



Restraint-Induced Glucocorticoid Receptor Downregulation is Dysregulated in High Fat Diet-Fed Rats Likely from Impairment of miR-142-3p Expression in the Hypothalamus and Hippocampus

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Abstract: High fat diet (HFD) induces dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis function. The HPA axis is controlled by the feedback of glucocorticoids on the hypothalamus, hippocampus and pituitary. At least three miRNAs (miR-101a, miR-124, miR-142-3p) have been reported to suppress glucocorticoid receptor (GR) translation. Because their relation to stress-induced downregulation of GR expression and dysregulation of its expression in HFD feeding are unclear, we studied to identify which miRNAs are involved in restraint-induced downregulation of GR expression in the hypothalamus and hippocampus, and to compare the basal and restraint-modified miRNA expressions in these tissues in HFD-fed rats. Rats exposed to HFD were divided into two groups, HFD-induced obese (HFD-ob) and obesity resistant (HFD-obR) rats. Basal plasma corticosterone concentrations were higher in HFD-ob than in standard chow-fed (SC) rats and in HFD-obR. Restraint-induced elevation of plasma corticosterone was higher in HFD-obR than in the other groups. Restraint decreased GR expressions and increased miR-142-3p expression in the hypothalamus and hippocampus without affecting others expressions. miR-142-3p expressions in both areas were increased by dexamethasone and restraint-induced miR-142-3p expression was blocked in adrenalectomy. The basal expression of GR or miR-142-3p expression in both areas of HFD-fed rats did not differ from those of SC, and restraint induced no changes in GR or miR-142-3p expression in both areas in HFD-ob and HFD-obR. These results suggest that impairment of glucocorticoid-induced increase in miR-142-3p may be involved in dysregulation of stress-induced downregulation of GR expression in the hypothalamus and hippocampus of HFD-fed rats.

Keywords: Stress, High Fat Diet, Hypothalamus, Hippocampus, microRNA

1. Introduction

Obesity is associated with lifestyle-related cardiovascular and metabolic disorders. In addition, recent epidemiological studies demonstrated that obesity is associated with an increased risk of developing depression (1). However, the precise mechanisms underlying the interaction between obesity and depression have not been elucidated. Excess or prolonged exposure to cortisol has been implicated in the development of stress-related disorders including depression. Stress activates the hypothalamic-pituitary-adrenal (HPA) axis. Glucocorticoid hormones are consistently increased in severely depressed patients and in rodent stress models (2-5).

It is thought that stressful life events and the consequent HPA axis hyperactivity can be a cause of depression. In addition to stressful life events, nutritional status changes HPA axis activity. A series of animal studies has shown that a high-fat diet (HFD)-feeding increases HPA axis activity; plasma corticosterone concentrations are significantly increased in HFD-fed rats (6) and HFD feeding also elevates both basal and restraint-induced HPA activity in rats (7).

Glucocorticoid receptor (GR) is widely distributed in the brain, including the regions for negative regulation of the HPA axis, such as the hypothalamus and hippocampus (8-10). Restraint decreases GR mRNA expression in the hypothalamus and the hippocampus (11). However, to the best

of our knowledge, there have been no studies on impairment of stress-induced GR downregulation in HFD-fed rats. We hypothesized that HFD feeding dysregulates stress-induced GR downregulation in the hypothalamus and the hippocampus because HFD feeding accentuates restraint-induced HPA activity.

MicroRNAs are small non-coding RNA molecules that regulate the expression of target genes at the post-transcriptional level (12). It has been reported that there are several microRNAs with sequences capable of binding to the GR 3' untranslated region (UTR) and that at least three miRNAs (miR-101a, miR-124, miR-142-3p) have been precisely examined for their regulatory role in GR expression in vitro (13, 14). However, their involvement in stress-induced downregulation of GR expression and dysregulation of HPA axis function in the HFD-fed rats is unclear. It is known that rats exposed to HFD can be divided into two groups; one half of rats is HFD-induced obese (HFD-ob) rats and the other is HFD-induced obesity resistant (HFD-obR) rats, gaining the almost the same amount of weight as standard chow (SC)-fed controls (15). However, the difference in HPA axis response to stress between HFD-ob and HFD-obR is unknown.

