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17 β -estradiol-induced regulation of the novel 5-HT_{1A}-related transcription factors NUDR and Freud-1 in SH SY5Y Cells

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Abstract

NUDR/Deaf-1 (nuclear deformed epidermal autoregulatory factor-1) and Freud-1 (Five prime repressor element under dual repression) are novel transcriptional regulators of the 5-HT_{1A} receptor, a receptor that has been implicated in the pathophysiology of various psychiatric illnesses. The antidepressant effect of 17 β -Estradiol (17 β E₂) is purported to involve the downregulation of this receptor. We investigated the possible role of NUDR and Freud-1 in 17 β E₂-induced downregulation of the 5-HT_{1A} receptor in the neuroblastoma cell line SH SY5Y. Cells were treated with 10nM 17 β E₂ for 3- or 48-h, followed by a 24-h withdrawal period. Proteins were isolated and analyzed by Western blotting. 17 β E₂ treatment increased NUDR immunoreactivity while Freud-1 and the 5-HT_{1A} receptor showed significant decreases. Upon withdrawal of 17 β E₂, protein expression returned to control levels, except for NUDR, which remained significantly elevated in the 3-h treatment. Taken together, these data support a non-genomic downregulation of 5-HT_{1A} receptor protein by 17 β E₂, which does not involve NUDR and Freud-1. Rather, changes in both transcription factors seem to be compensatory /homeostatic responses to changes in 5-HT_{1A} receptor induced by 17 β E₂. These observations further highlight the importance of NUDR and Freud-1 in regulating 5-HT_{1A} receptor expression.

Keywords

Estrogen; depression; NUDR; Freud-1; 5-HT_{1A}

Introduction

Nuclear deformed epidermal regulatory factor (NUDR/Deaf-1) and Five prime repressor under dual repressor binding protein (Freud-1; CC2D1A, coiled-coil C2 domain 1A) are novel 5-HT-related transcription factors that have been shown to modulate the expression of

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Conflict of Interest

None of the authors have conflict of interest.

the 5-HT_{1A} receptor gene (Albert and Lemonde 2004; Ou et al. 2003; Rogaeva et al. 2007; Rogaeva and Albert 2007). Although Freud-1 act as transcriptional repressors of the 5-HT_{1A} receptor gene, NUDR has a dual role; functioning as a transcriptional repressor in presynaptic serotonergic neurons and as an enhancer in postsynaptic non-serotonergic neurons (Czesak et al. 2006). Both transcription factors are found within serotonin neurons in the central nervous system (CNS) and are co-localized with the 5-HT_{1A} receptor (Ou et al. 2003; Szewczyk et al. 2009).

Alterations in the function and levels of the 5-HT_{1A} receptor have been reported in psychiatric illnesses including major depression and suicide (Albert and Lemonde 2004; Lemonde et al. 2003; Stockmeier et al. 1998). The recent reported decreases in both NUDR and 5-HT_{1A} receptor protein expression in a postmortem study of the PFC in depressed women but not men (Szewczyk et al. 2009), suggests that female sex hormones may play an important role in the regulation of this transcription factor. Given the above-mentioned gender specific changes in NUDR and 5-HT_{1A} receptor; the downregulation of the 5-HT_{1A} receptor by 17 β E₂ despite the absence of any known estrogen response elements (ERE) in its promoter region (Bethea et al. 2002), and the known transcriptional regulatory roles of NUDR and Freud-1 on 5-HT_{1A} receptor gene, we hypothesized that 17 β E₂ may regulate these transcription factors which may in turn contribute to the downregulation of the 5-HT_{1A} receptor seen in the presence of estradiol. We tested this hypothesis in a cell culture model using SH SY5Y neuroblastoma cells; our preliminary studies show that these cells produce serotonin and express major serotonergic proteins and mRNA including TPH2, 5-HT_{1A}, FEV/Pet-1 (a serotonin neuronal marker (Hendricks et al., 1999) and NUDR and Freud-1. SH SY5Y cells also express both ER α and ER β (Bang et al. 2004), making it ideal to study the effects of estradiol on serotonergic markers.

