Glucocorticoid pulsatility : implications for brain functioning

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Chapter 4

Disrupted corticosterone pulsatile patterns attenuate responsiveness to glucocorticoid signalling in rat brain

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Abstract

Chronically elevated circulating glucocorticoid levels are thought to enhance vulnerability to psychopathology. Here we hypothesised that such sustained glucocorticoid levels, disturbing corticosterone pulsatility, attenuate glucocorticoid receptor signalling and target gene responsiveness to an acute challenge in the rat brain.

Rats were implanted with vehicle, 40% or 100% corticosterone pellets known to flatten ultradian and circadian rhythmicity while maintaining daily average levels or mimic pathological conditions. Additionally, recovery from constant exposure was studied in groups that had the pellet removed 24 hours prior to the challenge. Molecular markers for receptor responsiveness (receptor levels, nuclear translocation, promoter occupancy and target gene expression) to an acute challenge mimicking the stress response (3 mg/kg ip) were studied in the hippocampal area.

Implantation of 40% and 100% corticosterone pellets dose-dependently down-regulated GR and attenuated MR and GR translocation to the acute challenge. Interestingly, while target gene Gilz expression to the challenge was already attenuated by tonic daily average levels (40%), Sgk-1 was only affected after constant high corticosterone exposure (100%) indicating altered receptor responsiveness due to treatment. Washout of 100% cort recovered all molecular markers (partial) while removal of the 40% cort pellet still attenuated responsiveness to the challenge.

We propose that corticosteroid pulsatility is crucial in maintaining normal responsiveness to glucocorticoids. While the results with 100% corticosterone are likely attributed to receptor saturation, subtle changes in the pattern of exposure (40%) induces changes at least as severe for glucocorticoid signalling as overt hypercorticism suggesting an underlying mechanism sensitive to the pattern of hormone exposure.

Introduction

Rhythmic secretion of glucocorticoids is one of the major characteristics of the hypothalamic-pituitary-adrenal (HPA) axis, which plays a pivotal role in maintenance of homeostasis (de Kloet et al. 2005, Herbert et al. 2006). This rhythmicity is based on approximately hourly ultradian bursts of corticosterone into the blood stream showing circadian variation in amplitude (Tapp et al. 1984, Jasper & Engeland 1991, Windle et al. 1998a) and also exists at the level of target tissues such as the brain (Droste et al. 2008). Pulse amplitude and frequency can be remarkably variable in different physiological states, (i.e. lactation and ageing (Lightman et al. 2000)). However, dysregulation of ultradian rhythmicity is also apparent in stress-related disease (Deuschle et al. 1997, Hartmann et al. 1997), though its precise function still remains unknown (Young et al. 2004, de Kloet & Sarabdjitsingh 2008, Lightman et al. 2008).

The actions of glucocorticoids are mediated by the high affinity mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR); the latter receptor has a ten-fold lower affinity for corticosterone (Reul & de Kloet 1985). Both nuclear receptors are abundantly expressed in the brain, especially in limbic areas such as the hippocampus (Herman 1993, De Kloet et al. 1998). MR and GR act as ligand-activated transcription factors that translocate to the nucleus upon ligand binding. In the nucleus the receptors modulate target gene expression via interactions with specific glucocorticoid responsive elements (GREs) in the regulatory region of glucocorticoid target genes or via protein-protein interactions with other transcription factors (Mangelsdorf et al. 1995, Datson et al. 2008). Nuclear translocation of GR is known to vary according to circadian fluctuations in corticosterone levels (Kitchener et al. 2004). Moreover, repeated rapid nuclear translocation of GR following intravenous corticosterone injections that mimic ultradian pulses was demonstrated in rats (Stavreva et al. 2009) while MR was continuously retained in the nucleus (Conway-Campbell et al. 2007), implying that disturbances in glucocorticoid pulsatility would mainly affect GR.

It is now being recognised that access and binding of steroid receptors to regulatory elements in the genome (Metivier et al. 2003), including GR, is highly dynamic (George et al. 2009, Stavreva et al. 2009). The individual steps of the receptor signalling cascade (i.e. nuclear translocation, chromatin binding and modulation of target gene expression) together determine cellular and/or tissue sensitivity to glucocorticoids and efficacy (Kino 2007). Still, little information is available on how rapidly fluctuating glucocorticoid levels in vivo affect receptor signalling, transcriptional output and hence target tissue sensitivity thereby determining the functional effects of glucocorticoid action. Changes in pattern of glucocorticoid exposure could possibly contribute to dysregulated glucocorticoid receptor signalling. Understanding the interplay between rapidly fluctuating glucocorticoid levels and

receptor signalling therefore could greatly contribute to our knowledge of cellular and tissue responses.

In the present study, we aimed to investigate the link between glucocorticoid pulsatility and target tissue sensitivity in response to an acute increase in glucocorticoids such as occurs during acute stress. Evidence from other hormonal systems suggests that the function of rapid ligand bursts is to maintain tissue sensitivity and receptor responsiveness (Hauffa 2001). Therefore we hypothesised that constant levels of glucocorticoids, thus loss of pulsatility, will similarly affect glucocorticoid signalling by changing the responsiveness of the glucocorticoid receptor and its proximal targets.

