Neonatal intramuscular injection of plasmid encoding glucagon-like peptide-1 affects anxiety behaviour and expression of the hippocampal glucocorticoid receptor in adolescent rats

Huitao Fan, Lina Wang, Feng Guo, Shi Wei and Ruqian Zhao*

Key Laboratory of Animal Physiology and Biochemistry, Nanjing Agricultural University, Nanjing 210095, P R China

**Corresponding author (Fax, +862584398669; Email, zhao.ruqian@gmail.com)*

Early-life endocrine intervention may programme hippocampal glucocorticoid receptor (GR) expression and cause psychiatric disorders in later life. Glucagon-like peptide-1 (GLP-1) has been implicated in the regulation of neuroendocrine and behavioural responses, but it is yet to be determined whether and how neonatal GLP-1 overexpression may modify hippocampal GR expression and thus programme adolescent behaviour in rats. Two-day-old pups were injected intramuscularly with vacant plasmid (VP) or plasmid DNA encoding secretory GLP-1 (GP). Anxiety-related behaviour was assessed in the elevated plus maze (EPM) test at 8 weeks of age. Plasma corticosterone levels were measured with enzyme immunoassay (EIA). Protein and mRNA levels were determined by western blot and real-time polymerase chain reaction (PCR), respectively. The DNA methylation status of the GR exon 1_7 promoter was determined by bisulphate sequencing PCR (BSP). GP rats exhibited anxiolytic behaviour compared with their VP counterparts. Hippocampal GLP-1 receptor (GLP-1R) and GR mRNA expression were significantly elevated in GP rats without a significant difference in plasma corticosterone. Significant reduction in DNA methyltransferase 1 (DNMT1) expression was observed in GP rats disconnected with alterations in DNA methylation of the GR exon 1_7 promoter. Nevertheless, mRNA expression of nerve growth factor-inducible protein A (NGFI-A) was significantly elevated in GP rats. These results suggest that neonatal intramuscular injection of plasmid DNA encoding GLP-1 affects anxiety behaviour in adolescent rats, probably through NGFI-A-activated upregulation of hippocampal GR expression.

[Fan H, Wang L, Guo F, Wei S and Zhao R 2010 Neonatal intramuscular injection of plasmid encoding glucagon-like peptide-1 affects anxiety behaviour and expression of the hippocampal glucocorticoid receptor in adolescent rats; *J. Biosci.* **35** 63–71] **DOI** 10.1007/s12038-010-0008-4

1. Introduction

Anxiety is one of the most prevalent psychiatric disorders in humans (Rouillon 1999; Krishnan 2003). Numerous studies indicate that this disorder is commonly caused by dysfunctions of the limbic–hypothalamic–pituitary–adrenal (LHPA) axis (Weinstock 2007; Sterlemann *et al.* 2008), which is highly susceptible to environmental or endocrine interventions during foetal and neonatal development (Weinstock 2007; Matthews 2000).

The glucocorticoid receptor (GR) in the hippocampus has been implicated as a major target for foetal/neonatal programming of the LHPA axis (Matthews 2000). It is also involved in a wide range of neural functions including

Keywords. Behaviour; DNA methylation; GLP-1; GR; hippocampus; NGFI-A

Abbreviations used: AVP, arg-vasopressin; BSP, bisulphate sequencing PCR; cAMP-PKA, cyclic adenosine monophosphate-dependent protein kinase A; CRF, corticotrophin-releasing factor; CRH, corticotrophin-releasing hormone; DNMT1, DNA methyltransferase 1; ECL, enhanced chemiluminescence; EIA, enzyme immunoassay; EPM, elevated plus maze; GLP-1, glucagon-like peptide 1; GLP-1R, GLP-1 receptor; GP, plasmid DNA encoding secretory GLP-1; GR, glucocorticoid receptor; ICV, intracerebroventricular; LHPA, limbic–hypothalamic–pituitary–adrenal; NGFI-A, nerve growth factor-inducible protein A; NTC, no template control; OE%, percentage of entries in open arms; OT%, percentage of time in open arms; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate; TTBS, Tween-Tris-buffer saline; VP, vacant plasmid

suppression of the HPA axis (De Kloet *et al.* 1998), memory (Fenoglio *et al.* 2005) and learning (Donley *et al.* 2005), as well as the pathogenesis of anxiety (Korte 2001).

Glucagon-like peptide 1 (GLP-1) is a brain–gut peptide involved in obesity and the stress response (Bojanowska 2005). Intracerebroventricular (ICV) injection of GLP-1 stimulates corticosterone secretion by activation of corticotrophin-releasing factor (CRF) neurons in the chicken (Tachibana *et al.* 2006). Intravenous infusion of GLP-1 or Exendin-4, a GLP-1 analogue, increases cortisol secretion in human subjects (Vella *et al.* 2002). Furthermore, GLP-1 induced HPA activation is found to be associated with increased levels of anxiety in the rat (Kinzig *et al.* 2003).

Glucagon-like peptide 1 receptor (GLP-1R) is expressed in the brain (Alvarez *et al.* 1996; Nussdorfer *et al.* 2000) and there is evidence suggesting a cross-talk between GR and GLP-1R in the hippocampus. GLP-1R activation could lead to stimulation of cyclic adenosine monophosphate-dependent protein kinase A (cAMP-PKA) (Ranta *et al.* 2006; Doyle and Egan 2007), which induces expression of nerve growth factor-inducible protein A (NGFI-A) (Kang *et al.* 2007; Kim *et al.* 2008), a transcription factor known to activate GR transcription (Weaver *et al.* 2004).

