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Rapid down-regulation of glucocorticoid gene expression in the dentate gyrus after acute stress *in vivo*: Role of DNA methylation and microRNA activity

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Abstract

Background Although glucocorticoid receptors (GRs) in the hippocampus play a vital role in the regulation of physiological and behavioural responses to stress, the regulation of receptor expression remains unclear. This work investigates the molecular mechanisms underpinning stress-induced changes in hippocampal GR mRNA levels *in vivo*. **Methods** Male Wistar rats were killed either under baseline conditions or after forced swim stress (FSS; 15min in 25°C water). Rat hippocampi were micro-dissected (for mRNA, microRNA, and DNA methylation analysis) or frozen whole (for chromatin immunoprecipitation (ChIP). In an additional experiment, rats were pre-treated with RU486 (a GR antagonist) or vehicle. **Results** FSS evoked a dentate gyrus-specific reduction in GR mRNA levels. This was associated with increased DNMT3a protein association with a discrete region of the *Nr3C1* (GR gene) promoter, shown here to undergo increased DNA methylation after FSS. FSS also caused a time-dependent increase in the expression of miR-124a, a microRNA known to reduce GR mRNA expression, which was inversely correlated with a reduction in GR mRNA levels at 30 min post-FSS. FSS did not affect GR binding to a putative negative glucocorticoid response element (GRE) within the *Nr3c1* gene. **Conclusions** Acute stress results in decreased GR mRNA expression specifically in the dentate gyrus. Our results indicate that a complex interplay of multiple molecular mechanisms including increased DNA methylation of discrete CpG residues within the *Nr3c1* gene, most likely facilitated by DNMT3a, and increased expression of miR-124a could be responsible for these changes.

Introduction

Acute stress results in secretion of glucocorticoid hormones (GCs) from the adrenals into the circulation. GCs act on areas of the brain, like the hippocampus, to evoke a number of biochemical, physiological and behavioural responses (1, 2). The hippocampus is a key area of the brain involved in the formation of contextual memories and is essential for appropriate adaptation to stress (3). The hippocampus can be divided into four principle anatomical subregions; the dentate gyrus (DG), and the cornu ammonis regions 1-3 (CA1-3). The hippocampus is characterized by a high expression of corticosteroid receptors, of which there are two types; mineralocorticoid receptors (MRs) that bind GCs with very high affinity, and glucocorticoid receptors (GRs), which bind GCs with lower affinity (4). Pioneering work investigating receptor occupancy by corticosterone clearly identified GR as the predominant receptor activated by excess ligand, with levels of receptor occupation rising from just 25% under baseline (home cage) conditions to over 75% at the peak of stress-induced corticosterone secretion. In contrast, MRs were already highly occupied under baseline conditions (>75%) and only rose marginally after stress (4, 5). Early binding studies using micro-punched hippocampal subregions or *in vitro* autoradiography showed a differential localisation of MRs and GRs across hippocampal subregions (4, 6). Laser capture micro-dissection of the dentate gyrus (DG) and CA3 subregions from young male Wistar rats and subsequent gene expression profiling analysis revealed a 1.7 fold higher expression of GR in the DG cf. CA3 (7). More detailed studies in male Sprague Dawley (SD) rats showed the same pattern of increased expression in the DG when compared with the CA3, but similar levels when compared to the CA1 and CA2 regions (8, 9). Subsequent works to elucidate the effect of stress on GR expression have been unable to identify a common effect of stress on GR expression in the hippocampus. Conflicting studies have shown GR mRNA levels remaining stable (10-14) or decreasing (10, 15, 16) after a variety of stressors including forced swimming, restraint, Morris water maze exposure, and novelty stress. Thus far, stress-induced GR mRNA expression has generally been measured by either polymerase chain reaction (PCR) analysis on whole hippocampus (10, 13) or via *in situ* hybridization (11, 12, 14, 15). The former method neglects differences in the subregional distribution of GR receptors and the latter is semi-quantitative and therefore may not be sensitive enough to detect moderate stress-related changes in GR expression.

Despite the ambiguity surrounding the effect of stress on GR expression in the hippocampus, progress has been made in determining how the GR gene is regulated. The *Nr3c1* gene consists of 8 coding exons (Exons 2-9) preceded

by a 5' leader sequence containing multiple sequences (Exons 1₁-1₁₁) that can be alternatively spliced to create tissue-specific first exon sequences (17). One of these exonic sequences (Exon 1₇) was the target of a landmark study from the Meaney group (18) who identified this region of the *Nr3c1* (GR) gene in the hippocampus as undergoing substantial DNA methylation if rat pups were reared in a 'stressful' environment (caused by low care-giving mothers). The region of differential methylation, exon 1₇, contained a binding site for early growth response protein 1 (Egr1, also termed NGFI-A or Zif268), a transcription factor involved in learning and memory processes (3, 19). Methylation of exon 1₇ prevented Egr1 binding and associated transcriptional activation of the GR gene (18). Similar phenomena have been observed in humans that experienced early life abuse and neglect and is associated with mental health problems including suicide and depression later in life (20, 21). Additional studies have gone on to show more transient changes in methylation at specific CpG sites within this exon after acute stress in both the hippocampus and amygdala, reviewed by Turecki & Meaney (22).

In addition to DNA methylation changes, GR mRNA can be the target for active degradation by microRNAs including miR-18 and miR-124a (13, 23, 24). MiR-124a is the predominant microRNA expressed in the brain, accounting for 25 - 48% of all brain microRNAs (25) and expression has been confirmed in the rat hippocampus during adolescence (23) and adulthood (13). Furthermore, acute stress has been shown to increase miR-124a expression and decreases GR protein expression in the corpus callosum of mice (26). Together these studies indicate a potential role for microRNAs in the regulation of GR mRNA expression *in vivo*.

The *Nr3c1* gene also contains multiple GREs, indicating potential for direct regulation by GR binding to these elements. One GRE, contained within exon 6 of the *Nr3c1* gene, has been associated with gene repression (27). In the absence of ligand this area of DNA (exon 6) containing the negative GRE site was directly associated with the proximal promoter region of the *Nr3c1* gene via a looping mechanism, confirmed by chromosome confirmation capture (3C) technology. The presence of ligand, however, activated GRs to recruit repressive cofactors such as nuclear receptor corepressor 1 (NCoR1) and histone deacetylase 3 (HDAC3) to this region of the proximal promoter to repress transcription of *Nr3c1* (27).

The work presented here shows a clear, DG-specific, down regulation of GR mRNA expression after FSS and provides evidence indicating the involvement of multiple mechanisms of GR regulation. Stress leads to increased methylation of a specific area of exon 1 within the *Nr3c1* gene, possibly due to the increased association of DNMT3a with this

region observed after FSS. Furthermore, we show a time-dependent increase of miR-124a, which correlated with reduced GR mRNA levels. Our results provide insight into the molecular mechanisms underpinning GR mRNA regulation, occurring specifically in the DG after stress. Suppression of GR mRNA levels after stress may be regarded as an adaptive mechanism to protect dentate neurons against the potentially damaging, GR-mediated effects of repeated stress.

Material and Methods

Animals

Male Wistar rats were purchased from Harlan (Oxon, UK) weighing 150-175 g upon arrival. Animals were housed two or three per cage under standard light (80-100 Lux, lights on 05:00-19:00) and environmentally controlled conditions (temperature $22 \pm 2^\circ\text{C}$; relative humidity $50 \pm 10\%$), food and water available *ad libitum*. After arrival, the animals were given 5 days to habituate to the housing conditions and then handled (2-3 min per rat per day) for a week before the experiment to reduce nonspecific stress. All animal procedures were approved by the University of Bristol Ethical Committee and the Home Office of the United Kingdom (UK Animal Scientific Procedures Act, 1986).

The Forced Swim Test

Experiments were carried out between 08:00-13:00. Rats were forced to swim in a glass beaker (height 35 cm, diameter 21.7 cm) containing water, ≈ 21 cm deep, at 25°C for 15 min. Rats were then killed immediately (FS15) or dried, returned to their home cage and placed in a recovery room with environmental conditions identical to the holding room until they were killed at 30 min (FS30), 60 min (FS60) or 180 min (FS180) after the start of forced swimming. To determine the effect of blocking GRs on GR mRNA levels some animals were injected with RU486 (GR antagonist, 100 mg/kg s.c.) or vehicle (20% DMSO/80% PEG 300) 30 min before forced swimming; rats were then killed 30 or 60 min after the start of forced swimming or 60 or 90 min after injection, respectively, for baseline controls. Animals were quickly anaesthetised with isoflurane (<15 s), decapitated and the brain removed. For micro-dissected samples the brain was cut into ~ 1 mm coronal sections which were immediately placed on ice cold steel boxes. Using a preparative light microscope (Leica, Milton Keynes, UK), from the dorsal hippocampus region, the DG and CA regions were micro-dissected into separate tubes and snap-frozen in liquid nitrogen. For whole hippocampal

dissections (ChIP studies) brains were dissected on ice cold steel boxes and whole hippocampi removed and snap-frozen in liquid nitrogen. All samples were stored at -80°C until analysis. Unless otherwise described all reagents used in sample preparation were from Sigma, Poole UK.

mRNA Analysis

RNA was extracted using TRI reagent (Sigma, Poole UK) following manufacturers guidelines. Briefly, tissue was homogenised in 1 ml of TRI reagent/100 mg tissue (\approx 50 mg dentate gyrus, \approx 100 mg CA regions, volumes adjusted accordingly), centrifuged (12,000 g, 10 min, 4°C) to remove insoluble material and left to stand at room temperature for 15 min. 100 μ l of 1-bromo-3-chloropropane was added and the sample shaken vigorously for 15 seconds. Samples were centrifuged (12,000 g, 15 min, 4°C) and the upper phase (containing RNA) retained. 2-propanol (500 μ l) was added, mixed and incubated for 10 min at room temperature. Precipitated RNA was pelleted by centrifugation (12,000 g, 15 min, 4°C) and washed twice with 1 ml 75% ethanol. The RNA pellet was air dried, resuspended in nuclease-free water (Life Technologies, Paisley, UK) and quantified using a NanoPhotometer P300 (Implen, München, Germany). RNA integrity was assessed in random samples with an Agilent 2100 Bioanalyser to confirm high quality RNA was extracted (RNA integrity numbers 8.5 ± 0.1 , $n=12$). Total RNA was reverse transcribed into cDNA using the QuantiTect reverse transcription kit (Qiagen, Manchester, UK) as per manufacturer's instructions (15 min, 42°C; 5 min, 95°C) using a BioRad T1000 thermal cycler. Absence of contaminating genomic DNA was ensured by including a step to remove DNA contamination by treatment with gDNA wipeout buffer (supplied with kit) immediately before the reverse transcription reaction. cDNA was diluted 4-fold and 2 μ l of diluted cDNA was used per reaction in quantitative polymerase chain reaction (qPCR) analysis detailed below. Expression of mRNA in samples was calculated based on the Pfaffl method of relative quantification (28) using primer/probes listed in Table S1 and standardised to the expression of housekeeping genes hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (*Ywhaz*). The data were expressed as fold change over baseline.

qPCR analysis

Mastermix for qPCR was prepared containing 900 nM forward and reverse primers, 200 nM probe, 1X TaqMan fast mastermix (Life Technologies, Paisley, UK) and made up to volume with nuclease-free water. Primers and dual-labelled probe with 6-FAM as the fluorescent dye and TAMRA as the quencher were designed using Primer Express

software (Version 3.0.1, Life Technologies, Paisley, UK). Standard curves were performed for each primer pair and the qPCR efficiency was calculated using the equation: $E = (10^{-1/\text{slope}} - 1) \times 100$ (where E is qPCR efficiency and the slope is the gradient of the standard curve). Only primer pairs with efficiencies greater than 90% were used. Quantitative PCR was performed using a StepOne Plus machine (Life Technologies, Paisley, UK). Taq enzymes were activated at 95°C for 20 s, then 40 cycles of 95°C (1 s) to 60°C (20 s) were performed to amplify samples.