Glucocorticoid pulsatility was abolished by subcutaneous corticosterone pellet implantation thereby exogenously inducing constant levels of glucocorticoids (Akana et al. 1985, Akana et al. 1992, Meijer et al. 1997). Consequently, we studied molecular markers for glucocorticoid signalling in the rat hippocampal area in response to an acute corticosterone challenge, superimposed on the chronically changed pattern of exposure. We also investigated whether these changes can be normalised after reinstatement of glucocorticoid pulsatility. Here we find that the transient response in glucocorticoid signalling is attenuated during exposure to constant corticosterone levels, but that this apparent resistance is reversible.

Experimental procedures

Animals

Male Sprague-Dawley rats (Harlan, Leiden, The Netherlands) weighing approximately 250 grams on the day of surgery, were group housed on a 12 h/12 h light/dark cycle (lights on at 07:00 AM) in a temperature controlled facility. Animals were handled daily for a week before the start of the experiment. Food and water were provided ad libitum. All experimental manipulations were done in the morning. Experiments were approved by the Local Committee for Animal Health, Ethics and Research of the University of Leiden (DEC nr. 07166). Animal care was conducted in accordance with the EC Council Directive of November 1986 (86/609/EEC).

Experimental design

Hormone levels were clamped at different constant levels using 40% and 100% corticosterone (cort) pellets (40 or 100 mg corticosterone; MP Biomedicals, Ohio, USA), and compared to 100 mg vehicle (cholesterol, Sigma-Aldrich, St. Louis, USA). Using isoflurane anaesthesia, pellets were subcutaneously implanted in intact animals randomly assigned to either

of the five treatment groups (n = 24): vehicle, 40%, 100%, 40% WO (washout) and 100% WO cort. Animals were weighed daily during the seven days after pellet implantation. On experimental day 7, blood samples were collected via the tail vein in the morning (08.00) and afternoon (17.00) to verify flattening of circadian cort levels in plasma without anaesthesia (Fluttert et al. 2000). Pilot studies indicated initial enhanced release of corticosterone from the pellet on the first two days after implantation and steady-state levels thereafter (Meijer et al. 1997). As described in the results section, corticosterone levels were indeed constant the day before the corticosterone challenge. In addition, high frequency blood sampling was used to verify flattened corticosterone rhythms with higher resolution [10 min intervals; (RA Sarabdjitsingh 2009)].

Early in the morning on post-surgery day 8 (08.00-09.00), animals from the WO groups were briefly anaesthetised during which the pellet was rapidly removed (washout) while the animals from the other groups were sham-operated. To ascertain normalisation in responsiveness of most of the available GR molecules, the washout period was decided to be 24 hours, according the half life time of GR redistribution to the cytoplasm following hormone withdrawal [t1/2 = 8-9 hours (Hache et al. 1999)]. After 24 hours, animals from all five groups were challenged with a high dose of corticosterone (3 mg/kg ip corticosterone-HBC; Sigma-Aldrich, USA). Tail blood samples were taken before and during the challenge to monitor corticosterone levels in blood. Animals were decapitated (n = 8 per time point per treatment group) 0, 60 and 180 min after injection. Brain tissue was collected, snap-frozen in isopentane on dry ice and stored at -80 ºC until further processing. Of each animal, one hemisphere of the brain was used for immunohistochemistry and in situ hybridisation while the hippocampus of the other hemisphere was isolated for Western blot analysis or chromatin immunoprecipitation (ChIP). Additionally, the thymus and adrenal glands were dissected, cleaned and weighed.

Corticosterone measurements

Blood samples were centrifuged for 15 min at 4000 rpm at 4 ˚C. Plasma was stored at -80 ˚C until processed using a commercially available radio immuno assay (RIA; MP Biomedicals Inc., CA., USA) according to the manufacturer's instructions.

Protein sample preparation

Hippocampal total protein samples (n = 12) were obtained by homogenising in ice-cold lysis buffer containing 1% NP-40, 1% sodium deoxycholate, 0,1% SDS, 0.15 M NaCl, 50mM Tris, pH 7.5 and protease inhibitors (Complete Mini cocktail tablets, Roche, The Netherlands). Protein concentrations were determined by a BCA protein assay (Pierce, Rockford, IL, USA).

Western blotting and analysis

Western blot was performed as described before (Conway-Campbell et al. 2007). Twenty μg protein samples were loaded on 5-8% polyacrylamide gels and transferred to PVDF membranes (Amersham Biosciences, Uppsala, Sweden). Membranes were probed with either primary anti-GR antibody (M20, Santa Cruz Biotechnology) or anti-MR antibody [MR1-18 1D5, kindly supplied by Dr. Gomez-Sanchez (Gomez-Sanchez et al. 2006)] and subsequently with anti-rabbit IgG-HRP (Santa Cruz Biotechnology) or anti-mouse IgG-HRP (Amersham Biosciences). Signal was detected using enhanced chemiluminescence reagent (ECLplus, Amersham Biosciences) and enhanced chemoluminescence hyperfilm (Amersham Biosciences). Membranes were also probed for α-tubulin as a loading control (Sigma Aldrich, USA).

Western blot bands were quantified by densitometry using an Epson perfection scanner (Epson Europe, NL) and ImageJ analysis software (NIH, USA; http://rsb.info.nih.gov/ij/). Optical density of GR and MR bands were corrected for film background and normalised for α-tubulin. Samples were compared within an individual blot, calculating fold induction from the control group loaded on each blot (vehicle t = 0). Fold inductions from all data sets were pooled to obtain group means ± SEM.