NGFI-A exerts its positive regulatory effect on GR gene transcription directly by interaction with the DNA regulatory element and elicits transactivation of promoter activity (Meaney *et al.* 2000; Weaver *et al.* 2007). It has been shown that NGFI-A binding and GR expression can be programmed by neonatal experiences through modification of the DNA methylation status of a GR gene promoter in the hippocampus (Weaver *et al.* 2004).

The present study was aimed to answer the following questions. First, would neonatal exposure to high GLP-1 programme anxiety behaviour in later life? Second, did such programming effects, if any, involve alterations in hippocampal GLP-1R and GR transcription? Third, did alteration in GR transcription involve modifications in NGFI-A expression or DNA methylation of the GR gene promoter? We first observed significant behavioural alterations in the elevated plus maze (EPM) test in 8-week-old adolescent rats neonatally treated with plasmid DNA encoding GLP-1. We then demonstrated, with real-time reverse transcription PCR (RT-PCR), significant upregulation of hippocampal GLP-1R and GR mRNA expression in neonatally GLP-1 treated rats. Furthermore, we found that the activation of GR transcription is not associated with alterations in DNA methylation in the GR exon 1_7 promoter region, despite significant reduction in DNA methyltransferase 1 (DNMT1) expression, but is accompanied by increased NGFI-A transcription, suggesting a *trans*-regulating mechanism in hippocampal GR activation programmed by neonatal GLP-1 treatment.

2. Materials and methods

2.1 *Animals*

Adult male and female Wistar rats were purchased from the Shanghai Laboratory Animal Center (SLAC) and bred to produce offspring for the present experiment in which only male pups were used. Rats were housed in a specific pathogen-free (SPF) facility with food and water *ad libitum*. The animal experiment protocol was approved by the Animal Ethics Committee of Nanjing Agricultural University.

2.2 *Plasmid construction and* in vivo *electroporation*

The recombinant plasmid expressing secretory GLP-1, sig-glp-1-pcDNA3, was constructed; it contained a signal peptide-coding sequence and full-length cDNA of rat glp-1. Plasmid DNA was extracted using EndoFree plasmid Kits (DP117, Tiangen Biotech Co. Ltd., Beijing, China). Newborn male pups of similar birth weight were divided at random into two groups, with one group receiving electroporation of vacant pcDNA3 plasmid (VP) and the other sig-glp-1-pcDNA3 plasmid (GP). Electroporation was performed on the second day after birth at four similar points on the lateral side of the left thigh. Plasmid DNA (120 μg in 120 μl phosphate-buffered saline [PBS] in total) was injected, and four electric pulses of 100 V at 50 ms followed by four more pulses of the opposite polarity were immediately delivered to the muscle. The electric pulse was delivered by an electric pulse generator (Ningbo Scienz Bio-tech Co. Ltd, Ningbo, China). Plasmid expression of GLP-1 mRNA in the gastrocnemius was verified 7 days after electroporation with RT-PCR.

2.3 *The elevated plus maze (EPM) test*

The EPM test was conducted in the 8th week after birth. The EPM was constructed of wood and had four arms (50 × 10 cm) shaped in the form of a plus, and was elevated 50 cm above the floor. Two opposing arms were enclosed by side and end walls (40 cm high) and the other two arms were open with only 1 cm high side and end walls. The connecting central area measured 10 cm × 10 cm. Each animal was placed in the centre of the EPM facing an open arm and was permitted to explore the maze freely for a 5-min period. The rat was videotaped and later scored by an unbiased observer blinded to the experimental conditions. Arm entry was defined as entering an arm with all four paws. The tapes were scored for the percentage of entries (OE%) in the open arms relative to total entries, and time (OT%) in open arms relative to total time and total entries in both arms (OE + CE). The maze was cleaned with 70% alcohol solution between animals.

2.4 *Tissue preparation*

Rats were killed by decapitation and trunk blood was collected, immediately placed on ice and subsequently centrifuged at 2000 × *g* for 15 min. Plasma samples were stored at –20°C until assayed. The hippocampus of each rat was dissected and immediately frozen in liquid nitrogen within 5 min of decapitation. Hippocampi were stored at –80°C until RNA and protein isolation. The adrenal gland of each rat was weighed and the index of adrenal weight was calculated by the formula: adrenal weight index = adrenal weight (mg)/body weight (100 g).

2.5 *Plasma corticosterone determination*

Plasma corticosterone levels were analysed using a commercially available competitive enzyme immunoassay (EIA) kit (Cayman Chemical Company, USA), following the instructions of the supplier. Measurements were carried out in duplicate with a microplate reader (Bio-Tek Instruments, Inc., USA). The kit sensitivity was 40 pg/ml and intra-assay variation was 9.5%.

2.6 *mRNA quantification*

Real-time PCR was performed in an Mx3000P system (Stratagene, USA). The cDNA amounts present in each sample were determined using the SYBR Green Master Mix (TOYOBO Ltd., Japan). Each RT-PCR quantification experiment was performed in duplicate. Mock RT and no template controls (NTC) were set to monitor the possible contamination of genomic DNA both at RT and PCR. The pooled sample made by mixing equal quantities of total RT products (cDNA) from all samples was used to optimize the PCR conditions and tailor standard curves for each target gene. Melting curves were performed to investigate the specificity of the PCR reaction. Cyclophilin (Marini *et al.* 2006), GR (Marini *et al.* 2006), GLP-1R (Li *et al.* 2003), DNMT1 (Desaulniers *et al.* 2005) and NGFI-A (Spessert *et al.* 2006) primers were synthesized by Invitrogen Biotechnology Co. Ltd. (Shanghai, China), according to the sequences used in cited publications. Amplification conditions were as follows: 45 cycles of 15 s at 95°C, 15 s at 62°C and 45 s at 72°C. Real-time PCR data were analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2006) with cyclophilin as an endogenous reference (Marini *et al.* 2006). The mRNA levels of target genes in GP rats were expressed as the fold of change relative to the mean value of VP rats.