Therefore, the first aim of this study is to identify which miRNAs are related to restraint-induced downregulation of GR expression in the hypothalamus and the hippocampus of SC-fed rats. The second aim is clarify how expression of candidate miRNAs is regulated by using dexamethasone treated rats and adrenalectomized rats. Finally, the third aim is to verify the hypothesis that HFD induces dysregulation of the HPA axis in stress by comparing the basal levels and restraint-induced changes of corticosterone concentration, GR mRNA and protein expressions and miRNA expression in the hypothalamus and hippocampus among SC-fed, HFD-ob and HFD-obR rats, and to further discuss the mechanisms underlying the HFD-induced dysregulation of the HPA axis in stress.

2. Materials and Methods

2.1. Animals

Wistar rats were maintained at $23 \pm 2^\circ\text{C}$ on a 12:12-h light-dark cycle (lights on at 0800 h, off at 2000 h). They were allowed *ad libitum* access to laboratory chow and distilled water. All experimental procedures were reviewed and approved by the Laboratory Animals Ethics Review Committee of Nippon Medical School. Four-week old male rats were fed either standard chow (SC; contains 3.85 kcal/g with 10% fat, 20% protein and 70% carbohydrate; D12451B; Research Diet, Inc., New Brunswick, NJ) or high-fat diet (HFD; contains 4.73 kcal/g with 45% fat, 20% protein, 35% carbohydrate; D12451 Research Diet, Inc.) for 4 weeks. Weekly food intake and body weight were measured throughout the 4 weeks. After 4 weeks on the HFD, rats with body weight gain above mean + 1SD of SC-fed rats were defined as HFD-obese (HFD-ob), while those with body weight gain within mean + 1 SD of SC-fed rats were defined

as HFD-obese resistant (HFD-obR). Mean body weights after 4-week HFD-loading were 338.6 ± 10.1 for HFD-ob (N=24), 306.1 ± 15.5 for HFD-obR (N=24) and 296.3 ± 15.3 for SC (N=24), and their daily calorie intakes per 100g bw were 36.0 ± 0.4 kcal for HFD-ob (N=24), 33.6 ± 0.5 kcal for HFD-obR (N=24) and 32.0 ± 0.4 kcal for SC (N=24).

2.2. Restraint Stress Exposure

HFD-ob, HFD-obR and SC rats were wrapped in a flexible wire mesh (12mm \times 12mm), and kept for 30, 90 or 120 min between 0900 h and 1200h in an isolated room (16). Rats were sacrificed in the adjacent room immediately after each stress exposure period and their trunk blood was collected for plasma hormone assay. Their brains were removed and hypothalamus and hippocampal slices (2-mm thickness) were dissected with a brain slicer (Muromachi kikai Co. Ltd, Tokyo, Japan). The paraventricular nucleus (PVN) of hypothalamus, CA1, CA3 and dentate gyrus (DG) of hippocampus were further punched out using a tissue puncher with 1.25 mm diameter (Brain Science Idea, Co., Osaka, Japan). Tissue samples were kept at -80°C until use for mRNA, miRNA and protein expression analyses. Non-stressed control rats were kept in a separate room from the stressed rats during restraint, and were treated the same way.

2.3. Dexamethasone Administration

Dexamethasone (30 or 100 $\mu\text{g}/\text{kg}$ bw) or saline was injected intraperitoneally to naive male rats (6 week old). Rats were killed at 2-h post-injection, brains were removed and the PVN and DG were punched out for mRNA, miRNA and protein expression analyses.

2.4. Adrenalectomy

Under pentobarbital sodium anesthesia, naive male rats (6 week old) were bilaterally adrenalectomized (ADX) and the same number of male rats were sham-operated (sham) as controls. All rats were returned to their home cages and immediately given a bottle of 0.9% NaCl. After 3-day recovery, rats were restrained as described in the restraint stress exposure session. Plasma corticosterone levels were measured on trunk blood samples to confirm successful adrenalectomy.