Immunohistochemistry

To visualise the subcellular distribution pattern of MR and GR immunoreactivity (IR) in hippocampal CA1, immunohistochemistry was performed on 20 μm slices as described previously (Sarabdjitsingh et al. 2009). Sections were incubated with primary GR (H300 Santa Cruz Biotechnology, USA) and MR antibodies (MR1-18 1D5). After washing, sections were incubated with secondary antibodies AlexaFluor- A488 and AlexaFluor-A594 IgG (Molecular Probes, USA). Nuclei were visualised with Hoechst 33258 (Molecular Probes, USA) and slides were mounted with Aqua Polymount (Polysciences, Inc). Controls included incubation of slides with equal amounts of normal rabbit and mouse IgG (Santa Cruz Biotechnology, USA), swapping of secondary fluorescent labels and incubation without any primary antibodies.

Confocal microscopy and image analysis

Nuclear translocation patterns were measured as previously described (van der Laan et al. 2005, Sarabdjitsingh et al. 2009). Briefly, the Hoechst stain was used as a template to identify the nucleus in sections. Fluorescence intensity values of nuclear IR were measured using ImageJ 1.32j analysis software (NIH, USA) on images (630x magnification, 155 x 155 μm) acquired with a Nikon confocal microscope (Nikon TE 2000-e). Per animal (n = 8 per time point), two frames with 30-40 cells on average were acquired. Non-specific binding (normal mouse and rabbit IgG) and background staining of the sections were also measured and

subtracted from the total signal to obtain the specific signal. Cells that had a clear ovally-shaped nucleus with a diameter of approximately 5-7 μm and showed IR clearly above background were included for analysis thereby excluding cells that were not in the plane of focus.

In situ hybridisation

In situ hybridisation was used to study expression patterns of glucocorticoid target genes Sgk-1 and Gilz in brain. In situ hybridisation was performed on 20 μm slices as previously published (Meijer et al. 2000). 33Phosporus end-labelled oligonucleotide probes (2 x 106 dpm) for Gilz (van der Laan et al. 2008b) and Sgk-1 (van Gemert et al. 2006) were used. The signal was quantified from films exposed 6-8 days (X-OMAT AR, Kodak, Rochester, NY) using ImageJ 1.32j analysis software. Relative expression levels were determined and related to standard curves of 14C (RPA 504 microscales, Amersham, UK), included to ensure that grey values were within the linear range between 0 and 255. Three hippocampal sections per animal (n =8 per time point) were analysed.

Chromatin immunoprecipitation and RT-qPCR

Chromatin immunoprecipitation to study Sgk-1 promoter occupancy by GR was adapted and modified from Van der Laan et al. (van der Laan et al. 2008b). Fixed chromatin derived from the hippocampi of three animals was pooled and sheared, yielding fragments of 100-500 bp (20 pulses of 30 seconds; Bioruptor, Diagenode). Immunoprecipitation was performed with either 6 μg of GR-specific H300 or normal rabbit IgG (Santa Cruz Biotechnology) overnight at 4 °C. After DNA recovery (Nucleospin, Macherey-Nagel), RT-qPCR was performed to study enrichment of Sgk-1 promoter fragments in the different treatment groups (LightCycler FastStart DNA Master PLUS SYBR Green I, Roche), according to manufacturer's instructions. Primers were designed around the glucocorticoid response element (GRE) of the rat Sgk-1 promoter (GGCTCAAAATTTATGCGGAA (Sgk-1 forward); CGGAAATAAGTCTCTGCGCT (Sgk-1 reverse). Myoglobulin was used a negative control for GR chromatin occupancy: CCTCACATGGGCAGCTATTT (myoglobin forward); GCTTGTGCAAGTCCAGACAG (myoglobulin reverse). PCR-products were analysed on an agarose-gel to check the length of the amplicon. Immunoprecipitation with a non-specific antibody (normal IgG) did not result in increased DNA recovery and was used to correct the GR immunoprecipitated samples (Wang et al. 2004, van der Laan et al. 2008b) .

Statistical analysis

Data are presented as mean ± SEM. Differences between groups were examined by one-way or two-way ANOVA. Tukey's post-hoc testing was applied to compare individual groups where applicable. Statistical significance was accepted at a p-value < 0.05.

Results

Controls for subcutaneous corticosterone pellet treatment

To validate that subcutaneous corticosterone pellet implantation effectively clamped circadian fluctuations at expected concentrations, morning and evening corticosterone samples were taken seven days after pellet implantation (n = 24). Vehicle animals showed typical circadian variation in corticosterone levels (21.4 ± 2.7 and 171.7 ± 20.1 ng/ml; morning and evening levels, respectively). No circadian variation in morning and evening corticosterone levels were found in the 40% (85.4 ± 6.6 and 90.0 ± 16.4 ng/ml) or 100% cort groups (254.8 ± 15.0 and 233.5 ± 14.5 ng/ml) indicating constant exposure around either daily average levels or at levels seen during stress-related disorders, respectively. Pellet removal in the morning of post-surgery day 8 resulted in rapid decrease in corticosterone levels (morning corticosterone levels the day after pellet removal: 12.5 ± 3.9 and 33.3 ± 6.9 ng/ml, 40% and 100% cort WO respectively). These values did not differ from vehicle animals (22.5 ± 3.0 ng/ml).