2.7 *Western blot analysis*

Forty micrograms of protein extract were diluted 4:1 with 5X loading buffer (10% glycerol, 2% sodium dodecyl sulphate [SDS], 5% 2-mercaptoethanol, 1% bromophenol blue) and denatured by boiling for 5 min before loading on a 12% SDS-PAGE gel. After electrophoresis, proteins were transferred to nitrocellulose membranes and the latter were then blocked with 5% fat-free milk in Tween-Tris-buffer saline (TTBS) for 2 h at room temperature. After repeated washing with TTBS, the membranes were cut into three parts according to protein marker and incubated with antibodies against GR (rabbit [rb]; 1:200; Bioreagents, USA), DNMT1 (mouse [ms]; 1 μg/ml; Imgenex, USA) and β-actin (Rb; 1:3000; Abcam, UK) respectively, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit for GR and β-actin, goat anti-mouse for DNMT1; 1:4000; Abcam, UK). Finally, the membrane was washed and the specific signals were detected by chemiluminescence using the LumiGlo substrate (Super Signal West Pico Trial Kit, PIERCE, Rockford, IL, USA). Enhanced chemiluminescence (ECL) signals recorded on X-ray film were scanned and analysed with a Kodak 1D Electrophoresis Documentation and Analysis System 120 (Kodak Photo Film Co. Ltd., Rochester, NY).

2.8 *DNA methylation detection*

Genomic DNA (1 μg) isolated from rat hippocampus was subjected to bisulphite modification using the CpGenome DNA Modification Kit (Chemicon, USA) according to the manufacturer's protocol. The GR exon 1_7 promoter region was PCR amplified with nested primer pairs (Weaver *et al*. 2004). The bands were purified with agarose Gel DNA Purification Kit (Promega, USA) and then subcloned into the pGEM T-easy vector (Promega, USA). Twenty-two positive clones from GP and fourteen positive clones from VP were sequenced using M13 primer by Invitrogen Biotechnology Co. Ltd., Shanghai. The final sequence results were processed by the online computer program: *http://biq-analyzer.bioinf.mpi-sb.mpg.de/* (Bock *et al*. 2005). Sequences with a conversion rate below 90% were excluded from analysis.

2.9 *Data analysis*

All data were presented as means ± SEM. Statistical analyses were carried out with SPSS13.0 for Windows. The differences were tested with ANOVA using the *t*-test for independent samples. The level of significance was set at $P<0.05$.

3. Results

3.1 *Behavioural data*

GP and VP rats demonstrated significantly different behaviour in the EPM test. Both the number of entries

(OE%, P=0.024) and the duration of stay (OT%, P=0.021) in the open arms were significantly increased in GP rats compared with VP rats, implicating an anxiolytic effect of neonatal GLP-1 exposure. It is noteworthy, however, that the total entries to both arms (OE + CE, P=0.011) were also significantly decreased in GP rats (table 1), indicating inhibited locomotor activity in GP rats.

3.2 *Adrenal weight index and plasma level of corticosterone*

No significant differences in adrenal weight index were observed between the VP (19.21 ± 1.04) and GP (20.94 ± 1.12) groups. Although a numeric increase was detected in plasma corticosterone concentration in GP rats compared with VP rats, there was no statistical difference between the two groups (P=0.42) (figure 1).

3.3 *Expression of GLP-1R, NGFI-A, GR and DNMT1 in the hippocampus*

As shown in figure 2, mRNA expression of GLP-1R (P=0.009) and NGFI-A (P=0.001) was significantly upregulated in hippocampi of GP rats, coinciding with significantly upregulated GR mRNA expression (figure 3A, P=0.01) and a tendency for increase in GR protein content (figure 3B and C, P=0.119). A significant downregulation in hippocampal expression of DNMT1 was observed in hippocampi of GP rats at the levels of both transcription (figure 4A, P=0.024) and translation (figure 4B and C, P=0.009), compared with VP counterparts.

3.4 *DNA methylation*

Despite the significant alterations in DNMT1 expression, no difference was found in the level of methylation in the exon 1_7 promoter sequence of the GR gene. Neither of the two CpGs (Weaver *et al.* 2004) within the NGFI-A binding site (CpG sites 16 and 17) was methylated in the 36 subjects investigated. Additionally, all the remaining 15 CpG sites in the examined exon 1_7 promoter sequence were rarely methylated except some sporadic methylated sites (figure 5).

Table 1. Behaviour of VP and GP rats in the elevated plus maze (EPM) test

	OE%	OT%	OE + CE
VP	51.8 ± 5.5	56.4 ± 13.3	7.8 ± 1.9
GP	87.5 ± 12.5	98.3 ± 1.8	2.0 ± 0.7

Remarkable differences were seen between rats in the VP (N = 5) and GP (N = 5) groups in OE% (P=0.024), OT% (P=0.021) and (OE + CE) (P=0.011) in the EPM test.

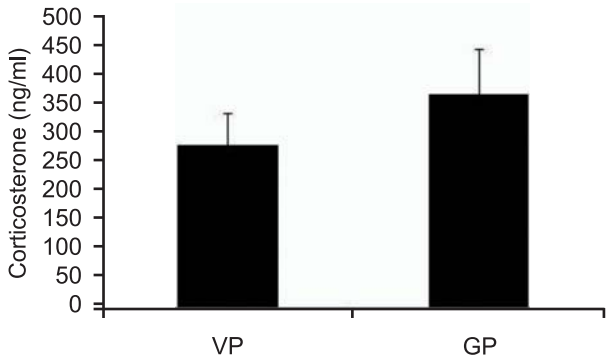

Figure 1. Plasma corticosterone levels in the group of rats receiving electroporation of vacant pcDNA3 plasmid (VP, N = 5) and those receiving electroporation of sig-glp-1-pcDNA3 plasmid (GP, N = 5) rats. No statistical difference was observed between two groups (P=0.42).

