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Fluoxetin Upregulates Connexin 43 Expression in Astrocyte

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ABSTRACT

Introduction: Recent studies have shown that astrocytes play major roles in normal and disease condition of the central nervous system including multiple sclerosis (MS). Molecular target therapy studies in MS have revealed that connexin-43 (Cx43) and Aquaporin-4 (AQP4) contents of astrocytes undergo expression alteration. Fluoxetine had some effects in MS patients unrelated to its known antidepressant effects. Some of fluoxetine effects were attributed to its capability of cAMP signaling pathway stimulation. This study aimed to investigate possible acute effects of fluoxetine on Cx43 and AQP4 expression in astrocyte.

Methods: Astrocytoma cells were treated for 24 hours with fluoxetine (10 and 20 µg/ml) with or without adenyl cyclase (AC) and protein kinase A (PKA) inhibition. Cx43 expression at both mRNA and protein levels and AQP4 expression at mRNA level were evaluated.

Results: Acquired results showed that fluoxetine with and without AC and PKA inhibition resulted in Cx43 up-regulation both in mRNA and protein levels, whereas AQP4 expression have not changed.

Discussion: In conclusion, data showed that fluoxetine alone and in the absence of serotonin acutely up-regulated Cx43 expression in astrocytes that can be assumed in molecular target therapy of MS patients. It seems that cAMP involvement in fluoxetine effects need more researches.

1. Introduction

Astrocytes are major cell types in brain with numerous important functions. In contrast to previous believes, they are not only supporting cells but also recent findings have shown their biological importance in physio- and pathophysiological conditions (He and Sun 2007). Multiple sclerosis is one of the diseases that based on recent findings astrocyte involvement have highlighted in progression of this disease. Although oligodendrocytes are known as myelinating cell type, astrocytes provide essential roles in establishing and maintaining a communication path within the inflamatory demyelinated lesions and so creating a favorable environment for remyelination (Keyser, Laureys

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et al. 2010). These communication networks provide by some astrocyte elements including Cx43 and AQP4 (Nedergaard, Ransom et al. 2003).

Studies have shown that expression of Cx43 and AQP4 altered in multiple sclerosis animal models and patients (Brand-Schieber, Beelitz et al. 2005; Li, Zhang et al. 2009).

Deletion of Cx43, the primary protein forming gap junction channels in astrocytes, has been shown that led to dysmyelinating Phenotype (Lutz, Zhao et al. 2009). In an Animal Model of Multiple Sclerosis, it was found that Cx43 is down-regulated in inflamed white matter of centeral nervous system (Brand-Schieber, Beelitz et al. 2005).

AQP4 is the most important member of the aquaporin family in astrocytes and has key roles in maintaining water and ion homeostasis. It was suggested that AQP4 may have other functions that is unrelated to its water transport role (Verkman 2009).

Possible involvement of AQP4 in CNS inflammatory diseases has been suggested based on evidences that have demonstrated auto-antibodies existed against AQP4 in patients with neuromyelitis optica (NMO) (Graber1, Levy et al. 2008). Moreover, up-regulation of AQP4 in CNS of animal model of MS provided more support for the possible involvement of AQP4 in CNS inflammatory diseases (Miyamoto, Nagaosa et al. 2009).

In addition, correction of Cx43 and AQP4 expression alterations had relationship with re-myelination. In a guinea pig model of experimental autoimmune encephalomyelitis (EAE), expression of Cx43 was almost absent in all demyelinated lesions, on the other hand, treated animals showed up-regulation of Cx43 in all re-myelinating lesions (Roscoe, Messersmith et al. 2007). With regards to AQP4 expression and EAE, it was shown that AQP4 knockout mice had more attenuated EAE severity (Li, Zhang et al. 2009).

Some previous researches have revealed that enhancing intracellular cAMP signalling pathways in astrocytes in patients with MS may reduces the severity of the disease (Keyser, Zeinstra et al. 2003; Mostert, Admiraal-Behloul et al. 2008). Further more, cAMP signaling pathway relationship with Cx43 and AQP4 expressions have been shown (TenBroek, Lampe et al. 2001; Kortenoeven, Trimpert et al. 2012). Fluoxetine, as a selective serotonin reuptake inhibitor (SSRI), widely used in depression (Rossi, Barraco et al. 2004), has been shown to elevate cAMP levels in astrocytes (Mostert, Admiraal-Behloul et al. 2008) and reduced the development of focal inflammatory lesions (Keyser, Laureys et al. 2010). Kong et al reported that fluoxetine needs AQP4 to induce antidepressive effect (Kong, Sha et al. 2009).

