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Original article

Hypothalamic insulin and glucagon-like peptide-1 levels in an animal model of depression and their effect on corticotropin-releasing hormone promoter gene activity in a hypothalamic cell line

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ABSTRACT

Background: In depression, excessive glucocorticoid action may cause maladaptive brain changes, including in the pathways controlling energy metabolism. Insulin and glucagon-like peptide-1 (GLP-1), besides regulation of glucose homeostasis, also possess neurotrophic properties. Current study was aimed at investigating the influence of prenatal stress (PS) on insulin, GLP-1 and their receptor (IR and GLP-1R) levels in the hypothalamus. GLP-1 and GLP-1R were assayed also in the hippocampus and frontal cortex – brain regions mainly affected in depression. The second objective was to determine the influence of exendin-4 and insulin on CRH promoter gene activity in *in vitro* conditions.
Methods: Adult male PS rats were subjected to acute stress and/or received orally glucose. Levels of hormones and their receptors were assayed with ELISA method. *In vitro* studies were performed on mHypoA-2/12 hypothalamic cell line, stably transfected with CRH promoter coupled with luciferase.
Results: PS has reduced GLP-1 and GLP-1R levels, attenuated glucose-induced increase in insulin concentration and increased the amount of phosphorylated IR in the hypothalamus of animals subjected to additional stress stimuli, and also decreased the GLP-1R level in the hippocampus. *In vitro* studies demonstrated that insulin is capable of increasing CRH promoter activity in the condition of stimulation of the cAMP/PKA pathway in the applied cellular model.
Conclusion: Prenatal stress may act as a preconditioning factor, affecting the concentrations of hormones such as insulin and GLP-1 in the hypothalamus in response to adverse stimuli. The decreased GLP-1R level in the hippocampus could be linked with the disturbances in neuronal plasticity.

Introduction

Many of the hormones involved in the maintenance of glucose homeostasis, such as insulin and glucagon-like peptide-1 (GLP-1) are also known to exert neurotrophic action and enhance neurotrophic processes [1]. However, some of factors engaged in the regulation of energy homeostasis, depending on the period of development and duration of action, may also exert unfavorable effects on neuronal plasticity. A typical example of such a bidirectionally acting hormone is corticosterone, which at physiological concentrations exerts neuroprotective properties, whereas at high levels or in case of chronic exposure, may aggravate nerve damage [2]. Adverse effects associated with excessive glucocorticoid action on neuronal plasticity are considered to be important in the pathogenesis of depression; however, the mechanism of this action is poorly explored. Some studies suggest that these effects may result from the inhibition of glucose and glutamate uptake or downregulation of growth factor synthesis. Thus far, little attention has been directed at the action of glucocorticoids on brain pathways, involved in energy metabolism in the development of depression.

Abbreviations: cAMP, cyclic adenosine monophosphate; CREB, cAMP response elementbinding protein; CRH, Corticotropinreleasing hormone; GLP-1glucagon, like peptide-1; GLP-1R, glucagonlike peptide-1 receptor; HPA, hypothalamic – pituitary – adrenal axis; IR, insulin receptor; PI3K, phosphatidylinositol3-kinase; PKA, protein kinase A.

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The frequent co-occurrence of depression and diabetes and similar brain changes, observed in animal models of diabetes and depression indicate that, in both of these illnesses, metabolic disturbances in the central nervous system may occur [3]. Glucocorticoids are known to enhance gluconeogenesis and evoke peripheral insulin resistance [4]. In addition to the peripheral action of glucocorticoids, they have also been observed to act in the hypothalamus to impair insulin signaling in an animal model of depression [5].

Our previous research, performed in prenatal stress model of depression in rats, demonstrated not only increases in plasma glucose and insulin concentrations but also changes in the concentrations of metabolic enzymes in the frontal cortex and hippocampus [6,7], as well as a decrease in the level of active phosphorylated insulin receptor in the hippocampus in animals after oral glucose administration [6]. However, in our previous study, the action of insulin was apparent only in frontal cortex and hippocampus – brain regions mainly affected in depression, and not in the hypothalamus which is a structure that plays major role in coordinating neuroendocrine processes and can better reflect the changes associated with excessive hypothalamic – pituitary – adrenal (HPA) axis activation during the prenatal period.

In addition to brain insulin action, in the context of both depression and diabetes, particular interest has been focused on the role of GLP-1, which is an incretin hormone, synthetized in intestinal L-cells and the brain. Stable GLP-1 analogs are currently used in therapies for type 2 diabetes. On the one hand, GLP-1 exhibits insulinotropic, neuroprotective and antidepressant properties [8], but on the other hand, it is known to enhance HPA axis activity, and *via* this action, it may potentiate the adverse effects of glucocorticoids and increase anxiety-like behavior [9].

Therefore, the first objective of the present study was to determine the levels of insulin, the total and phosphorylated form of the insulin receptor, GLP-1 and the GLP-1 receptor (GLP-1R) in the hypothalamus in a prenatally stressed rats. Additionally, to determine the influence of adverse factors that act in adult life, these parameters were also determined in control and prenatally stressed animals subjected to acute stress and glucose loading. Since GLP-1 exerted antidepressant effect in experimental animals, expression of this peptide and its receptors was also measured in the hippocampus and frontal cortex.

