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Stress-induced alterations in 5-HT1A receptor transcriptional modulators NUDR and Freud-1

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Abstract

The effect of stress on the mRNA and protein level of the 5-HT1A receptor and two of its key transcriptional modulators, NUDR and Freud-1, was examined in the prefrontal cortex (PFC) and hippocampus (Hp) using rodent models: olfactory bulbectomy (OB) and prenatal stress (PS) in male and female rats; chronic mild stress in male rats (CMS) and pregnancy stress.

In PFC, CMS induced the most widespread changes, with significant reduction in both mRNA and protein levels of NUDR, 5-HT1A receptor and in Freud-1 mRNA; while in Hp 5-HT1A receptor and Freud-1 protein levels were also decreased. In male, but not female OB rats PFC Freud-1 and 5-HT1A receptor protein levels were reduced, while in Hp 5-HT1A receptor, Freud-1 and NUDR mRNA's but not protein were reduced. In PS rats PFC 5-HT1A receptor protein was reduced more in females than males; while in Hp Freud-1 protein was increased in females. In pregnancy stress, PFC NUDR, Freud-1 and 5-HT1A protein receptor levels were reduced, and in HP 5-HT1A receptor protein levels were also reduced; in HP only NUDR and Freud-1 mRNA levels were reduced. Overall, CMS and stress during pregnancy produced the most salient changes in 5-HT1A receptor and transcription factor expression, suggesting a primary role for altered transcription factor expression in chronic regulation of 5-HT1A receptor expression. By contrast, OB (in males) and PS (in females) produced gender-specific reductions in PFC 5-HT1A receptor protein levels, suggesting a role for post-transcriptional regulation. These and previous data suggest that chronic stress might be a key regulator of NUDR/Freud-1 gene expression.

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Statement of Interest

None.

Keywords

chronic mild stress; Freud-1; 5-HT1A receptor; NUDR; olfactory bulbectomy; prenatal stress

Introduction

Dysfunction of the serotonergic system has been suggested as a factor for depressive disorder (Jans et al., 2007). Depression and suicide appear to result from reduced serotonergic activity, while antidepressants are thought to enhance serotonergic neurotransmission (Albert and Lemonde, 2004; Savitz et al., 2009). Among multiple receptors through which serotonin acts, the 5-HT1A receptor subtype has been suggested as a key determinant for the development and treatment response of depression. These receptors located in the raphe nuclei function as key presynaptic regulators of serotonergic activity, but also play important roles as postsynaptic receptors expressed in non-serotonergic cortical and limbic neurons mediating the local responses to serotonin (Albert and Lemonde, 2004). As was revealed in clinical and rodent studies both pre- and post-synaptic 5-HT1A receptors are implicated in depression, depressive-like behaviour and mechanism of action of antidepressant drugs (Savitz et al., 2009). A growing body of evidence indicates that dysregulation in this receptor system is observed in depression and could be associated with transcriptional regulation of the 5-HT1A receptor gene (Albert and Lemonde, 2004). Previous studies have identified two novel serotonin-related transcription factors that regulate the expression of the 5-HT1A receptor gene in neurons: nuclear deformed epidermal autoregulatory factor (NUDR/Deaf-1) and five-prime repressor element under dual repression/coiled-coil C2 domain-1A (Freud-1/CC2D1A) (Lemonde et al., 2003; Ou et al., 2003; Albert and Lemonde, 2004; Czesak et al., 2006).

NUDR is a human homolog of Drosophila Deformed epidermal autoregulatory factor-1 (Deaf-1), which was identified as a DNA-binding protein and potential gene regulator (Huggenvik et al., 1998). NUDR/Deaf-1 represses 5-HT1A receptor promoter-luciferase constructs and reduces the levels of endogenous 5-HT1A receptor-binding sites and mRNA levels in raphe cells (Lemonde et al., 2003). Interestingly, the negative regulation of both 5-HT1A receptor gene transcription and protein expression by NUDR is restricted to the serotonergic cells but in non-serotonergic cells such as postsynaptic neurons, in which NUDR was found to acts as a transcriptional enhancer of 5-HT1A receptor (Czesak et al., 2006). This dual activity of Deaf-1 (NUDR) on endogenous 5-HT1A receptor expression has been demonstrated recently *in vivo* (Czesak et al., 2012). It was found that Deaf-1 knockout mice displayed increased levels of 5-HT1A receptor mRNA and protein in dorsal raphe but reduced levels in the frontal cortex, with no significant change in hippocampal 5-HT1A receptor mRNA and protein level (Czesak et al., 2012).

Freud-1, in turn, belongs to a gene family consisting of two homologous genes; Freud-1 and Freud-2 (Nakamura et al., 2008; Hadjighassem et al., 2009). Freud-1 binds to the 5′14-bp element (FRE) of the 5-HT1A-receptor promoter and negatively regulates basal expression of the 5-HT1A receptor both in serotonergic and non-serotonergic neurons. Freud-1 exists as two isoforms, a short form (Freud-1S), which is the predominant isoform in rodent cells, and

a long form (Freud-1L), which occurs mainly in human cells (Rogaeva et al., 2007). Beside the action as a transcriptional repressor of 5-HT1A receptor, Freud-1 has been also found to bind to the promoter of the dopamine D2 receptor and to act as a negative regulator of D2 receptor expression (Rogaeva et al., 2007). In addition, a recent study using human embryonic kidney cells reported that Freud-1 can function as a novel scaffolding protein selectively activating the 3-phosphoinositide-dependent protein kinase 1 (PDK1)/Akt pathway (Nakamura et al., 2008).

Studies in rodents have shown that both NUDR and Freud-1 are detected in the prefrontal cortex (PFC), hippocampus and dorsal raphe nucleus and are co-localized with 5-HT1A receptors in neurons in these brain areas (Lemonde et al., 2003; Ou et al., 2003).