Genomic DNA extraction and sodium bisulfite treatment of DNA

Tissue was lysed in 10 volumes of lysis buffer (0.5% SDS, 0.1 M EDTA, 10 mM Tris pH 8.0), incubated with RNase A (50 µg/ml) for 1 hour at 37°C and proteinase K (250 µg/ml) overnight at 50°C. Samples were incubated for one additional hour with proteinase K (125 µg/ml) before being heated to 65°C for 30 min to inactivate the enzyme.

Genomic DNA was purified using a standard phenol-chloroform method as follows - an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each sample, vortexed and transferred into a phase lock gel tube (5 PRIME, Hamburg, Germany). Samples were centrifuged for 15 min (13,000 rpm, room temperature) and the supernatant carefully transferred into a fresh phase lock gel tube. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the sample and the tube inverted 20 times before being centrifuged (13,000 rpm, room temperature). The chloroform:isoamyl alcohol step was repeated a second time and after centrifugation the supernatant was collected.

To precipitate DNA, one quarter volume of ammonium acetate (10 M) was added followed by 2 volumes of ice cold 100% ethanol and samples were stored overnight at -20°C. After centrifuging (13,000 rpm, 4°C, 30 min) the pellet was washed in 70% ethanol, centrifuged (13,000 rpm, 4°C, 5 min) and left to air dry. Finally, DNA was resuspended in nuclease-free water and dissolved overnight at 37°C before quantification using a NanoPhotometer (Implen, München, Germany). A 0.8% agarose gel (w/v, dissolved in TAE buffer, 0.0075% ethidium bromide) confirmed the presence of undegraded, contaminant-free genomic DNA.

Rat genomic DNA samples were diluted to a standard concentration and 500 ng subjected to sodium bisulfite treatment using the EZ 96-DNA methylation kit (Zymo Research CA, USA) in accordance with the manufacturer's kit instructions. Negative controls (water) and positive controls (artificially methylated and artificially unmethylated rat DNA, EpigenDx, MA, USA) were included in the sodium bisulfite treatment step. The presence of converted DNA was

confirmed with nanodrop analysis and a control bisulfite PCR. The UCSC genome browser (<http://genome.ucsc.edu/>) was used to derive nucleotide sequences for the genomic regions of interest associated with the *Nr3c1* gene, which were used for amplicon region selection and primer design (Table S2). Primers were optimised and samples amplified by PCR in duplicate, with positive and negative controls, using a MJ PTC-225 thermal cycler (MJ Research, City, country). Taq polymerase was activated at 95°C for 15 mins, then 45 cycles of 94°C (30 s) to 56°C (30 s) 72°C (30 s) were performed to amplify samples, followed by 10 min at 72°C. Successful amplification of the target region was confirmed using agarose gel electrophoresis, which allows visualisation of the PCR products.

Pyrosequencing was carried out using the PyromarkQ24 Pyrosequencer (Qiagen, City, USA) using the standard manufacturer's protocol. Pyromark Q24 software (Qiagen) was used to analyse the data generated by the pyrosequencer. Stringent quality control was implemented at this stage; samples displaying poor signal quality were removed. Following quality control, statistical data for analysis was obtained using this software to generate quantitative methylation percentage data at each CpG site for each sample.

Chromatin preparation and chromatin immuno-precipitation (ChIP) analysis

To assess whether DNA methyltransferases are associated with the region interrogated for DNA methylation after stress, we performed ChIP with antibodies against DNMT3a and DNMT3b. Hippocampus tissues were cross-linked in buffer containing 1% formaldehyde, 0.1 mM PMSF, 5mM Na⁺ Butyrate (NaBut), and PhosSTOP phosphatase inhibitor cocktail tablet (1 per 10 ml; Roche, Burgess Hill, UK) in 1X PBS at room temperature and left on a rotator for 10 min to facilitate the crosslinking process. Crosslinking was terminated by addition of glycine (final concentration 200 µM) and samples rotated for an additional 5 min at room temperature before centrifugation (5 min, 6000 g, 4°C). Pellets were washed 3 times in PBS with inhibitors [5 mM NaBut, 1mM Na₃VO₄, 0.1 mM PMSF, PhosSTOP phosphatase inhibitor cocktail tablet (1 per 10 ml; Roche)]. Pellets were re-suspended in ice-cold Lysis buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 5mM EDTA pH 8.0, 0.5% v/v Igepal, 0.5% Na deoxycholate, 1% SDS, 5mM NaBut, 2 mM AEBSF, 1mM Na₃VO₄, Complete ultra EDTA-free protease inhibitor tablets and PhosSTOP phosphatase inhibitor cocktail tablet (both 1 per 10 ml, Roche, Burgess Hill, UK) and lysis continued for 15 min at 4°C on a rotator. Each sample was aliquoted into 1.5 ml microcentrifuge tubes and sonicated at high power for 3 sets of 10 cycles (30 s ON, 60 s OFF) using a water-cooled (4°C) bioruptor (UCD-300, Diagenode, Liège, Belgium), vortexing samples between sets. Remaining tubes were kept on ice until all sonication was complete. All tubes were centrifuged (10 min, 20,000

g, 4°C) and supernatants (containing the sheared chromatin) recombined, and then re-aliquoted into fresh tubes for subsequent ChIP analysis, for assessment of input DNA (i.e. the starting material), and for checking of sonication efficiency on a 1% agarose gel (to confirm the chromatin was sonicated to approximately 3 nucleosome-lengths (approximately 450 bp). Before checking the chromatin on a gel, the crosslinks were reversed by addition of NaCl (Final concentration 200 mM) and overnight incubation at 65 °C.

For ChIP analysis, aliquots of chromatin were diluted 10-times in ice-cold dilution buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA pH 8.0, 1% v/v Triton, 0.1% Na deoxycholate 5mM NaBut, 1 mM AEBSF, Complete Ultra EDTA-free protease inhibitor tablets and PhosSTOP phosphatase inhibitor cocktail tablet (both 1 per 10 ml, Roche). 10 µl of antibody, either **GR (H-300 Sc-8992 X, Santa Cruz, Heidelberg, Germany)**, DNMT3a (ab2850, Abcam, Cambridge, UK) or DNMT3b (ab13604, Abcam) was added to each sample and tubes were rotated overnight at 4°C. Dynabeads® Protein A (150 µl/sample) were aliquoted into microcentrifuge tubes and washed once before blocking overnight in 0.5% BSA/PBS. The following day pre-blocked beads were washed once in dilution buffer (see above), resuspended in the antibody:chromatin mix, and allowed to incubate for 3 h at 4°C to allow binding of beads to antibody:chromatin complexes. After 3 h the unbound fraction was discarded and beads were washed 3-times with ice-cold RIPA buffer [10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 7.5, 0.1% SDS, 0.5mM EGTA, 1% Triton, 0.1% Na Deoxycholate, 140 mM NaCl, 1mM AEBSF, Complete ultra EDTA-free protease inhibitor tablets and PhosSTOP phosphatase inhibitor cocktail tablet (both 1 per 10 ml; Roche)] and washed twice with ice-cold Tris-EDTA buffer. Bound DNA was eluted in a two-stage process; first with 200 µl Elution buffer 1 (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 1.5% SDS) for 15 min with intermittent vortexing and second with 100 µl Elution buffer 2 (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.5% SDS), again for 15 min with intermittent vortexing. Crosslinks were reversed by addition of NaCl (final concentration 200 mM) and overnight incubation at 65°C. Samples were incubated first with RNase A (60 µg/ml, 37°C, 1 h), followed by proteinase K (250 µg/ml, 37°C, 3.5 h). DNA was purified using QIAquick PCR purification kit (Qiagen) as per manufacturer's instructions. Input samples were incubated overnight at 65°C with 200 mM NaCl to reverse crosslinks. The next day samples were sequentially incubated with RNase A (100 µg/ml, 37°C, 1 h) and proteinase K (250 µg/ml, 37°C, overnight). Finally DNA from input samples was purified as detailed above. Total double-stranded DNA content was determined with a high-sensitivity DNA assay kit (Life Technologies) as per manufacturer's instructions and quantified using a Qubit 2.0 fluorometer. All samples (Bounds and Inputs) were diluted to a standardised concentration with nuclease free water and analysed by qPCR as previously described

above using primers/probes listed in Table S3 and the inclusion of a standard curve created from serial dilutions of rat brain genomic DNA (Biochain, CA, USA). The obtained Ct values ranged between 30 and 36.5. Binding of GR or DNA methyltransferases to regions of interest is expressed as quantity of bound DNA divided by the respective quantity of input DNA to give a measure of enrichment. For the DNA methyltransferase ChIPs, this data was then expressed relative to baseline levels to allow direct comparison between the two antibodies, DNMT3a and DNMT3b.

MicroRNA Analysis

Expression of miR-18 is barely detectable in the adult rat hippocampus (13). In contrast, miR-124a is the predominant miRNA expressed in the brain (25). MicroRNA analysis was therefore targeted against miR-124a only. Total RNA (extracted as detailed above) was converted into cDNA using TaqMan[®] MicroRNA Reverse Transcription Kit including RNase inhibitor, dNTPs, and primers against mmu-miR-124a (Assay ID 001182) and snoRNA (Assay ID 01718) (Life Technologies) as per manufacturer's instructions (30 min, 16°C; 30 min, 42°C; 5 min, 85°C) using a BioRad T1000 thermal cycler. cDNA was diluted 10-fold and 1.6 µl of diluted cDNA was used per reaction in the qPCR. Mastermix for qPCR was prepared containing 1X TaqMan[®] MicroRNA assay (mmu-miR-124a Assay ID 001182 or snoRNA assay ID 01718), 1X TaqMan[®] Universal Master Mix II, No AmpErase[®] UNG (Life Technologies) and made up to volume with nuclease-free water. qPCR was performed using a StepOne Plus machine (Life Technologies). Taq enzymes was activated at 95°C for 10 min, then 40 cycles of 95°C (15 s) to 60°C (60 s) were performed to amplify samples. Expression of miRNA in samples was calculated based on the Pfaffl method of relative quantification (28) and standardised to the expression of control miRNA snoRNA. The data were expressed as fold change over baseline.

