

Epigenetic regulation of the *glucocorticoid receptor promoter 1₇* in adult rats

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Abbreviations: GR/Gr, glucocorticoid receptor; HPA, hypothalamus pituitary adrenal axis; MR/Mr, mineralocorticoid receptor; Ngfi-a, nerve growth factor-induced protein A

Regulation of glucocorticoid receptor (GR) levels is an important stress adaptation mechanism. Transcription factor Nfya and environmentally induced *Gr* promoter 1₇ methylation have been implicated in fine-tuning the expression of *Gr* 1₇ transcripts. Here, we investigated *Gr* promoter 1₇ methylation and *Gr* 1₇ expression in adult rats exposed to either acute or chronic stress paradigms. A strong negative correlation was observed between the sum of promoter-wide methylation levels and *Gr* 1₇ transcript levels, independent of the stressor. Methylation of individual sites did not, however, correlate with transcript levels. This suggested that promoter 1₇ was directly regulated by promoter-wide DNA methylation. Although acute stress increased *Ngfi-a* expression in the hypothalamic paraventricular nucleus (PVN), *Gr* 1₇ transcript levels remained unaffected despite low methylation levels. Acute stress had little effect on these low methylation levels, except at four hippocampal CpGs. Chronic stress altered the corticosterone response to an acute stressor. In the adrenal and pituitary glands, but not in the brain, this was accompanied by an increase in methylation levels in orchestrated clusters rather than individual CpGs. PVN methylation levels, unaffected by acute or chronic stress, were significantly more variable within- than between-groups, suggesting that they were instated probably during the perinatal period and represent a pre-established trait. Thus, in addition to the known perinatal programming, the *Gr* 1₇ promoter is epigenetically regulated by chronic stress in adulthood, and retains promoter-wide tissue-specific plasticity. Differences in methylation susceptibility between the PVN in the perinatal period and the peripheral HPA axis tissues in adulthood may represent an important “trait” vs. “state” regulation of the *Gr* gene.

Introduction

Regulation of glucocorticoid receptor (GR) expression, particularly in the brain, is an important mechanism of adaptation to stress. The 5' region of the *GR* gene (OMIM + 138040; *NR3C1*) consists, in humans, of nine untranslated alternative first exons (1A to 1I),^{1–3} each with its own promoter.⁴ The *Gr* promoter region in rats has a similar structure with 11 untranslated first exons (1₁ to 1₁₁). Eight of these exons (1₄ to 1₁₁) are highly homologous in rats and humans.^{2,5} The human exon 1F and its rat homolog exon 1₇ represent only a small fraction of all *GR* transcripts, but epigenetic regulation of these two exons in the brain by environmental influences has recently received much attention. Promoters 1F/1₇ are among the first gene promoters shown to be susceptible to environmentally induced DNA methylation, and this has been associated with a decrease in *Gr* transcript levels both in rodents⁶ and in

humans,⁷ suggesting that it represents a more general epigenetic regulation process.

In rodents, a specific post-natal environment such as low maternal care is associated with low levels of adult *Gr* expression in the hippocampus and the paraventricular hypothalamic nucleus (PVN).⁸ Low *Gr* expression was associated with levels of 80–100% methylation of a fully conserved key CpG site in promoter 1₇ in the hippocampus (rat CpG 16; human CpG 37), thought to correspond to a binding site of the transcription factor Ngfi-a (also known as Egr-1; zif-268; Krox-24), which is believed to be implicated in 1₇ transcript expression.^{6,9,10} However, in subsequent rodent studies, such high levels of methylation have not been observed at CpGs 16 and 17 in this Ngfi-a binding site.^{11,12}

Most of the literature on the epigenetic regulation of the glucocorticoid receptor is from the perinatal period. However, the HPA axis maintains its plasticity during adolescence and even

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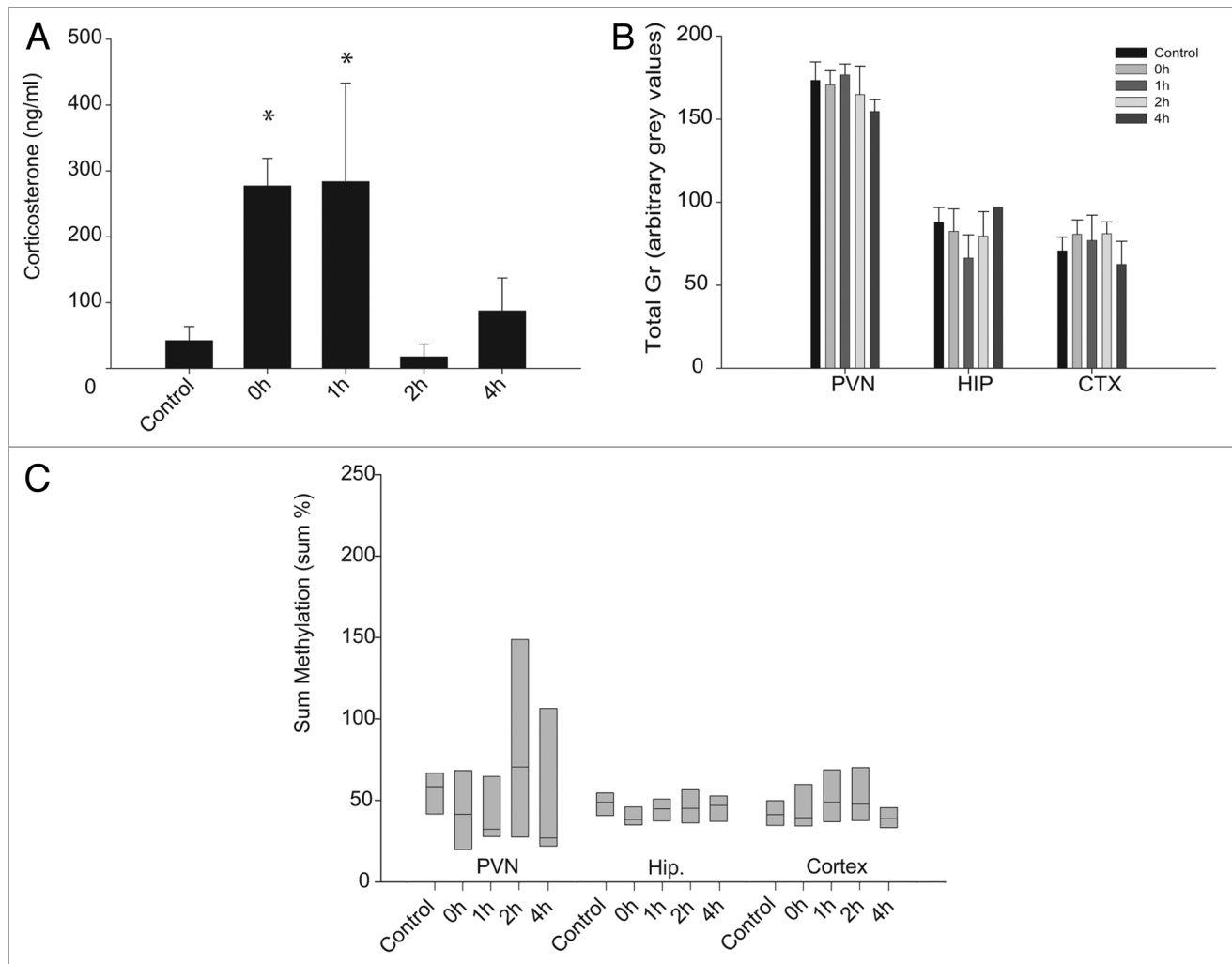


Figure 1. Plasma corticosterone levels (A), total *Gr* levels (B) and sum of methylation levels (C) after exposure to a single acute stressor. Significance levels were in comparison to the no stress group. * $p < 0.05$. Panels A and B are mean \pm SD. Panel C box plots show the 25th percentile, median and 75th percentile.