Our recent, human post-mortem studies have shown reduced protein expression of NUDR and of 5-HT1A receptors in the PFC of female but not male subjects with major depressive disorder (MDD) (Szewczyk et al., 2009). In contrast, Freud-1 protein levels were not significantly different in the female subjects diagnosed with MDD but were significantly decreased in the PFC of male MDD subjects compared to gender-matched controls (Szewczyk et al., 2010). Based on these studies we have put forward the hypothesis that reduced Freud-1 level in the male MDD subjects may reflect an adaptive response to maintain normal levels of 5-HT1A receptor (bearing in mind that Freud-1 functions as a repressor of the 5-HT1A receptor). Further, since Freud-1 was not significantly decreased in the female MDD subjects (only a trend for reduced expression was observed), such adaptive response in Freud-1 was not sufficient to override the deficit in NUDR expression (enhancer of 5-HT1A receptor) and the corresponding reduction in 5-HT1A receptors in women with MDD (Szewczyk et al., 2009, 2010). These data suggest that alterations in NUDR/Freud-1 transcription factors in depressed individuals might be gender specific and could contribute to an underlying biological mechanism associated with the higher incidence of depression in women. In addition, data obtained from animal studies examining the alterations in these transcription factors in different stress procedures shown decreased Freud-1 protein and mRNA expression but not NUDR in the PFC of male rats subjected to chronic restrain stress alongside with the elevated 5-HT1A receptor mRNA (Iyo et al., 2009). In turn, a study performed by Kieran et al., found no changes in both Freud-1 and NUDR mRNA expression and a decreased level of 5-HT1A receptor mRNA level in chronic social defeat stress (Kieran et al., 2010).

Based on these data the present study was designed to further clarify the sex, stress or brain region specific regulation of the expression of NUDR and Freud-1 and its possible role in the regulation of 5-HT1A receptor in depression using the olfactory bulbectomy (OB), prenatal stress (PS) and chronic mild stress (CMS) models as well as female rats subjected to stress during pregnancy.

Method

All procedures were undertaken in accordance with the guidelines of the National Institutes of Health Animal Care and Use Committee and were approved by the Ethic Committee of the Institute of Pharmacology Polish Academy of Science in Krakow. All the procedures

were performed with a minimization of animal suffering and as few animals were used as was possible.

Olfactory bulbectomy model

Male and female Sprague-Dawley rats (Charles River, Germany), weighing 250–270 g, were kept under standard laboratory conditions of lighting (12/12 h light/dark cycle) and temperature (23 °C). Food and water were freely available. Each experimental group consisted of 8–12 animals. The experiment was done according to the procedure published in the paper by Nowak et al., 2003. Briefly the following was done: 1 wk after arrival in the laboratory, a bilateral olfactory bulbectomy was performed on rats under ketamine (100 mg/kg)/xylazine (10 mg/kg) anaesthesia. Metoxicam (0.05 mg/kg, s.c.) was given as an analgaesic and anti-inflammatory drug 60 min both before the operation and for the following two days after surgery. Following exposure of the skull, burr holes were drilled using the coordinates 7 mm anterior to the bregma and 2 mm (on either side) from the middle line, i.e. at a point corresponding to the posterior margin of the orbit of the eye. The olfactory bulbs were removed by suction, and the burr holes were filled with a haemostatic sponge (Ferrosan, Poland). The skin was closed. Sham-operated animals were similarly treated, but the bulbs were left intact. The animals were allowed to recover for 14 d following surgery; they were handled daily by the experimenter throughout the recovery period to eliminate any aggressiveness that would otherwise develop (Leonard and Tuite, 1981). On day 15 the open-field test was performed both in male and female rats to examine depression-like behaviour (hyperactivity) induced by olfactory bulbectomy (data not shown). Only rats with observed hyperactivity and rats with completely removed olfactory bulbs but without significant damage to the frontal cortex (as visually assessed post-sacrifice), were selected for the biochemical studies.

Prenatal stress

Female Sprague-Dawley rats (Charles River, Germany), weighing 250–270 g, were kept under standard animal housing conditions (a room temperature of 23 °C, 12/12 h light/dark cycle, lights on at 08:00 h), with food and water available *ad libitum*. One week after their arrival, vaginal smears from the female rats were checked daily in order to determine the phase of the oestrous cycle. On the pro-oestrous day they were placed with males for 12 h and the presence of sperm in vaginal smears was subsequently checked. Pregnant females were then randomly assigned to the control and stress group (n =15 in each group).

Stress procedure

Prenatal stress was performed as previously described by Morley-Fletcher et al. (2003a, b) (Morley-Fletcher et al., 2003a, b) Briefly, pregnant rats were subjected daily to three stress sessions starting at 09:00, 12:00 and 17:00 h, during which they were placed in plastic cylinders (7/12 cm) and exposed to a bright light for 45 min. Stress sessions, were performed from day 14 of pregnancy until delivery (8 d). Control pregnant females were left undisturbed in their home cages. Only offspring from litters containing 10–14 pups with a similar number of males and females were kept. Female and male offspring were selected from 21-day-old litters. Eight animals per group (1–2 animals from each litter) were used for biochemical experiments. They were housed in groups of four animals per cage under

standard conditions. Pregnant female rats were decapitated 5 mth after delivery, female offspring 5 mth and male offspring 4 mth after birth.

Chronic mild stress

Male Wistar rats (Charles River, Germany), weighing 250–270 g, were kept under standard laboratory conditions of lighting (12/12 h light/dark cycle) and temperature (23 °C). Food and water were freely available. Each experimental group consisted of eight animals. Chronic mild stress procedure was described in detail in our previous paper (Pochwat et al., 2013). Briefly, after a 2-week period of adaptation to laboratory and housing conditions, the male rats were first trained to consume a 1% sucrose solution; the training consisted of 9 1 h baseline tests in which sucrose was presented, in the home cage, following 14 h of food and water deprivation. The sucrose intake was measured by weighing bottles containing the sucrose solution, at the end of the test. Subsequently, sucrose consumption was monitored under similar conditions, at weekly intervals throughout the experiment. On the basis of sucrose intake in the final baseline test, the animals with equal average sucrose consumption were divided into two matched groups. One group of animals was subjected to the chronic mild stress procedure for a period of seven consecutive weeks. Each week of the stress regime consisted of: two periods of food or water deprivation, two periods of cage tilt, two periods of intermittent illumination, two periods of soiling of the cage, one period of paired housing, two periods of low intensity stroboscopic illumination and three periods without stress. All of the stressors were of 10–14 h duration and were applied individually and continuously, day and night. The control animals were housed in separate rooms and had no contact with the stressed animals. Rats were deprived of food and water for the 14 h preceding each sucrose test, but otherwise food and water were freely available in the home cage. For the biochemical studies animals were selected on the basis of positive results obtained from individual sucrose consumption test (data not shown).