Statistical analysis

GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis. Graphs show group means ± SEM and sample sizes are indicated in the figure legends. Multiple comparisons were analysed by appropriate one-way or two-way ANOVA and if significant, an appropriate post hoc analysis was performed. ChIP data (Figure 5) was analysed by Student's two-tailed t-test. Individual statistical results are provided in the figure legends. A significance value of 95% ($p < 0.05$) was applied in all cases.

Results

Acute stress causes a significant down regulation of GR mRNA specifically in dentate gyrus neurons

To assess stress-induced changes in GR mRNA in hippocampal regions, male Wistar rats were killed either under baseline conditions or 15, 30, 60 or 180 min after the start of FSS (15 min, 25°C water). Forced swimming resulted in a significant down regulation of GR mRNA expression in the dentate gyrus (Figure 1A). The stress-induced decline in GR mRNA was significant 30 min after the start of forced swimming, with maximal reduction (~75% of baseline levels) occurring at FS60 and was maintained at this level at FS180. No significant effect of stress on GR mRNA expression was found in the CA regions at any of the time points studied (Figure 1B). This indicates that, within the (dorsal) hippocampus, the dentate gyrus is the primary target for stress-induced GR mRNA changes. There were no significant changes in MR mRNA any time point after forced swim stress in either the dentate gyrus or CA regions (Figure S1).

Negative GRE in exon 6 of *Nr3c1* gene does not play a role in forced swimming-induced GR mRNA down regulation

In a recent study *in vitro*, a negative GRE (nGRE) binding site was described in exon 6 of the *Nr3c1* gene, exerting an inhibitory influence on GR mRNA expression (27). Therefore, ChIP analysis was performed **on whole hippocampus** for GR binding to this nGRE after forced swim stress (Figure 2). **We found low enrichment for GR binding at this site under baseline conditions (Bound/Input (B/I) \approx 1.25) and despite significant increases in plasma corticosterone (Table S4), there was no increase in binding of GR to this region after forced swimming (Figure 2). There was an apparent decrease in GR binding to the negative GRE at FS180 (~20 % reduction) but this decrease from baseline did not reach statistical significance.** **The failure of GR to bind to this GRE was not due to a technical issue as we have previously observed substantial, stress-sensitive binding of this steroid receptor to well-known GC-inducible genes (e.g. *Per1*, *SGK1*) (2) (Mifsud & Reul, unpublished observations).**

Down regulation of GR mRNA after forced swimming is increased by the GR antagonist RU486

Previous studies have reported that GR mRNA depletion, induced by the administration of synthetic glucocorticoids, was attenuated by administration of the GR antagonist RU486 due to reduced nuclear localisation of RU486-bound receptor and/or the inability of RU486-bound receptor to repress GR gene transcription (29, 30). Therefore we investigated if the stress-induced reduction of GR mRNA in the dentate gyrus at FS30 or FS60 could be prevented by prior s.c. administration of RU486 (100 mg/kg) 30 min before the onset of forced swimming. Surprisingly, RU486 treatment resulted in a highly significant reduction of GR mRNA in the DG 30 min after stress (Figure 3A). There was no significant stress-induced reduction in GR mRNA in the vehicle-treated group at this time point in this experiment, most likely due to the restraint/injection stress dampening the magnitude of the response to forced swimming (31). The FS-induced GR mRNA reduction is apparent at FS60 (Figure 3B) at which point there is no additional effect of RU486 on GR mRNA in the dentate gyrus. This indicates that, instead of **blocking the FS-induced decrease in GR mRNA levels**, treatment with GR antagonist RU486 appears to potentiate the stress-effect on GR mRNA reduction.

Forced swimming is associated with site-specific hypermethylation in the *Nr3c1* gene promoter

DNA methylation status around the transcriptional start site is known to affect *de novo* gene transcription in a variety of genes including the GR gene (18, 32, 33). We therefore selected two areas for DNA methylation analysis to investigate if FSS evoked significant changes in the DNA methylation status of *Nr3c1*, which may explain the observed stress-evoked reduction in GR mRNA. The first area (referred to as GR area 1 throughout this paper, see Figure 4A) was upstream of the translational start site (ATG) located in exon 2 of the *Nr3c1* gene. In addition, early life stress has been widely shown to alter the methylation status of the *Nr3c1* gene at a specific exon in both rodents (Exon 1₇) and humans (Exon 1_F) (18, 20, 22, 34). We therefore selected a second region for DNA methylation analysis targeting Exon 1₇ (referred to as GR area 2 throughout this paper, see Figure 4A).

Forced swimming did not affect CpG methylation levels within GR area 1 in either the dentate gyrus (Figure 4B) or CA regions (Figure 4C). In contrast, GR area 2 underwent significant methylation changes in response to stress, especially in the dentate gyrus where FSS caused an overall increase in the percentage CpG methylation (Figure 4D), confirmed by two-way ANOVA analysis ($p < 0.0001$; see figure legends for statistical data). Post-hoc analyses identified CpG 4 and CpG 8 as having significantly increased DNA methylation after forced swimming, and a trend for

increased DNA methylation post-stress was observed at CpG 5. In the CA regions, FSS resulted in an overall decrease in DNA methylation of GR Area 2, however, the stress effect was CpG-dependent as revealed by the significant ANOVA interaction term (Figure 4E).

Forced swimming increases association of DNMT3a with stress-sensitive GR area 2 of *Nr3c1* gene

To determine if DNA methyltransferases could be functionally linked to this area of DNA methylation within GR area 2 we performed CHIP for DNMT3a and DNMT3b on whole hippocampal chromatin from baseline and FS60 rats. A significant increase in the association of DNMT3a with the area of forced swimming-induced CpG methylation in the *Nr3c1* gene (GR area 2) was observed 60 min after the start of forced swimming (Figure 5A). Levels of DNMT3b association with this area remained unchanged after forced swimming (Figure 5B).

Up regulation of miR-124a after forced swimming is associated with down regulation of GR mRNA

MicroRNAs are small non-coding RNA molecules involved in gene silencing and post-transcriptional regulation (35). Previously, miR-124a has been shown to be associated with GR down regulation in cultured cells (23). Furthermore, environmental enrichment of the Indian field mouse resulted in increased miR-124a expression which was associated with reduced GR mRNA and protein expression in the amygdala (24). We therefore investigated the effect of forced swimming on miR-124a expression in the dentate gyrus to elucidate a putative role of this microRNA in the observed stress-induced decline in GR mRNA in this hippocampal region. Forced swimming caused a time-dependent increase in miR-124a expression in the dentate gyrus (Figure 6A). Correlation analysis between miR-124a expression and GR mRNA showed a strong negative correlation specifically at FS30 (Figure 6B) – i.e. the time point of the onset of the stress-evoked increase in miR-124a levels and when GR levels have significantly decreased (see Figure 1A). This inverse correlation indicates that these responses may be causally linked at this time point. Other time points (FS15, FS60, FS180) did not show a significant correlation between miR124a expression and GR mRNA levels (data not shown); this may reflect additional mechanisms (e.g. DNA methylation) contributing to the stress-induced GR mRNA decrease at later time points.

Given that RU486 enhanced the forced swimming-induced reduction in GR mRNA (Figure 2A) we sought to determine if that was due to an ability of the GR antagonist to increase miR-124a expression. RU486, however, failed to induce a significant rise in miR-124a expression at FS30 (Figure 7A), the time point at which RU486 administration led to a significant decrease in GR mRNA levels (see Figure 2A). This potentiation by RU486 of the stress-response on GR mRNA levels is, therefore, unlikely to be solely mediated by RU486 increasing miR-124a levels. Interestingly, in rats killed 60 min after the start of forced swimming, RU486 significantly increased miR-124a levels in the dentate gyrus (Figure 7B). These observations indicate that an RU486-evoked enhancement in miR-124a expression may participate in the heightened GR mRNA down regulation after administration of the GR antagonist.

Discussion

Despite the importance of GR's role in controlling the stress response (36) and stress-related memory formation (3), little is understood about the regulatory mechanisms acting on *Nr3c1* gene transcription after acute stress. Our data show that acute stress, in the form of a single 15-min forced swim challenge, causes a strong down regulation of GR mRNA expression specifically within the dentate gyrus of the hippocampus. No effects of acute stress were found on MR mRNA levels. DNA methylation analysis of this hippocampal sub-region revealed a stress-induced increase in methylation in an area of the gene previously shown to be a target of stress-induced DNA methylation changes with long-term consequences for HPA axis and behavioural function (14, 34, 37). This observation corresponds with the increased binding of the *de novo* methyltransferase DNMT3a with the stress-sensitive region GR Area 2 in the *Nr3c1* gene. Furthermore, forced swimming significantly increased the expression of miR-124a, a microRNA previously shown to reduce GR expression *in vitro* (23). We also provide evidence that this increase is correlated with reduced GR mRNA expression in individual animals. Based on our work a picture is emerging indicating that acute stress impacts on GR mRNA expression through different epigenetic mechanisms acting in parallel, including DNA methylation changes and microRNA production.

We explored whether GRs activated by endogenously secreted glucocorticoids after the stressful challenge (4, 5) could be directly involved in the regulation of its mRNA levels. Recently, it has been reported that exon 6 of the *Nr3c1* gene contains a negative GRE which after GR binding facilitates the recruitment of a repressive transcriptional complex *in vitro* (27). We found, however, relatively poor enrichment in GR binding to this region, which did not

alter significantly after forced swimming; this is in contrast to the effect of DEX *in vitro* which induced a 5-fold rise in GR binding to this region (27). This is most likely due to differences in molecular and genomic processes controlling chromatin accessibility in mouse cell lines *in vitro* and in the intact rat *in vivo*.

One of the central findings of this work is evidence for sub-region specificity for the transcriptional response of *Nr3c1* to stress, occurring exclusively in the dentate gyrus at the time points observed. An earlier study by Paskitti et al (15) using *in situ* hybridisation failed to show any significant decrease in GR mRNA in the dentate gyrus at 15 min, 30 min, 60 min, or 180 min after restraint stress (30 min) but did find significant decreases in all three hippocampal areas studied (dentate gyrus, CA1 and CA3) at 2 h. The lack of a dentate gyrus-specific reduction in GR mRNA after stress at earlier time points may be due to the semi-quantitative nature of *in situ* hybridisation. Sub-regional differences in hippocampal RNA expression have been previously documented and include differences in the expression of GR mRNA between DG and CA3 (7). Furthermore, sub-regional differences in the translocation patterns of GR protein after a corticosterone stimulus have also been documented by the same group (9). Interestingly, GR levels are reduced in the dentate gyrus of depressed individuals compared with unaffected controls but this reduction is not significant in other hippocampal sub-regions (38). Thus, it appears that GR gene expression and GR-related cell biological processes are differentially regulated between the different hippocampal sub-regions.