Apart from neuroprotective effects of GLP-1, the stimulatory action of this peptide on the HPA axis is currently being investigated. In the present state of knowledge, it seems that this effect may result from the stimulation of catecholamine neurons in the hindbrain regions that project to the hypothalamic paraventricular nucleus (PVN) and from the direct action of GLP-1 on the gene encoding corticotropin-releasing hormone (CRH) [10]. Current scientific data also note the possible involvement of insulin in the modulation of HPA axis activity [11]; however, it is not known whether this effect is exerted *via* the direct action of insulin on CRH-expressing neurons. Our past study [12] and the research conducted by other authors [13] has demonstrated that stimulation of the cyclic AMP-protein kinase A (cAMP/PKA) pathway is the major path involved in CRH promoter activation and that only this pathway is activated by the GLP-1R. Therefore, our second aim was to determine the influence of the stable GLP-1 analog: exendin-4 and insulin on the activity of the CRH gene promoter in a hypothalamic cell line in basal and forskolin-stimulated conditions which would allow for an understanding of whether GLP-1 and insulin may directly influence CRH synthesis and furthermore distinguish possible effects of these hormones in basal and stress conditions.

Material and methods

Animals

Sprague-Dawley rats were purchased in Charles River (Germany) and maintained at standard conditions with food and water available *ad libitum*. Vaginal smears from females were collected daily to determine the phase of the estrous cycle. During *proestrus*, female rats were placed with males for 12 h. Next day vaginal smears were tested for the presence of sperm. The pregnant females were than randomly assigned to the control and stress groups.

All experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Local Ethics Committee in Kraków, Poland.

Prenatal stress procedure

Prenatal stress was performed as described by Morley-Fletcher et al. [14]. Pregnant female rats were subjected to three stress sessions daily (at 9:00; 12:00 and 17:00), beginning from 14th day of pregnancy till delivery. Females were placed in plastic cylinders (7/12 cm) and exposed to a bright light for 45 min, while the control females were left undisturbed in their home cages. Twenty-one days after birth, male offspring from litters containing 10–14 pups with a comparable number of males and females were separated for the experiments.

Forced swimming test (FST)

FST was performed on 3-month old male rats accordingly to the procedure described by Detke et al. [15]. The animals were forced to swim in a cylinder (40 cm high, 18 cm in diameter) filled with water (22 °C ± 1 °C). Rats were subjected to two trails in 24-hour interval. The first trial (pre-test) lasted for 15 min, and the second trial lasted for 5 min. The total durations of immobility and climbing time were measured throughout the second trial.

Acute immobilization stress and oral glucose administration in adult animals

Half of the animals from the control and prenatally stressed groups were subjected to acute stress by immobilization in plastic cylinders for 1 h.

Immediately after acute stress, half of the animals from each of the control and stressed groups with and without acute stress were administered glucose (1 g/kg) by oral gavage. The remaining animals received water in the volume equal to glucose solution.

Tissue collection

Two hours after glucose administration, the animals were sacrificed by rapid decapitation. Brain structures were dissected on ice-cold glass plates, frozen on dry ice and stored at −80 °C.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of GLP-1, GLP-1 receptor (GLP-1R), insulin and the concentrations of active (phosphorylated at tyrosine$^{1162/1163}$) and total amount of insulin receptor (phospho-IR and IR) in selected brain structures were determined with ELISA method with commercially available assay kits (GLP-1: EGLP-35 K, Merck-Millipore; GLP-1R: MBS2031967, MyBioSource; insulin: RI-13 K, Merck-Millipore; phospho-IR: 17-484, Merck-Millipore; IR: 17-483, Merck-Millipore). The sensitivity of selected kits were as

follow: 2 pM for GLP-1; 23.44 pg/mL for GLP-1R; 0.1 ng/mL for insulin; 1 U/mL for phosphor-IR and 0.75 ng/mL for IR.

The concentrations of selected markers were subsequently divided by protein content in a given sample. Total protein concentrations was determined with bicinchoninic acid method [16].

Cell culture conditions

Hypothalamic cell line mHypoA-2/12 (CLU172; CELLutions-Cedarlane, Burlington, Ontario, Canada) was maintained in DMEM (5796, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) with the addition of 50 units/ml of penicillin and 50 μg/ml of streptomycin in an atmosphere composed of 5% CO_2/95% O_2 at 37° C. The cells were detached by washing once PBS free of Ca^{2+} and Mg^{2+} and subsequently trypsinized 1× in trypsin-EDTA. One day before each experiment the cells were washed and serum-starved overnight in the culture medium containing 0.2% FBS.

Plasmid construction and cell transfection

The coding sequence of the luciferase hR*luc* gene was obtained from psiCHECK-2 plasmid (Promega, #C8021; Madison, WI, USA) by PCR reaction with the usage of following primers: h*luc*F: 5′-GCATCGAGGATCCCATATGGCCGATGCTAAGAACATT–3′; and h*luc*R: 5′–CAGTACCTCGAGTTATTACACGGCGATCTTGCC-3′. After amplification and purification of the obtained PCR product (1700 bp), the luciferase gene sequence was cloned into the pCRHYFAP plasmid. This plasmid contained a fragment (from –663 to +124 bp) of the human CRH gene promoter has been described in our previous work [12]. The sequence coding EYFP was replaced with luciferase using *XhoI* and *NdeI* and subsequently ligated. The obtained pCRH*luc* plasmid was analyzed by restriction digestion and sequencing. Plasmid DNA was than amplified in *E.coli* DH5α strain (ThermoFisher Scientific, Waltham, MA, USA) and purified using QIAfilter Plasmid Maxi Kit (Qiagen, 12263; Hilden, Germany).