Corticosterone pellet implantation led to a dose-dependent decrease in body and thymus weight (Fig. 1A-B; $F(4, 115) = 158.9$; $p < 0.05$ and $F(4, 115) = 208.4$; $p < 0.001$) and decreased adrenal weight (Fig. 1C; $F(4, 114) = 21.1$; $p < 0.01$), indicating that corticosterone pellet implantation was effective. Pellet removal and 24 hours of washout of exogenous corticosterone did not result in recovery of any of the parameters measured (Fig. 1; dashed bars).

Cort pellet implantation down-regulates MR and GR protein in hippocampus

Western blot analysis for MR and GR was performed on hippocampal homogenate (Fig. 2). MR protein showed a trend towards reduction in the 100% cort group (Fig. 2A; $p = 0.07$). Pellet removal reversed any trend towards decreased MR protein levels in the 100% WO cort group. MR protein levels were not affected by 40% cort pellet implantation or washout.

Cort pellet implantation reduced GR protein levels in the 40% and significantly in the 100% cort pellets groups to 0.76 ± 0.07 and 0.65 ± 0.08, respectively compared to vehicle ($F(2, 31) = 5.1$; $p < 0.01$; Fig. 2B). GR protein levels in the 40% WO cort group were still reduced after washout (0.74 ± 0.1), but in the 100% WO cort group a recovery was noted as values were not different from vehicle treated animals (0.88 ± 0.13; $F(2, 27) = 1.2$; $p = 0.33$).

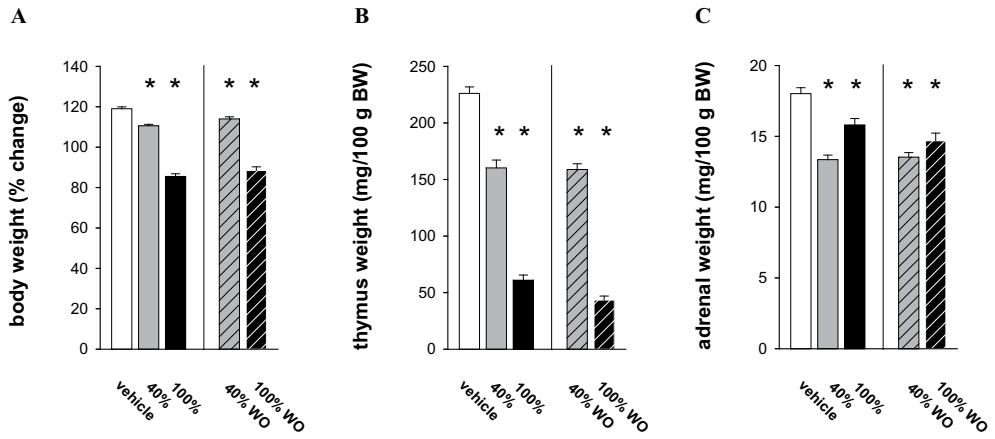

Figure 1 | Corticosterone pellet implantation affects glucocorticoid sensitive parameters. Animals implanted for 7 days with vehicle, 40% or 100% cort pellets (n=24). The dashed bars depict animals in which the cort pellet was removed (40% WO and 100% WO) and show dose-dependent decreases in A | relative body weight gain, B | thymus and C | adrenal weight as indicated with one-way ANOVA (* $p < 0.05$ compared to vehicle; Tukey's post-hoc test).

Next to the effect of cort pellet implantation, we studied whether there was an acute effect of the corticosterone injection (cort challenge) on MR and GR protein levels. Western blot analysis of hippocampal homogenate of rats from the time groups 0, 60 and 180 min after the cort challenge of all treatment groups, showed no effect of time on MR ($p = 0.35$) or GR protein levels ($p = 0.49$; two-way ANOVA; data not shown) indicating that acute changes in gene expression were not complicated by concomitant changes in receptor expression.