Systemic administration of RU486, failed to inhibit the stress-induced reduction in GR mRNA, in fact the drug further enhanced the reduction in GR mRNA caused by stress. This could be in part due to a decrease in GR half-life as a result of binding of GRs by increased total amount of ligand (i.e. endogenous GC plus RU486), as opposed to binding GC only (39). Alternatively, partial agonist properties of RU486 presenting in the presence of certain activated signalling factors like Protein Kinase A (PKA) are well-documented (30). Given that PKA is implicated in forced swimming-induced synaptic plasticity, the protein kinase could be triggering mechanisms allowing RU486 to switch from an antagonist to an agonist and therefore be in part responsible for the enhanced down regulation of GR mRNA by RU486 (40, 41).

The decrease in GR mRNA after acute stress corresponds with the observed increase in DNA methylation of CpGs across GR area 2 in the dentate gyrus. In contrast, the CA regions, which do not show any significant forced swimming-induced reduction in GR mRNA at any of the time points studied, showed an overall decrease in DNA methylation in this area after forced swimming, which was clearly CpG-dependent. Apparently, this decline in

methylation levels did not translate into increased mRNA levels, possibly due to the lack of uniformity in the DNA methylation changes after stress or potentially due to additional regulatory mechanism occurring in this region *in vivo*. GR area 2 was selected as an area susceptible to stress-induced DNA methylation as shown in the pioneering studies by Weaver et al. (34). Weaver et al. (15) showed that, of the nine CpGs we examined for methylation status, five were stress-sensitive (CpGs 12, 13, 14, 15 and 16 in the Weaver et al., 2004 study). Of these five stress-sensitive CpGs we show significant increases in methylation occurring at two of these in the dentate gyrus after acute FSS (CpGs 4 and 8 corresponding with CpGs 12 and 16 in the Weaver et al. (2004) study) and a trend towards a significant increase in CpG 5 (corresponding to CpG 13 in Weaver et al., 2004). CpG 8 is especially interesting as it is the 5' CpG located within the Egr1-binding region, thought to be important in mediating the effects of early life programming and has been linked to changes in HPA axis function and increased risk of depression and suicide in adulthood (20, 42). The distinctly different effects of forced swimming on CpG methylation in GR Area 2 in the CA regions (c.f. DG) underline that sub-region-specific mechanisms are controlling GR mRNA expression in the hippocampus. For instance, the contrasting effects of the stressor on CpG 8 methylation in the dentate gyrus and the CA regions may impact on the likelihood of Egr1 binding to exon 1₇ of the *Nr3c1* gene with possible consequences for GR mRNA expression. This notion should be an avenue for future research.

Overall levels of methylation observed in our study of this area (ranging from 1-2%) are very similar to DNA methylation level of this region from postnatal day 90 in pups from high licking grooming/arched back nursing (LG/ABN) mothers, indicating that this area of the *Nr3c1* gene in our rats is largely unmethylated in the majority of cells. The relatively small increase in methylation, however, may reflect the fact that DNA methylation is a binary event for each DNA molecule so even a large effect of DNA methylation occurring in only in a small sub-population of cells would be reflected as a very low overall percentage methylation. Therefore, given that environmental stimuli are known to activate only a few percent of all dentate gyrus neurons (3, 43, 44), the small percentage changes, in absolute terms, after forced swimming may very well be of physiological relevance.

The mechanisms for stress-related changes in DNA methylation are poorly investigated, however, a screen of DNMT mRNA expression following FSS recently identified a transient rise in DNMT3a mRNA specifically in the dentate gyrus (31). A direct role of DNMT3a in the increased methylation of the *Nr3c1* gene was further implicated in our ChIP studies, showing a clear increase in the association of DNMT3a with the site of forced swimming-induced *Nr3c1*

methylation at FS60. The transient rise in DNMT3a mRNA may therefore be an adaptive response aimed to maintain DNMT3a protein stores.

Forced swimming evoked a significant time-dependent increase in miR-124a, a microRNA known to interact with the 3' UTR region of the *Nr3c1* gene to regulate GR expression (23, 24). Furthermore, we also found a strong negative correlation between miR-124a expression and GR mRNA levels in the dentate gyrus at FS30, the time point at which stress-induced miR-124a levels are beginning to rise and the decline in GR levels has just reached statistical significance. This observation supports the studies of stress-related miR-124a expression in the amygdala (24) and corpus callosum (26) confirming that up regulation of miR-124a is associated with the suppression of GR mRNA expression *in vivo*. Given the FS-induced correlation between miR-124a and the decrease in GR mRNA we investigated if a RU486-induced increase in miR-124a expression could explain the enhanced suppression of GR mRNA observed in the presence of the antagonist. RU486, when combined with FS, evoked a robust increase in miR-124a expression in the dentate gyrus. This response was only significant 60 min after forced swimming and as such does not account for the enhanced reduction in GR mRNA observed at FS30 in the presence of RU486. Thus, the suppressive effect of RU486 on dentate gyrus GR mRNA levels at 60 min after forced swimming may involve the enhanced expression of miR-124a at this time point; however, the expression of this microRNA is not altered at 30 min therefore it is not likely to be involved in the antagonist's effect on GR mRNA at this time point. This observation underlines that the mechanisms regulating GR mRNA levels in the dentate gyrus are not only multifarious but also their relative contributions change over time.

To summarise, acute stress causes a dentate gyrus-specific reduction in the expression of GR mRNA, but not MR mRNA. This reduction is mediated by multiple complex mechanisms, presumably orchestrated in order to fine-tune GR-mediated effects in the dentate gyrus to repeated stressful events. The increased methylation observed at discrete CpGs within exon 1, after stress, combined with the increase in DNMT3a expression and its increased association with these methylation sites after stress supports DNA methylation as a mechanism to confer subtle regulation of the *Nr3c1* gene *in vivo*. Furthermore, enhanced miR-124a expression presents an additional mechanism in balancing GR expression levels after stress. This apparent redundancy in GR mRNA control mechanisms underscores the physiological importance of fine-tuning the expression and possibly function of this glucocorticoid-binding receptor.

References

1. Reul JMHM. Making memories of stressful events: a journey along epigenetic, gene transcription, and signaling pathways. *Front Psychiatry*. 2014;5:5.
2. Reul JMHM, Collins A, Saliba RS, Mifsud KR, Carter SD, Gutierrez-Mecinas M, et al. Glucocorticoids, epigenetic control and stress resilience. *Neurobiology of Stress*. 2015;1:44-59.
3. Gutierrez-Mecinas M, Trollope AF, Collins A, Morfett H, Hesketh SA, Kersante F, et al. Long-lasting behavioral responses to stress involve a direct interaction of glucocorticoid receptors with ERK1/2-MSK1-Elk-1 signaling. *Proc Natl Acad Sci U S A*. 2011;108(33):13806-11.
4. Reul JMHM, de Kloet ER. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. *Endocrinology*. 1985;117(6):2505-11.
5. Reul JMHM, van den Bosch FR, de Kloet ER. Relative occupation of type-I and type-II corticosteroid receptors in rat brain following stress and dexamethasone treatment: functional implications. *J Endocrinol*. 1987;115(3):459-67.
6. Reul JMHM, de Kloet ER. Anatomical resolution of two types of corticosterone receptor sites in rat brain with in vitro autoradiography and computerized image analysis. *J Steroid Biochem*. 1986;24(1):269-72.
7. Datson NA, Meijer L, Steenbergen PJ, Morsink MC, van der Laan S, Meijer OC, et al. Expression profiling in laser-microdissected hippocampal subregions in rat brain reveals large subregion-specific differences in expression. *Eur J Neurosci*. 2004;20(10):2541-54.
8. Droste SK, Chandramohan Y, Hill LE, Linthorst AC, Reul JMHM. Voluntary exercise impacts on the rat hypothalamic-pituitary-adrenocortical axis mainly at the adrenal level. *Neuroendocrinology*. 2007;86(1):26-37.
9. Sarabdjitsingh RA, Meijer OC, Schaaf MJ, de Kloet ER. Subregion-specific differences in translocation patterns of mineralocorticoid and glucocorticoid receptors in rat hippocampus. *Brain Res*. 2009;1249:43-53.
10. Karandrea D, Kittas C, Kitraki E. Forced swimming differentially affects male and female brain corticosteroid receptors. *Neuroendocrinology*. 2002;75(4):217-26.
11. Veenema AH, Meijer OC, de Kloet ER, Koolhaas JM, Bohus BG. Differences in basal and stress-induced HPA regulation of wild house mice selected for high and low aggression. *Horm Behav*. 2003;43(1):197-204.
12. Aguilar-Valles A, Sanchez E, de Gortari P, Balderas I, Ramirez-Amaya V, Bermudez-Rattoni F, et al. Analysis of the stress response in rats trained in the water-maze: differential expression of corticotropin-releasing hormone, CRH-R1, glucocorticoid receptors and brain-derived neurotrophic factor in limbic regions. *Neuroendocrinology*. 2005;82(5-6):306-19.
13. Uchida S, Nishida A, Hara K, Kamemoto T, Suetsugi M, Fujimoto M, et al. Characterization of the vulnerability to repeated stress in Fischer 344 rats: possible involvement of microRNA-mediated down-regulation of the glucocorticoid receptor. *Eur J Neurosci*. 2008;27(9):2250-61.
14. Witzmann SR, Turner JD, Meriaux SB, Meijer OC, Muller CP. Epigenetic regulation of the glucocorticoid receptor promoter 1(7) in adult rats. *Epigenetics*. 2012;7(11):1290-301.
15. Paskitti ME, McCreary BJ, Herman JP. Stress regulation of adrenocorticosteroid receptor gene transcription and mRNA expression in rat hippocampus: time-course analysis. *Brain Res Mol Brain Res*. 2000;80(2):142-52.
16. Nishimura K, Makino S, Tanaka Y, Kaneda T, Hashimoto K. Altered expression of p53 mRNA in the brain and pituitary during repeated immobilization stress: negative correlation with glucocorticoid receptor mRNA levels. *J Neuroendocrinol*. 2004;16(1):84-91.
17. McCormick JA, Lyons V, Jacobson MD, Noble J, Diorio J, Nyirenda M, et al. 5'-heterogeneity of glucocorticoid receptor messenger RNA is tissue specific: differential regulation of variant transcripts by early-life events. *Mol Endocrinol*. 2000;14(4):506-17.
18. Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, et al. Epigenetic programming by maternal behavior. *Nat Neurosci*. 2004;7(8):847-54.
19. Jones MW, Errington ML, French PJ, Fine A, Bliss TV, Garel S, et al. A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nat Neurosci*. 2001;4(3):289-96.
20. McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonte B, Szyf M, et al. Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat Neurosci*. 2009;12(3):342-8.
21. van der Knaap LJ, Riese H, Hudziak JJ, Verbiest MM, Verhulst FC, Oldehinkel AJ, et al. Glucocorticoid receptor gene (NR3C1) methylation following stressful events between birth and adolescence. The TRAILS study. *Transl Psychiatry*. 2014;4:e381.
22. Turecki G, Meaney MJ. Effects of the Social Environment and Stress on Glucocorticoid Receptor Gene Methylation: A Systematic Review. *Biol Psychiatry*. 2014;79(2):87-96.