Cell transfection was performed according to our earlier protocol [12] using Lipofectamine® 2000 reagent (Thermo Fisher Scientific; Waltham, MA, USA). The dose-response assay with mHypoA-2/12 cells indicated that 400 μg/ml of selection antibiotic zeocin was the lowest concentration that killed 100% of the non-transfected cells. Four weeks after transfection, the zeocin resistant colonies were selected, cultured in the medium with 200 μg/ml of zeocin and subsequently assayed for reporter gene activity.

Drug treatment

Stably transfected mHypoA-CRH/luc-663 cells were treated with the appropriate vehicle, forskolin (BioShop, Burlington, Ontario, Canada; final concentration: 10 μM), exendin-4 (Tocris, final concentrations: 0.1 μM 1 μM), insulin (Tocris, final

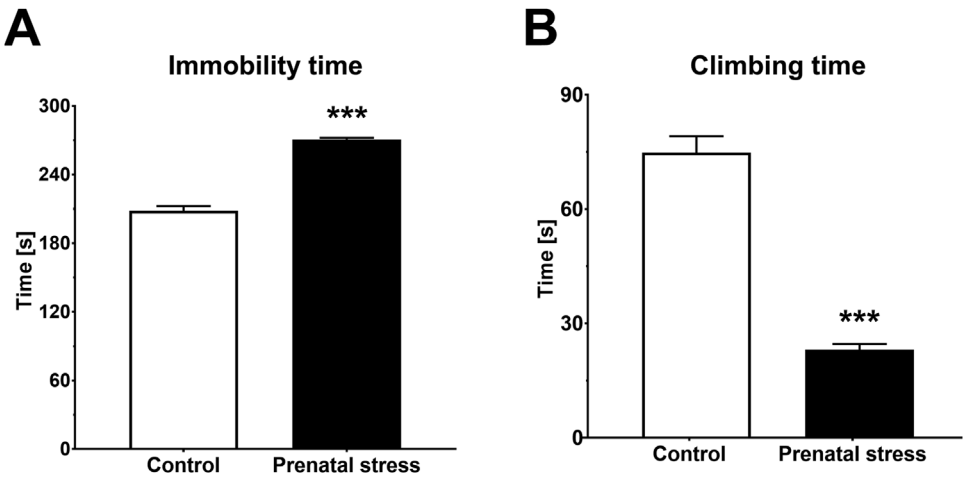

Fig. 1. The effects of prenatal stress on the immobility time (A) and climbing time (B) in the forced swim test, n = 40; ***$p < 0.001$ *vs.* control group.

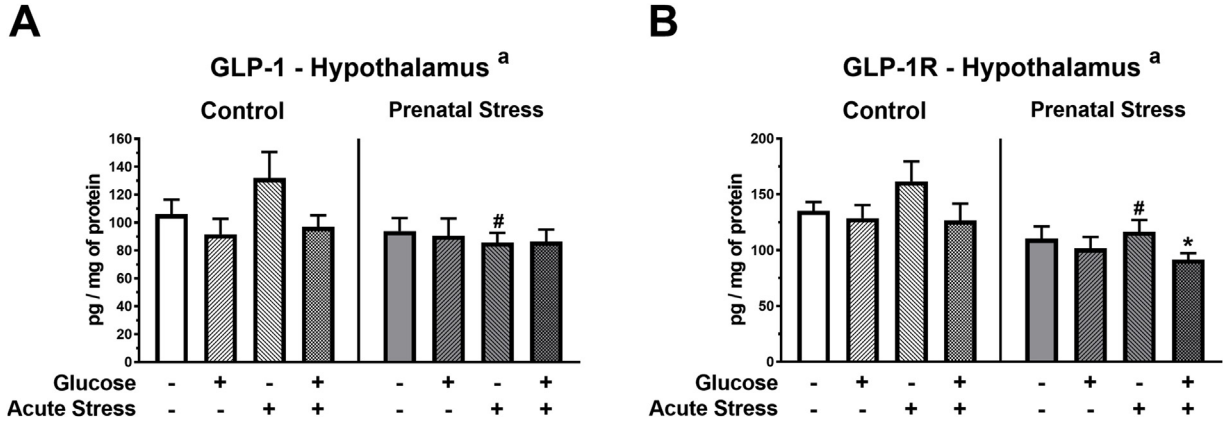

Fig. 2. The effects of prenatal stress, acute stress and glucose administration on GLP-1 (A) and GLP-1R (B) levels in the hypothalamus, n = 7–9, a – Statistically significant main effect of prenatal stress; *$p < 0.05$ *vs.* control (without prenatal stress, acute stress or glucose loading), #$p < 0.05$ *vs.* appropriate control group (control animals subjected to similar stress conditions).

concentrations: 0.1 and 1 μM) and wortmannin (Sigma-Aldrich; final concentrations: 0.1 and 1 μM).