2.5. RNA Extraction and Real-Time RT-PCR Analysis

Total RNA was extracted from the hypothalamus and hippocampus of rats using ISOGEN II (Nippon Gene, Tokyo, Japan). For miRNA expression analysis, first-strand cDNA was synthesized using 1 μg of denatured total RNA at 37°C for 1 h, and was then terminated at 85°C for 5 min using Mir-X[®] miRNA First-Strand Synthesis and SYBR[®] qRT-PCR kit (Clontech Laboratories Inc., Mountain View, CA). For mRNA expression analysis, the first-strand cDNA was generated using 0.5 μg of denatured total RNA at 37°C for 15 min, 84°C for 5 s, and 4°C for 5 min using a PrimeScript[®] RT reagent kit with gDNA Eraser (Takara Bio, Shiga, Japan). PCR was performed by denaturation at 94°C for 5 s and

annealing-extension at 60 °C for 30 s for 40 cycles, using SYBR premix Ex Taq (Takara) and specific primers for rat GR (NR3c1) (NM_012576.2, forward primer, 5'-CATGTTAGGTGGGCGTCAAGTG, and reverse primer, 5'-GGTCATTTGGTCATCCAGGTGTAAG-3') and GAPDH (NM_017008.3, forward primer, 5'-GGCACAGTCAAGGCTGAGAATG3, and reverse primer, 5'-ATGGTGGTGAAGACGCCAGTA). To normalize each sample for RNA content, GAPDH, a house-keeping gene, or U6 small nuclear RNA (Clontech Laboratories, Inc.) were used for mRNA and miRNA expression analyses, respectively. Diluted normal rat pituitary cDNA and the 2nd derivative method were used as the standard and for calculating Ct values, respectively.

2.6. Western Blotting of GR

Tissues were lysed with TNE buffer (10 mM Tris-HCl, PH 7.8, 1% NP-40, 150 mM NaCl and 1mM EDTA) containing the Complete™ proteinase inhibitor cocktail (Roche Diagnostics). After centrifugation to remove debris, protein concentrations in the supernatant were measured. Protein extracts (20 g) were mixed with 3XSDS-sample buffer (200 mM Tris-HCl, pH 6.8, 30% glycerol, 6% SDS, 0.03% bromophenol blue and 3% β-mercaptoethanol) and boiled for 5 min. They were then separated by Mini-PROTEAN TGX gel (Bio-Rad Laboratories) and transferred onto PVDF membranes. Membranes were blocked with 5% nonfat dried milk and primed with anti-GR antibodies (1:200; MA1-510; Thermo Scientific, Rockford, IL). Membranes were washed and then incubated with HRP-conjugated anti-mouse IgG (Jackson Immuno Research Laboratory, West Grove, PA). Immunoreactivity was detected and quantified with ChemiDoc XRS (Bio-Rad Laboratories, Inc.) using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific). After detecting GR signals, membrane were incubated to strip those antibodies using restore PLUS western blot stripping buffer (Thermo Scientific), and were re-primed with HRP-conjugated mouse anti-actin monoclonal antibody (Progen Biotechnik, Heidelberg, Germany). Signals were detected and quantified to normalize for equal loading and blotting efficiency.

2.7. Plasma Corticosterone Measurement

Trunk blood was collected into tubes containing EDTA 2Na (1 mg/ml blood) and centrifuged at 3000 rpm for 20 min at 4°C. A 1-ml aliquot of plasma was transferred into 1.5-ml Eppendorf tubes and stored at -80 °C for later measurement. Plasma corticosterone concentrations were measured using a corticosterone EIA kit (500655, Cayman Chemical Company, Ann Arbor, MI).

2.8. Statistical Analysis

Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Turkey's *post-hoc* test and two-way ANOVA with Bonferroni multiple comparisons using Prism 6.0 software (GraphPad Software, Inc., La Jolla,

CA). For real-time RT-PCR data, all results were expressed as percent of control values. Statistical significance was set at the $p < 0.05$ level.