Tissue collection

Animals were killed under non-stress conditions by rapid decapitation 24 h after the last test session (sucrose test in CMS and open-field test in OB) or handling (prenatal stress). Brains were rapidly removed and the prefrontal cortex and hippocampus were dissected on an ice-cold glass plate. The tissues were frozen in dry ice and stored at −80 °C.

Western blot analysis

Tissue samples from PFC and hippocampus were homogenized by ultrasonication in 2% solution of sodium dodecyl sulfate (10 mg/100 ul). After that, homogenates were denatured at 95 °C for 10 min and finally centrifuged for 5 min at 10000 rpm at 4 °C. After centrifugation, the supernatant was collected and the protein content was determined. For this assay, bicinchonic acid was used (Pierce, USA). Next, the samples were fractionated by 10% SDS-polyacrylamide gel electrophoresis. In a further step, proteins were transferred to the nitrocellulose membrane (Invitrogen, UK). To block non-specific binding, a 1% blocking solution was used [BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit) made by Roche, USA]. After blocking, the membranes were incubated overnight at 4 °C with the respective antibodies. The following antibodies were used: rabbit polyclonal anti-5-HT1A receptor antibody (1:1000, Abcam); rabbit anti-NUDR polyclonal antibody [1:5000

(Szewczyk et al., 2009)] and rabbit anti-Freud-1 polyclonal [(1:1000, (Szewczyk et al., 2010)]. All of the antibodies were dissolved in 0.5% blocking reagent (Roche). The next day, the membranes were washed three times for 10 min in Tris-buffered saline with Tween (TBS-T) and incubated for 30 min with secondary anti-rabbit- IgG-peroxidase conjugated antibody (1:7000, Roche). After incubation, the membranes were washed three times for 10 min with TBS-T. In the last step, the blots were incubated with detection reagent (Roche). The signal from the tested proteins was visualized using the Fuji-Las 1000 system. To check the transfer and loading, β-actin was indicated on each blot. For this, a primary β-actin monoclonal antibody (Millipore; 1:8000) was used. Further procedures were the same as for the other proteins. The Western blot band optical density analysis was performed using Image Gauge v.4.0 software. The obtained results are given as the ratio of the optical density of particular proteins to an optical density of β-actin.

Statistical analysis—Differences between control and experimental groups were analysed by Student t-test (GraphPad Prism Software). A value of $p < 0.05$ was considered statistically significant.

RNA isolation and real-time RT-PCR

Total RNA was extracted from homogenized tissue samples with TRIzol reagent (Invitrogen). The quality of the obtained RNA was evaluated by gel electrophoresis. RNA purity and concentration were assessed with Nanodrop spectrophotometer (Thermo Scientific, USA). One microgram of total RNA of each sample was digested with DNase I (Sigma-Aldrich, USA) and then reverse transcribed to single-stranded cDNA using a High Capacity cDNA Reverse Transcription Kit (random primers; Applied Biosystems). Real-time Polymerase Chain Reactions (PCRs) were performed in triplicate in a final volume of 18 μl using CFX96 Real-Time System (Bio-Rad), C1000 Touch Thermal Cycler (Bio-Rad) and Power SYBR Green Master Mix (Applied Biosystems). Concentration of primers in all reactions was 200 nM each. Standard cycling conditions were applied: polymerase activation (95 °C, 10 min) and 40 PCR cycles (denaturation: 95 °C, 15 s; annealing-elongation: 60 °C, 1 min) followed by melt curve analysis (at ramp +0.5 °C). Primer sequences are shown in the Table 1. The specificity of each primer set was confirmed by checking melting temperature and the product size by gel electrophoresis. Using glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as the endogenous reference gene, ΔCt values were obtained for all samples. For graphical presentation and statistical analyses, relative mRNA level index of each target gene was generated with $2^{-\Delta Ct}$ formulae (Livak and Schmittgen, 2001).

Statistical analysis—Statistical analyses of mRNA levels were performed on $2^{-\Delta Ct}$ values using the Student t-test (for equal variances). Significance was assumed at $p<0.01$.

Results

Effect of OB on NUDR, Freud-1 and 5-HT1A receptor protein and mRNA level

As shown in Fig. 1(*a–f*), OB procedure did not induce any significant effect on mRNA levels of NUDR, Freud-1 or 5-HT1A receptor in male or female rat PFC. As shown in Fig. 2(*a–f*) the OB procedure reduced the protein level of Freud-1 (p=0.0432) and 5-HT1A receptor (p=

0.0378) in the male PFC but did not influence the protein level of NUDR, Freud-1 and 5-HT1A receptor in the female PFC rats.

In the hippocampus (Table 2) OB procedure decreased the mRNA level of NUDR in both female (p =0.003) and male (p=0.001) rats and the level of Freud-1 and 5-HT1A receptor in male rats (p=0.037 and p=0.002, respectively).

Effect of prenatal stress procedure on the NUDR, Freud-1 and 5-HT1A receptor protein and mRNA level

As shown in Fig. 3(*a–f*) prenatal stress had no effect on the mRNA level of NUDR, Freud-1 or 5-HT1A receptor in the PFC of male or female offspring. As shown in Fig. 4(*a–f*) prenatal stress did not induce any changes in the protein level of NUDR, Freud-1 and 5-HT1A receptor in males; however, it decreased the protein level of the 5-HT1A receptor (p=0.024) in the PFC of stressed females when compared to non-stressed animals.

In the hippocampus (Table 3) prenatal stress induced a decrease in NUDR mRNA level in males (p=0.006) and increased Freud-1 protein level in females.

Effect of stress applied to pregnant female rats on NUDR, Freud-1 and 5-HT1A receptor protein and mRNA level

As shown in Fig. 5(*a–c*) repeated restraint stress applied to the pregnant female rats did not influence the level of mRNA of NUDR, Freud-1 and 5-HT1A receptor in the PFC, however, it induced a significant decrease in NUDR (p=0.024), Freud-1 (p=0.001) and 5-HT1A receptor (p=0.040) protein levels (Fig. 5*d–f*) when compared to non-stressed pregnant female rats.