during adulthood,¹³⁻¹⁵ with neuroendocrine changes persisting for more than 12 mo after exposure to chronic stress.¹⁴ This HPA axis plasticity is associated with significantly reduced MR and GR levels in certain hippocampus regions,^{13,15} possibly due to methylation of the receptor genes. Infusion of L-Methionine, a precursor of the methyl-group donor S-adenosyl-methionine, directly into the brain of rats having experienced high maternal care altered the functional and behavioral phenotype to that of the hypermethylated low maternal care group. These experimental results further confirm that the HPA axis plasticity in adulthood is associated with changes in DNA methylation and GR or MR levels and that these changes directly affect the long-term stress response.⁹

We hypothesized that in young adult rats HPA axis plasticity can result from a similar balance between upregulation of transcription by *Ngfi-a* and downregulation by promoter methylation. Since acute stress rapidly induces *Ngfi-a* in the PVN,¹⁶⁻¹⁸ and chronic stress is thought to epigenetically alter the HPA axis, we investigated *Gr* 1₇ expression and promoter methylation in

adult rats after both acute and chronic stress exposure. Our data suggest that *Ngfi-a* does not immediately regulate 1₇ transcripts, and that promoter 1₇ methylation retains its plasticity into adulthood in the peripheral components of the HPA axis.

Results

Acute stress induces a corticosterone response and has no effect on *Gr* levels. To confirm HPA axis activation after a single session of acute restraint stress, plasma corticosterone levels were measured immediately after decapitation (Fig. 1A). Animals decapitated immediately after restraining had corticosterone levels > 10-fold higher than the unrestrained controls. Animals decapitated 1 h after the onset of the stress had equally elevated corticosterone levels. Corticosterone levels returned to baseline 2 h after stress. A minor, but not significant ($p > 0.05$) increase in corticosterone was observed 4 h after restraint initiation, corresponding to the start of the afternoon circadian increase in corticosterone levels of these nocturnal animals.

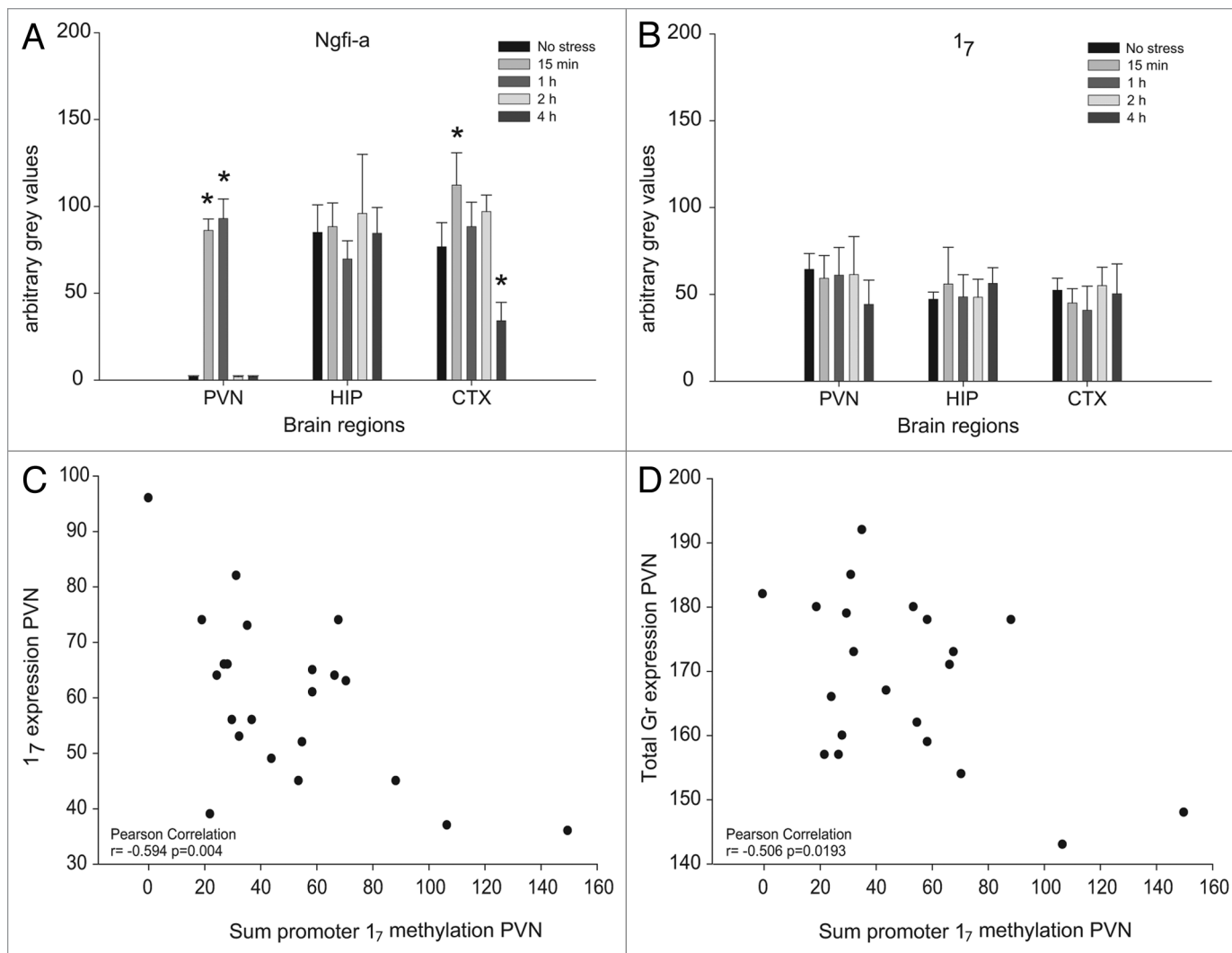


Figure 2. Quantification of *Ngfi-a* mRNA (A), *Gr17* mRNA (B) and the correlation of *Gr17* (C) and total *Gr* (D) transcripts with the sum of methylation throughout the promoter after acute stress by in situ hybridization in the paraventricular nucleus of the hypothalamus (PVN), the prefrontal cortex (CTX) and in the CA1 area, representing the hippocampus (HIP) and pyrosequencing. Panels A and B are mean \pm SD. Significance levels were in comparison to the no stress group. * $p < 0.05$.

In the above animals, a single session of acute restraint stress had no significant effect on total *Gr* mRNA levels in any of the brain tissues investigated at least up to the 4 h time point (Fig. 1B). Similarly, after a single acute stressor, there was no ANOVA group effect on the sum of methylation levels in promoter *Gr17* of any of these tissues (Fig. 1C).

Acute stress-induced *Ngfi-a* does not induce *Gr* exon *Gr17* expression. Since a single exposure to acute restraint stress is known to induce *Ngfi-a* expression in the PVN, in situ hybridization of both *Ngfi-a* and *Gr17* was performed at different time points after acute stress. Restraint stress immediately induced *Ngfi-a* in the PVN ($p < 0.05$), and levels were still elevated 1h later (Fig. 2A). After 2h, *Ngfi-a* levels were back to pre-stress levels. Within 4 h after the onset of stress, the *Gr* transcript *Gr17*, immediately downstream of promoter *Gr17* (which contains an *Ngfi-a* recognition site),⁶ was not upregulated in the PVN (Fig. 2B). Also, total *Gr* expression was not affected within the 4 h after restraint stress (Fig. 1B). *Ngfi-a* and

Gr17 levels were also measured in the hippocampus and the cortex (Fig. 2A and B). Also in the cortex, *Ngfi-a* levels were induced by 15 min of restraint stress ($p < 0.05$). After 4 h in their home cage, the rats had significantly reduced *Ngfi-a* levels compared with the unstressed group ($p < 0.05$). *Gr17* and total *Gr* transcript levels, however, remained unchanged throughout the 4 h period. In the hippocampus, *Ngfi-a*, *Gr17* and total *Gr* levels remained unaffected by the exposure to acute restraint stress. These results from the PVN and the cortex suggest that *Gr17* transcription is independent of *Ngfi-a* expression within this time frame.