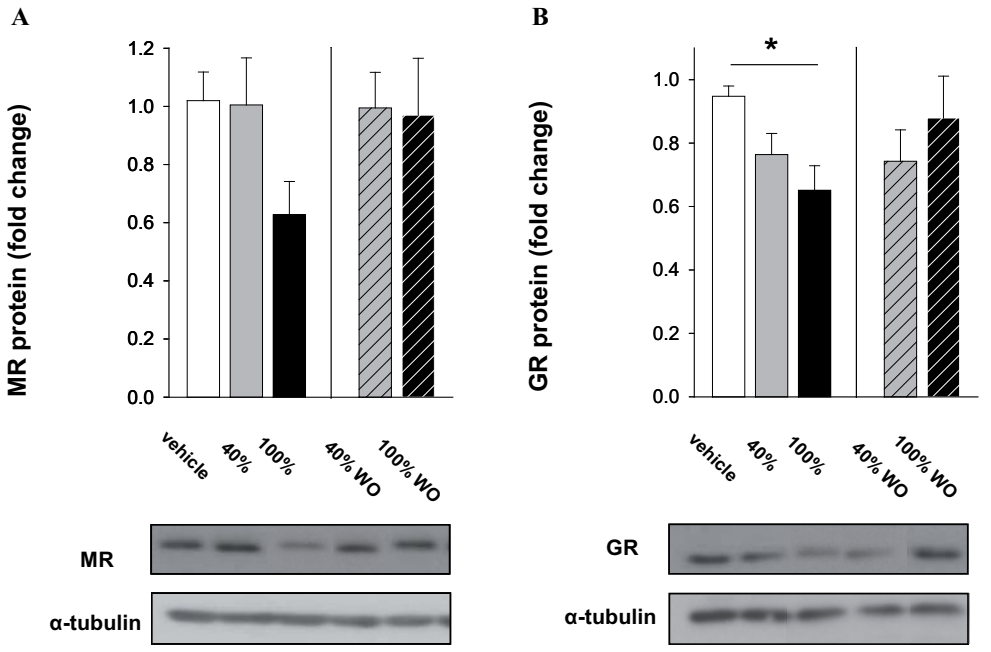

Figure 2 | Corticosterone pellet implantation decreases MR and GR protein levels. Western blot analysis of receptor protein levels in hippocampal homogenate (n = 12) after 7 days of vehicle, 40%, and 100% cort pellet implantation shows no significant effect on A | MR protein levels. B | A dose-dependent decrease was observed for GR protein levels in the 100% cort group. Interestingly, after washout (dashed bars), GR levels recovered in the 100% WO group, while the 40% cort group was still affected. Receptor protein levels were normalized against α-tubulin. * $p < 0.01$ compared to vehicle; one-way ANOVA and Tukey's post-hoc test.

Corticosterone pellet implantation attenuates GR translocation in CA1 cells

To study a different aspect of the receptor protein, we visualised corticosterone-induced MR and GR translocation patterns to the nucleus, rather than total protein hippocampal levels (Fig. 2), using immunohistochemistry and confocal imaging. MR immunoreactivity (IR) in CA1 pyramidal cells of the rat hippocampus was mostly localised in the nucleus before the cort challenge in all conditions (Fig. 3A and C, left panel). No difference in baseline levels of the different treatment groups was detected ($F(2, 19) = 1.5$; $p = 0.24$). Nuclear MR IR showed a very modest but significant increase after the challenge only in vehicle pellet rats at 60 minutes (Fig. 3A; $F(2, 16) = 3.6$; $p = 0.049$); no MR translocation was observed in the other treatment groups. Washout of corticosterone levels did not result in recovery of MR nuclear translocation in the 40% and 100% WO groups.

In contrast, low levels of nuclear GR IR were observed before the challenge in the vehicle group (Fig. 3B and C, right panel). After the challenge, a strong transient increase in nuclear

GR IR was observed ($F(2, 18) = 8.8$; $p < 0.01$) which returned back to baseline levels after 180 min, clearly indicating nuclear translocation of GR in CA1 cells. Implantation of the 40% cort pellet did not affect basal levels of nuclear GR IR, but completely abolished the increase in nuclear GR signal at 60 minutes after the acute injection (Fig. 3B). The 100% cort pellet increased 'basal' levels of nuclear GR significantly ($F(2, 18) = 8.7$; $p < 0.01$), but no additional nuclear GR localisation was observed after the cort challenge. Washout of corticosterone levels did not result in recovery of GR nuclear translocation in the 40% WO group. However, a trend towards recovery in GR translocation ($p = 0.1$) was found in the 100% WO group (Fig. 3B).

Corticosterone pellet implantation affects glucocorticoid target gene expression

To monitor primary receptor responsiveness we studied the expression profiles of two primary glucocorticoid-responsive genes: Gilz (Yachi et al. 2007, van der Laan et al. 2008b) and Sgk-1 (van Gemert et al. 2006) in the rat hippocampal area.