23. Vreugdenhil E, Verissimo CS, Mariman R, Kamphorst JT, Barbosa JS, Zweers T, et al. MicroRNA 18 and 124a down-regulate the glucocorticoid receptor: implications for glucocorticoid responsiveness in the brain. *Endocrinology*. 2009;150(5):2220-8.
24. Durairaj RV, Koilmani ER. Environmental enrichment modulates glucocorticoid receptor expression and reduces anxiety in Indian field male mouse *Mus booduga* through up-regulation of microRNA-124a. *Gen Comp Endocrinol*. 2014;199:26-32.
25. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. *Curr Biol*. 2002;12(9):735-9.
26. Shimizu S, Tanaka T, Tohyama M, Miyata S. Yokukansan normalizes glucocorticoid receptor protein expression in oligodendrocytes of the corpus callosum by regulating microRNA-124a expression after stress exposure. *Brain Res Bull*. 2015;114:49-55.
27. Ramamoorthy S, Cidlowski JA. Ligand-induced repression of the glucocorticoid receptor gene is mediated by an NCoR1 repression complex formed by long-range chromatin interactions with intragenic glucocorticoid response elements. *Mol Cell Biol*. 2013;33(9):1711-22.
28. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001;29(9):e45.
29. Hoeck W, Rusconi S, Groner B. Down-regulation and phosphorylation of glucocorticoid receptors in cultured cells. Investigations with a monospecific antiserum against a bacterially expressed receptor fragment. *J Biol Chem*. 1989;264(24):14396-402.
30. Cadepond F, Ulmann A, Baulieu EE. RU486 (mifepristone): mechanisms of action and clinical uses. *Annu Rev Med*. 1997;48:129-56.
31. Saunderson EA, Spiers H, Mifsud KR, Gutierrez-Mecinas M, Trollope AF, Shaikh A, et al. Stress-induced gene expression and behavior are controlled by DNA methylation and methyl donor availability in the dentate gyrus. *Proc Natl Acad Sci USA* 2016; In Press.
32. Irvine RA, Lin IG, Hsieh CL. DNA methylation has a local effect on transcription and histone acetylation. *Mol Cell Biol*. 2002;22(19):6689-96.
33. Weaver IC. Epigenetic programming by maternal behavior and pharmacological intervention. Nature versus nurture: let's call the whole thing off. *Epigenetics*. 2007;2(1):22-8.
34. Weaver IC, Diorio J, Seckl JR, Szyf M, Meaney MJ. Early environmental regulation of hippocampal glucocorticoid receptor gene expression: characterization of intracellular mediators and potential genomic target sites. *Ann N Y Acad Sci*. 2004;1024:182-212.
35. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281-97.
36. De Kloet ER, Reul JM. Feedback action and tonic influence of corticosteroids on brain function: a concept arising from the heterogeneity of brain receptor systems. *Psychoneuroendocrinology*. 1987;12(2):83-105.
37. Tran L, Chaloner A, Sawalha AH, Greenwood Van-Meerveld B. Importance of epigenetic mechanisms in visceral pain induced by chronic water avoidance stress. *Psychoneuroendocrinology*. 2013;38(6):898-906.
38. Webster MJ, Knable MB, O'Grady J, Orthmann J, Weickert CS. Regional specificity of brain glucocorticoid receptor mRNA alterations in subjects with schizophrenia and mood disorders. *Mol Psychiatry*. 2002;7(9):985-94, 24.
39. McIntyre WR, Samuels HH. Triamcinolone acetonide regulates glucocorticoid-receptor levels by decreasing the half-life of the activated nuclear-receptor form. *J Biol Chem*. 1985;260(1):418-27.
40. Nordeen SK, Bona BJ, Beck CA, Edwards DP, Borrer KC, DeFranco DB. The two faces of a steroid antagonist: when an antagonist isn't. *Steroids*. 1995;60(1):97-104.
41. Roberson ED, English JD, Adams JP, Selcher JC, Kondratieff C, Sweatt JD. The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. *J Neurosci*. 1999;19(11):4337-48.
42. Perroud N, Rutembesa E, Paoloni-Giacobino A, Mutabaruka J, Mutesa L, Stenz L, et al. The Tutsi genocide and transgenerational transmission of maternal stress: epigenetics and biology of the HPA axis. *World J Biol Psychiatry*. 2014;15(4):334-45.
43. Chandramohan Y, Droste SK, Reul JM. Novelty stress induces phospho-acetylation of histone H3 in rat dentate gyrus granule neurons through coincident signalling via the N-methyl-D-aspartate receptor and the glucocorticoid receptor: relevance for c-fos induction. *J Neurochem*. 2007;101(3):815-28.
44. Chandramohan Y, Droste SK, Arthur JS, Reul JM. The forced swimming-induced behavioural immobility response involves histone H3 phospho-acetylation and c-Fos induction in dentate gyrus granule neurons via activation of the N-methyl-D-aspartate/extracellular signal-regulated kinase/mitogen- and stress-activated kinase signalling pathway. *Eur J Neurosci*. 2008;27(10):2701-13.

Figure legends

Figure 1. Expression of GR mRNA in hippocampal sub-regions after forced swimming. Rats were killed direct from home cage (Baseline, BL) or 15 min, 30 min, 60 min or 180 mins after the start of forced swimming (15 min, 25°C-water). The graphs show GR mRNA levels normalised to the expression of housekeeping genes *Hprt1* and *Ywhaz* represented as mean fold change over baseline mRNA levels (\pm SEM, n=8-9 per group) in dentate gyrus (A) and CA regions (B). Statistical analysis: One-way ANOVA; (A) $F_{(4, 38)} = 11.32$, $p < 0.0001$, (B) $F_{(4, 38)} = 1.376$, $p = 0.261$. Dunnett's multiple comparison test: ** $p < 0.01$, *** $p < 0.001$ compared with baseline.

Figure 2. Association of GR with a putative negative GRE within the *Nr3c1* gene under baseline conditions and after forced swimming. Rats were killed direct from home cage (Baseline, BL) or 15 min, 30 min, 60 min and 180 mins after the start of forced swimming (15 min, 25°C). ChIP assays were conducted for GR binding to a putative negative GRE (nGRE) within exon 6 of the *Nr3c1* gene. Enrichment is calculated as the quantity of DNA bound to GR divided by respective quantity of input DNA (Bound/Input (B/I); mean \pm SEM, n=3-4), as determined by qPCR. Statistical analysis: One-way ANOVA; $F_{(4, 14)} = 1.908$, $p = 0.165$.

Figure 3. Effect of RU486 on expression of GR mRNA in the dentate gyrus at baseline and after forced swimming. Rats were injected with either RU486 (100 mg/kg, s.c.) or vehicle (20% DMSO/80% PEG300) and killed 30 or 60 min after the start of forced swimming (15 min, 25°C), or at matched time points after injection for baseline controls. The effect of RU486 on GR mRNA normalised to the expression of the housekeeping genes *Hprt1* and *Ywhaz* is presented as mean fold change over baseline mRNA levels (\pm SEM, n=6-9 per group) in the dentate gyrus at FS30 (A) and FS60 (B) Statistical analysis: Two-way ANOVA; (A) effect of RU486: $F_{(1, 26)} = 9.962$, $p = 0.004$; effect of stress $F_{(1, 26)} = 31.08$, $p < 0.0001$; interaction RU486 x stress $F_{(1, 26)} = 7.339$, $p = 0.0118$ (B) effect of RU486: $F_{(1, 26)} = 0.3582$, $p = 0.5547$; effect of stress $F_{(1, 26)} = 57.66$, $p < 0.0001$; interaction RU486 x stress $F_{(1, 26)} = 4.874$, $p = 0.0363$. Bonferroni post-hoc test ** $p < 0.01$, *** $p < 0.001$ compared with respective baseline group; $^{\$ \$ \$}$ $p < 0.0001$ compared with respective vehicle group.

Figure 4. Effect of forced swimming on DNA methylation levels within two areas (GR area 1 and GR area 2) upstream of the *Nr3c1* translational start site (ATG) in hippocampal sub-regions. Rats were killed straight from home cages (Baseline, BL) or 60 min after the start of forced swimming (FS60, 15 min, 25°C). The location of GR Area 1, GR Area 2 and translational start site (ATG) in the *Nr3c1* rat gene is shown in (A). Gene trace was adapted from the Rat Nov 2004 (Baylor 3.4/rn4) assembly (<http://genome.ucsc.edu/>). The graphs show DNA methylation changes at CpGs in GR area 1 and GR area 2 in the dentate gyrus (B and D, respectively) and CA regions (C and E, respectively). Data are shown as percentage methylation (mean \pm SEM, n=4-6). Statistical analysis: Two way ANOVA; (B) GR Area 1 – Dentate Gyrus; effect of CpG site: $F_{(2, 27)} = 106.2$, $p < 0.0001$; Effect of stress: $F_{(1, 27)} = 0.017$, $p = 0.8969$; interaction CpG site x stress: $F_{(2, 27)} = 1.286$, $p = 0.293$. (C) GR Area 1 – CA regions; effect of CpG site: $F_{(2, 27)} = 27.10$, $p < 0.0001$; Effect of stress: $F_{(1, 27)} = 3.682$, $p = 0.066$; interaction CpG site x stress: $F_{(2, 27)} = 0.049$, $p = 0.952$. (D) GR Area 2 – Dentate Gyrus; effect of CpG site: $F_{(8, 61)} = 16.02$, $p < 0.0001$; Effect of stress: $F_{(1, 61)} = 24.223$, $p < 0.0001$; interaction CpG site x stress: $F_{(8, 61)} = 0.341$, $p = 0.946$. (E) GR Area 2 – CA regions; effect of CpG site: $F_{(8, 71)} = 24.852$, $p < 0.0001$; effect of stress: $F_{(1, 71)} = 9.082$, $p = 0.004$; interaction CpG site x stress: $F_{(8, 71)} = 3.314$, $p = 0.003$. Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and trend $^{\dagger} < 0.1$ $p > 0.05$ compared with respective baseline group.