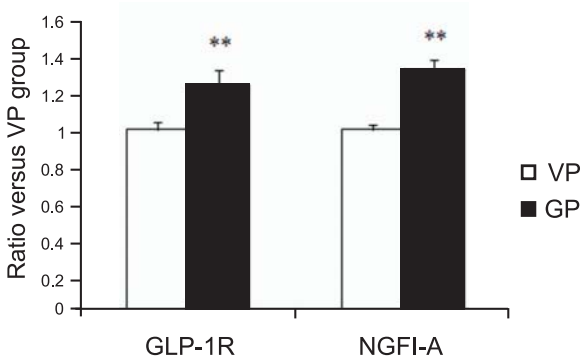

Figure 2. mRNA expression of glucagon-like peptide-1 receptor (GLP-1R) and nerve growth factor-inducible protein A (NGFI-A) of the hippocampus in VP (N = 5) and GP (N = 5) rats. GLP-1R (P=0.009) and NGFI-A (P=0.001) gene mRNA expressions were significantly upregulated in the GP group when compared with the VP group. **P<0.01.

4. Discussion

In the present study, we have demonstrated that neonatal GLP-1 exposure produces long-term behavioural alterations in adolescent rats, which is associated with increased hippocampal GLP-1R and GR mRNA expression. The upregulation of GR transcription is not associated with *cis*-activation by modification of GR exon 1_7 promoter DNA methylation, but may be attributed to transactivation through increased expression of NGFI-A.

It has been shown that anxiolytic compounds increase, whereas anxiogenic compounds decrease, the number of entries and the time of permanence in the open arms of the EPM (Pellow *et al.* 1985; Cole *et al.* 1995). We observed a significant increase in the OE% and OT% in GP rats, indicating an anxiolytic effect of neonatal GLP-1 exposure. In keeping with our results, GLP-1R

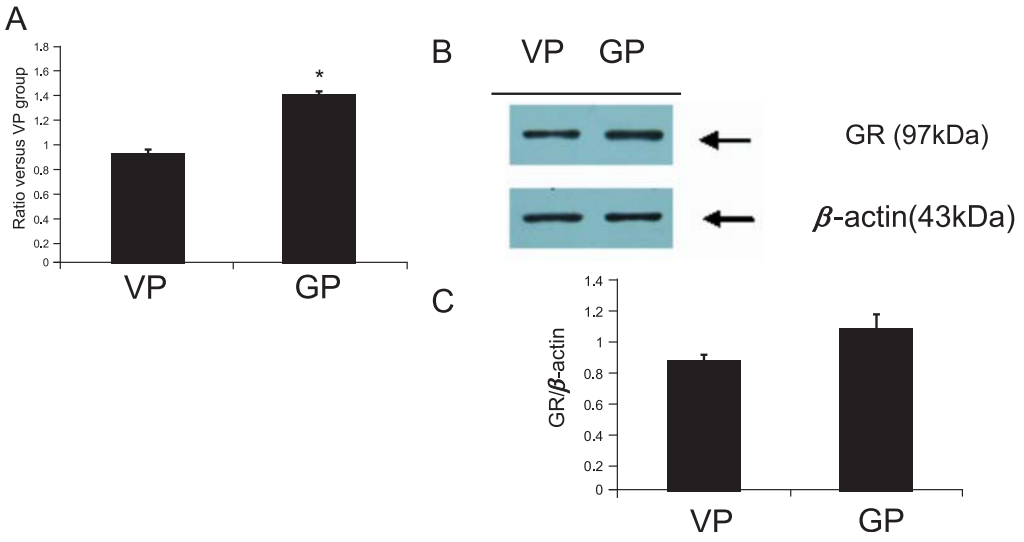

Figure 3. Determination of glucocorticoid receptor (GR) mRNA and protein levels in whole hippocampi. **(A)** mRNA expression in the GR of the hippocampus in VP ($N = 5$) and GP ($N = 5$) rats (P=0.01). **(B)** Representative bands of the total levels of GR and β-actin protein lysates of hippocampal tissue from VP or GP rats. **(C)** Ratio of relative protein levels of total GR expressed to β-actin in hippocampi of VP ($N = 5$) or GP ($N = 5$) rats. No statistically significant difference was found between the two groups (P=0.119). *P<0.05.