Luciferase reporter gene activity assay

Luciferase activity was determined using a Luciferase Assay System (Promega, Madison, WI, USA). The cells were washed with PBS without Ca^{2+} and Mg^{2+}, harvested with potassium phosphate buffer (pH = 7,8) with added 0,2% Triton® X-100 according to the procedure described by Hampf and Gossen [17] and subsequently centrifuged (10 000 × g; 2 min). The light emission of each sample was measured for 10 s in MicroBeta Trilux 1450 LSC and Luminescence Counter (PerkinElmer; Waltham, MA, USA).

Statistical analysis

The results are displayed as means ± SEM. Data were analyzed using the STATISTICA program. The statistical analyses were performed using factorial ANOVA and then Duncan's *post hoc* test, $p < 0.05$ was considered significant.

Results

Measurement of immobility and climbing time in FST

The results of FST were analyzed using one-way ANOVA and Duncan's *post hoc* test. When compared to control group, prenatally stressed rats demonstrated significantly elevated total immobility time (270.33 ± 1.46 *vs.* 208.25 ± 4.12; $p = 0.000113$) and lower climbing time (78.03 ± 4.33 *vs.* 26.30 ± 1.55; $p = 0.000113$) (Fig. 1A, B).

Hypothalamic GLP-1 and GLP-1 receptor levels

Factorial ANOVA shown significant main effect of prenatal stress on the concentration both of GLP-1 and its receptor in the hypothalamus. *Post hoc* analysis however revealed, that the statistically significant decreases ($p < 0.05$) in the levels of GLP-1 and GLP-1R were evident only in the group of prenatally stressed rats subjected to acute stress *vs.* control group treated with the same stressor in the adulthood (Fig. 2A, B). Additionally, the level of GLP-1R in the hypothalamus was significantly lower in prenatally stressed animals to both additional stress factors (acute stress, oral glucose administration) in comparison with untreated control (without acute stress and glucose loading).

Hypothalamic insulin levels

Three-way analysis of variance shown significant main effects of prenatal stress and glucose loading. As displayed in Fig. 3A, the concentrations of insulin were significantly increased in both the control and prenatally stressed animals, which received orally glucose *vs.* water-receiving control animals ($p < 0.001$–0.01). Moreover, *post hoc* Duncan's test shown, that in prenatally stressed group which received glucose, the level of insulin was significantly decreased ($p < 0.05$), when compared to the corresponding control group (control animals after glucose administration, without acute stress).

Total and phosphorylated forms of the insulin receptor

Post hoc analysis revealed significant augmentation of the active form of insulin receptor β subunit (phosphorylated at $Tyr^{1162/1163}$) in the prenatally stressed group that was subjected to both acute stress and receiving glucose *vs.* control group subjected to similar stressors (Fig. 3B). Furthermore, glucose loading significantly increased the total amount of the insulin receptor β subunit in the prenatally stressed groups that received glucose, when compared to animals from the control group (without acute stress and glucose loading) (Fig. 3C).

Levels of GLP-1 and its receptor in the hippocampus and frontal cortex

Three-way ANOVA revealed no significant effect of all stress factors (prenatal stress, acute stress and glucose administration) on the concentration of GLP-1 in the hippocampus and frontal cortex (Fig. 4A and C) or GLP-1R level in the frontal cortex (Fig. 4B).