In the hippocampus (Table 4) decreased levels were found in NUDR (p=0.027), Freud-1 (p=0.011) mRNA level and 5-HT1A receptor protein level (p=0.047).

Effect of CMS procedure on NUDR, Freud-1 and 5-HT1A receptor protein and mRNA level

As shown in Fig. 6(*a–c*) CMS induced a significant reduction in the mRNA of NUDR (p=0.0001), Freud-1 (p=0.001) and 5-HT1A receptor (p=0.003) in the PFC of stressed animals compared to controls. CMS also significantly reduced NUDR (p=0.031) and 5-HT1A receptor (p=0.032) protein levels in the PFC (Fig. 6*e–f*) relative to control rats, while the level of Freud-1 protein was unchanged.

In the hippocampus (Table 5) CMS induced a significant decrease in the protein level of Freud-1 (p=0.024) and 5-HT1A receptor (p=0.047).

Discussion

Animal models of depression are most often based on exposure to stress during development or in adulthood, genetic manipulations, or destruction of brain circuits, which creates the neurochemical, neuroendocrinological or neuroimmunological changes that may mimic changes in major depression in humans (Willner, 1990). However, current animal models incompletely reproduce the symptoms of depression seen in humans. These behavioural

models were often developed using male rodents and then applied to females to study gender effects in depression, which may not appropriate for females. Gender differences might also occur only after stress exposure or drug administration (Palanza, 2001). Nevertheless, Chourbaji et al. (2008, 2011) reported the important role of environmental conditions and BDNF (brain-derived neurotrophic factor) genotype in the induction of the sex-specific alterations in emotional phenotype (Chourbaji et al., 2008, 2011). These studies also show that sex-specific changes in levels of BDNF and 5-HT metabolites depend on the brain region (e.g. PFC or hippocampus) (Chourbaji et al., 2012). Thus, we have used rodent models that utilize different mechanisms to elicit a depression phenotype, and examined changes in 5-HT1A receptor and its regulators in both male and female PFC and hippocampus (summarized in Table 6).

Olfactory bulbectomy (OB)

Olfactory bulb ablation is known to bring a specific syndrome that includes stress-induced hyperactivity, irritability, impaired avoidance learning and disturbances in social and sexual behaviours (van Riezen et al., 1976; Willner, 1990). Beyond behavioural changes, OB induces multiple biochemical alterations especially in the serotonergic system (Nesterova et al., 1997).

The OB procedure decreased the protein level of Freud-1 and the 5-HT1A receptor in the PFC of male but not female rats, while no changes in mRNA levels were detected in PFC. In contrast to PFC, in the hippocampus OB decreased the gene expression of NUDR, Freud-1 and the 5-HT1A receptor in male rats, but only NUDR expression was decreased in female rats. The absence of changes in gene expression of NUDR and Freud-1 in the PFC despite decreased levels of 5-HT1A receptor protein observed in OB male rats may be related to post-transcriptional mechanisms such as receptor down-regulation to reduce protein content (Harrington et al., 1994; Albert and Lemonde, 2004). Since in PFC NUDR enhances 5-HT1A receptor transcription while Freud-1 represses (Albert, 2012), the reduction of both appears to have antagonist effect yielding no change in 5-HT1A receptor RNA. By contrast, in the hippocampus reduced transcriptional activity at the 5-HT1A receptor and its regulators in male rats may involve direct genomic effects of glucocorticoids, as the hippocampus is highly sensitive to glucocorticoids, which directly suppress 5-HT1A receptor transcription (Meijer et al., 2000; Ou et al., 2001). The selective decrease in PFC 5-HT1A receptor mRNA in OB males but not females suggests that female hormones may antagonize the stress-induced reduction in 5-HT1A receptor transcription.

Prenatal and pregnancy stress

Prenatal stress is one of the best-characterized animal models of depression. It is established that chronic stress during prenatal brain development has been associated with various learning, behavioural and mental disturbances in later life (Huizink et al., 2004). In animals, prenatal stress is associated with increased immobility time in the forced swim test, impaired sleep and cognitive functions, decreased sexual behaviours and disturbances in the hypothalamic-pituitary-adrenal (HPA) axis (Koehl et al., 1999; Rao et al., 1999; Lemaire et al., 2000; Morley-Fletcher et al., 2003a, b). Prenatal stress has been also shown to influence the serotonergic system in the brain of rat offspring, including 5-HT1A receptors (Peters,

1988; Hayashi et al., 1998). Interestingly, the only observed changes induced by prenatal stress were reduced 5-HT1A receptor protein levels in female PFC and decreased NUDR mRNA in the male hippocampus. Because the tissues were assessed in adulthood, it is possible that early alterations may have become normalized; nevertheless the persistence of reduced 5-HT1A receptors in females suggests an increased susceptibility of the females to this stressor, perhaps via dysregulation of HPA function observed after prenatal stress (Weinstock, 2005). The preferential reduction of 5-HT1A receptors in the PFC could be, in part, consistent with the finding that prenatal maternal stress increases susceptibility of female offspring to anhedonia in adulthood, but induces alterations in presynaptic serotonin function predominantly in male offspring (van den Hove et al., 2014).

In agreement with the increased susceptibility of female offspring to prenatal stress, we found that exposure of pregnant female rats to restraint stress during the last week of gestation resulted in significant reductions in PFC NUDR, Freud-1 and 5-HT1A receptor protein levels, but no change in mRNA level. On the other hand, decreased levels of NUDR and Freud-1 mRNA along with Freud-1 protein were observed in the hippocampus of pregnant female rats. The more pronounced changes induced by stress during pregnancy compared to prenatal stress the shorter latency to tissue analysis in the former condition, but the common effect seems to involve a reduction in 5-HT1A receptor levels. As suggested above for OB males, the PFC 5-HT1A receptor and transcription regulators appear to be down-regulated through post-translational effects of stress. Conversely, the hippocampus appears to be more sensitive to transcriptional repression of these genes. The lack of change in 5-HT1A receptor genes in hippocampus may reflect antagonistic effects of derepression via reduced Freud-1 levels *vs.* glucocorticoid mediated repression of the 5-HT1A receptor promoter.