Methods

Cell culture

SH-SY5Y cells were cultivated according to vendor's instructions (ATCC, Manassas, VA, USA). Cells were seeded in either 6-well plates or 5cm dishes at densities of 0.5×10^6 cells/well or 1.15×10^6 cells/dish respectively (an approximately equal number of cell/area). Twenty-four hours after seeding, medium was removed and replaced with medium containing 10nM 17 β E₂ (Sigma-Aldrich, St. Louis MO, USA) and 0.0001% ethanol, control medium contained only 0.0001% ethanol. Cells were incubated for either 3-h (5cm dishes) or 48 h (6-well plates) after which they were harvested with Trypsin-EDTA and processed for western blotting. Our preliminary/optimization experiments have shown that 10nM is the lowest effective concentration and that the 3-h treatment is the optimum exposure time that showed significant effects on the proteins we are studying. The treatment durations were thus chosen to represent short term and long term effects. Another set of cells treated in the same manner were not harvested immediately. In these set, the estradiol-containing medium was aspirated and the cells were carefully washed with PBS before the control medium was added. After twenty-four hours, cells were then harvested. All cell culture supplies were obtained from Invitrogen, Carlsbad CA, USA.

Western blotting

Protein was extracted and concentration was determined using the bicinchoninic acid (BCA) Kit (Pierce Biotechnology, Inc, Rockford, IL, USA). Twenty micrograms of each sample was used for Western Blot analysis as previously described (Iyo et al., 2009). Primary antibodies and the respective concentrations used are as follows: rabbit anti-NUDR polyclonal antibody (1: 3000) (Szewczyk et al., 2009); rabbit anti-Freud-1 polyclonal antibody (1:5000) (Rogaeva et al. 2007); rabbit anti-5-HT_{1A} receptor polyclonal antibody

(Aviva Systems Biology, LLC, San Diego, CA) (1:5000) and anti β -Actin monoclonal antibody (Chemicon International Inc., Temecula, CA USA (1:3000) for sample loading control. The following protein weights were detected by the various antibodies: 5-HT_{1A} 45 kDa; Freud-1, 130 kDa, and NUDR, 60 kDa and β -actin 43 kDa.

Data analysis

Data for Western blotting is presented as individual protein band immunoreactivity (relative optical density; ROD) relative to those of the respective β -actin for each sample. Treatment effect was analysed using ANOVA followed by Dunnett's post-hoc test. Statistical significance was set $p < 0.05$.

Results

17 β E₂ resulted in significant increase in NUDR protein levels while both Freud-1 and 5-HT_{1A} receptor immunoreactivities were significantly reduced (Figs. 1, 2, and 3). All protein levels essentially returned to control levels after withdrawal, except NUDR protein, which remained elevated in the 3 h treatment (Fig. 1a).

Discussion

In this study, we show that treatment of SH SY5Y neuroblastoma cells with 17 β E₂ results in a rapid and extensive downregulation of the 5-HT_{1A} receptor protein. On the other hand NUDR protein levels increased while Freud-1 protein levels decreased. These effects were reversed upon withdrawal of 17 β E₂ but the increase in NUDR was sustained for at least 24hrs (Fig. 1a)

Our data (NUDR increases with 17 β E₂ treatment) may be related to the reported gender-specific decrease in NUDR protein expression in depressed women (Szewczyk et al. 2009) in the light of significantly lower levels of estrogen in some depressed women (Young et al. 2000). Considering that NUDR is a known transcriptional enhancer of the 5-HT_{1A} receptor in postsynaptic locations (Czesak et al., 2006) and the fact that the SH-SY5Y used in this study are considered postsynaptic (Hadjigassem et al. 2009; Biedler et al. 1978), one would expect a corresponding increase in 5-HT_{1A} protein expression. On the contrary 5-HT_{1A} protein decreased. This scenario may be an attempt to compensate for the decrease in the 5-HT_{1A} receptor through increasing the expression of the transcriptional enhancer (NUDR). However, it is possible that the expected increase may not have set in at the time points we examined in the current study. It is noteworthy to mention at this point that the SH-SY5Y cell culture model may not mirror what obtains in a complex system as the brain. However, the possibility that NUDR might be acting as a repressor does not seem likely since 5-HT_{1A} immunoreactivity completely returned to control levels after the withdrawal of 17 β E₂ despite sustained NUDR increase (Fig. 1a).