Motivated by the potential effects of fluoxetine on biological pathways in astrocytes, we studied possible effects of fluoxetine on expression of Cx43 and AQP4 in astrocytic cell lines through cAMP and its newly described downstream Epac (exchange protein directly activated by cAMP) (Kawasaki, Springett et al. 1998). As a model system, we used the human astrocytoma cell lines 1321N1 and U87MG, which have been shown to be useful cell lines to investigate signal transduction (Harden, Cotton et al. 1980).

2. Methods

2.1. Materials

All drugs in this study including Fluoxetine hydrochloride, (F132), adenyl cyclase inhibitor, SQ 22,536 (S 153), PKA specific inhibitor (H-89) (B1427) were purchased from Sigma-Aldrich (USA). Qiazol and reverse transcriptase were purchased from Qiagen and vivantis respectively.

2.2. Cell Culture

The human astrocytoma cell lines including 1321N1 and U87MG were obtained from the Stem Cell Technology Research Center, (Tehran, Iran) and were grown in DMEM containing 10% FBS, incubated at 5% CO2 and 37˚C temperature. Cells were plated in six-well plates for RNA extraction analysis. Treatment was done when the cell confluency reach to 70-80% following replacement of medium with DMEM containing 1% FBS.

2.3. Real-time Reverse Transcription-Polymerase Chain Reaction Assay

Relative expressions of Cx43 and AQP4 genes were measured on treated and controls cells 24 hours after treatment. Total cellular mRNA was extracted using the Qiazol (Qiagen) then used for cDNA synthesis by reverse transcriptase (vivantis Cat No: RTPL12). HPRT1 used as the housekeeping gene. The primers were used in the reactions had the following sequences:

Cx43 forward: 5′- GAT GAG GAA GGA AGA GAA G -3′,

Cx43 reverse: 5′- CGC TAG CTT GCT TGT TGT AA -3′,

AQP4 forward: 5′- CTG ATG TCA CTG GCT CAA TAG-3′,

AQP4 reverse :5′- ACC CAA TAT ATC CAA TGG-3′,

Epac forward: 5′- CGA TTC ACT GAC TCC CTT AC -3′,

Epac reverse: 5′- CTT CCA AAT GTG TGA TAG ATT AG -3′,

HPRT1 forward: 5′- CCT GGC GTC GTG ATT AGT G -3′,

HPRT1 reverse: 5′- TCA GTC CTG TCC ATA ATT AGT CC -3′.

Gene expression levels were quantified by Rotor Gene 6000 (Corbett, Concorde, NSW, Australia). The relative expression ratio of genes was calculated using the relative expression software tool (REST).

2.3. Western Blot Analysis

Immunoblotting assay were performed using correspond antibodies for detection of Cx43 and EPAC. Total cell lysates were collected by using ice-cold PBS and homogenization in cold lysis buffer (RIPA buffer). lysate clarified by centrifugation at 12,000 rpm for 10 min, supernatant transferred to a new tube. Protein content was measured using the BCA protein kit (Pierce). Equal amount of protein (25µg) in each sample was loaded to 12.5% standard SDS-PAGE. By using a wet system (Bio-Rad) the proteins were transferred to PVDF membranes. After blocking, membranes were incubated over night with primary antibodies (1/1000), washed three times with TBST 20 (0.5%) incubated with secondary antibody (1/5000) for 1 hour at room temperature. After three washes, detection was performed by applying Pierce ECL Plus Western Blotting Substrate (#32134), emanating chemiluminescence from the membrane for manual x-ray film development. Protein levels were normalized to β-actin.

3. Results

3.1. EPAC expression in Cell Lines

To evaluate possible effect of fluoxetine on expression of EPAC in U87MG and 1321N1 cell lines, we performed Real- time PCR analysis on cDNA obtained from these cells using specific primers for detection of EPAC. We found that fluoxetine did not change the expression of EPAC mRNA in both cell lines (Fig. 1).