***Gr* promoter *Gr17* methylation levels are low in the hippocampus and PVN.** Methylation levels throughout the PVN of acutely stressed rats (Fig. 3A) were below 20%, indicating that binding of *Ngfi-a* to promoter *Gr17* was not inhibited. This suggests that methylation was not the reason why promoter *Gr17* did not respond to stress-induced increases in *Ngfi-a* levels as described in the previous section.

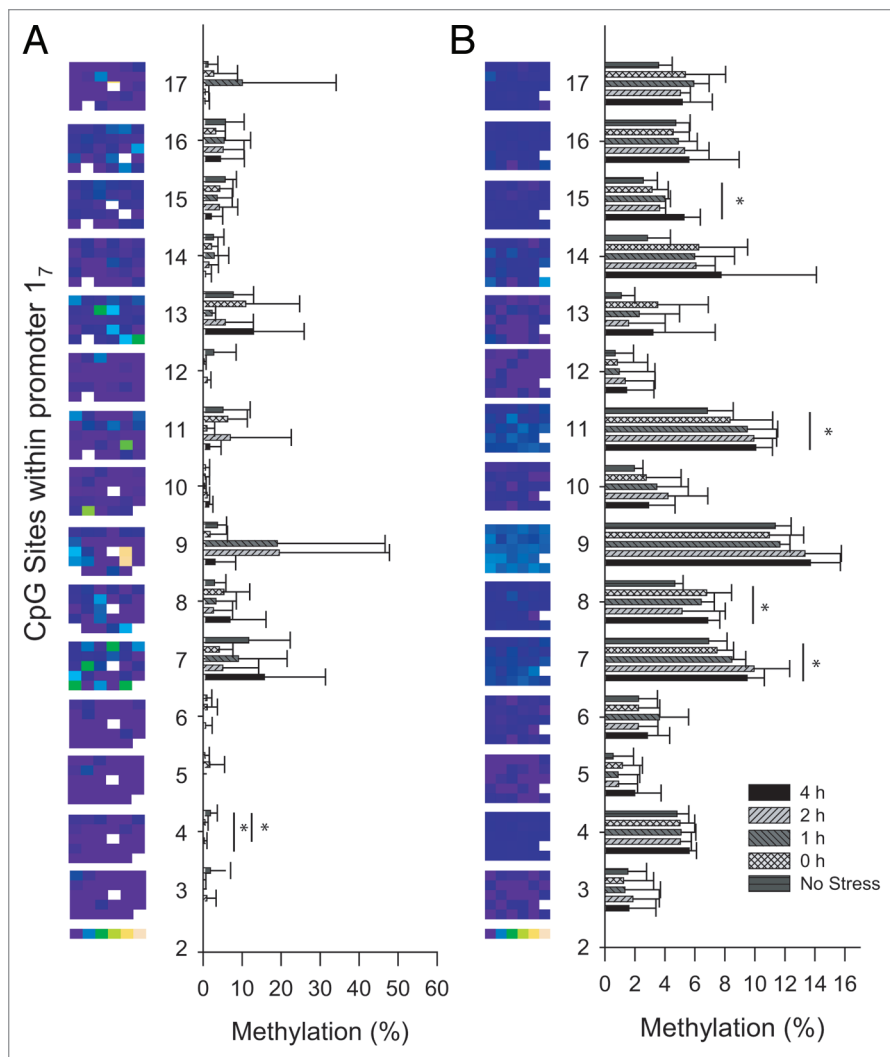


Figure 3. Methylation levels of individual CpG dinucleotides in the 1_7 promoter in the PVN (A) and the hippocampus (B) after a single acute stressor. Methylation levels are expressed for all animals in columns by color [0% (blue) to 75% (yellow)] and as the group mean \pm SD. Significance levels were in comparison to the no stress group. * $p < 0.05$. CpG sites are numbered according to Weaver et al.⁶

While there were no group effects on the sum methylation level in the PVN or the hippocampus, several individual CpGs showed a significant response to acute stress. In the PVN, a significant demethylation of CpG 4 ($p = 0.024$ after post-hoc correction) 1 h and 2 h after acute stress compared with controls (Fig. 3A) was observed, although basic methylation levels were already very low ($< 5\%$). In the hippocampus, 2 h and 4 h after stress, 4 of the 15 investigated CpG dinucleotides, CpGs 7, 8, 11 and 15, showed a significant increase ($p < 0.05$) in methylation levels after post-hoc corrections (Fig. 3B). One of these CpGs, CpG 11 in rats, was suggested by McGowan et al. to be part of a putative NGFI-A binding site in humans (CpG 30–32).⁷ In the hippocampus, this CpG increased from 6.8 ± 1.7 to $10.1 \pm 1.1\%$ ($p < 0.05$) after acute stress, suggesting that it was still available for binding. Thus, low and stress-modulated methylation levels do not explain the unresponsiveness

of 1_7 transcripts to stress induced Ngfi-a expression.

Gr promoter 1_7 methylation levels are variable in the PVN but not the hippocampus. Methylation levels of most CpG sites in the PVN were highly variable between animals, and significantly less variable in the hippocampus (Fig. 3B). The D'Agostino and Pearson omnibus normality test revealed that the sum of methylation of all CpGs in promoter 1_7 showed a normal distribution in the hippocampus ($K2 = 2.726$; $p = 0.2559$) but not in the PVN ($K2 = 12.78$; $p = 0.0017$), indicating more variability in the latter region. The large intra-group differences, together with a lack of difference between the groups, suggests that the variability in PVN sum methylation levels was established prior to the acute stressor.

Promoter 1_7 methylation correlates with transcript expression. Both the sum and individual CpG methylation levels, as well as exon 1_7 transcript levels, were more variable in the PVN than in any other organ tested, and these variabilities were independent of exposure to acute stress. Therefore, the link between the methylation levels and the observed exon 1_7 transcript level was further investigated in this brain region. The Pearson's correlation coefficients showed no significant link between methylation levels of individual CpG position and 1_7 transcript levels (r values varied between -0.396 and 0.05 and p values between 0.068 and 0.825). However, the sum of the methylation level throughout the promoter showed a significant negative correlation (Fig. 2C) with expression levels of the 1_7 transcript ($r = 0.594$; $p = 0.004$). This correlation was

essentially independent of the first five CpG positions (CpG 3–7) including CpG 4, which was stress sensitive, as described above. This suggests that the methylation of this shorter region covering CpG 3–7 does not contribute to the regulation of 1_7 transcripts. The correlation of the sum of methylation from CpG 8–17 with the total Gr level was weaker than for the 1_7 transcript, although still significant ($r = -0.503$, $p = 0.02$; Fig. 2D). In the cortex and the hippocampus of acutely stressed rats, methylation levels of neither the individual CpG sites nor the sum of all CpG sites correlated significantly with either 1_7 transcripts or the total Gr level. No correlation was found between PVN, hippocampus and cortex methylation levels.

Chronic stress alters growth and organ weight. Animals were chronically stressed either by a daily restraint stress routine (chronic restraint stress) or by daily randomization of their home cage and cage mates (chronic psychosocial stress).

Control animals were only weighed daily. After the 21 d of chronic stress exposure, animals with chronic restraint stress showed only 55.8 +/- 10.74 g weight gain, compared with 69.7 +/- 5.05 g for the psychosocial stress group or 77.3 +/- 5.03 g for the handling control group (ANOVA $p < 0.05$, all pairwise comparisons significant; Fig. 4A). On day 22, the restraint stress group showed a trend toward an increase in adrenal gland weight ($p = 0.08$; Fig. 4B) and a trend toward a decrease in pituitary weight in animals subjected to social stress (ANOVA, $p = 0.07$; Fig. 4C).

