quantitativa em tempo real. Animais com estresse crônico tiveram níveis aumentados de mRNA para tirosina hidroxilase e a expressão diminuída de ambos os transportadores. A fluoxetina aumentou a tirosina hidroxilase e diminuiu a expressão do gene transportador de norepinefrina tanto em ratos não estressados quanto em CUMS. Estes resultados sugerem que o tratamento crônico com fluoxetina aumentou os níveis plasmáticos de catecolaminas por afetar alterações opostas na síntese e absorção de catecolaminas.

Palavras-chave: medula adrenal, antidepressivos, expressão gênica, transportador de norepinefrina, tirosina hidroxilase.

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