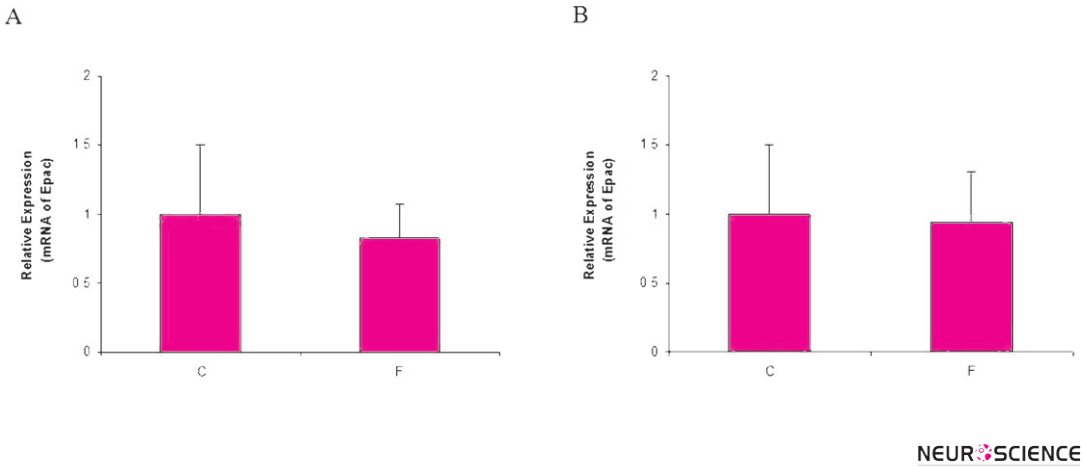

Figure 1. Relative expression of Epac in 1321N1 (Fig. 1A) and U87MG (Fig. 1B) cell lines in un-treated (C) and treated cells with 10 µg/ml fluoxetine (F) HPRT1 was used as internal control.

* P<0.05; ** P<0.01; PTZ, Pentylenetetrazol; Ctrl, Control; SB, SB334867; ORX, Orexin-A; Lido., lidocaine

3.2. Effect of fluoxetine on Cx43 expression

Treatment of cells with 10 and 20 µg/ml of fluoxetine for 24 hours resulted in up-regulation of Cx43 both in mRNA (Fig. 2) and protein (Fig. 3) levels. These effects were not dose dependent. As our data revealed that in 40 µg/ml of fluoxetine complete cell death occurred. To evaluate the specificity of this finding cells pretreated for 45 minutes with SQ 22,536 (20 µg/ml) as an AC inhibitor, and H-89 (10 µg/ml), as PKA inhibitor, these

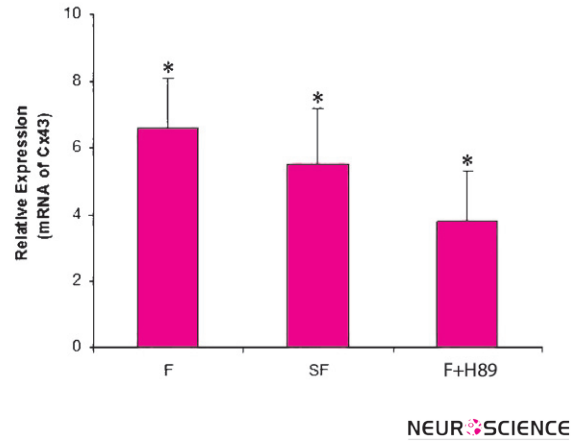

Figure 2. Fluoxetine effects on Cx43 mRNA expression with and without cAMP-PKA signaling pathway. 1321N1 cells treated with F (Fluoxetine), SF (AC inhibitor+ Fluoxetine) and F+H89 (Fluoxetine + PKA inhibitor) for 24 hours and changes in transcript amount expression were determined by real-time qPCR. It revealed an up-regulation of Cx43 in all of groups (*p < 0.01 compared to untreated controls).

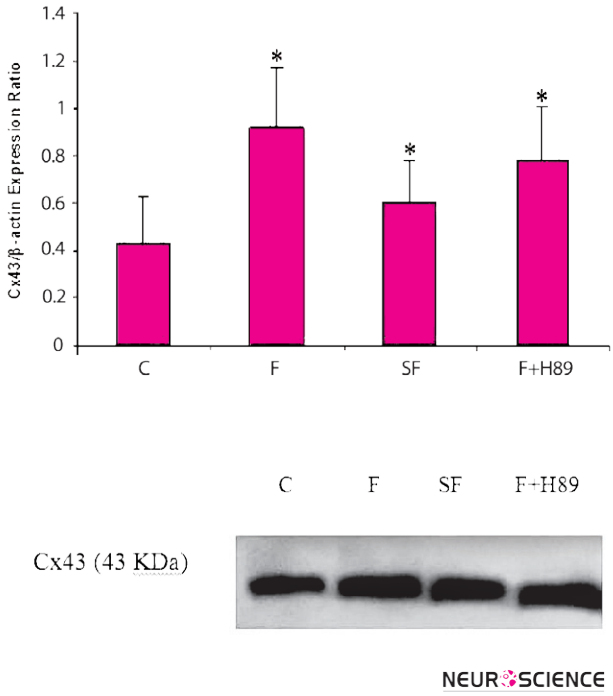

Figure 3. Expression of Cx43 protein in the 1321N1 cells. Cx43 protein levels were normalized to that of β-actin protein. Data are expressed as means ± S.E.M. Each immunoblotting was performed in duplicate to increase the reliability of the measurements. Lower Panels show representative immunoblots of Cx43 (43 kDa). C (un-treated), F (