Chronic stress induces an altered HPA axis. After 14 d of either chronic restraint or psychosocial stress, all animals were restrained for 1h. Corticosterone levels pre- and post-restraining showed that the control group mounted a normal HPA axis response, with a > 7-fold increase in corticosterone (t-test $p < 0.01$). Animals subjected to daily restraint stress had a significant corticosterone response, although levels 30 min post-stress on day 14 were significantly lower than on day 1 (mean difference -183.4 ng/ml; t-test $p < 0.05$). Conversely, animals subjected to chronic psychosocial stress had an increased response to restraint stress on day 14 (mean difference +44.8 ng/ml $p < 0.05$; Fig. 5A) compared with day 1. There was no statistical difference in the corticosterone levels pre-stress between days 1 and 14 in any of the groups ($p > 0.05$).

Chronic stress affects *Gr* levels only in peripheral leucocytes. After 21 d, there was no difference in total *Gr* mRNA levels observed in the PVN, hippocampus, cortex, adrenal glands or pituitary glands. However, lower total *Gr* levels were observed in the peripheral leucocytes of the psychosocial stress group (ANOVA, $p < 0.05$; Fig. 5B) but not in the chronically restrained group.

Chronic stress alters the sum of the methylation levels in promoter 1₇ in the adrenal and pituitary glands. As the sum of methylation throughout promoter 1₇ correlated with *Gr* 1₇ transcript levels in our acute stress paradigm, we also investigated the effect of chronic stress on this promoter. A significant ANOVA group effect on the sum of methylation was found in both the adrenal and the pituitary glands (Fig. 5C; $p < 0.05$) after exposure to both chronic stress paradigms. In both tissues, psychosocial stress induced promoter 1₇ methylation; however, restraint stress decreased methylation in the adrenal gland but increased it in the pituitary. There was no effect of either stressor on methylation levels in the PVN, hippocampus or cortex. However, leucocytes showed a trend to higher methylation in both paradigms, although there was significant variability in methylation levels after both restraint and psychosocial stress.

Promoter 1₇ methylation correlates with transcript expression. Since there was an ANOVA group effect of chronic stress on the sum of methylation levels in the adrenal and pituitary glands, we measured their correlations with the total *Gr* transcript levels. In both tissues, there was a negative correlation between aggregated *Gr* transcript levels and the sum of the methylation, although this did not reach statistical significance ($r = -0.31$, $p = 0.127$ and $r = -0.21$, $p = 0.11$ respectively; Fig. 5D). Without the outlier in Figure 5D, the correlation becomes statistically significant in the adrenal gland ($r = 0.553$, $p < 0.01$).

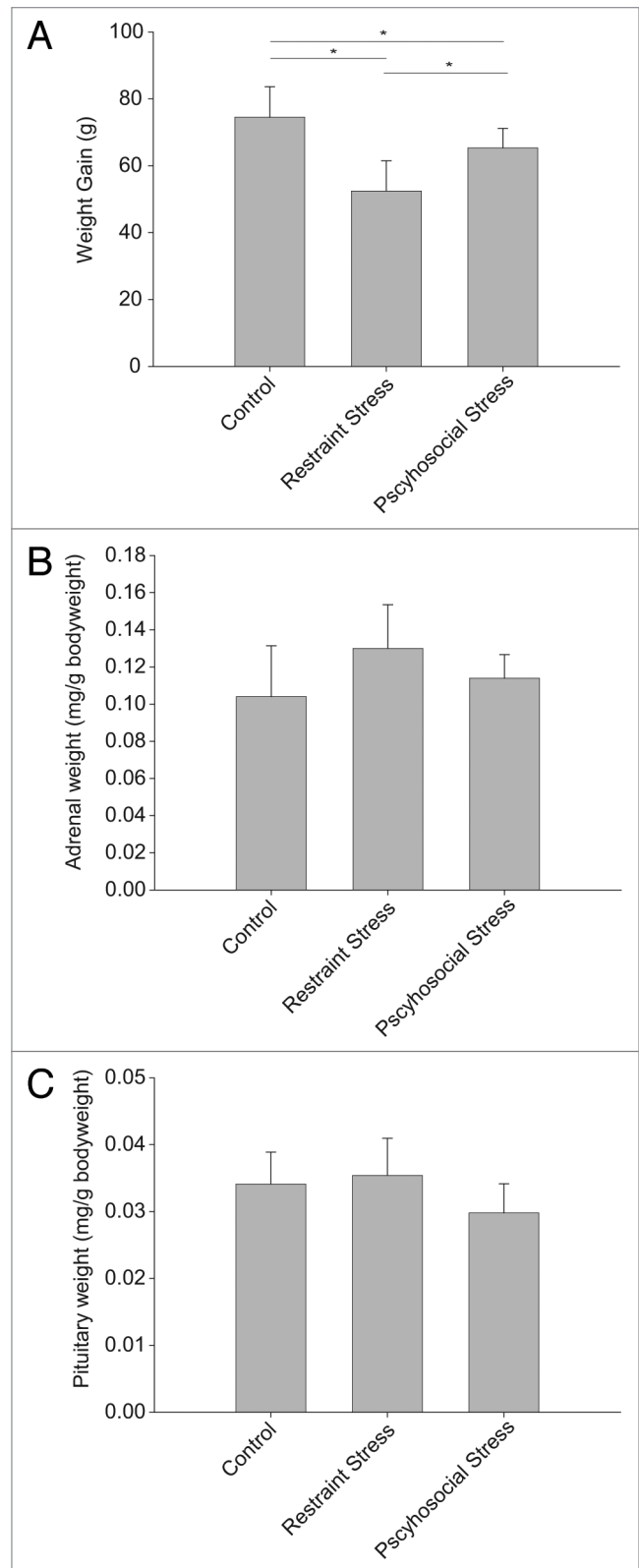


Figure 4. Exposure to 21 d chronic stress alters weight gain (A), relative adrenal gland weight (B) and relative pituitary weight (C). All panels are mean +/- SD. Significance was assessed by ANOVA, and * $p < 0.05$ for pairwise Tukey post-hoc comparison and correction.