3. Results

3.1. HFD-Induced Prolonged Elevated Plasma Corticosterone Levels in Restrained HFD-ob and HFD-obR Rats

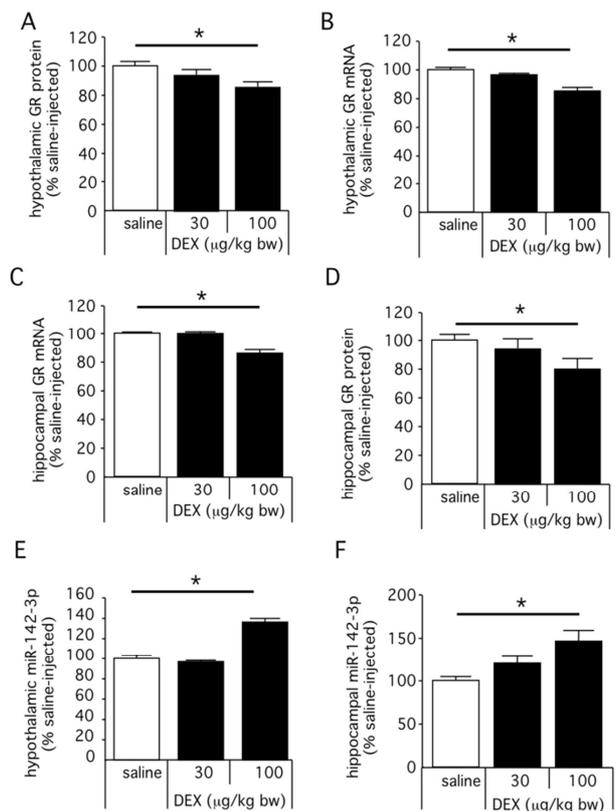


Figure 1. SC, HFD-ob and HFD-obR rats were exposed to restraint stress for 30, 90 or 120 min. Plasma corticosterone (A) and GR mRNA (B and D), GR protein (C and E) and miR-142-3p (F and G) expression in the hypothalamus (B, C and F) and hippocampus (D, E and G) were assayed. Expression levels of protein, mRNA and miRNA are given as % of non-restrained controls (non). N=6. * $p < 0.05$ vs. non-restrained controls, # $p < 0.05$ vs. SC and HFD-ob rats.

Basal plasma corticosterone concentrations at 0900AM were higher in HFD-ob (22.6 ± 2.8 ng/ml) than in HFD-obR (19.1 ± 1.4 ng/ml) and SC-rats (12.0 ± 2.4 ng/ml, $p < 0.05$) (N=8). Restraint significantly increased plasma corticosterone concentrations at 30 min (1.5 ± 0.2 µg/ml, N=6, $p < 0.05$), and these were then significantly lower at 90 and 120 min in SC rats (Fig. 1A). In HFD-ob rats, restraint significantly increased corticosterone concentrations at 30 min (2.5 ± 0.7 µg/ml, N=6, $p < 0.05$) and these were significantly lower at 90 and 120 min. However, the concentrations at 30, 90 and 120 min were significantly higher than those in SC rats (2.5 ± 0.7 ng/ml for HFD-ob vs 1.5 ± 0.2 ng/ml for SC at 30 min, 1.1 ± 0.7 ng/ml for HFD-ob vs 0.3 ± 0.1 ng/ml for SC at 90 min and 0.9 ± 0.2 ng/ml for HFD-ob vs 0.2 ± 0.05 for SC at 120 min, N=6, $p < 0.05$) (Fig. 1A). In HFD-obR rats, restraint increased

corticosterone concentrations and these were significantly higher at 30 min ($2.8 \pm 0.9 \mu\text{g/ml}$), 90 min ($3.2 \pm 0.9 \mu\text{g/ml}$) and 120 min ($3.2 \pm 0.2 \mu\text{g/ml}$) than in S SC-rats ($N=6$, $p<0.05$). Restraint-induced elevation of plasma corticosterone concentrations at 90 min and 120 min was significantly higher in HFD-obR than in HFD-ob and SC-rats (Fig. 1A).