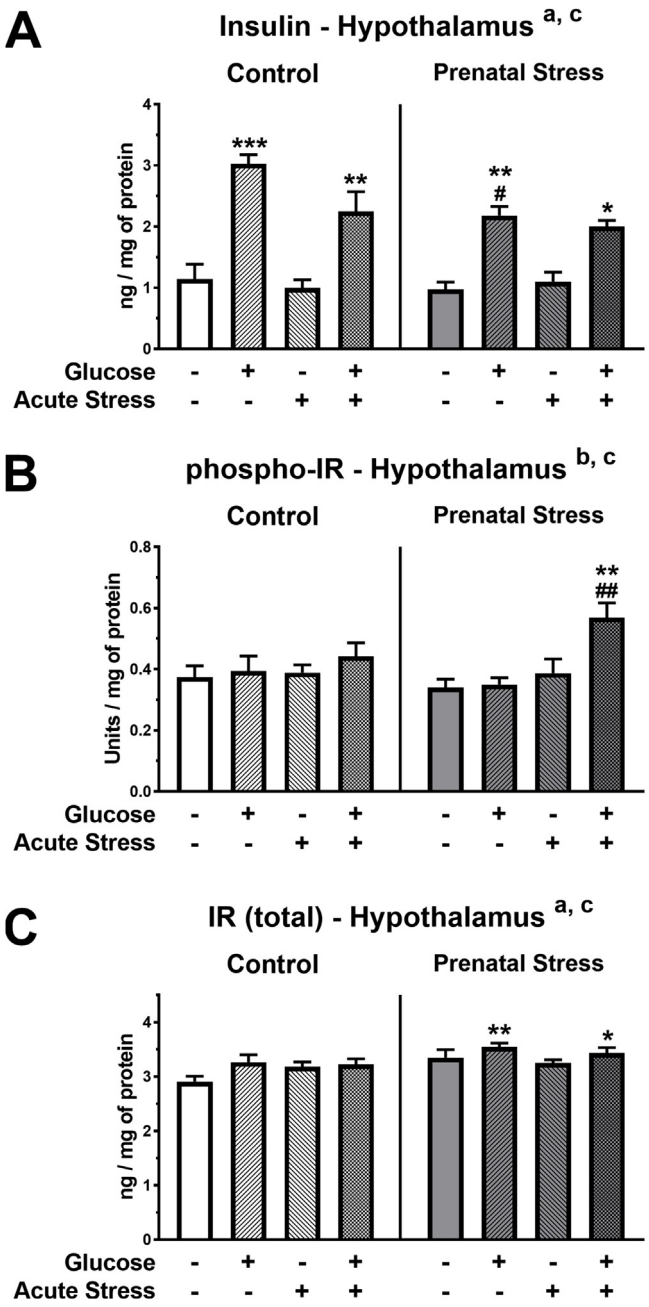

Fig. 3. The effects of prenatal stress, acute stress and glucose administration on insulin levels (A) and the levels of phosphorylated (Tyr 1162/1163) (B) and total (C) insulin receptor β subunit in the hypothalamus, n = 7–9; a – Statistically significant main effect of prenatal stress, b – Statistically significant main effect of acute stress, c – Statistically significant main effect of glucose loading. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control group, # $p < 0.05$, ## $p < 0.01$ *vs.* appropriate control group (control animals subjected to similar stress conditions).

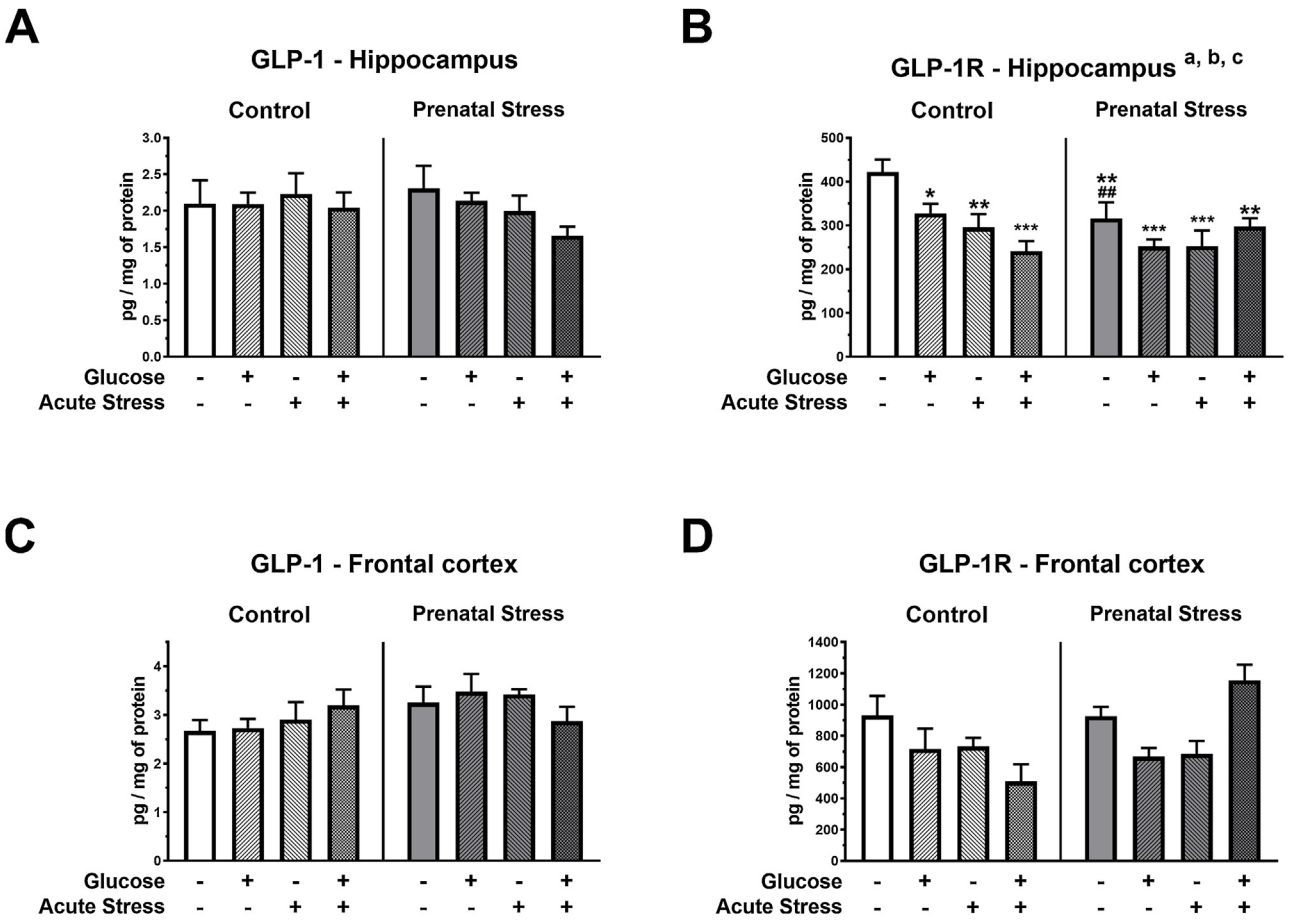

Fig. 4. The effects of prenatal stress, acute stress and glucose administration on GLP-1 and GLP-1 levels in the hippocampus (A, B) and frontal cortex (C, D), n = 6–8; a – Statistically significant effect of prenatal stress, b – Statistically main significant effect of acute stress, c – Statistically significant main effect of glucose loading $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control group; $\#\#p < 0.01$ *vs.* appropriate control group (control animals subjected to similar stress conditions).