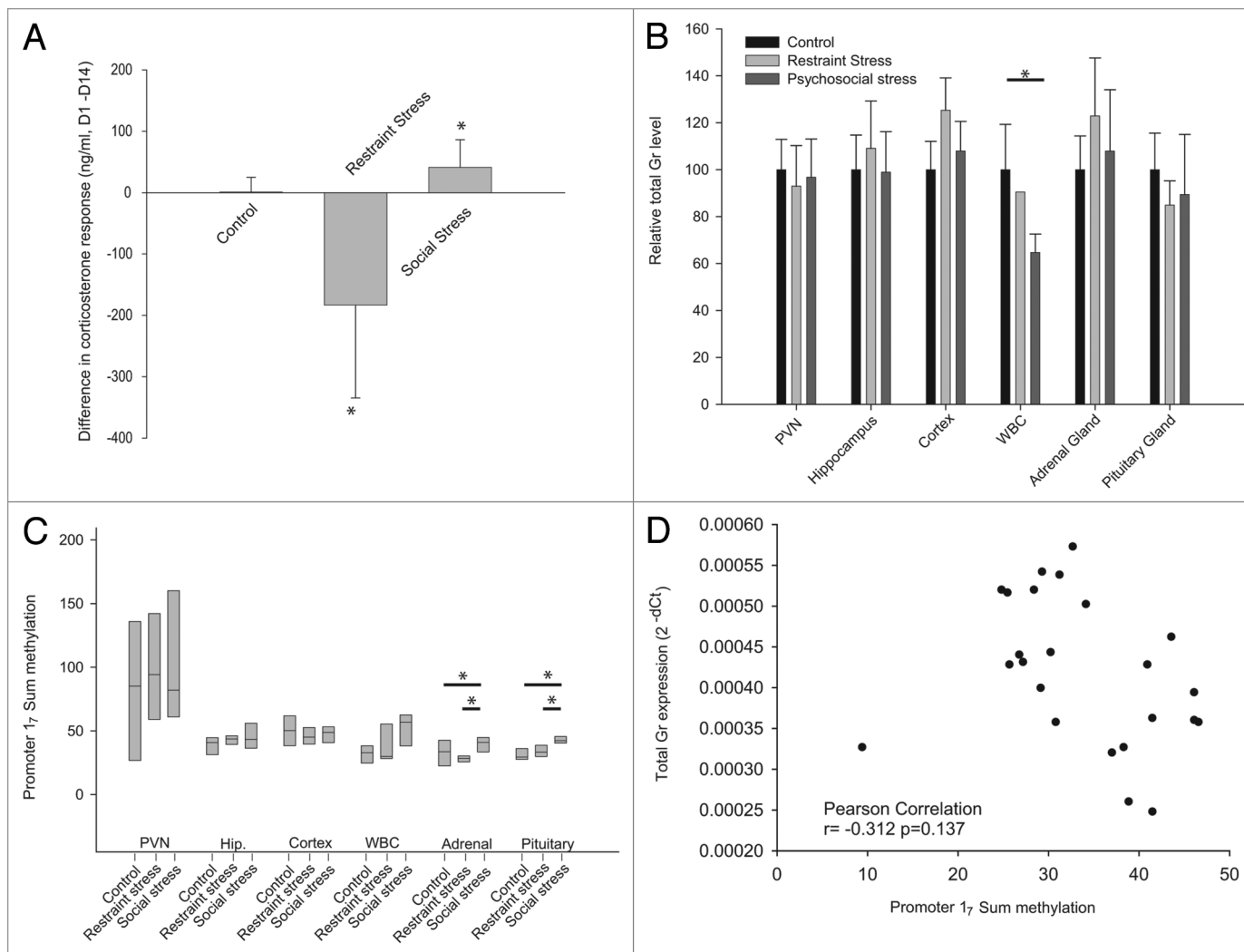


Figure 5. Change in plasma corticosterone response to a single acute stressor after 14 d exposure to chronic stress (A), total *Gr* mRNA levels (B) and the sum of methylation levels throughout promoter 1₇ (C) after chronic restraint or psychosocial stress. Panels A and B are mean \pm SD. Panel C box plots show the 25th percentile, median and 75th percentile.

PVN methylation levels are variable and independent of chronic stress. Similar to our acute stress observations above, methylation levels appeared to be much more variable in the PVN than in all other tissues (Fig. 5C). In both groups of chronically stressed rats, the D'Agostino and Pearson omnibus normality test revealed that the sum of the methylation shows a normal distribution (hippocampus: $K_2 = 0.068$; $p = 0.987$, PVN: $K_2 = 3.572$; $p = 0.168$). PVN methylation levels showed a greater skewedness and kurtosis than the hippocampus, although they did not deviate significantly from a normal distribution. Thus, as for acute stress exposure above, the variability in PVN methylation levels would appear to be determined prior to the stressor.

The *Ngfi*-a binding site remains unmethylated after chronic stress. As CpGs 16 and 17, the core *Ngfi*-a binding site, were poorly methylated (< 10%) and unaffected by a single acute stressor, we investigated the effect of chronic stress on their methylation levels. Although the sum of methylation, at least in the adrenal and pituitary glands, is significantly affected by chronic

stress, in both chronic stress paradigms methylation of CpGs 16 and 17 was consistently < 10% in all six tissues investigated (Fig. 6; Fig. S1; $p > 0.05$). This is in line with previous reports from other rat stress models,^{11,12} but in contrast to an earlier publication.¹⁹ Methylation levels of CpG 16 increased only in the adrenal gland ($p < 0.05$; Fig. S1).

Methylation levels of individual CpG positions in promoter 1₇ correlate after chronic stress. Since in chronically stressed animals ANOVA group effects on the sum of the methylation levels were only observed in the adrenal and pituitary glands, the individual CpG positions in promoter 1₇ of these tissues were examined in more detail. In the adrenal gland, 7 of the 15 CpGs investigated showed a significant increase in methylation levels (ANOVA $p < 0.05$; Fig. S1). An additional three CpGs showed a trend toward higher methylation levels. In the pituitary gland, methylation levels of promoter 1₇ were increased in six of the 15 CpGs, and a trend was observed in two additional CpGs. In both tissues, methylation levels were increased in the same CpGs

(CpGs 7, 8, 9, 11, 13 and 15). Methylation was additionally increased at CpG 16 in the adrenal gland. Pearson correlations performed on the aggregated adrenal gland data revealed a high degree of correlation (Fig. 7A) between individual CpGs in a pairwise comparison as well as between most CpGs and the sum of methylation levels. A similar pattern was found in the pituitary gland (Fig. 7B), although CpGs 3 and 4 appeared to correlate less well with the sum of methylation levels and with the other individual CpGs within promoter 1₇ (p value from 0.02 to 0.38). The high correlation between individual CpGs suggests that methylation is a promoter-wide phenomenon, with levels increasing across multiple CpGs in close proximity.

Leucocyte sum methylation levels may represent a surrogate or biomarker for peripheral HPA axis tissues. Aggregating all animals irrespective of their group, the sum CpG methylation data (Fig. 7C) shows that there is a strong and significant correlation between the leucocytes and the pituitary gland methylation ($r = 0.511$, $p = 0.011$; Fig. 7D). There was, however, only a trend toward a correlation between the pituitary and adrenal gland methylation, as well as between cortex and leucocytes ($r = 0.371$, $p = 0.075$ and $r = 0.368$, $p = 0.077$, respectively; Fig. 7C).

Discussion

Gr 1₇ promoter methylation and *Ngfi-a* transcripts were measured to investigate their antagonistic roles in the production of appropriate Gr level. Exposure to a single acute stressor induced the expected corticosterone response and upregulated *Ngfi-a* expression in the PVN, as previously reported,¹⁶⁻¹⁸ as well as in the cortex but not in the hippocampus, setting the stage for investigating the subsequent regulation of the *Gr* 1₇ transcript. Upregulation of its expression confirmed that *Ngfi-a* is an important stress induced immediate early gene in these brain regions and that it may play a role in the subsequent stress response.¹⁷ However, upregulation of *Ngfi-a* had no effect on 1₇ transcription or on total Gr expression. Also, in previous studies, even two hours of restraint stress did not alter total *Gr* transcript levels in the PVN or the hippocampus.²⁰ This suggests that the *Ngfi-a* binding site previously identified in the rat promoter 1₇ is not functional in the PVN during acute stress. In contrast, acute stress downregulated *Gr* in the dentate gyrus (DG) and the CA1 region of the hippocampus.^{21,22}

Methylation of CpG 16 in the core of the *Ngfi-a* binding site has previously been shown to affect the regulation of *Gr* 1₇ transcripts by *Ngfi-a*. This CpG site was rarely methylated in the hippocampus of offspring of high caring mothers, but highly methylated (> 80%) after low maternal care.⁶ Therefore, binding

of *Ngfi-a* to the *Gr* 1₇ promoter was thought to be inhibited in the maternal care paradigm by this methylation, reducing, as a result, *Gr* 1₇ expression.¹⁰ However, in our study, none of the animals in the acute stress experiments showed methylation levels > 10% at this CpG site, confirming that the *Ngfi-a* site was available for binding. Nevertheless, methylation of the promoter appeared to be important, especially in the PVN, since the sum of methylation levels, in particular of CpGs 8–17, correlated negatively with transcript 1₇ expression, indicating repression of *Gr* 1₇ transcription by methylation. Importantly, there was no correlation when only CpG 16 or 17 in the *Ngfi-a* binding site were considered. Thus, the absence of a response of 1₇ transcription to acute stress-induced *Ngfi-a* is not caused by methylation within the core of the *Ngfi-a* binding site but rather due to methylation across a much wider region of the promoter, indicative of an important role of other transcription factor binding sites and perhaps transcription factors. This suggests that *Ngfi-a* expression, at least within the 4 h of this study, was not sufficient to drive 1₇ transcription.