3.2. Impaired Regulation of GR mRNA and Protein Expression in the Hypothalamus and Hippocampus of Restrained HFD-ob and HFD-obR Rats

Hypothalamic GR mRNA and protein expression was significantly decreased in restraint-exposed SC-rats at 120 min ($77.8 \pm 7.1\%$ of non-stressed for mRNA, and $65.4 \pm 10.5\%$ of non-stressed for protein, $N=6$, $p<0.05$), while there were no significant changes in hypothalamic GR mRNA and protein expressions in HFD-ob and HFD-obR rats (Fig. 1 B and C). Expression of GR mRNA and protein was significantly decreased in the dentate gyrus of the hippocampus of SC rats at 120 min ($80.8 \pm 3.3\%$ of non-stressed for mRNA, and $78.6 \pm 5.9\%$ of non-stressed for protein, $N=6$, $p<0.05$), while there were no changes in the expressions of GR mRNA and protein in HFD-ob or HFD-obR rats (Fig. 1 D and E). Similarly,

expression of GR protein and mRNA was significantly decreased in CA1 and 3 of the hippocampus of SC rats at 120min, while they were not changed in HFD-ob and HFD-obR rats (data not shown in figure).

3.3. Impaired Regulation of miR-142-3p Expression in the Hypothalamus and Hippocampus of Restrained HFD-ob and HFD-obR Rats

Hypothalamic and the hippocampal miR-142-3p expression was significantly increased in restraint-exposed SC rats at 90 min (1.2 ± 0.3 -fold of non-stressed for hypothalamus and 1.2 ± 0.7 -fold of non-stressed for hippocampus, $N=6$, $p<0.05$) and 120 min (1.3 ± 0.3 -fold of non-stressed for hypothalamus and 1.4 ± 0.6 -fold of non-stressed for hippocampus, $N=6$, $p<0.05$) (Fig. 1 F and G), while there were no changes in miR-101a and miR-124 expression in these tissues (data not shown in figure). In HFD-ob and HFD-obR rats, there were no significant changes in miR-101a, miR-124 and miR-142-3p expression in the hypothalamus and hippocampus (Fig. 1F and G).

3.4. Impaired Regulation of GR mRNA and Protein Expression in the Hypothalamus and Hippocampus of Restrained HFD-ob and HFD-obR Rats

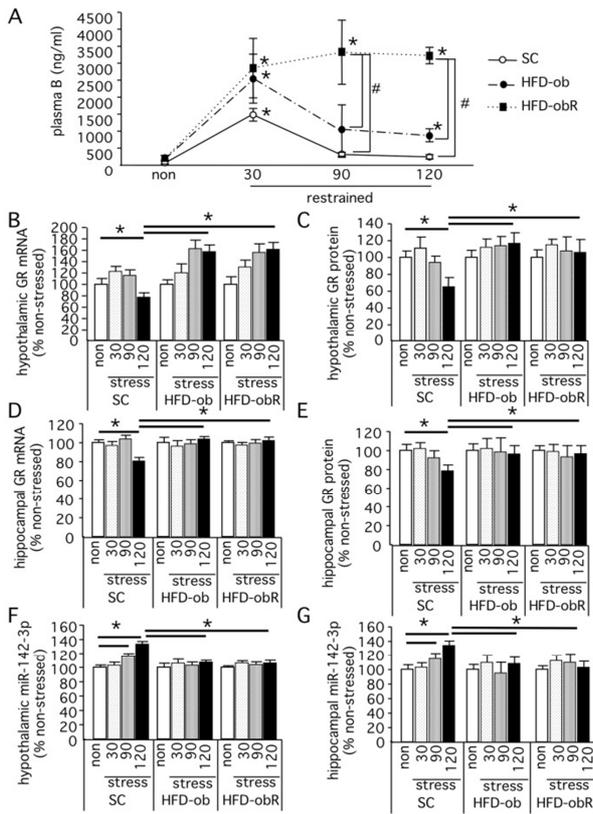


Figure 2. Dexamethasone (DEX) or saline was administered intraperitoneally to rats. Hypothalamic (A, B and E) and hippocampal (C, D and F) GR mRNA (A and C), GR protein (B and D) and miR-142-3p (E and F) expression were assayed. mRNA, protein and miRNA expression levels are given as % of saline group. $N=8$. * $p<0.05$ vs. saline group.