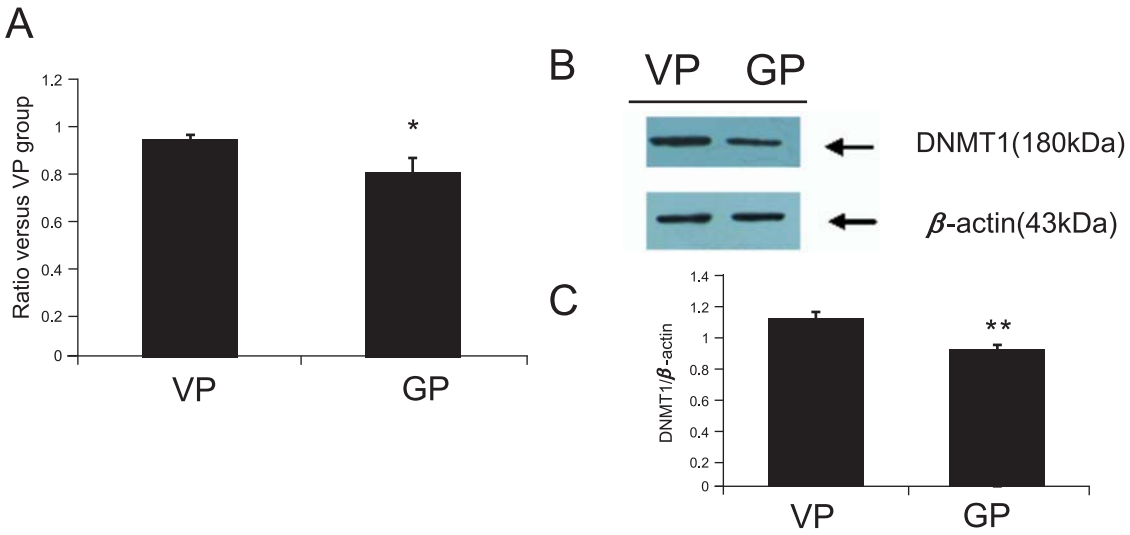

Figure 4. Determination of DNA methyltransferase 1 (DNMT1) mRNA and protein levels in whole hippocampi. **(A)** mRNA expression of DNMT1 in hippocampi of VP ($N = 5$) and GP ($N = 5$) rats (P=0.024). **(B)** Representative immunoblots of the total levels of DNMT1 and β-actin protein lysates in hippocampal tissues from VP or GP rats. **(C)** Ratio of relative protein levels of total DNMT1 expressed to β-actin in hippocampi of VP ($N = 5$) or GP ($N = 5$) rats (P=0.009). *P<0.05.

knock-out (GLP-1R -/-) mice display increased anxiety-like behaviour when placed in an EPM compared with wild-type (GLP-1R +/+) mice (MacLusky *et al.* 2000). Conversely, rats that were administered a GLP-1R agonist (Exendin-4) into the third ventricle spent significantly more time in the open arms than control animals. However, the effect of GLP-1 on anxiety seems to be dependent on the site of administration in the brain. When administered directly into the central nucleus of the amygdala, GLP-1 actually increases the levels of anxiety in the EPM accompanied by elevated levels of corticosterone (Kinzig *et al.* 2003).

The discrepancy in the central action of GLP-1 on anxiety might be attributed to the activity and reactivity of the HPA axis. Adrenal hypertrophy and increase of plasma corticosterone levels are associated with increased anxiety-like behaviour in the EPM (Song *et al.* 2008; Sterlemann *et al.* 2008). In the present experiment, ectopic overexpression of GLP-1 was achieved by intramuscular transfection of the plasmid expressing secretory GLP-1. Therefore,