Chronic mild stress (CMS)

CMS is a rodent model developed to induce decreased responsiveness to reward (anhedonia), a common symptom of human depression (Willner, 1997; Russo and Nestler, 2013), in order to study stress-related mechanisms involved in the pathophysiology of depression (Willner, 1990; Duncko et al., 2001; Kubera et al., 2001; Papp, 2012). Importantly, CMS induces neurobiological alterations pertaining to the serotonergic system (Willner, 2005). In male rats, CMS significantly decreased both mRNA and protein levels of NUDR and 5-HT1A receptor in PFC, and only mRNA but not the protein level of Freud-1. Given that NUDR acts as enhancer of PFC 5-HT1A receptor gene expression (Czesak et al., 2006, 2012), the reduction in NUDR could mediate reduced 5-HT1A receptor expression observed in the PFC. In the hippocampus CMS did not affect the mRNA level of any of the genes studied, but decreased the level of Freud-1 and 5-HT1A receptor protein, suggesting a post-transcriptional mechanism. In the CMS model rats were subjected to the stress procedure for seven weeks, after seven weeks of adaptation, so the persistent reduction in NUDR may suggest a direct effect of adult stress on NUDR expression. By comparison, male rats subjected to severe chronic restraint stress for three weeks showed reduced Freud-1 but not NUDR, resulting in increased 5-HT1A receptor mRNA but reduced protein, the latter involving post-transcriptional mechanisms (Iyo et al., 2009; Kieran et al., 2010). Similarly, chronic social defeat in male rats reduced PFC 5-HT1A receptor protein levels

without changes in 5-HT1A receptor mRNA, NUDR or Freud-1 (Kieran et al., 2010). By contrast, in human subjects with long-term (months, years) depression, reductions in both NUDR and 5-HT1A receptor were observed in females but not males (Szewczyk et al., 2009). Thus persistent mild stress appears to reduce cortical NUDR level to transcriptionally reduce 5-HT1A receptor expression, while severe stress (restraint during pregnancy, prenatal stress, OB) appears to exert a post-transcriptional effect on PFC 5-HT1A receptor levels. Conversely, in the hippocampus, mild (CMS) and severe (stress during pregnancy) reduced 5-HT1A receptor protein post-transcriptionally.

Conclusions

The present study provides the first account of the effects of OB, prenatal stress, stress during pregnancy and CMS models of depression on the level of NUDR and Freud-1, known 5-HT1A receptor transcriptional regulators (Table 6). Generally, these data support the hypothesis that regulatory factors NUDR and Freud-1 might be involved in stress-induced alterations of 5-HT1A receptor observed in depression. However, changes in NUDR or Freud-1 appear to play significant but opposite roles in the pathogenesis of some models but not in others. The decreased level of NUDR observed in PFC of male rats subjected to CMS and in female rats subjected to repeated stress during pregnancy indicates that NUDR expression is sensitive to stress and seems to be more susceptible to adult stress.

Our results indicate that the role of gender in 5-HT1A receptor expression depends on the model studied: OB (in males) and PS (in females) produced gender-specific reductions in PFC 5-HT1A receptor protein levels, suggesting a role for post-transcriptional regulation. Previously, we found that reduced expression of NUDR in PFC was correlated with reduced 5-HT1A receptor expression in female but not male depression (Szewczyk et al., 2009) and that NUDR expression is up-regulated by oestrogen in cell culture studies (Adeosun et al., 2012). However, our data suggest that NUDR dysregulation is not restricted to females, as NUDR expression was decreased following chronic mild stress in male rats. However, as mentioned above, animal models of depression do not fully replicate human depression. These concerns had already been raised by Hellweg et al., 2007 who found up-regulation of BDNF in the OB model in mice, contrary to the general neurotrophin hypothesis of depression (Hellweg et al., 2007).

Our studies of different depression models show that multiple transcriptional and post-transcriptional mechanisms lead to the brain region-specific alterations in 5-HT1A receptor levels that may contribute to the depression phenotype. Reductions in NUDR levels appear to contribute to reduced PFC 5-HT1A receptor expression in chronic stress models, while reductions in Freud-1 appear to be a compensatory mechanism to counteract stress-induced reduction in 5-HT1A receptor expression (Szewczyk et al., 2010). On the other hand, post-transcriptional mechanisms appear to down-regulate PFC 5-HT1A receptor protein levels in severe acute or prolonged stress models, and in the hippocampus in acute or chronic stress.

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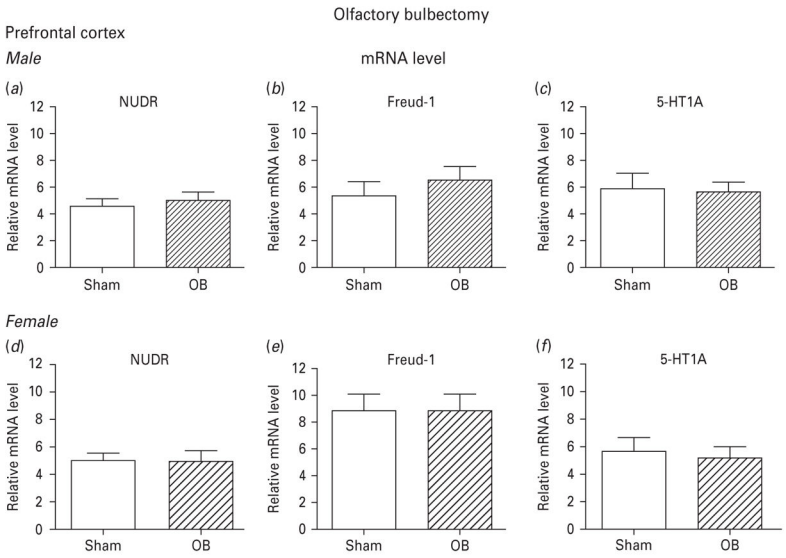

Fig. 1.