On the other hand, all the examined factors influenced GLP-1R protein level in the hippocampus (Fig. 4D). In basal conditions *post hoc* analysis shown significant decrease in the amount of GLP-1R in prenatally stressed group of animals without acute stress and glucose loading *vs.* control group ($p < 0.05$). Also in animals subjected to acute stress and/or receiving glucose, both control and prenatally stressed, the GLP-1 receptor level was significantly lower than in the control group (animals without acute stress and glucose loading).

The effect of exendin-4 and insulin on the basal and forskolin-stimulated activity of the CRH promoter gene

Forskolin, when added to the culture medium for six hours, significantly increased the basal activity of luciferase reporter gene in transfected mHypoA-CRH/*luc*-663 cells.

None of the studied concentrations of exendin-4 were shown to influence CRH promoter gene sequence, neither in basal conditions or after stimulation with forskolin (Fig. 5A–C).

In basal conditions (in groups without the treatment with forskolin), insulin did not influence the activity of the selected CRH promoter at any of the chosen time points (Fig. 5D–F). However, when administered for 7 and 24 h, it elicited a stimulatory effect on the CRH promoter gene in the forskolin-treated groups. *Post hoc* analysis revealed, that seven- and twenty-four-hour incubations with insulin at concentrations of 0.1 and 1 μM evoked significant increases ($p < 0.001$) in luciferase reporter gene activity in forskolin-stimulated cells in comparison with the groups treated with forskolin alone (Fig. 5E, F).

Effects of wortmannin on CRH promoter gene activity in transfected cells stimulated with insulin and forskolin

Wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3K), was added to the culture medium 30 min prior to the treatment of the cells with forskolin. When administered to the culture medium at the concentration of 0.1 μM, it did not inhibit the action of insulin (added to the cells in the final concentration of 0.1 μM) in the forskolin-stimulated group (Fig. 6A). However, when given at higher concentration of 1 μM, wortmannin managed to reverse the stimulatory effect of insulin. *Post hoc* analysis shown a significant decrease of reporter gene activity ($p < 0.001$) in wotmannin treated +forskolin +insulin group *vs.* + forskolin + insulin treated vehicle (Fig. 6B).

Discussion

The obtained results indicate that prenatal stress in rats can decrease the levels of GLP-1 and/or its receptor in the hypothalamus and hippocampus, but not in the frontal cortex. Our findings also revealed marked alterations in hypothalamic insulin level as well as changes in the expression and insulin receptor phosphorylation, however these effects were not evident in basal condition, but observed in animals subjected to additional stress factors