Our chronic stress model altered the HPA axis, as would be expected from previous reports.^{13,15} While the acute stressor

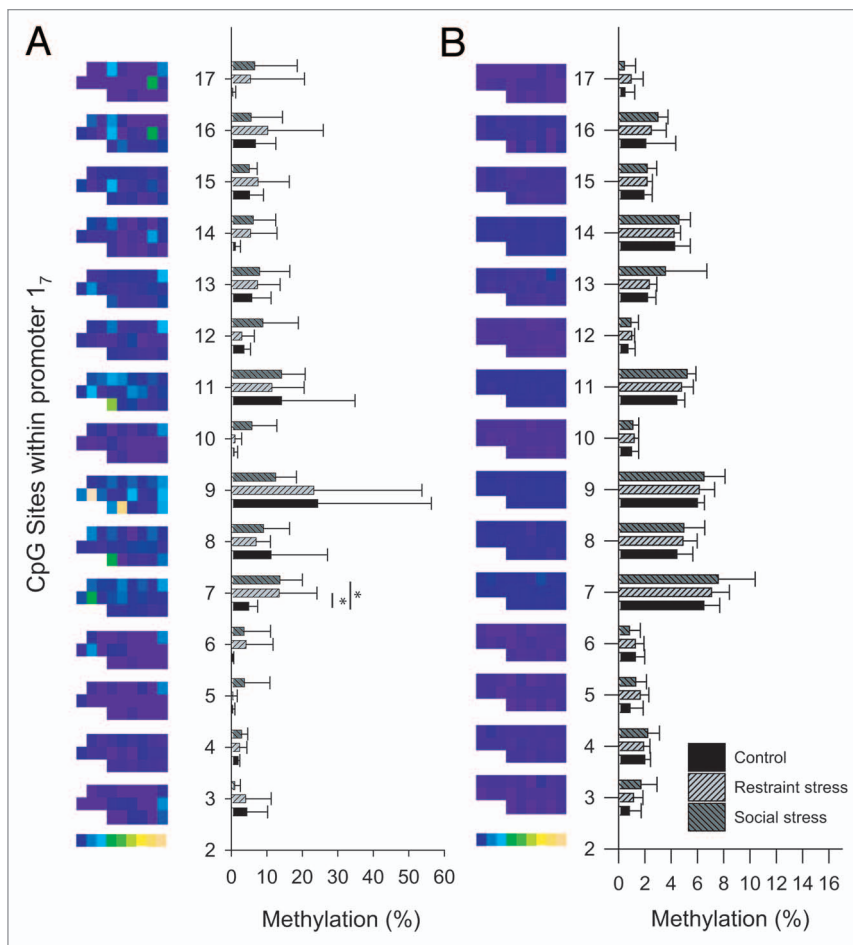


Figure 6. Methylation levels of individual CpG dinucleotides in the 1₇ promoter in the PVN (A) and the hippocampus (B) after 21 d chronic stress. Methylation levels are expressed for all animals in columns by color [0% (blue) to 75% (yellow)] and as the group mean \pm SD. Significance levels were in comparison to the no stress group. * $p < 0.05$. CpG sites are numbered according to Weaver et al.⁶

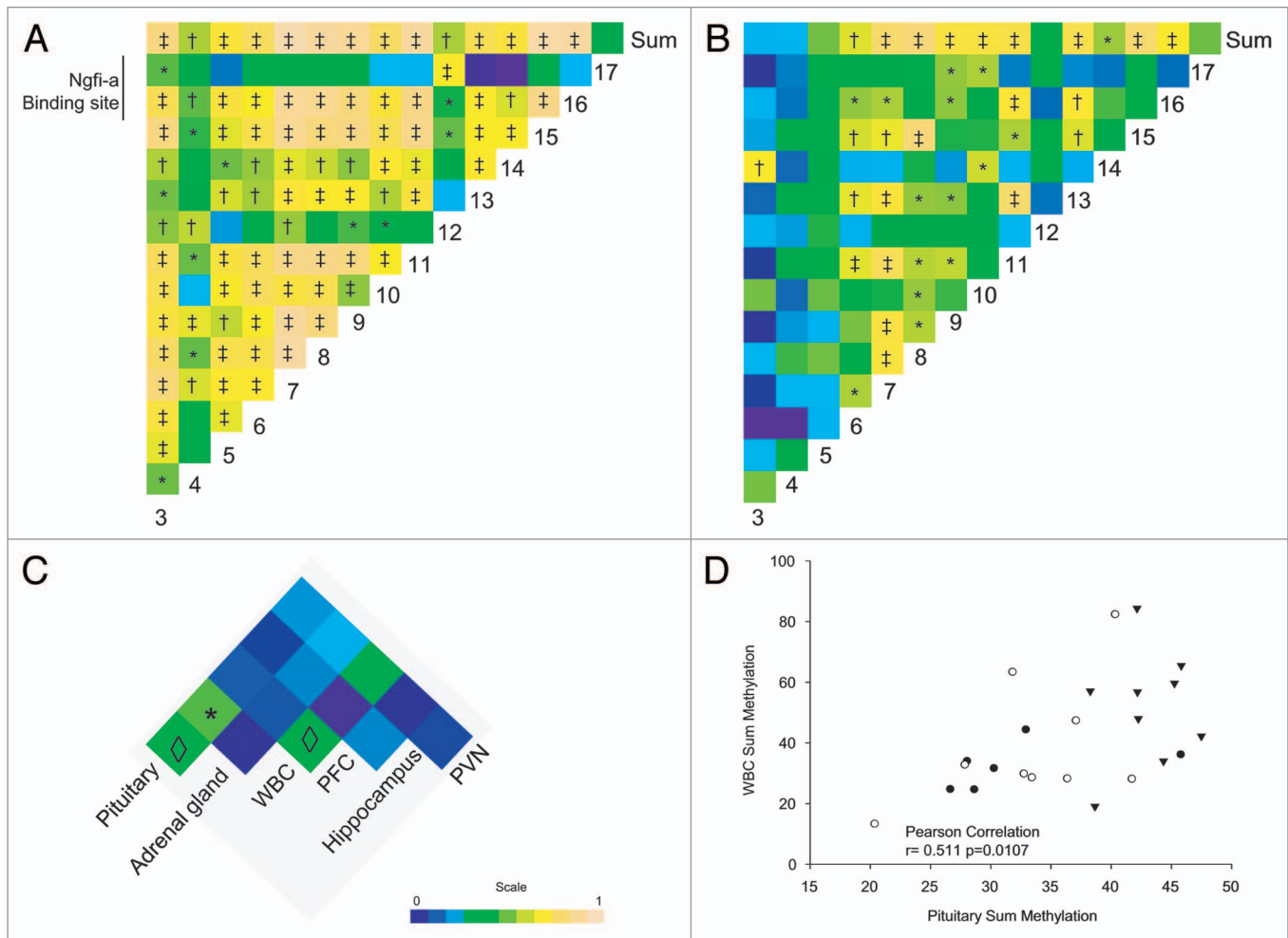


Figure 7. Pearson correlation heat maps for individual CpGs in the pituitary gland (A) the adrenal gland (B), and for the sum of methylation between all tissues (C) after exposure to chronic stress. The sum of methylation levels for individual animal in (filled circles, controls; empty circles, restraint stress; triangles, psychosocial stress) (D) Pearson correlation coefficients (panels A–C) are expressed by color [0 (blue) to 1 (yellow)]. * $p < 0.01$, $\diamond p < 0.012$.