Real-time PCR analysis of the expression of the NUDR, Freud-1 and 5-HT1A receptor mRNA in the PFC of male (*a*–*c*) and female (*d*–*f*) rats subjected to the procedure of olfactory bulbectomy. Data are expressed as mRNA of NUDR, Freud-1 and 5-HT1A receptor in relation to endogenous reference gene GAPDH ($2^{-\Delta Ct}$ values×103±S.D.), n=6–9 animals. Data were analysed by Student t-test. Significance was assumed at $p<0.01$.

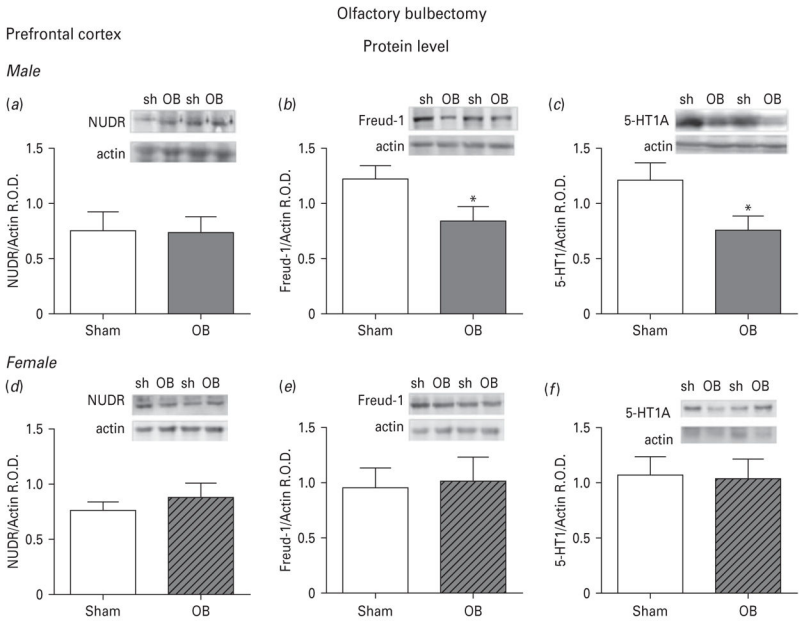

Fig. 2.

Representative Western blots and analysis of the protein expression of the NUDR, Freud-1 and 5-HT1A receptor in the PFC of male (*a*–*c*) and female (*d*–*f*) rats subjected to the procedure of olfactory bulbectomy. Data are expressed as protein level of NUDR, Freud-1 and 5-HT1A receptor in relation to actin (R.O.D values±S.E.M), n=6–9 animals. Data were analysed by Student t-test. Significance was assumed at $p<0.05$.

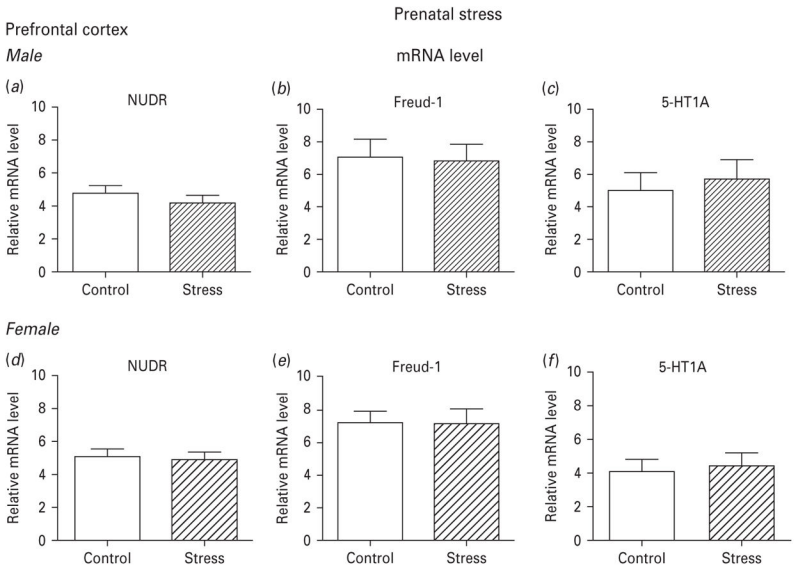

Fig. 3.

Real-time PCR analysis of the expression of the NUDR, Freud-1 and 5-HT1A receptor mRNA in the PFC of male (*a*–*c*) and female (*d*–*f*) rats subjected to the procedure of prenatal stress. Data are expressed as mRNA of NUDR, Freud-1 and 5-HT1A receptor in relation to endogenous reference gene GAPDH ($2^{-\Delta Ct}$ values×103±S.D.), n=6–8 animals. Data were analysed by Student t-test. Significance was assumed at $p<0.01$.

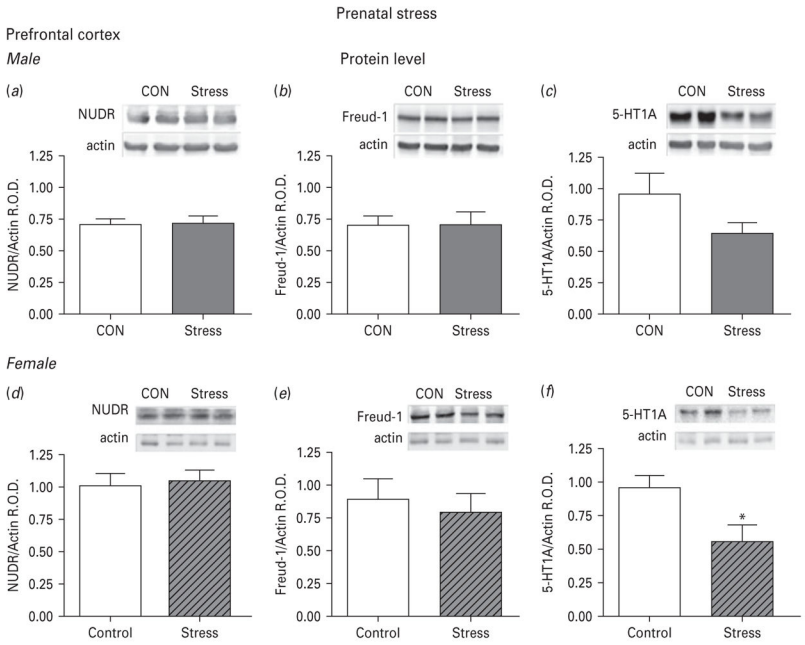

Fig. 4.