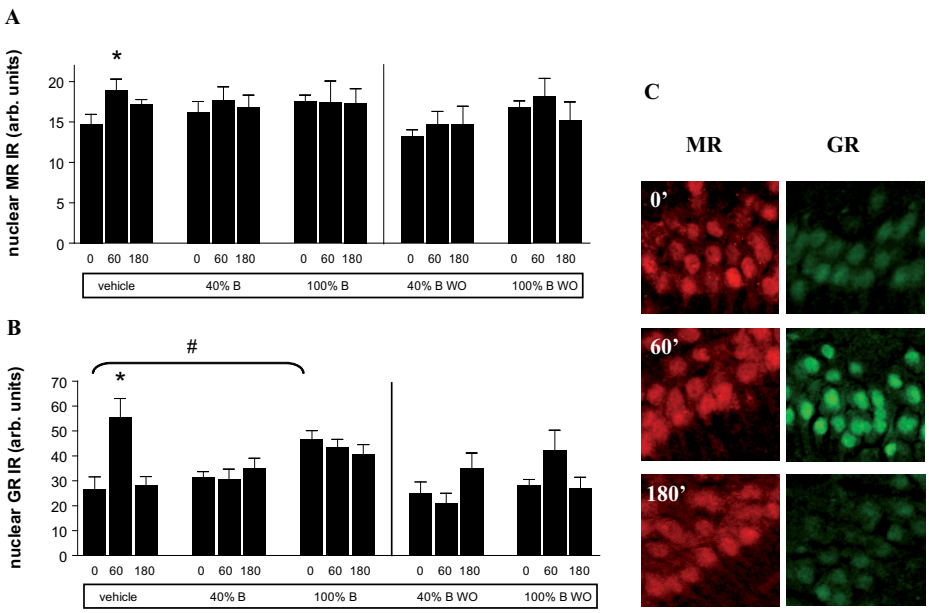

Figure 3 | Subcellular localisation of MR and GR in pyramidal CA1 cells is affected by cort pellet implantation. A | Quantification of nuclear intensity visualised by C | immunohistochemistry and confocal imaging only showed an effect of the cort challenge on MR nuclear localisation in the vehicle group (* $p < 0.05$). B | A clear transient increase in nuclear GR IR was observed in vehicle animals after the challenge (* $p < 0.01$). This effect was abolished in the 40% and 100% cort groups while basal levels were increased in the 100% cort group (# $p < 0.01$). Removal of 100% cort pellets resulted in a trend ($p = 0.1$) towards recovery in GR translocation. N = 8 animals per time point.

Gilz expression in rat hippocampus

In vehicle pellet animals, low basal expression of Gilz was observed in the CA1 pyramidal cell layer, while relatively high expression was found in CA3, dentate gyrus and cortex (Fig. 4A). In CA1 in vehicle animals, the cort challenge led to increased expression levels at 60 min ($F(2, 19) = 3.6$; $p < 0.05$). Gilz mRNA expression in CA1 in the 40% and 100% cort pellet groups resembled the GR translocation data. In the 100% cort, but not the 40% group, pellet implantation resulted in increased 'basal' expression levels ($F(2, 20) = 6.4$; $p < 0.05$) but neither group responded to the additional corticosterone challenge (Fig. 4B). Though pellet removal and washout of exogenous corticosterone decreased baseline levels back to vehicle, the pattern of gene expression did not fully recover while the kinetics of the response in the 100% WO group seemed changed: a trend towards recovery was found for the 100% cort WO group at 60 and 180 minutes after injection ($p = 0.07$). In CA3 (Fig. 4C), expression levels did not change as a consequence of time or treatment.

Sgk-1 expression in rat hippocampal area

Under basal conditions, Sgk-1 mRNA was expressed in all hippocampal regions, but highest in CA3. The corticosterone challenge led to a marked increase in the CA1 hippocampal subregion but also in the white matter surrounding the hippocampus which dropped back to baseline after 180 min (Fig. 5A). This increase has been reported before and probably reflects regulation in oligodendrocytes (van Gemert et al. 2006). Sgk-1 expression in white matter (Fig. 5B; corpus callosum) was maximally sustained in the 100% cort group but expression in response to constant and acute changes in corticosterone in the 40% cort pellet implantation group ($F(2,19) = 46.9$; $p < 0.001$) was identical to the vehicle pellet group ($F(2,20) = 185.5$; $p < 0.001$). Washout of exogenous corticosterone recovered the transient response in Sgk-1 expression in the 100% WO group (Fig. 5B; $F(2,20) = 130.1$; $p < 0.001$). Similar results were observed for CA1, but in the 100% cort group, induction of 'basal' Sgk-1 mRNA levels was absent. In contrast to the data for Gilz mRNA, responsiveness remained present in the 40% cort pellet group (Fig. 5C). Similar to Gilz mRNA, in CA3 no effect of constant and acute cort exposure was observed for Sgk-1 mRNA (Fig. 5D).