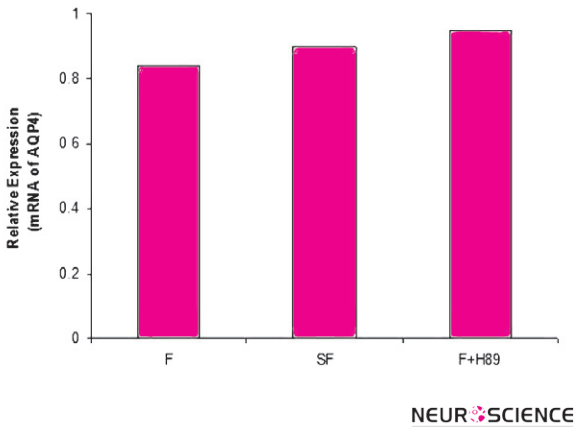

Figure 4. Fluoxetine effects on AQP4 mRNA expression with and without cAMP-PKA signaling pathway. U87MG cells treated with F (Fluoxetine), SF (AC inhibitor+ Fluoxetine) and F+H89 (Fluoxetine + PKA inhibitor) for 24 hours and changes in transcript amount expression were determined by real-time qPCR. There were no significant differences in treated groups in compared to untreated controls.

pretreatments declined the fluoxetine effects on Cx43 expression, but not returned it to basal level. Suggesting that up-regulation of Cx43 seen in the presence of fluoxetine may have gone partially via cAMP-Epac and without PKA pathway.

3.3. Effect of Fluoxetine on AQP4 Expression

We reported in our previous study (not published yet) that AQP4 was not expressed in 1321N1 cells using RT-PCR and real time PCR. So we used U87MG glioma cells to study fluoxetine possible effect on AQP4 expression in this cell line. In U87MG cells, although 24 hours treatment with fluoxetine (10 and 20 μg/ml) decreased AQP4 expression, but this effect was not significant. Pretreatment of cells with AC inhibitor and PKA inhibitor had no effect on AQP4 expression level (Fig. 4). These data suggested that expression of AQP4 in this cell line have other regulatory pathway.

4. Discussion

The roles of astrocytes in CNS disorders are under intense researches. Alteration of astrocyte roles as loss of normal functions or the gain of abnormal functions may involve in disease initiation and progression, such as multiple sclerosis. Consistent with this idea recent findings have demonstrated that Cx43 and AQP4 contents of astrocytes are changed in MS animal models or patients (Brand-Schieber, Beelitz et al. 2005; Miyamoto, Nagaosa et al. 2009). Besides the new finding about molecular mechanisms underling MS pathophysiology, new possible molecular targeted therapy has both clinical and experimental interest. Some reported studies have

demonstrated that fluoxetine could reduce formation of new enhancing lesions and had neuroprotective effects against axonal degeneration (Mostert, Admiraal-Behloul et al. 2008). Since fluoxetine has been shown to have relationship with the cAMP signaling pathway and have regulatory effects on Cx43 and AQP4 (Darrow, Fast et al. 1996; Kortenoeven, Trimpert et al. 2012), this study aimed to investigate possible fluoxetine effects on Cx43 and AQP4 expression through cAMP-Epac pathway in astrocyte. We showed that 24 hours treatment of astrocytoma cells with fluoxetine resulted in up-regulation of Cx43 both in mRNA and protein levels. Investigation of the signaling pathway demonstrated that inhibition of cAMP production by AC inhibitor decline the fluoxetine effects on Cx43 expression, but not returns it to basal level. This can be attributed to partial effect of cAMP in total effect of fluoxetine on Cx43 expression. We have seen the same effect of PKA inhibitor on Cx43 level.

Our results are in line with previous study that showed Cx43 protein expression was significantly increased following treatment with fluoxetine (Fatemi, Folsom et al. 2008). In this study, we want to investigate acute effect of fluoxetine in the absence of serotonin. Acute effect of fluoxetine on MAP kinase pathway activation in cultured astrocytes has been showed (Mercier, Lennon et al. 2004). Our data showed that pre-inhibition of AC and PKA decreased fluoxetine effects on Cx43 expression but this change was not significant. Although previous studies have reported that antidepressant treatment resulted in activation of AC and increased cAMP activity (Donati, Thukral et al. 2001), but our results have not confirmed AC and cAMP involvement in fluoxetine effects. We think that dosage and duration of treatment have role in this discrepancy observed in our study.

With regards to AQP4 expression, fluoxetine had no significant effect. we used U87MG cell, which have higher glioma grade (Stiborová, Rupertová et al. 2011). So ineffectiveness of fluoxetine in alteration of AQP4 expression may attribute to higher glioma grade of U87MG.

In conclusion, we showed that 24 hours treatment with fluoxetine that can be assumed as acute treatment increased Cx43 expression. Our data suggested that some fluoxetine effects can be attributed to its acute effects on Cx43 expression. So in MS patients that commonly have depression and based on researches have down-regulated level of Cx43, fluoxetine as an antidepressant with capability of crossing the blood-brain-barrier can have dual benefit effects.

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