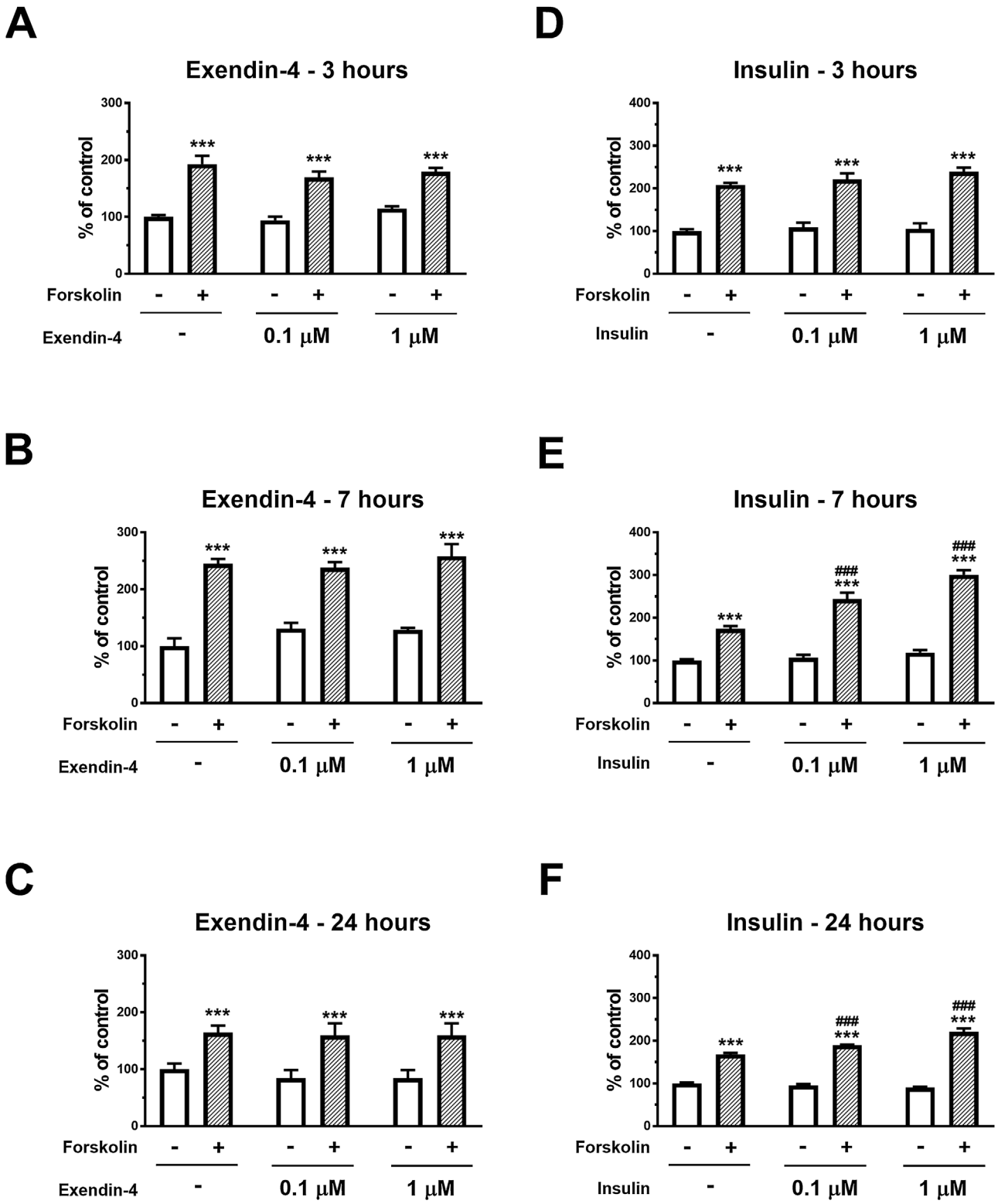

Fig. 5. Effects of exendin-4 (A, B and C) and insulin (D, E and F) on the basal and forskolin-stimulated luciferase activity in stably transfected mHypoA-CRH/*luc*-663 cells. Both compounds were added to the culture medium for 3 h, 7 h, and 24 h at final concentrations of 0.1 and 1 μM. The cells were treated in triplicate, and the averages of at least three independent experiments are shown. The light counts per second (LCPS) of each sample were normalized to the protein concentration and are finally displayed as the % of the control. $^{***}p < 0.01$ *vs.* control group; $^{###}p < 0.01$ *vs.* control group stimulated with forskolin.

which is consistent with our previous results, displaying that stress in the prenatal period may change the sensitivity of the brain tissue to adverse stimuli during the course of life [6,7].

In the hypothalamus, weakened response of GLP-1 release to acute stress observed in prenatally stressed rats may correspond to the failure in the adaptation to stress conditions – a phenomenon often seen in depression. On the other hand, observed down-regulation of GLP-1R expression in prenatally stressed group after acute immobilization may be considered an adaptive mechanism, aimed at weakening of HPA axis hyperactivity, which was

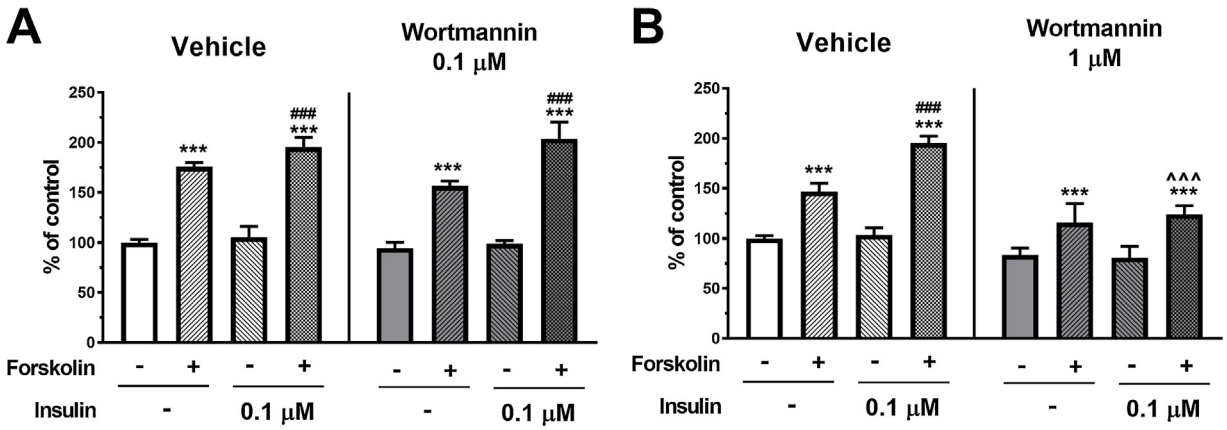

Fig. 6. The effect of wortmannin on insulin action in forskolin-stimulated activity in stably transfected mHypoA-CRH/luc-663 cells. Wortmannin was added to the culture medium in final concentrations of 0.1 μM (A) and 1 μM (B) 30 min prior to forskolin treatment. The cells were treated in triplicate, and the averages of at least three independent experiments are shown. The light counts per second (LCPS) of each sample were normalized to the protein concentration and are finally presented as the % of control. ***$p < 0.001$ *vs.* control group; ###$p < 0.001$ *vs.* forskolin-treated control group; ^^^$p < 0.001$ *vs.* forskolin and insulin treated control group.

previously observed in the applied depression model [18]. GLP-1 has been shown to stimulate the HPA axis activity, among others by its direct effect on CRH-expressing neurons in the PVN and this action of GLP-1 on CRH synthesis [10]. It is therefore possible that as a result of acute stress in adult prenatally stressed rats, GLP-1R receptor expression is suppressed in order to prevent excessive CRH secretion.