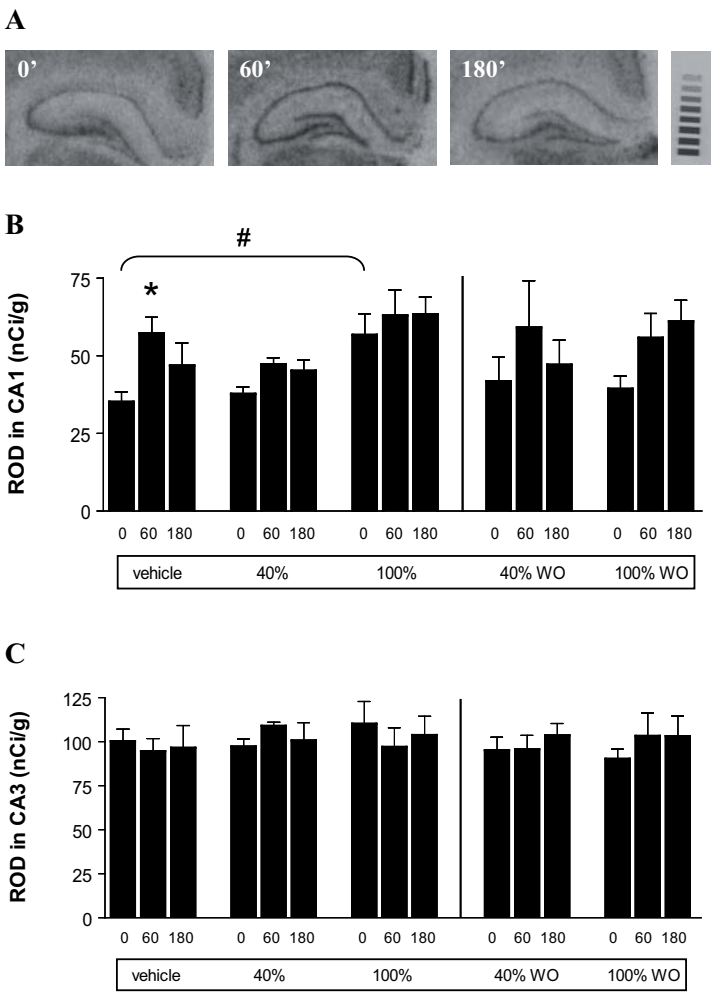

Figure 4 | Gilz mRNA expression profiles in rat hippocampus. A | Gilz expression was visualised in vehicle animals 0, 60 and 180 min after the cort challenge (n = 8 per time point) using ISH. Values were calibrated against a C14 grey-scale ladder. Quantification of relative optical density (ROD) in B | CA1 pyramidal cells show transient responses in vehicle animals (* $p < 0.05$) but sustained responses after 40% and 100% cort pellet implantation. Cort pellet implantation increased 'basal' levels significantly in the 100% cort group (# $p < 0.05$). C | No significant effect of time or treatment was found in the CA3 pyramidal cell layer.

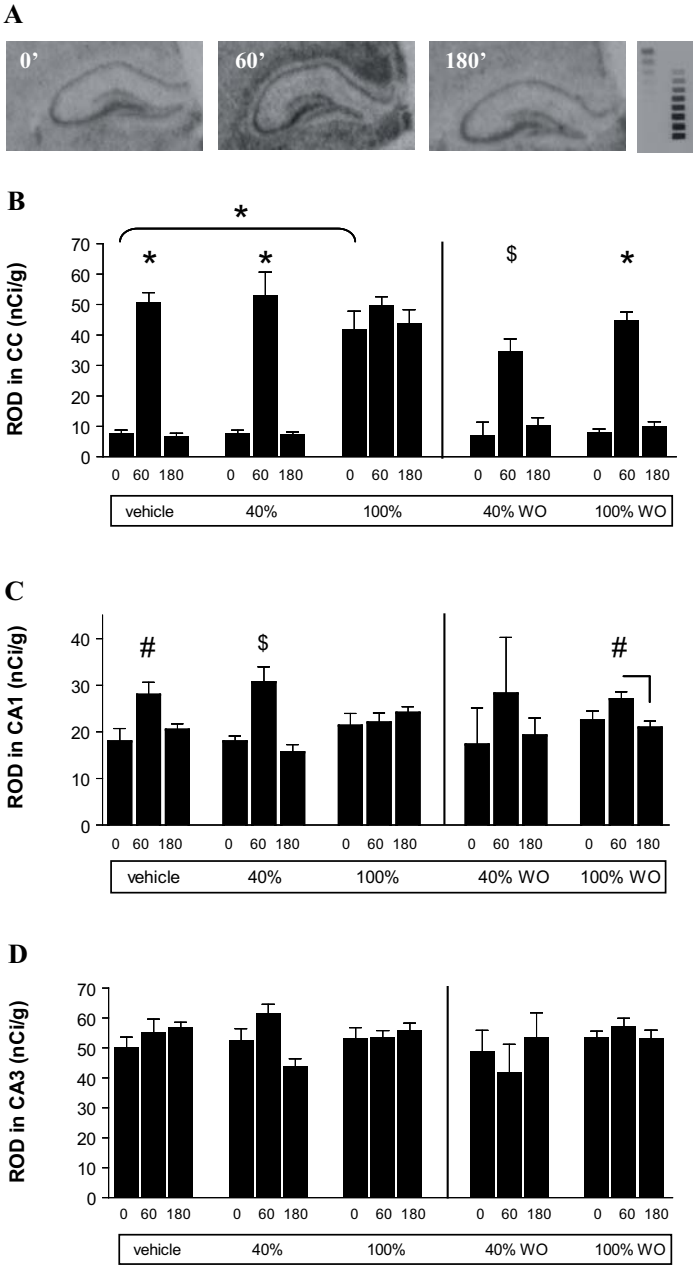

Figure 5 | Sgk-1 mRNA expression profiles in rat hippocampus. A | ISH was used to visualise Sgk-1 expression in vehicle animals 0, 60 and 180 min after the cort challenge (n = 8 per time point). Values were calibrated against a C14 grey-scale ladder. Quantification ROD in B | white matter corpus callosum and C | CA1 pyramidal cells show transient increases in response to the challenge in vehicle and 40% treated animals, but not in 100% cort animals. D | No significant effect of time or treatment was found in the CA3 (* $p < 0.001$, \$ $p < 0.01$, # $p < 0.05$).

Sgk-1 promoter occupancy by GR correlates with expression profiles

Because the gene expression profiles were different between the treatment groups, we determined whether the altered profiles were also accompanied by changes at the level of GR chromatin occupancy using chromatin immunoprecipitation (ChIP) for the Sgk-1 gene. Since no significant difference in Sgk-1 expression profiles between vehicle and 40% cort animals was observed, we chose to compare vehicle and 100% cort treated animals. In vehicle pellet animals, GR occupancy increased 3.6 ± 0.2 fold at 60 min, and returned back to baseline 180 min after the challenge ($p < 0.001$; Fig. 6), indicating that GR binding at response elements correlates with the response profile of this target gene. Challenging the 100% cort group did not result in increased DNA recovery in the GR-specific groups since levels were already increased before injection. Again, we observed elevated basal levels compared to vehicle ($p < 0.01$) which was still slightly elevated after washout. Washout of corticosterone resulted in recovery of the transient increase in Sgk-1 promoter occupancy by GR by 3.1 ± 0.1 fold 60 min after the challenge ($p < 0.001$; Fig. 6). Control measurements for the negative control locus of the myoglobulin gene showed no enrichment of GR in the vehicle group after the challenge ($p = 0.95$; data not shown), indicating that the observed increase in promoter occupancy of glucocorticoid target gene Sgk-1 is specific.