induced a weaker corticosterone response in animals exposed to chronic restraint, it induced a stronger corticosterone response in psychosocially stressed rats. HPA axis hypo-responsiveness after chronic restraint stress was associated with a trend toward an enlarged adrenal gland, as previously reported by Schmidt et al. in a similar murine stress paradigm.¹⁵ Conversely, psychosocial stress-induced hyper-responsiveness was associated with a tendency toward a smaller pituitary gland, together with an enlarged adrenal gland, as previously reported after a similar exposure to restraint stress²³ suggesting that after chronic stress the rats had not completely habituated to the stressor.²⁴ In both of these tissues, total *Gr* levels were unchanged compared with control values after either restraint or psychosocial stress. Also, hippocampal *Gr* levels remained unaffected by either psychosocial or restraint stress. This is in contrast with the downregulation previously reported in some hippocampal subregions, e.g., CA1 or dentate gyrus, which were not independently investigated by us.²² Both of our chronic stress paradigms induced HPA axis changes reminiscent of those observed in human major depression (MDD)

studies. Previously, we reported that hippocampal total GR levels were unaffected in MDD,²⁵ although transcript 1F levels were slightly lower.²⁶ Similarly, the adrenal glands in MDD and in suicide victims are larger, have an increased adrenal cortex, and an exaggerated cortisol response after ACTH administration.²⁷⁻³⁰ These human observations have led several authors to suggest that peripheral HPA axis elements are responsible for the observed hypercortisolemia in MDD.^{27,31,32} Our psychosocial stress paradigm also mirrors the increased stress reactivity of the HPA axis in MDD. Previously, such neuroendocrine changes have been shown to be prevented by concurrent treatment with antidepressants such as paroxetine.¹⁵

The peripheral organs of the HPA axis were further implicated in the altered stress response by our observation that epigenetic methylation of the *Gr* promoter 1₇ was preferentially induced by chronic stress in the adrenal gland and the pituitary. Methylation levels in the pituitary and adrenal glands were significantly increased by chronic stress, and showed a trend toward higher levels in peripheral leucocytes, but were unchanged in the

PVN, hippocampus and cortex. Thus, our chronic stress models showed that epigenetic methylation of the *Gr* can be altered in peripheral tissues. Similar observations of *Gr* methylation have been made in the liver after a protein restricted diet³³ in small cell lung tumors³⁴ and in the maternal side of the placenta.³⁵ This is compatible with the notion that DNA methylation patterns are still variable during adulthood and can be modulated by environmental challenges.³⁶

Throughout promoter 1₇, irrespective of the stressor and for all tissues except the PVN, average methylation levels never exceeded 15%, measured by highly quantitative pyrosequencing.³⁷ These low levels concur with previous reports of hippocampal 1₇ methylation in rats.^{11,12} Similar low levels of methylation were also seen in the human 1F promoter in peripheral leucocytes both in newborns³⁸ and adults,^{39,40} as well as in post mortem adult brains²⁶ and the placenta.³⁵

While the range of methylation levels observed was small, aggregating the data from control, restraint and psychosocially stressed animals we find a high degree of correlation between methylation levels of individual CpG dinucleotides within promoter 1₇ in the adrenal and the pituitary glands. A similar pattern was previously found in promoter 1F of placentas from large for gestational age births.⁴¹ Methylation levels were increased in all promoter 1F CpG positions, although statistical significance was reached in only a single position after post-hoc correction for multiple testing. The importance of promoter-wide methylation is further supported both by our observation that the sum of methylation levels throughout promoter 1₇ correlated with *Gr* 1₇ transcript levels, and the co-regulation of CpGs in close proximity. Recently a similar pattern of increased methylation levels of CpGs in close proximity has been observed for the human *GR* 1H promoter in suicide completers, although the effect of distance on co-regulation was not analyzed statistically.⁴² Co-regulation of methylation levels at CpG dinucleotides in close proximity has previously been reported from high-throughput methylation assays of human biopsies. In genome wide studies in both normal and cancerous colon tissue, irrespective of the genomic locus, Nautiyal et al. observed a very high correlation in methylation levels that decreased as the distance between dinucleotides increased.⁴³ Over the scale of a typical promoter, they observed a linear decrease in the Pearson's *r* from > 0.8 for dinucleotide pairs less than 50 bp apart to < 0.4 for pairs 1 kb apart. Chronic stress increased methylation levels significantly in the pituitary and adrenal glands, and in both tissues a promoter-wide regulation was observed. In contrast, only single CpG dinucleotides, such as CpG16, were reported to be altered by maternal care.^{6,7} We suggest that *Gr* 1₇ transcripts were regulated by promoter-wide methylation, and that the role of individual CpGs in the non-functional *Ngfi*-a binding site, and perhaps the role of the site itself, is more limited than previously thought.

The role of methylation throughout promoter 1F is supported by the recent observation that average methylation levels rather than individual CpG levels throughout promoters 1B and 1H correlated significantly with the expression of their corresponding exons.⁴² Also, in promoter 1F, mean promoter-wide methylation levels also correlated with GR expression levels in the

placenta (Spearman's rho = -0.28) although not reaching statistical significance.³⁵

As methylation levels increased promoter-wide after chronic stress in a tissue specific manner, we investigated the correlation between the different tissues. The strong correlation between the sum of methylation levels in the pituitary gland and in leucocytes suggests that the latter may be suitable surrogates or potential biomarkers for methylation in peripheral HPA axis organs. In contrast, the hippocampus and the PVN did not correlate with any other tissue, confirming the tissue specificity of their methylation patterns. Peripheral leucocytes have been investigated in the majority of human studies so far.^{38,40,44,45} Methylation levels in leucocytes of CpG 37 (ortholog of the rat CpG 16), although uniformly low, were shown to be associated with both prior childhood adversity as well as maternal mood during pregnancy,^{38,45} and reflected functional differences in the HPA axis. In these studies increased leucocyte methylation was associated with a decreased salivary cortisol stress response.³⁸ Since in these and our studies leucocyte methylation levels seem to reflect those in the pituitary and adrenal glands, it may be hypothesized that the functional differences in these infants were the result of altered methylation levels in peripheral HPA axis tissues.

The PVN is unique in that highly variable methylation patterns ranging from 0% to 68% were found in the different animals from both the acute and chronic stress experiments. These levels and variability were not reached in any other tissue. Since the within-group differences clearly exceeded between-group differences in both experiments, we interpret these as unrelated to the stressor, but rather as pre-existing individual differences. Thus, in these outbred rats, environmental influences that were not identified here seem to determine 1₇ promoter activity through methylation of CpG dinucleotides throughout the 1₇ promoter of the PVN. Recently, it has been shown that, for instance, the amount of maternal care received varies considerably between litter mates, and individual care levels correlated positively with hippocampal synaptic plasticity and *Gr* expression levels in this brain region.⁴⁶ We suggest that this PVN variability may be established in the litter prior to our experiment and may represent an individual trait, in agreement with the early observations on the effects of maternal care.⁸ The methylation patterns in the PVN, together with the adrenal and pituitary glands, suggest that tissue-specific plasticity is retained from the perinatal period until adulthood.

In conclusion, we report that acute stress induced *Ngfi*-a did not affect *Gr* 1₇ transcript levels. Promoter 1₇ methylation, particularly in the *Ngfi*-a binding site, did not explain this lack of activity, although overall methylation levels throughout promoter 1₇ regulated exon 1₇ transcript levels in the PVN. Chronic stress altered the corticosterone stress response to a subsequent acute stressor; however, hippocampal and PVN methylation levels were unaffected. In contrast, methylation levels were increased in the pituitary and adrenal glands. These increased methylation levels were orchestrated in promoter-wide clusters of proximal CpGs rather than individual CpG dinucleotides in the non-functional *Ngfi*-a binding site. The *Gr* 1₇ promoter showed tissue-specific methylation plasticity remaining epigenetically sensitive in

peripheral HPA axis tissues even in adults. In contrast, methylation levels in the PVN would appear to be unaffected by acute or chronic stress and were significantly more variable within than between groups, suggesting that they were instated before stress exposure as a pre-established trait, probably during the perinatal period.