Representative Western blots and analysis of the protein expression of the NUDR, Freud-1 and 5-HT1A receptor in the PFC of male (*a*–*c*) and female (*d*–*f*) rats subjected to the procedure of prenatal stress. Data are expressed as protein level of NUDR, Freud-1 and 5-HT1A receptor in relation to actin (R.O.D values±S.E.M), n=6–8 animals. Data were analysed by Student t-test. Significance was assumed at $p<0.05$.

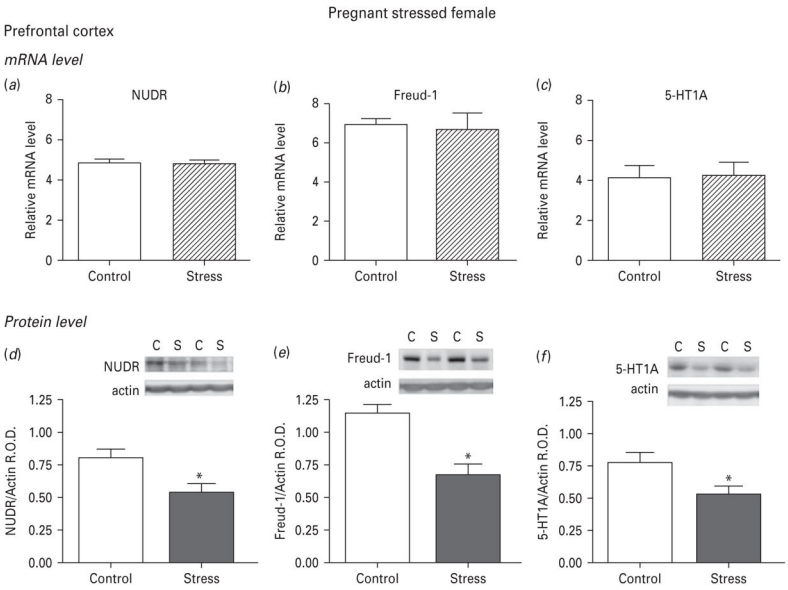

Fig. 5.

(*a*–*c*) Real-time PCR analysis of the expression of the NUDR, Freud-1 and 5-HT1A receptor mRNA in the PFC of pregnant stressed female rats. All data are expressed as mRNA of NUDR, Freud-1 and 5-HT1A receptor in relation to endogenous reference gene GAPDH ($2^{-\Delta Ct}$ values×103±S.D.), n=4–5 animals. (*d*–*f*) Representative Western blots and analysis of the expression of the NUDR, Freud-1 and 5-HT1A receptor protein level. Data are expressed as protein level of NUDR, Freud-1 and 5-HT1A receptor in relation to actin (R.O.D values±S.E.M), n=4–5 animals. Data were analysed by Student t-test. Significance was assumed at p<0.01 for mRNA and p<0.05 for protein levels. *C*, control*; S*, stress.

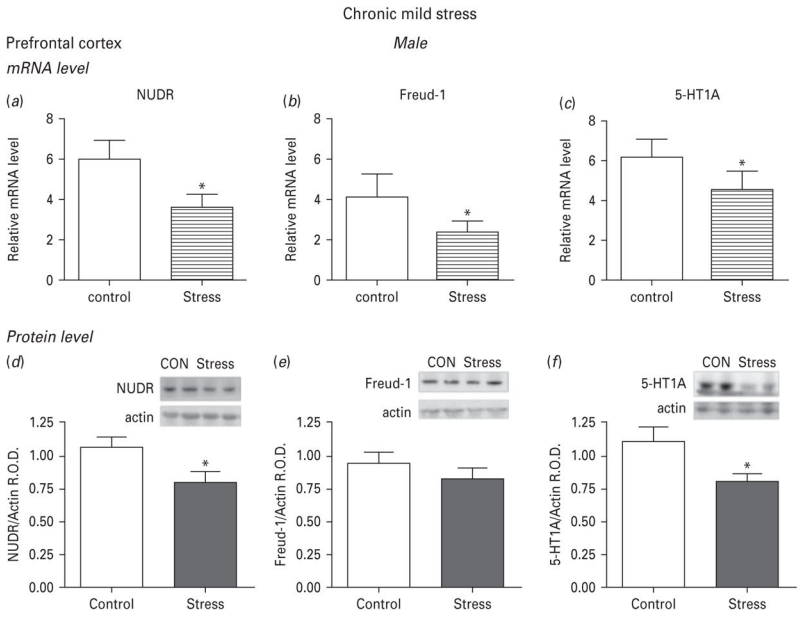

Fig. 6.

(*a*–*c*) Real-time PCR analysis of the expression of the NUDR, Freud-1 and 5-HT1A receptor mRNA in the PFC of male rats subjected to chronic mild stress. All data are expressed as mRNA of NUDR, Freud-1 and 5-HT1A receptor in relation to endogenous reference gene GAPDH ($2^{-\Delta Ct}$ values×103±S.D.), n=7–8 animals. (*d*–*f*) Representative Western blots and analysis of the expression of the NUDR, Freud-1 and 5-HT1A receptor protein level. Data are expressed as protein level of NUDR, Freud-1 and 5-HT1A receptor in relation to actin (R.O.D values±S.E.M), n=7–8 animals. Data were analysed by Student t-test. Significance was assumed at p<0.01 for mRNA and p<0.05 for protein levels.