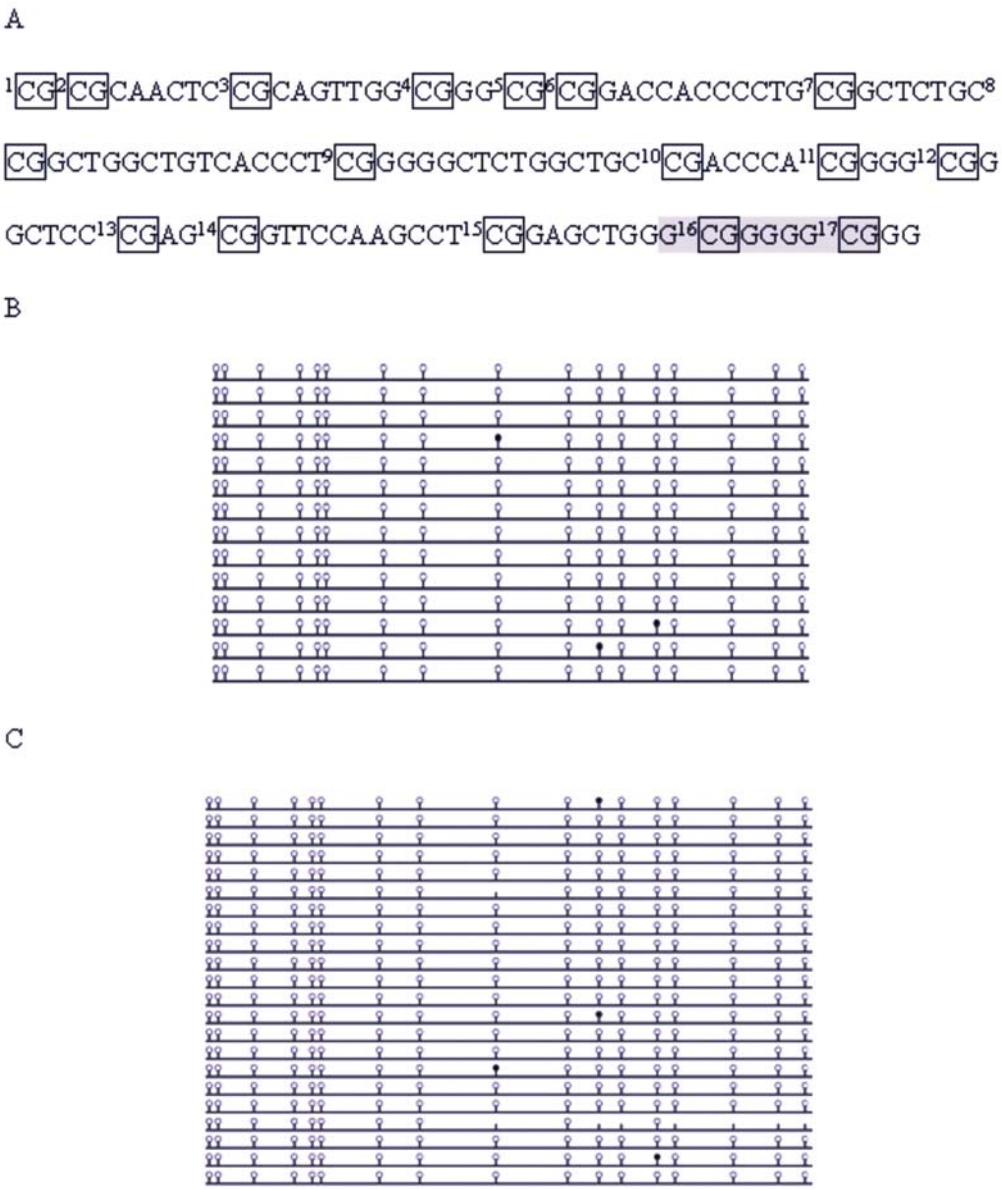

Figure 5. Methylation profiles in the exon 1_7 promoter region of the hippocampal glucocorticoid receptor (GR). **(A)** Genomic sequence with numbered CpG dinucleotides. Each CpG site is numbered in the rectangle. Nerve growth factor-inducible protein A (NGFI-A) response element is indicated in grey shading. **(B)** Methylation profiles of the exon 1_7 promoter in 14 positive clones of the VP group ($N = 5$); **(C)** Methylation profiles of the exon 1_7 promoter in 22 positive clones of the GP group ($N = 5$). Filled (black) circles correspond to methylated Cs and unfilled (white) circles correspond to unmethylated Cs. Neither of the two CpGs within the NGFI-A binding site (CpG sites 16 and 17) was methylated in the 36 subjects investigated. In contrast, all the remaining 15 CpG sites of the corresponding exon 1_7 promoter were sparsely methylated other than some sporadic methylated sites.

GLP-1 was temporally elevated systemically during the neonatal period. It was not possible to determine the site of action of systemically increased GLP-1. However, neither plasma corticosterone level nor adrenal weight index was significantly different between the two groups, implying unaltered or even blunted HPA activity in neonatally GLP-1-exposed rats.

Endocrine interventions during the foetal and neonatal period could programme the 'set point' of the HPA axis. Attenuated HPA responsiveness is often caused by an increase in GR expression in the hippocampus (De Kloet *et al.* 1998). In the present study, we found a significant upregulation of hippocampal GR expression in neonatally GLP-1-exposed rats, suggesting increased negative feedback on the HPA

axis. The increased sensitivity of the HPA axis to feedback inhibition would thus permit normalization of the HPA axis and a more efficient termination of Arg-vasopressin (AVP) and corticotrophin-releasing hormone (CRH) in various regions of the brain postulated to be involved in the causality of anxiety (Larsen *et al.* 1997; Sarkar *et al.* 2003).

Nevertheless, the correlations between hippocampal GR expression and anxiety-related behaviour are inconsistent. Enhanced hippocampal GR mRNA expression was found in parallel with stress-induced anxiety (Jakovcevski *et al.* 2008), whereas other studies have suggested that impairment of GR was concurrent with an increased anxiety-like state (Sterlemann *et al.* 2008). Elevated as well as reduced GR can lead to anxiety. The relationship between GR expression and anxiety-related behaviour remains unclear. However, in the present study, we have shown that increased hippocampal GR expression was associated with anxiolytic behaviour.

The total number of entries (OE + CE) is a measure of locomotor activity (Calfa *et al.* 2006). ICV administration of GLP-1 has been shown to suppress locomotor activity (Moller *et al.* 2002). Our results also indicate decreased locomotor activity in GP rats. There is evidence that blunted HPA reactivity is usually associated with inhibited exploratory behaviour and decreased physical activity in chicken (Saito *et al.* 2005) and rat (Darnaudery and Maccari 2008). Therefore, the decreased locomotion observed in GP rats may represent blunted HPA responsivity induced by augmented GR-mediated negative feedback on the HPA axis.

The mechanism by which neonatal GLP-1 programmes hippocampal GR expression has been explored in the present study. As neonatal experience could programme hippocampal GR expression through modification of DNA methylation status at a GR gene promoter (Weaver *et al.* 2004), we evaluated hippocampal DNMT1 expression and DNA methylation status of the GR exon 1_7 promoter. GLP-1 programmed rats exhibited a robust reduction both in mRNA and protein levels of hippocampal DNMT1, suggesting decreased DNA methylation. However, in contrast to the findings of Weaver *et al.* (2004), neither of the two CpGs within the NGFI-A binding site was methylated in the 36 subjects investigated, which is in line with the observations of Moser *et al.* (2007) that the hippocampal GR exon 1-F promoter is not methylated at the NGFI-A binding site in human (Moser *et al.* 2007). All the remaining 15 CpG sites present in the promoter fragment investigated were sparsely methylated, except some sporadic methylated sites in both groups. These results suggest that GLP-1 programmed activation of GR transcription in the hippocampi of GP rats does not involve alterations of DNA methylation on the GR exon 1_7 promotor. The downregulation of DNMT1 expression in the hippocampus could be the consequence, rather than the cause, of enhanced GR expression. As a matter of fact, DNMT1 establishes and maintains patterns of genomewide DNA methylation in mammalian cells, but not in a locus-specific manner (Geiman *et al.* 2004; Tsumura *et al.* 2006). Alterations in DNMT1 availability do not always correlate with variations in DNA methylation levels (Eads *et al.* 1999). The fact that GR could inhibit the activity of the DNMT1 promoter decouples the cause-and-effect relationship between GR and DNMT1 expression (Rouleau *et al.* 1995).

It has been shown that GLP-1 present in the bloodstream can enter the brain (Orskov *et al.* 1996) and we have demonstrated a marked upregulation of hippocampal GLP-1R expression in GP rats, indicating the presence of functional GLP-1R. It has been shown that GLP-1R and its potent agonist Exendin-4 could activate transcription and induce expression of NGFI-A through the cAMP–PKA pathway (Kang *et al.* 2007; Kim *et al.* 2008). NGFI-A could exert its transcriptional regulatory effect directly on the GR gene by interaction with the DNA regulatory element and elicit transactivation of promoter activity (Meaney *et al.* 2000; Weaver *et al.* 2007). Indeed, we detected dramatically upregulated NGFI-A mRNA expression in the hippocampi of GP rats, indicating a transactivation of hippocampal GR transcription in rats neonatally exposed to high levels of GLP-1.