Methods

Acute and chronic stress protocols. Ten- to twelve-week-old male Sprague Dawley rats were obtained from Harlan and acclimatized for 1 week. Animals were kept under natural light at $22 \pm 2^\circ\text{C}$ and $40 \pm 5\%$ relative humidity. Food and water were available ad libitum. Animals were housed in groups of 3 in $48 \times 37.5 \times 21$ cm clear plastic cages. All animal experiments were performed in compliance with the rules of the European Communities Council Directive of 24 November 1986 (86/609/EEC), and all national ethical guidelines.

Animals subjected to the acute stress paradigm were randomly divided into five groups of six animals. Unrestrained animals served as a control group. The restraint groups were immobilized for 15 min and decapitated immediately or returned to their home cages for 1–4 h before decapitation. Isolated brains from all animals were frozen in isopentane cooled in an ethanol-dry ice bath and stored at -80°C . EDTA anti-coagulated decapitation blood was collected and plasma was stored at -20°C .

Animals subjected to the chronic stress paradigm were randomly divided into three groups. The control group contained six animals and both the groups exposed to chronic restraint and psychosocial stress contained nine animals. Control animals were weighed daily and returned to their home cage without further manipulation. The chronic restraint stress group was weighed daily and subsequently immobilized for 1 h before being returned to their home cage. The chronic psychosocial stress group was weighed daily, and randomly returned to one of three cages, as outlined in Table S1. All experimental and control animals were restrained for 1 h on day 1 and 14 and tail vein blood was drawn to assess corticosterone levels. All animals were sacrificed on day 21. Right hippocampus and prefrontal cortex were dissected from isolated brains, and the remainder frozen in isopentane cooled in an ethanol-dry ice bath and stored at -80°C . EDTA anti-coagulated decapitation blood was collected and plasma was stored at -20°C . Thymus, pituitary, and adrenal glands were extracted, weighed, and stored in RNAlater (Qiagen) at -20°C until analyzed.

Corticosterone ELISA. Plasma corticosterone levels were measured by ELISA (IBL) following the manufacturer's instructions. Analyses were performed on 20 μl undiluted or 1:10 diluted plasma as necessary. Intra- and inter-assay variability were 5.2% and 6.4% respectively, and the sensitivity was < 1.63 nmol/L.

Nucleic acid extractions. Serial tissue sections (14 μm) of all brains were cut on a cryostat (Reichert-Jung Frigocut 2800) and thaw-mounted onto Superfrost Plus glass slides and stored at -80°C . One cryostat section per animal was selected for dissection at room temperature under a stereomicroscope. This section was chosen on the basis of the presence of the PVN

and the hippocampus in stained adjacent sections. A surface of 3×3 mm including bilateral PVNs was scraped into separate RNase-free tubes for subsequent isolation of genomic DNA using the QIAamp[®] DNA Micro kit (Qiagen, Venlo, The Netherlands) and RNA using the RNeasy Micro Kit (Qiagen) and stored at -20°C . Genomic DNA from hippocampal tissue was isolated using the QIAamp[®] DNA Mini kit (Qiagen) following the manufacturer's instructions.

In situ hybridization. In situ hybridization was performed using ³⁵S UTP labeled riboprobes as described previously.⁴⁷ Slides were processed to measure the expression of *Nggf-a*, *Gr* exon 1₇, and *Gr* total mRNA in the PVN, cortex and hippocampus. The 230 bp *Nggf-a* probe used was a kind gift of Prof. J. Milbrandt. For total *Gr* we used a 500 bp fragment (exon 2, coding for N-terminus of the receptor—courtesy of Dr M. Bohn, Northwestern University) of the original full-length rat *Gr* clone (courtesy of Dr K. Yamamoto, University of California, San Francisco). mRNA containing exon 1₇ was quantified with a specific riboprobe kindly provided by Dr K. Chapman (University of Edinburgh).⁵ Sense probes were used as negative controls. After hybridization, slides were put in a cassette and a Biomax-MR film (Kodak) was exposed for 3 d (*Gr*) to 13 d (exon 1₇) and ¹⁴C microscaler (GE healthcare) were used to calibrate the signal. Films were scanned and quantified using the Image J software. Average optical densities were corrected for film and tissue background.

Quantitative RT-PCR. First-strand (cDNA) synthesis was performed in a 40 μl reaction containing 375 mM KCl, 250 mM Tris-HCl, 15 mM MgCl₂, 10 mM dithiothreitol and 500 μM deoxynucleoside triphosphates (dNTPs) at 42°C for 50 min using 200 U SuperScript II Reverse Transcriptase (Invitrogen) and 2.5 μM dT16 primer (Eurogentec).

Duplicate amplifications of cDNA by PCR were performed in 25 μl reactions containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 mM deoxynucleoside triphosphates (dNTP), 2.5 U Platinum Taq DNA polymerase (Invitrogen) and 1 \times concentrated SYBR Green (Cambrex). Thermal cycling, (Opticon 2, BioRad) conditions were: 95°C , 2 min; followed by 45 cycles each at 95°C , 20 sec; annealing, 20 sec; 72°C , 30 sec. All PCR products were separated on a 2% agarose gel and visualized with SYBR Safe (Invitrogen) under UV illumination. Primer sequences, annealing temperatures, and MgCl₂ concentrations are given in Table S2. All primers were synthesized by Eurogentec.

Methylation analysis. The EpiTect Bisulphite kit (Qiagen) was used to convert unmethylated cytosine residues to uracil, whereas methylated cytosines remained unmodified as previously described.^{26,40} The bisulphite-modified DNA was used to amplify promoter 1₇ with the primers and under the conditions shown in Table S2. PCR was performed using 20 mM TRIS-HCl (pH 8.4), 50 mM KCl, 200 mM deoxynucleoside triphosphates, 1 \times SYBR Green (Cambrex) and 2.5 U Diamond Taq DNA polymerase (Bioline) on an Opticon 2 thermal cycler (BioRad). Cycling conditions were as follows: 95°C for 2 min, 40 cycles, at 95°C for 20 sec, annealing temperature for 20 sec and 72°C for 25 sec. Products of first round PCRs were used as a template for nested amplifications using the primers and conditions in Table S2. The region homologous to the human *GR* promoter

1A was used as a positive pyrosequencing control. PCR was performed using Platinum *Taq* polymerase (Invitrogen) with the primers and conditions described in Table S2. Specific primer sets for rat homolog 1A and all pyrosequencing primers have been selected using the PSQ Assay Design software (Biotage). All PCR products were pyrosequenced on a Pyromark ID using Pyrogold reagents (Biotage) to allow quantitative analysis of DNA methylation patterns.

Pyrosequencing controls. As the methylation levels detected in all tissues were on average below 20%, we used the region homologous to the human *GR* promoter 1A as a positive methylation control in all pyrosequencing experiments. This region is outside of the CpG island and therefore highly methylated (89%).⁴⁸ The bisulphite conversion efficiency was 93% and the total average methylation was 90%.

Statistical analysis. All results were analyzed using a one way analysis of variance (ANOVA) followed by Tukey post-hoc correction. The relationship between promoter methylation and *1 γ* expression levels was analyzed using Pearson's correlation coefficient. Normal distribution of overall methylation levels in the hippocampus and the PVN were tested using the D'Agostino-Pearson omnibus test. Statistical analyses were performed using SPSS Version 18 (SPSS Inc.) and the Sigma Stat software for

Windows. Differences were considered to be significant when $p < 0.05$ after post-hoc correction. Sum methylation data are the sum of methylation levels at all individual CpG positions of an individual animal. Aggregated methylation and aggregated sum methylation data refer to the data of all animals combined irrespective of their experimental group.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/epigenetics/article/22363

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