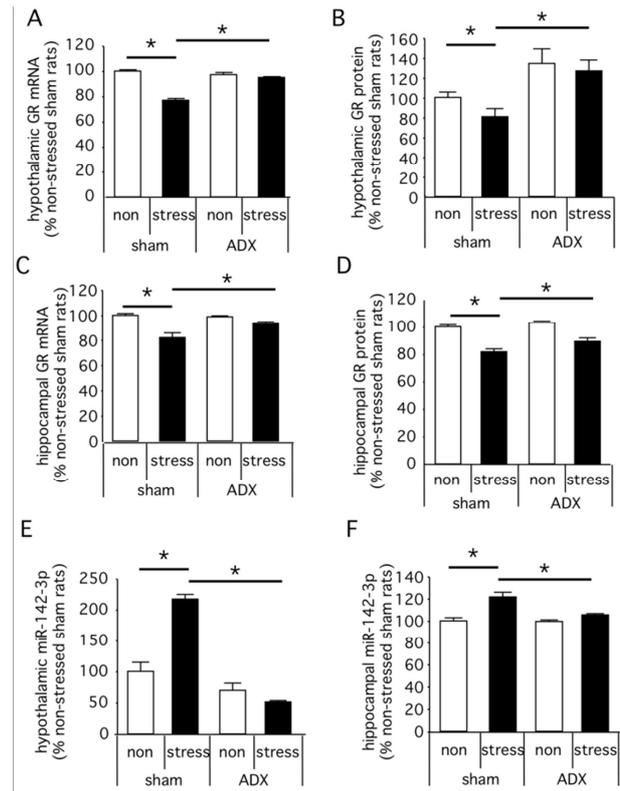


Figure 3. Adrenalectomized (ADX) or sham-operated (sham) rats were restrained for 90 min (stress). Hypothalamic (A, B and E) and hippocampal (C, D and F) GR mRNA (A and C), GR protein (B and D) and miR-142-3p (E and F) expression were assayed. mRNA, protein, and miRNA expression levels are given as % of non-restrained (non) sham rats. $N=6$. * $p<0.05$ vs. non-restrained controls.

Intraperitoneal injection of DEX at a dose of 100 µg/kg b.w. significantly decreased the expression of GR mRNA and protein in the hypothalamus and hippocampus ($85.2 \pm 2.4\%$ and $86.0 \pm 2.5\%$ of saline-injected for mRNA, and $85.0 \pm 3.8\%$ and $80.3 \pm 7.0\%$ of saline-injected for protein in hypothalamus and the hippocampus, respectively, $N=8$, $p<0.05$) and significantly increased miR-142-3p expression, but not miR-101a and miR-124, in the hypothalamus and the hippocampus (1.36 \pm 0.04-fold for hypothalamus and 1.46 \pm 0.12-fold for the hippocampus, $N=8$, $p<0.05$) (Fig. 2).

ADX blocked the restraint-induced decrease in the expression of GR mRNA and protein in the hypothalamus and hippocampus, and restraint-induced increase in miR-142-3p in the hypothalamus and hippocampus (Fig. 3).