Table 1

Primer sequences used for real-time RT-PCR

Gene	Forward	Reverse	Product size
5HT1A (Htr1a)	5′-atcatgggcaccttcatcctctg-3′	5′-gctttcacagaaaggcaggaccag-3′	72 bp
Freud-1 (Cc2d1a)	5′-accaggacgtagtacagcgtag-3′	5′-ctccagatggcttgcatactcc-3′	92 bp
NUDR (Deaf1)	5′-cggctgccacaaggttaactac-3′	5′-tgctgatggtctttccagtccttg-3′	66 bp
Gapdh	5′-agccgcatcttcttgtgcagtg-3′	5′-tggtaaccaggcgtccgatacg-3′	96 bp

Table 2

The effect of olfactory bulbectomy on the expression of the NUDR, Freud-1 and 5-HT1A receptor mRNA and protein levels in the rat hippocampus

Olfactory bulbectomy						
	mRNA level			**Protein level**		
Hippocampus	**Sham**	**OB**	***p***	**Sham**	**OB**	***p***
NUDR						
Male	8.48±1.42	5.91±0.83	**0.001***	0.89±0.27	0.64±0.09	0.422
Female	5.91±0.55	5.04±0.53	**0.003***	1.00±0.09	1.04±0.09	0.763
Freud-1						
Male	7.63±1.97	5.92±0.72	**0.037***	0.77±0.10	0.97±0.09	0.194
Female	9.09±1.14	9.05±1.01	0.937	0.82±0.17	0.89±0.16	0.764
5-HT1A						
Male	22.08±2.47	17.83±1.99	**0.002***	1.09±0.24	0.83±0.13	0.403
Female	21.29±2.35	20.44±2.25	0.445	0.75±0.05	0.71± 0.03	0.530

mRNA level of NUDR, Freud-1 and 5-HT1A receptor in relation to endogenous reference gene GAPDH ($2^{-\Delta Ct}$ values$\times 10^3$±S.D.), n=8–9 animals. Protein level of NUDR, Freud-1 and 5-HT1A receptor in relation to actin (R.O.D values±S.E.M), n=8–9 animals. Data were analysed by Student t-test. Significance was assumed at $p<0.01$ for mRNA and $p<0.05$ for protein levels.

Table 3

The effect of prenatal stress on the expression of the NUDR, Freud-1 and 5-HT1A receptor mRNA and protein levels in the rat hippocampus

Prenatal stress						
	mRNA level			**Protein level**		
Hippocampus	**Sham**	**OB**	***p***	**Sham**	**OB**	***p***
NUDR						
Male	5.58±0.76	4.45±0.64	**0.006***	1.14±0.23	1.02±0.27	0.741
Female	5.97±0.60	6.50±1.32	0.392	0.81±0.09	0.94±0.21	0.586
Freud-1						
Male	8.31±1.44	6.72±1.87	0.079	0.81±0.12	0.85±0.14	0.835
Female	7.51±1.12	8.17±0.97	0.236	0.71±0.05	1.15±0.10	**0.002****
5-HT1A						
Male	19.80±5.52	20.30±7.00	0.876	0.73±0.18	0.55±0.07	0.343
Female	18.91±7.09	18.21±3.01	0.944	0.93±0.13	0.79±0.057	0.340

mRNA level of NUDR, Freud-1 and 5-HT1A receptor in relation to endogenous reference gene GAPDH ($2^{-\Delta Ct}$ values$\times 10^3$±S.D.), n =8 animals. Protein level of NUDR, Freud-1 and 5-HT1A receptor in relation to actin (R.O.D values±S.E.M), n=8 animals. Data were analysed by Student t-test. Significance was assumed at $p<0.01$ for mRNA and $p<0.05$ for protein levels.

Table 4

The effect of stress induced during pregnancy on the expression of the NUDR, Freud-1 and 5-HT1A receptor mRNA and protein levels in the hippocampus female rats

Pregnant stressed females						
	mRNA level			**Protein level**		
Hippocampus	**CON**	**Stress**	***p***	**CON**	**Stress**	***p***
NUDR	6.49±0.20	6.01±0.34	**0.027***	0.77±0.08	0.73±0.04	0.708
Freud-1	8.57±0.80	7.26±0.43	**0.011***	0.94±0.10	0.86±0.08	0.581
5-HT1A	19.98±6.70	14.88±1.30	0.152	1.10±0.12	0.72±0.08	**0.047***

mRNA level of NUDR, Freud-1 and 5-HT1A receptor in relation to endogenous reference gene GAPDH ($2^{-\Delta Ct}$ values$\times 10^3$±S.D.), n =5 animals. Protein level of NUDR, Freud-1 and 5-HT1A receptor in relation to actin (R.O.D values±S.E.M), n=5 animals. Data were analysed by Student t-test. Significance was assumed at $p<0.01$ for mRNA and $p<0.05$ for protein levels.

Table 5

The effect of chronic mild stress on the expression of the NUDR, Freud-1 and 5-HT1A receptor mRNA and protein levels in the rat hippocampus

Chronic mild stress male						
	Relative mRNA level			**Protein (% of control)**		
Hippocampus	**CON**	**Stress**	***p***	**CON**	**Stress**	***p***
NUDR	9.17±1.02	8.85±1.14	0.597	0.41±0.05	0.40±0.03	0.908
Freud-1	8.55±1.14	8.17±1.21	0.557	0.61±0.10	0.36±0.02	**0.024***
5-HT1A	18.50±1.64	17.75±1.62	0.414	0.32±0.09	0.11±0.03	**0.047***

mRNA level of NUDR, Freud-1 and 5-HT1A receptor in relation to endogenous reference gene GAPDH (2−ΔCt values×103±S.D.), n=6–8 animals. Protein level of NUDR, Freud-1 and 5-HT1A receptor in relation to actin (R.O.D values±S.E.M), n=6–8 animals. Data were analysed by Student t-test. Significance was assumed at $p<0.01$ for mRNA and $p<0.05$ for protein levels.

Table 6

Chart of stress-induced changes

	PFC-1A	PFC-NUDR	PFC-F1	Hp-1A	Hp-NUDR	Hp-F1
OB						
M	↓P		↓P	↓R	↓R	↓R
F					↓R	
PS						
M	↓Pns				↓R	
F	↓P					→P
PREG						
F	↓P	↓P	↓P	↓P	↓R	↓R
CMS						
M	↓RP	↓RP	↓R Pns	↓P		↓P

Statistically significant changes (reduction ↓ or increase →) or trends (ns) in the protein (P) or mRNA (R) levels of 5-HT1A (1A), NUDR, or Freud-1 (F1) are shown for the different stress/depression models: olfactory bulbectomy (OB), prenatal stress (PS), pregnancy stress (PREG) and chronic mild stress (CMS) in males (M) or females (F).