Freud-1 protein levels were surprisingly downregulated but returned to control levels 24-h after 17 β E₂ withdrawal in both the 3 and 48-h treatments. Considering its function as a repressor and its downregulation in 17 β E₂ treated cells we would expect an increase in the expression of the 5-HT_{1A} receptor protein rather than a decrease. At this point it is safe to assume that since Freud-1 was downregulated it may not play any role in the downregulation of the 5-HT_{1A} receptor protein seen in this study. Rather, its reduction may also represent a compensatory mechanism to elevate 5-HT_{1A} receptor protein levels. Indeed such a compensatory mechanism has been adduced for Freud-2, which is also a negative regulator of the 5-HT_{1A} (Hadjigassem et al. 2009). It was suggested that the reduction could be a compensatory adaptive mechanism following the downregulation of the 5-HT_{1A} receptor, as seen in chronic mild stress (Lopez et al. 1999); G/G mutation in a region

(G-1019) of the 5-HT_{1A} receptor promoter and in 5-HTT sec/sec genotype (Lemondé et al., 2004; David et al., 2005). Given the structural similarity and the possible overlap of functions between Freud-1 and Freud-2, it is likely that the same scenario holds true for Freud-1.

The rapid, dramatic decrease and complete recovery of 5-HT_{1A} protein following estradiol treatment and withdrawal respectively and the fact that 5-HT_{1A} has no estrogen response element (ERE) in its promoter region (Bethéa et al., 2002), suggest that the decrease in 5-HT_{1A} protein may be due to 'non-genomic' effects of estradiol (Mize et al., 2001; Mize & Alper, 2002). A possible 'non-genomic' mechanism has been proposed to be mediated by a putative membrane bound G-protein estrogen receptor, coupled to Gα_q which activates protein kinase Cδ and A (PKCδ and PKA) (Picotto et al., 1999; Qui et al., 2003; Malyala et al., 2005). The activation of PKA and PKC is reported to lead to the phosphorylation and decoupling (Mize & Alper, 2002), and possibly also, the eventual downregulation of the 5-HT_{1A} receptor following exposure to 17βE₂. There may however be the possibility of the contribution of other indirect or ERE-independent transcriptional mechanisms in the long term (48-h) effect, which in any case does not seem to include NUDR and Freud-1.

In conclusion, the results of the present study provide evidence that estradiol may regulate both NUDR and Freud-1. The results further suggest that the increase in NUDR and decrease in Freud-1 proteins may represent compensatory/homeostatic adjustments to prevent excessive, non-genomic decrease of 5-HT_{1A} receptor induced by 17βE₂. These results provide further evidence of the importance of NUDR and Freud-1 in regulating 5-HT_{1A} receptor expression by maintaining homeostasis. Future studies, especially in intact animals are required to further delineate how estradiol regulates NUDR and Freud-1 in vivo.

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Fig. 1. NUDR protein levels in SH-SY5Y cells after 3-h (A) and 48-h (B) Estradiol treatment (TX) and after 24-h withdrawal (TW). Data represent mean relative optical densities (ROD) $\pm$ SEM where sample size (N) for each treatment group (Control, TX and TW) is 3. ANOVA p values for 3-h and 48-h are 0.0020, 0.0111 respectively. Post hoc Dunnet's Test (TX or TW vs. Control) $p < 0.05$ (*); $p \leq 0.01$ (**).

Fig.2.

Freud-1 protein levels in SH-SY5Y cells after 3-h (A) and 48-h (B) Estradiol treatment (TX) and after 24hr withdrawal (TW). Data represent mean relative optical densities (ROD)±SEM where sample size (N) for each treatment group (Control, TX and TW) is 3. ANOVA *p* values for 3-h and 48-h are 0.0294, 0.0288 respectively. Post hoc Dunnet's Test (TX and TW vs. Control) *p*<0.05 (*) *p*<0.01(**).

Fig.3. 5-HT_{1A} receptor protein levels in SH-SY5Y cells after 3-h (A) and 48-h (B) Estradiol treatment (TX) and after 24-h withdrawal (TW). Data represent mean relative optical densities (ROD)±SEM where sample size (N) for each treatment group (Control, TX and TW) is 3. ANOVA *p* values for 3-h and 48-h are 0.0007 and 0.0222 respectively. Post hoc Dunnett's Test (TX and TW vs. Control) *p*<0.05 (*) *p*<0.01(**).