4. Discussion

The present study demonstrated that restraint-induced increase in plasma corticosterone concentrations show prolonged elevation in HFD-obR rats and are significantly higher in HFD-ob when compared with SC-fed rats, and that GR mRNA and protein levels in the hypothalamus and hippocampus are significantly lowered after 120 min of restraint only in the SC-fed rats, but not in HFD-ob or HFD-obR rats. The present study also showed that restraint significantly increases miR-142-3p, but not miR-101a or miR-124, in these tissues in SC-fed rats, while it does not increase expression of these miRNAs in either HFD-ob or HFD-obR rats. It has been demonstrated that the miRNAs examined in the present study have sequences capable of binding to the GR 3'-UTR and that overexpression of those miRNAs decreases GR expression (13, 14). As miR-142-3p expression in the hypothalamus and hippocampus was increased by DEX and restraint-induced increase in miR-142-3p was blocked by adrenalectomy in the present study, miR-142-3p expression appears to be upregulated by glucocorticoids. Therefore, taken together with these results, the present study suggests that stress-induced miR-142-3p expression in the hypothalamus and hippocampus through corticosterone decreases GR expression in these tissues in normal rats. However, HFD feeding impairs the increase in miR-142-3p expression in the hypothalamus and hippocampus in stress and this impairment may be involved in dysregulation of restraint-induced downregulation of GR in HFD-fed rats.

GR in the hypothalamus and hippocampus is thought to play a role in negative regulation of the HPA axis (8-10). Decreases in GR expression in the hypothalamus are associated with elevated HPA axis activity (17). Moreover, the hippocampus is also known to be an important site of negative feedback of the HPA axis (18) (19). Prolonged stress is associated with a decrease in corticosterone binding to the GR in the hippocampus (20) (21). Thus, dysregulated GR downregulation in the hypothalamus and hippocampus may prolong restraint-induced elevation of plasma corticosterone levels in HFD-fed rats in the present study.

NPY stimulates corticosterone release through increased

CRF release. Although leptin suppresses NPY expression/release in the hypothalamus in lean rats (22-24), HFD-induced leptin resistance failed to suppress NPY expression/secretion (25, 26). We showed elevation of basal corticosterone concentrations in both HFD-ob and HFD-obR rats. Plasma leptin concentrations in HFD-ob rats (27.1 ± 9.0 ng/ml, $N=8$) were significantly higher than those of SC rats (7.1 ± 1.5 ng/ml, $N=8$, $p<0.05$), but the plasma leptin concentrations in HFD-obR rats (10.3 ± 3.4 ng/ml) did not significantly differ from those of SC rats. Therefore, some factors other than leptin stimulate CRF expression or release in the hypothalamus of HFD-obR rats.

In addition to regulation of GR mRNA by a variety of neurotransmitters (27, 28), several *in vitro* studies have demonstrated a glucocorticoid-induced decrease in GR transcription (29-31). In the present study, we showed that the expression of miR-142-3p is increased by glucocorticoids. This is the first report outlining a novel pathway through which miRNA suppresses GR expression in addition to the glucocorticoid-induced or neurotransmitter-mediated suppression of GR transcription in stress. However, the mechanisms underlying the impairment of miR-142-3p expression in restraint-exposed HFD-fed rats still remain unclear. It has been reported that HFD-fed affects various signal transduction pathways, as well as metabolism and neurotransmission in the brain (32). Therefore, several changes in intracellular signaling pathway and/or neurotransmitter release in the brain may impair miR-142-3p expression in the hypothalamus and hippocampus.

Diet-induced obesity is associated with a higher risk of metabolic disorders such as diabetes. It has been reported that pathophysiology resembling metabolic syndrome or type 2 diabetes can be rapidly induced in young HFD-fed rats by chronic exogenous glucocorticoids administration (33). Therefore, it seems likely that the prolongation of restraint-induced elevation of glucocorticoid levels in HFD-fed rats increases the risk of metabolic disorders. The mechanisms underlying the prolonged elevation of plasma corticosterone concentration in restraint-exposed HFD-fed rats still remain unclear. Further studies are necessary in order to clarify the mechanisms underlying the prolonged elevation of plasma corticosterone concentration in restrained HFD-ob and HFD-obR rats.

5. Conclusion

The results suggest that miR-142-3p is probably involved in restraint-induced downregulation of GR expression in SC rats and that HFD causes dysregulation of restraint-induced GR downregulation, probably through impairment of glucocorticoid-induced miR-142-3p expression in the hypothalamus and hippocampus.

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