Figure 5. ChIP and qPCR analysis of DNMT3a and DNMT3b association with GR area 2, the site of FS-induced methylation within the *Nr3c1* gene under baseline conditions and after forced swimming. Rats were killed straight from home cages (Baseline, BL) or 60 min after the start of forced swimming (FS60, 15 min, 25°C). ChIP assays were conducted for DNMT3a (A) and DNMT3b (B) followed by qPCR of the genomic region of GR area 2. Data are expressed as the enrichment of the respective enzymes at GR area 2 after FS60 relative to mean enrichment at baseline levels (mean \pm SEM, n=3-4). Statistical analysis: Student's two-tailed t-test; ** $p=0.009$ (A), $p=0.502$ (B).

Figure 6. Changes in the expression of miR-124a in the dentate gyrus after forced swimming; correlation with changes in GR mRNA expression. Rats were killed direct from home cage (Baseline, BL) or immediately (15 min), 30 min, 60 min or 180 mins after forced swimming. (A) miR-124a expression levels normalised to the expression of snoRNA (control microRNA) represented as mean fold change over baseline levels (\pm SEM, n=8-9 per group) in dentate gyrus. (B) Expression levels of miR-124a were plotted against expression of GR mRNA at the time point FS30.

Statistical analysis: (A) One-way ANOVA; $F_{(4, 39)} = 3.295$, $p = 0.0216$. Bonferroni post-hoc test $^*p < 0.05$ compared with baseline. (B) Pearsons correlation analysis; $R^2 = 0.6459$, $p = 0.0091$.

Figure 7. Effect of RU486 on expression of miR-124a microRNA in the dentate gyrus at baseline and after forced swimming. Rats were injected with either RU486 (100 mg/kg, s.c.) or vehicle and killed 30 or 60 min after the start of forced swimming (15 min, 25°C-water), or at matched time points after injection for baseline controls. The effects of RU486 on miR-124a expression levels normalised to the expression of snoRNA (control microRNA) are presented as mean fold change over baseline levels (\pm SEM, $n=6-9$ per group) in dentate gyrus at FS30 (A) or FS60 (B). Statistical analysis: Two-way ANOVA; (A) effect of RU486: $F_{(1, 29)} = 0.172$, $p = 0.682$; effect of stress $F_{(1, 27)} = 0.672$, $p = 0.419$; interaction RU486 x stress $F_{(1, 29)} = 1.006$, $p = 0.324$ (B) effect of RU486: $F_{(1, 24)} = 6.676$, $p = 0.016$; effect of stress $F_{(1, 24)} = 0.941$, $p = 0.342$; interaction RU486 x stress $F_{(1, 24)} = 0.611$, $p = 0.442$; Bonferroni post-hoc test $^5p < 0.05$ compared with respective vehicle group.

Supplementary legends

Figure S1. Expression of MR mRNA in hippocampal sub-regions after forced swimming. Rats were killed direct from home cage (Baseline, BL) or 15 min, 30 min, 60 min or 180 mins after the start of forced swimming (15 min, 25°C-water). The graphs show MR mRNA levels normalised to the expression of housekeeping genes *Hprt1* and *Ywhaz* represented as mean fold change over baseline mRNA levels (\pm SEM, $n=8-9$ per group) in dentate gyrus (A) and CA regions (B). Statistical analysis: One-way ANOVA; (A) Dentate gyrus, $F_{(4, 38)} = 1.443$, $p = 0.2386$, (B) CA regions, $F_{(4, 39)} = 1.058$, $p = 0.3901$.

Supplementary Tables

Table S1. mRNA primer and probe sequences for qPCR

Identifier	Gene name	Accession number	Forward primer sequence	Reverse primer sequence	Probe sequence
GR mRNA (Figure 1)	<i>Nr3c1</i>	NM_012576.2	CGGCTTCTGGAAGAAAGCATT	CCAGTTGCAGACGTTGAACTCT	[6FAM]CTCAATAGGTCGACCAGCGTTCCAGAGA[TAM]
HPRT1 mRNA	<i>Hprt1</i>	NM_012583.2	CCTCCTCAGACCGCTTTTCC	CATAACCTGGTTCATCATCACTAATCA	[6FAM]CATGTCGACCCTCAGTCCCAGCG[TAM]
YWHAZ mRNA	<i>Ywhaz</i>	NM_013011.3	TGCTCGTGGTGATGACAAGAA	CATCTCCTTTTGCTGATTTCAAA	[6FAM]TGGACCAGTCACAGCAAGCATACCAAGAA[TAM]

Table S2. Primer sequences for bisulfite sequencing

Identifier	Gene name	Accession number	Forward primer sequence	Reverse primer sequence	Sequence primer
GR area 1 (Figure 4)	<i>Nr3c1</i>	NC_005117.2	GGAGGAATTTTGAAAGGTTTAGAAG	BIOTIN-CCCATCTACAAAACAACCTCATT	ATTTTGAAAGGTTTAGAAG T
GR area 2 (Figure 4)	<i>Nr3c1</i>	NC_005117.2	BIOTIN-ATTTGGTTTGGGAGGGAAA	ACCATCCTATAACCCCTCTACTA ATATAA	ACTAACTATCACCCCTC

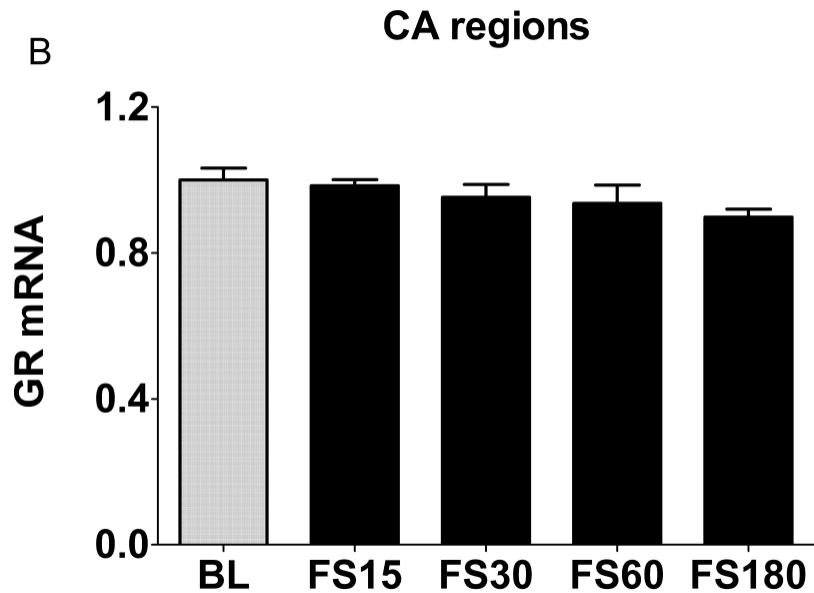
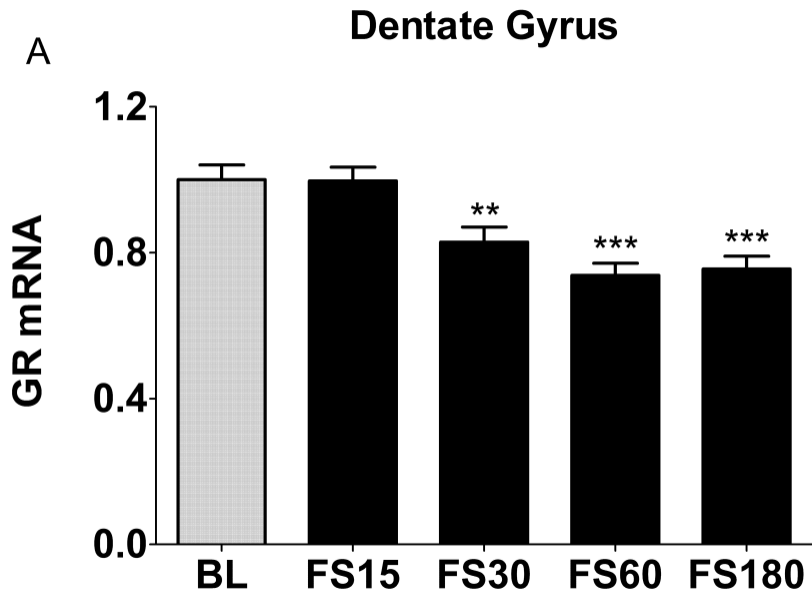
Table S3. Primer and probe sequences for ChIP DNA analysis

ChIP	Identifier	Gene name	Accession number	Forward primer sequence	Reverse primer sequence	Probe sequence
For GR ChIP (Figure 2)	GR nGRE (Ex6)	<i>Nr3c1</i>	NC_005117.4	TGACCAAATGACCCTGCTACAGT	CACTTGATTGTCTGTATGATCTCCAA	FAM-CTCATGGATGTTTCTCATGGCATTGTC-TAMRA
For DNMT ChIP (Figure 5)	GR area 2	<i>Nr3c1</i>	NC_005117.4	GGGCGGGAGGGAGCC	TCGCCTTTCCCAACC	FAM-CACCCGCTGCCGACTTTTCTC-TAMRA

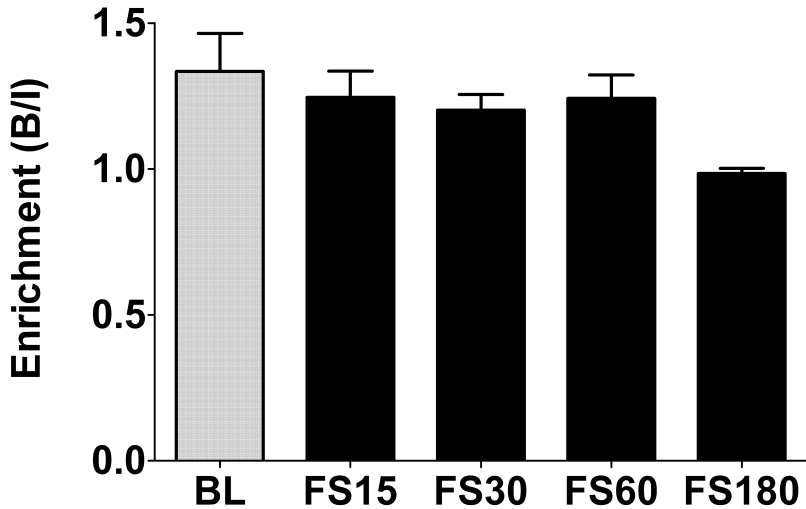
Table S4. Corticosterone concentration in plasma (ng/ml) under baseline conditions and following forced swim stress