Taken together, our findings provide the first evidence that neonatal GLP-1 exposure exerts long-term programming effects on hippocampal GR expression and anxiety behaviour in adolescent rats which, if transferred to the human situation, may lead to novel insights and clues into the pathophysiology of and therapeutic approach to the treatment of anxiety. However, further experiments are required to clarify the exact signalling pathway for GR transcriptional activation by GLP-1 exposure.

Acknowledgements

This work was supported by the National Basic Research Program of China (2004CB117505) and National Nature Science Foundation of China (Project No. 30430420).

References

Alvarez E, Roncero I, Chowen J A, Thorens B and Blazquez E 1996 Expression of the glucagon-like peptide-1 receptor gene in rat brain; *J. Neurochem.* **66** 920–927

Bock C, Reither S, Mikeska T, Paulsen M, Walter J and Lengauer T 2005. BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing; *Bioinformatics* **21** 4067–4068

Bojanowska E 2005 Physiology and pathophysiology of glucagon-like peptide-1 (GLP-1): the role of GLP-1 in the pathogenesis of diabetes mellitus, obesity, and stress; *Med. Sci. Monit.* **11** RA271–RA278

Calfa G, Volosin M and Molina V A 2006 Glucocorticoid receptors in lateral septum are involved in the modulation of the emotional sequelae induced by social defeat; *Behav. Brain. Res.* **172** 324–332

Cole B J, Hillmann M, Seidelmann D, Klewer M and Jones G H 1995 Effects of benzodiazepine receptor partial inverse agonists in the elevated plus maze test of anxiety in the rat; *Psychopharmacology (Berl.)* **121** 118–126

Darnaudery M and Maccari S 2008 Epigenetic programming of the stress response in male and female rats by prenatal restraint stress; *Brain Res. Rev.* **57** 571–585

De Kloet E R, Vreugdenhil E, Oitzl M S and Joels M 1998 Brain corticosteroid receptor balance in health and disease; *Endocrinol. Rev.* **19** 269–301

Desaulniers D, Xiao G H, Leingartner K, Chu I, Musicki B and Tsang B K 2005 Comparisons of brain, uterus, and liver mRNA expression for cytochrome p450s, DNA methyltransferase-1, and catechol-o-methyltransferase in prepubertal female Sprague-Dawley rats exposed to a mixture of aryl hydrocarbon receptor agonists; *Toxicol. Sci.* **86** 175–184

Donley M P, Schulkin J and Rosen J B 2005 Glucocorticoid receptor antagonism in the basolateral amygdala and ventral hippocampus interferes with long-term memory of contextual fear; *Behav. Brain. Res.* **164** 197–205

Doyle M E and Egan J M 2007 Mechanisms of action of glucagon-like peptide 1 in the pancreas; *Pharmacol. Ther.* **113** 546–593

Eads C A, Danenberg K D, Kawakami K, Saltz L B, Danenberg P V and Laird P W 1999 CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression; *Cancer Res.* **59** 2302–2306

Fenoglio K A, Brunson K L, Vishai-Eliner S, Stone B A, Kapadia B J and Baram T Z 2005 Enduring, handling-evoked enhancement of hippocampal memory function and glucocorticoid receptor expression involves activation of the corticotropin-releasing factor type 1 receptor; *Endocrinology* **146** 4090–4096

Geiman T M, Sankpal U T, Robertson A K, Zhao Y, Zhao Y and Robertson K D 2004 DNMT3B interacts with hSNF2H chromatin remodeling enzyme, HDACs 1 and 2, and components of the histone methylation system; *Biochem. Biophys. Res. Commun.* **318** 544–555

Jakovcevski M, Schachner M and Morellini F 2008 Individual variability in the stress response of C57BL/6J male mice correlates with trait anxiety; *Genes Brain Behav.* **7** 235–243

Kang J H, Kim M J, Jang H I, Koh K H, Yum K S, Rhie D J, Yoon S H and Hahn S J *et al.* 2007 Proximal cyclic AMP response element is essential for exendin-4 induction of rat EGR-1 gene; *Am. J. Physiol. Endocrinol. Metab*. **292** E215–E222

Kim M J, Kang J H, Chang S Y, Jang H J, Ryu G R, Ko S H, Jeong I K and Kim M S *et al.* 2008 Exendin-4 induction of Egr-1 expression in INS-1 beta-cells: interaction of SRF, not YY1, with SRE site of rat Egr-1 promoter; *J. Cell. Biochem.* **104** 2261–2271

Kinzig K P, D'Alessio D A, Herman J P, Sakai R R, Vahl T P, Figueiredo H F, Murphy E K and Seeley R J 2003 CNS glucagon-like peptide-1 receptors mediate endocrine and anxiety responses to interoceptive and psychogenic stressors; *J. Neurosci.* **23** 6163–6170

Korte S M 2001 Corticosteroids in relation to fear, anxiety and psychopathology; *Neurosci. Biobehav. Rev.* **25** 117–142

Krishnan K R 2003 Comorbidity and depression treatment; *Biol. Psychiatry* **53** 701–706

Larsen P J, Tang-Christensen M and Jessop D S 1997 Central administration of glucagon-like peptide-1 activates hypothalamic neuroendocrine neurons in the rat; *Endocrinology* **138** 4445–4455

Li B, Xi X, Roane D S, Ryan D H and Martin R J 2003 Distribution of glucokinase, glucose transporter GLUT2, sulfonylurea receptor-1, glucagon-like peptide-1 receptor and neuropeptide Y messenger RNAs in rat brain by quantitative real time RT-PCR; *Brain Res. Mol. Brain Res.* **113** 139–142