Interestingly, in the present study, reduced GLP-1R protein expression level was found also in the hippocampus - brain region which is most often severely affected in animal models of depression and depressed patients. Some data indicate that excess glucocorticoid action can impair brain GLP-1 signaling [19]. Our previous research demonstrated, that prenatal stress and acute stress may elevate corticosterone level in the frontal cortex, while in the hippocampus, the increase in the concentration of this hormone was recorded only after acute stress [6]. Therefore, it is suggested that the observed downregulation of GLP-1R expression in the hippocampus is not due to the effect of corticosterone in adult animals, and it may be rather a consequence of early epigenetic programming caused by glucocorticoids in the prenatal period. Outside of the hypothalamus, GLP-1Rs are expressed in multiple brain regions, particularly in structures involved in cognitive function and mood regulation *i.e.* prefrontal cortex, anterior cingulate cortex, hippocampus and amygdala [20]. The function of GLP-1 in these brain structures is less clearly defined, however numerous research data emphasizes its neurotrophic and neuroprotective properties, *i.e.* by the facilitation of long-term potentiation (LTP) [21,22], upregulation of neurotrophic factor expression and the prevention of neuronal apoptosis [23]. Thus, a reduction of GLP-1 receptor level in the hippocampus observed in the current study may be a change related to pro-depressive behavior and may therefore facilitate in the impairment of neurotrophic factor expression, which was observed in prenatal stress model [24].

Like GLP-1, also insulin is known to exhibit neuroprotective and neurotrophic effects. In the hypothalamus, we found a significant increase in insulin levels in both control and stressed animals after oral glucose administration when compared to water-receiving controls; however, it has also been demonstrated that prenatal stress attenuated the glucose-induced increase in insulin levels. The hypothalamus is the main brain region involved in energy homeostasis, which regulates metabolic processes *via* its reciprocal projections to other brain regions and to the periphery. Since hypothalamic insulin signaling is required for inhibition of glucose production [25], thus a lower level of this hormone in glucose-loaded prenatally stressed rats may be the reason for the increase in plasma glucose concentration in rats subjected to prenatal stress, as well as a rise in serum insulin levels occurring in prenatally stressed groups that received glucose in our previous experiments [6]. This hypothesis seem to be additionally supported by the increased expression and phosphorylation of the insulin receptor in the hypothalamus in prenatally stressed animals, which received glucose.

In the present *in vitro* study, we have also examined whether GLP-1 can directly affect the activity of CRH promoter gene. Despite growing evidence describing the involvement of GLP-1 in the stimulation of CRH gene expression in the hypothalamus, we did not manage to observe this effect in the stably transfected hypothalamic mHypoA-CRH/*luc*-663 cells since different concentrations of exendin-4 had no effect on the activity of the (+124 to -663 bp) CRH promoter gene sequence. The applied fragment of the CRH promoter sequence contained the cAMP-responsive element (CREB) binding site, which bound the main transcription factor involved in stimulation of CRH gene transcription in mammalian cells [26]. These results may suggest that the involvement of GLP-1 in the activation of the CRH gene is not direct; however, other researchers have managed to prove a direct stimulatory effect of GLP-1 on the CRH promoter in the different, transiently transfected hypothalamic cell line 4B [27]. Therefore, it may be possible that the lack of changes in the activity of the CRH promoter in our experiments may either be a result of the differences in the length of the chosen promoter gene sequence or may simply emerge from phenotypic differences between individual cell lines.

Interestingly, the obtained results indicate that insulin may stimulate CRH synthesis in mHypoA-CRH/luc-663 cells. This effect however, was only demonstrated in the conditions of CRH gene promoter activation by the stimulation of the cAMP/PKA pathway by forskolin. Moreover, the observed stimulatory effect of insulin was reversed with wortmannin, which is an inhibitor of phosphatidylinositol 3-kinase (PI3-K), the classical insulin-activated intracellular pathway. Additionally, there are studies that have demonstrated either direct or indirect roles of PI3-K/AKT in the CREB pathway [28]. Most of the current experimental data suggest that insulin has an inhibitory rather than stimulatory effect on HPA axis activation [11]. However, there are also studies demonstrating that the administration of supraphysiological insulin concentrations can increase HPA axis activity. A group of male patients given a high dose of insulin displayed increased plasma ACTH and cortisol levels over the course of six hours

[29,30]. However, these reports do not provide a definitive answer, whether this effect is mediated by a direct action of insulin on the hypothalamus.

The results of *in vitro* studies revealed that insulin may increase the activity of the CRH promoter in stressful conditions. This finding may provide new data concerning the influence of insulin on HPA axis activity; however, at this point, it is not clear whether this effect can be translated into *in vivo* conditions, and additional experiments are needed to determine the physiological significance of this mechanism.

Moreover, present study demonstrated that prenatal stress in rats acted mostly as a preconditioning factor in the hypothalamus, affecting insulin, GLP-1 and their receptor concentrations in response to adverse stimuli during adulthood. In turn, the prenatal stress-induced decrease in GLP-1 receptor level in the hippocampus may be responsible for disturbances in neuronal plasticity, observed in this model of depression i.e. by contributing to the attenuation of growth factor synthesis. At this point, however, it remains difficult to determine whether the decrease in the expression of this receptor in the hippocampus may be one of the primary causes of disturbances observed in this model of depression, or is it just a secondary change resulting from the impairment of other molecular mechanisms in the hippocampus caused by chronic stress in the prenatal period. The results of insulin and its receptor measurements in the hypothalamus may suggest that prenatal stress may decrease its distribution in this part of the brain, but at the same time contribute to the increase in the number and activity of insulin receptors in response to sudden increase in glucose supply.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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