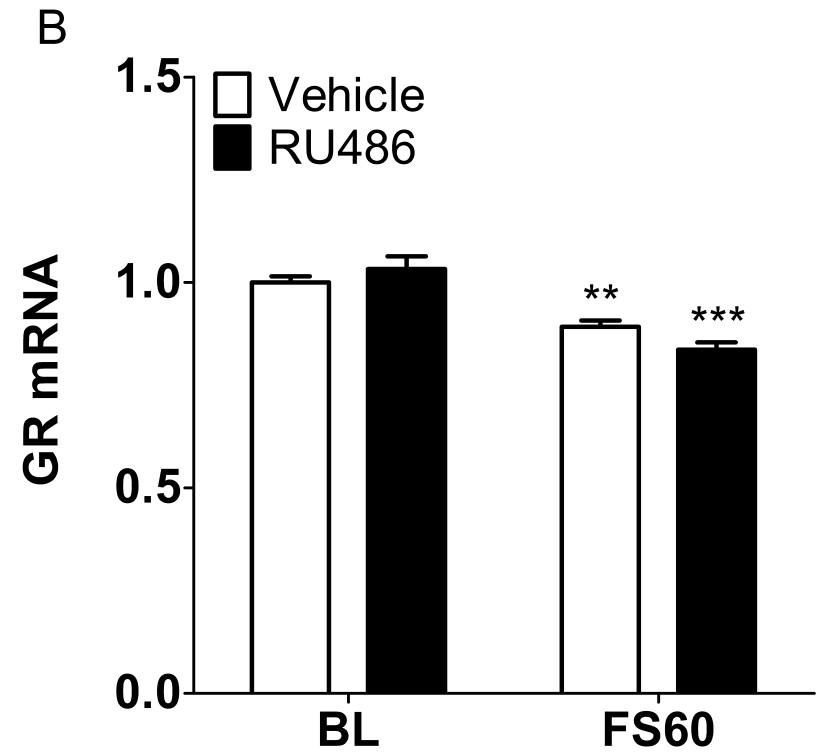
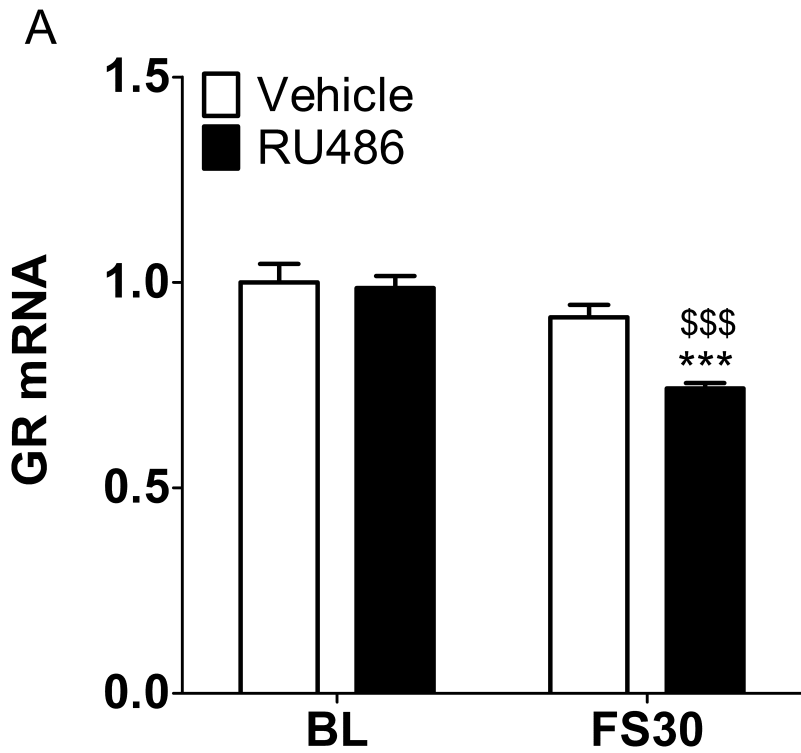
Plasma corticosterone (ng/ml)				
Baseline	FS15	FS30	FS60	FS180
7 ± 1, n=8	782 ± 29, n=8*	1158 ± 41, n=9*	225 ± 27, n=9*	23 ± 3, n=9

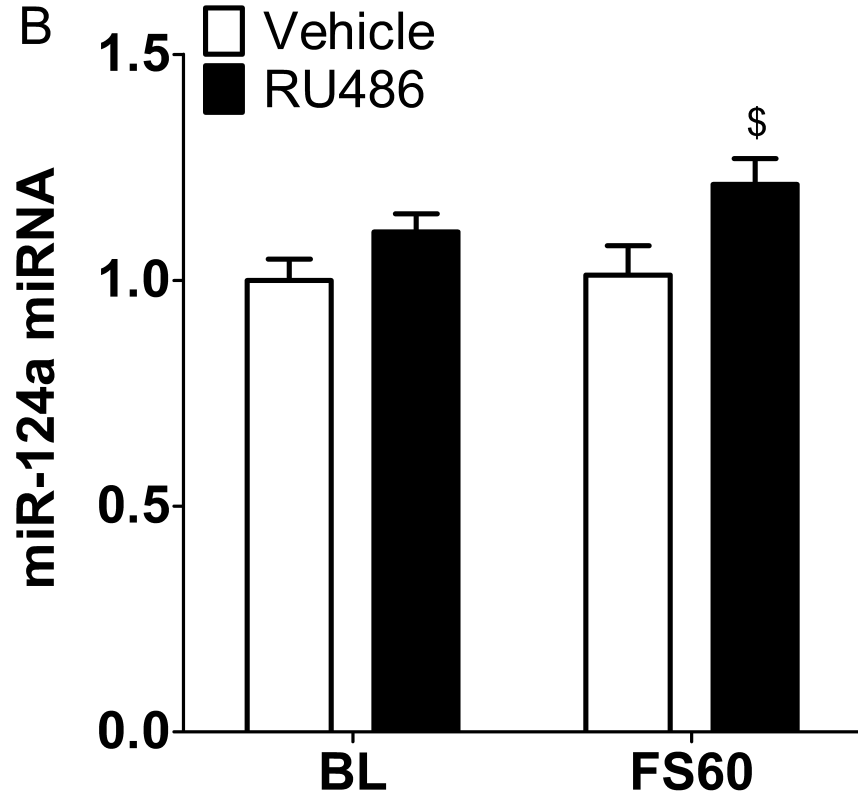
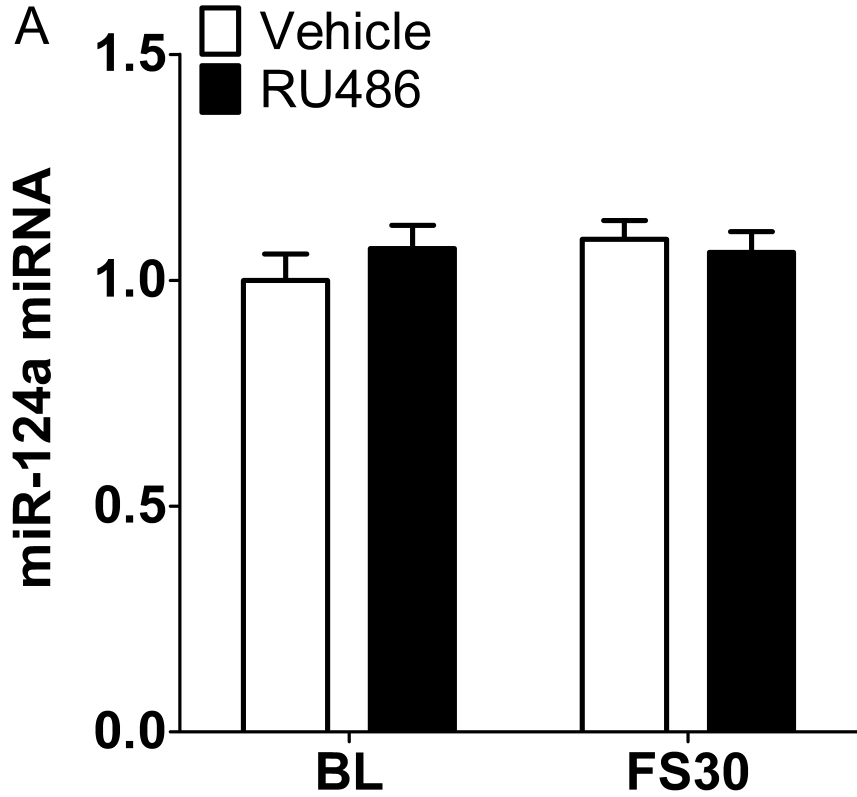
- *Significantly different compared with baseline, $p < 0.0001$, One way ANOVA and post-hoc Bonferroni test