Livak K J and Schmittgen T D 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method; *Methods* **25** 402–408

MacLusky N J, Cook S, Scrocchi L, Shin J, Kim J, Vaccarino F, Asa S L and Drucker D J 2000 Neuroendocrine function and response to stress in mice with complete disruption of glucagon-like peptide-1 receptor signaling; *Endocrinology* **141** 752–762

Marini F, Pozzato C, Andreetta V, Jansson B, Arban R, Domenici E and Carboni L 2006 Single exposure to social defeat increases corticotropin-releasing factor and glucocorticoid receptor mRNA expression in rat hippocampus; *Brain Res.* **1067** 25–35

Matthews S G 2000 Antenatal glucocorticoids and programming of the developing CNS; *Pediatr. Res*. **47** 291–300

Meaney M J, Diorio J, Francis D, Weaver S, Yau J, Chapman K and Seckl J R 2000 Postnatal handling increases the expression of cAMP-inducible transcription factors in the rat hippocampus: the effects of thyroid hormones and serotonin; *J. Neurosci.* **20** 3926–3935

Moller C, Sommer W, Thorsell A, Rimondini R and Heilig M 2002 Anxiogenic-like action of centrally administered glucagon-like peptide-1 in a punished drinking test; *Prog. Neuropsychopharmacol. Biol. Psychiatry* **26** 119–122

Moser D, Molitor A, Kumsta R, Tatschner T, Riederer P and Meyer J 2007 The glucocorticoid receptor gene exon 1-F promoter is not methylated at the NGFI-A binding site in human hippocampus; *World J. Biol. Psychiatry* **8** 262–268

Nussdorfer G G, Bahcelioglu M, Neri G and Malendowicz L K 2000 Secretin, glucagon, gastric inhibitory polypeptide, parathyroid hormone, and related peptides in the regulation of the hypothalamus–pituitary–adrenal axis; *Peptides* **21** 309–324

Orskov C, Poulsen S S, Moller M and Holst J J 1996 Glucagon-like peptide I receptors in the subfornical organ and the area postrema are accessible to circulating glucagon-like peptide I; *Diabetes* **45** 832–835

Pellow S, Chopin P, File S E and Briley M 1985 Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat; *J. Neurosci. Methods* **14** 149–167

Ranta F, Avram D, Berchtold S, Dufer M, Drews G, Lang F and Ullrich S 2006 Dexamethasone induces cell death in insulin-secreting cells, an effect reversed by exendin-4; *Diabetes* **55** 1380–1390

Rouillon F 1999 Anxiety with depression: a treatment need; *Eur. Neuropsychopharmacol. (Suppl. 3)* **9** S87–S92

Rouleau J, MacLeod A R and Szyf M 1995 Regulation of the DNA methyltransferase by the Ras-AP-1 signaling pathway; *J. Biol. Chem.* **270** 1595–1601

Saito S, Tachibana T, Choi Y H, Denbow D M and Furuse M 2005 ICV CRF and isolation stress differentially enhance plasma corticosterone concentrations in layer- and meat-type neonatal chicks; *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **141** 305–309

Sarkar S, Fekete C, Legradi G and Lechan R M 2003 Glucagon like peptide-1 (7-36) amide (GLP-1) nerve terminals densely innervate corticotropin-releasing hormone neurons in the hypothalamic paraventricular nucleus; *Brain Res.* **985** 163–168

Song C, Manku M S and Horrobin D F 2008 Long-chain polyunsaturated fatty acids modulate interleukin-1beta-induced changes in behavior, monoaminergic neurotransmitters, and brain inflammation in rats; *J. Nutr.* **138** 954–963

Spessert R, Gupta B B, Rohleder N, Gerhold S and Engel L 2006 Cyclic AMP-inducible genes respond uniformly to seasonal lighting conditions in the rat pineal gland; *Neuroscience* **143** 607–613

Sterlemann V, Ganea K, Liebl C, Harbich D, Alam S, Holsboer F, Muller M B and Schmidt M V 2008 Long-term behavioral and neuroendocrine alterations following chronic social stress in mice: implications for stress-related disorders; *Horm. Behav*. **53** 386–394

Tachibana T, Sato M, Oikawa D and Furuse M 2006 Involvement of CRF on the anorexic effect of GLP-1 in layer chicks; *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **143** 112–117

Tsumura A, Hayakawa T, Kumaki Y, Takebayashi S, Sakaue M, Matsuoka C, Shimotohno K and Ishikawa F 2006 Maintenance of self-renewal ability of mouse embryonic stem cells in the absence of DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b; *Genes Cells* **11** 805–814

Vella A, Shah P, Reed A S, Adkins A S, Basu R and Rizza R A 2002 Lack of effect of exendin-4 and glucagon-like peptide-1-(7,36)-amide on insulin action in non-diabetic humans; *Diabetologia* **45** 1410–1415

Weaver I C, Cervoni N, Champagne F A, D'Alessio A C, Sharma S, Seckl J R, Dymov S and Szyf M *et al.* 2004 Epigenetic programming by maternal behavior; *Nat. Neurosci.* **7** 847–854

Weaver I C, D'Alessio A C, Brown S E, Hellstrom I C, Dymov S, Sharma S, Szyf M and Meaney M J 2007 The transcription factor nerve growth factor-inducible protein a mediates epigenetic programming: altering epigenetic marks by immediate-early genes; *J. Neurosci.* **27** 1756–1768

Weinstock M 2007 Gender differences in the effects of prenatal stress on brain development and behaviour; *Neurochem. Res.* **32** 1730–1740

MS received 7 September 2009; accepted 16 January 2010

*e*Publication: 29 January 2010

Corresponding editor: VIDITA A VAIDYA