GR CHIP

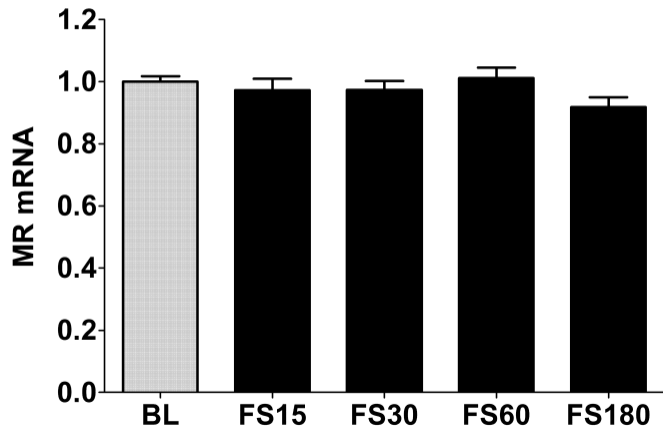






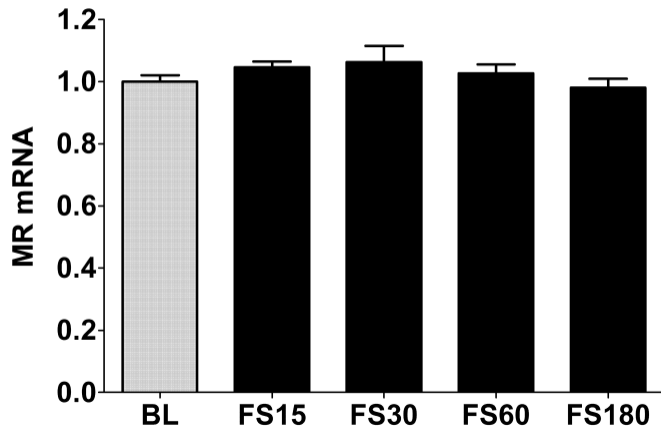
A

Dentate Gyrus

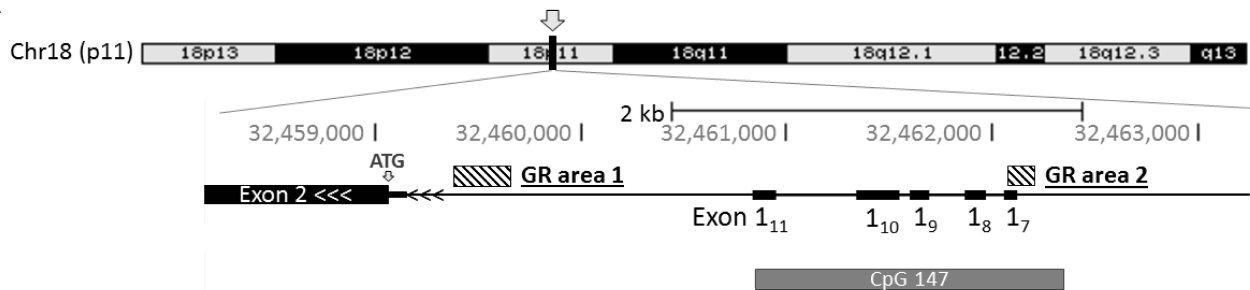


B

CA regions

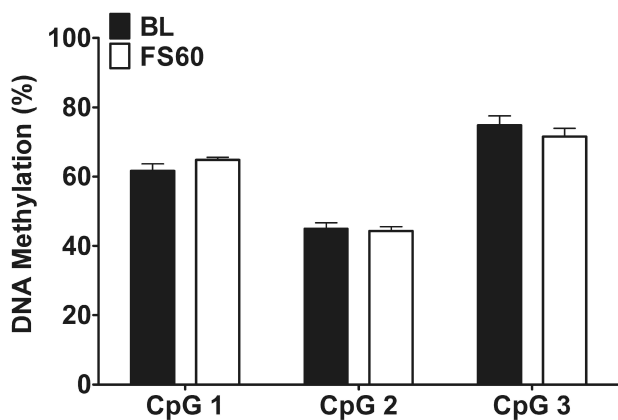


A



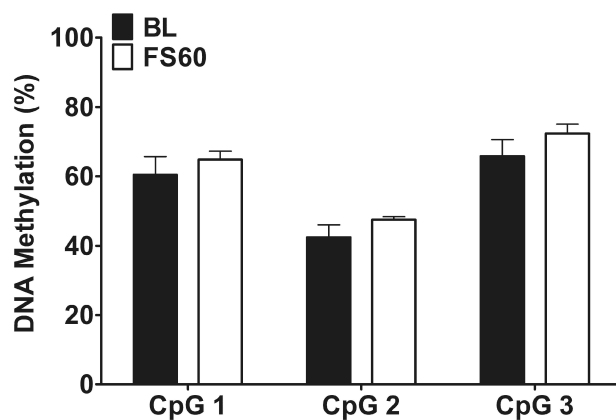
B

GR area 1 - Dentate Gyrus



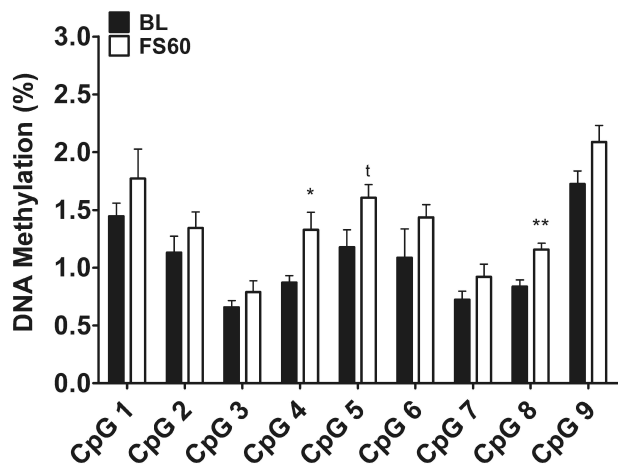
C

GR area 1 - CA regions



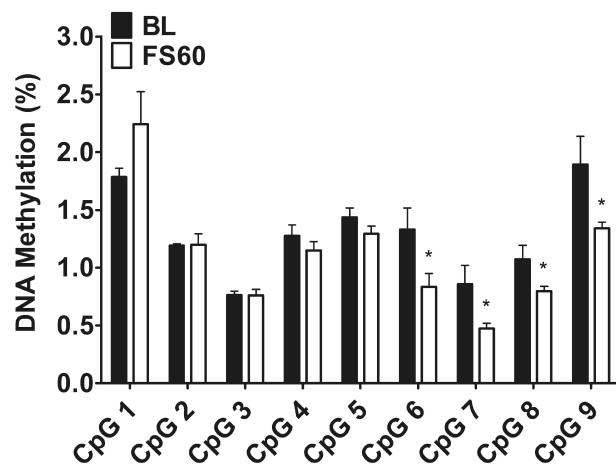
D

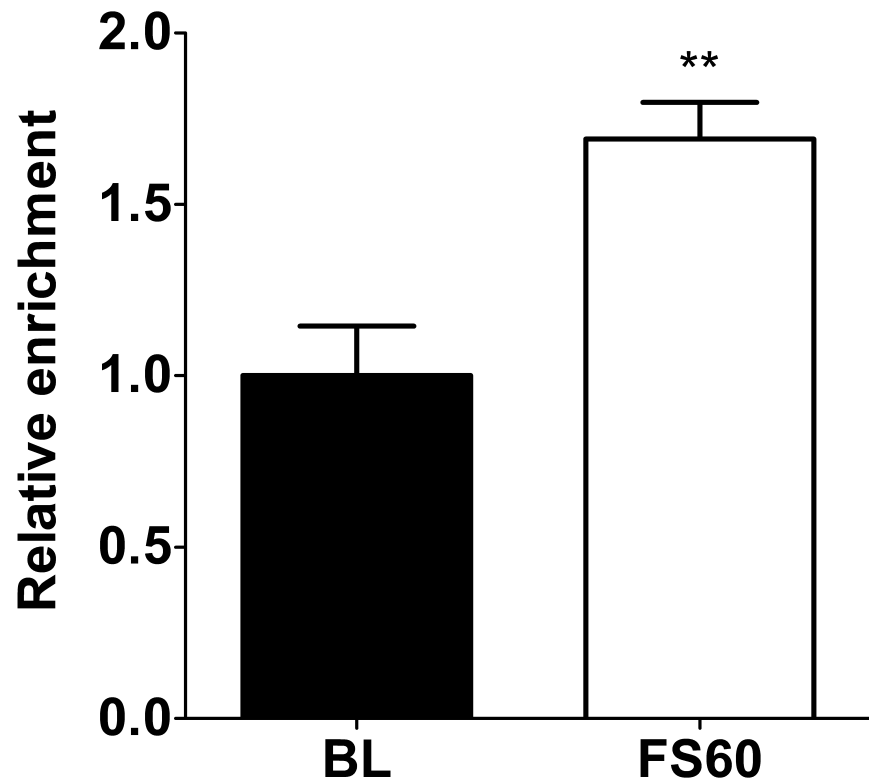
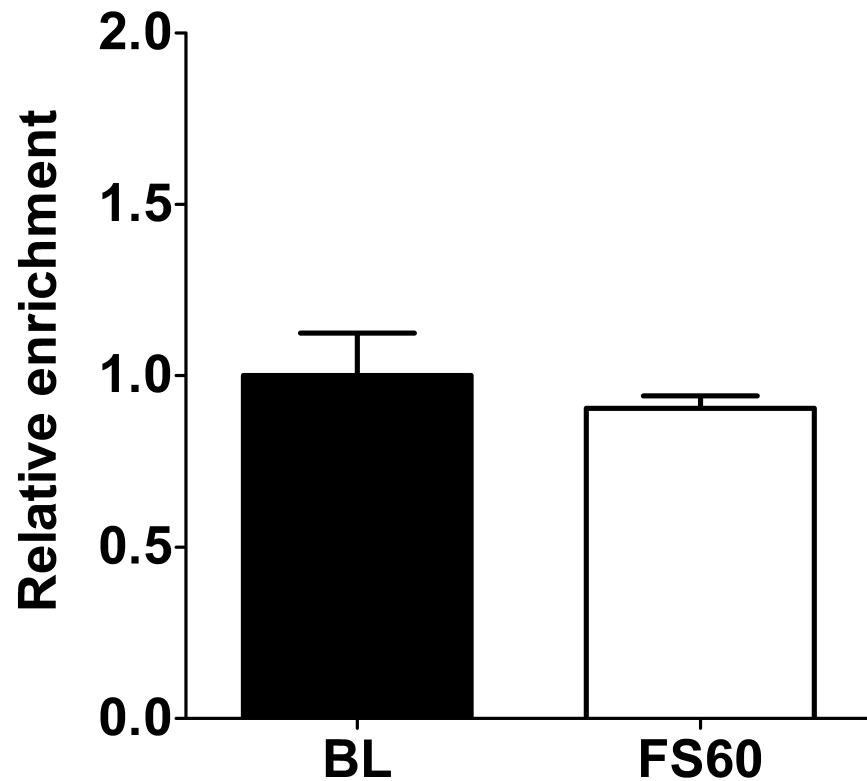
GR area 2 - Dentate Gyrus



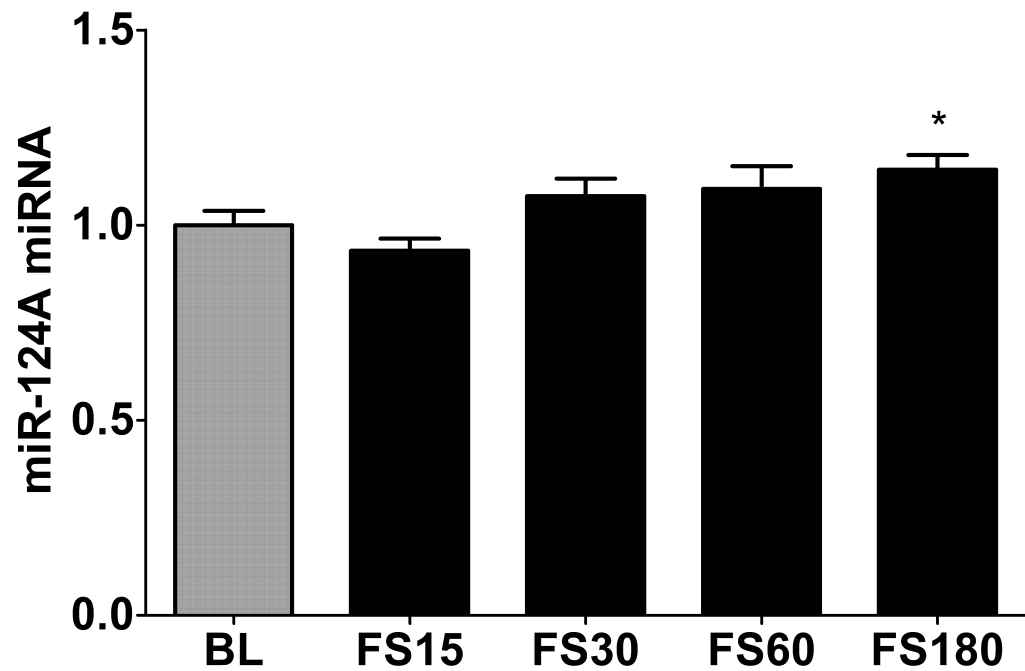
E

GR area 2 - CA regions



A**DNMT3a CHIP****B****DNMT3b CHIP**

A



B