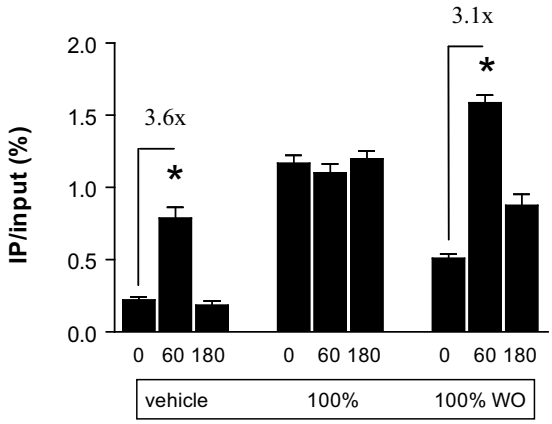

Figure 6 | Sgk-1 promoter occupancy in rat hippocampus. Chromatin immunoprecipitation and RT-qPCR to represent Sgk-1 promoter occupancy by GR for vehicle and 100% cort pellet animals (3 hippocampi pooled per time point). In the vehicle animals the cort challenge resulted in a transient increase in promoter occupancy by GR 60 min after while this response was attenuated in the 100% cort animals. Basal levels were increased compared to vehicle ($p < 0.01$). Washout of corticosterone shows a recovery in the transient response to the challenge. (* $p < 0.001$).

Discussion

In the present study we used two different concentrations of corticosterone pellets that abolish normal corticosterone pulsatility by clamping hormone levels around daily average (40% cort pellet) or at supra-physiological levels as may be seen during pathology (100% cort pellet). We have shown considerable changes in molecular markers for both chronic and acute glucocorticoid action in the rat hippocampus. Moreover, we demonstrate that the consequences of subtle variations in glucocorticoid pattern are at least as severe as those with overt hypercorticism. We therefore propose that pulsatile glucocorticoid levels are essential in maintaining receptor responsiveness, particularly of the GR, and prevent desensitisation of some targets. Consequently, we suggest that the pattern of hormone exposure is a major determinant in the adaptive capacity of target tissues in the face of acute stress.

Validation of experimental design

Subcutaneous corticosterone pellet implantation, providing a constant signal in blood plasma, mimics some of the risk factors for stress-related disease by disrupting normal glucocorticoid variation (Young et al. 2004, de Kloet et al. 2005, Herbert et al. 2006, Lightman et al. 2008). In line with previous studies that have shown flattening of circadian corticosterone rhythms (Akana et al. 1985, Akana et al. 1992, Meijer et al. 1997), we effectively clamped corticosterone levels around the daily average or at levels seen during chronic stress [40% and 100% cort pellet, respectively (RA Sarabdjitsingh 2009)]. This allowed us to directly compare the impact of a corticosterone challenge on molecular targets in the context of either constant of fluctuating hormone patterns.

Typically, subcutaneous corticosterone pellet implantation results in dose-dependent decreases in body, thymus and adrenal gland weight (Spencer et al. 1991, Akana et al. 1992). This decrease was also present in the 40% cort group, though to a lesser extent than the 100% cort group, suggesting mild hypercorticism. However, we cannot exclude that the observed decreases in the 40% group are due to alterations in the pattern of plasma corticosterone since we assume that changed pulsatility may affect glucocorticoid sensitive parameters. We interpret our groups as the 40% cort lacking pulsatility with daily cort exposure in the normal range and the 100% group lacking pulsatility while having overt hypercorticism.

100% corticosterone pellet treatment

MR and GR are both regulated by glucocorticoids (De Kloet et al. 1998). In the present data, the dose-dependent down-regulation of hippocampal GR rather than MR protein (Spencer et al. 1991), was more pronounced after constant corticosterone exposure. Additionally GR nuclear translocation patterns were clearly more attenuated after the challenge.

We assume that MR is already fully saturated under the conditions of 40% cort treatment (Reul & de Kloet 1985, Meijer et al. 1997) as no additional increase in nuclear MR translocation was observed in our study. Also, we did not specifically address MR and MR-targets as the dose of corticosterone (3 mg/kg i.p.) that was used to challenge the adrenally intact animals is more suitable to study GR-dependent effects. This is supported by the lack of glucocorticoid regulation in the CA3 pyramidal cell layer of the hippocampus. This region has very low levels of GR but expresses MR (Arriza et al. 1988, Ahima & Harlan 1990, Sarabdjitsingh et al. 2009). Even though the target genes Gilz and Sgk-1 that were used in this study are both expressed in this area under basal conditions, and are regulated by glucocorticoids in adrenalectomised animals (van der Laan et al. 2008b), we did not observe any change in transcriptional output after constant or acute corticosterone treatment. Thus, our data show that both the 40% and 100% pellets in particular affected GR signalling.

GR homologous down-regulation after chronic high glucocorticoid treatment has been observed before (Sapolsky et al. 1984, Reul et al. 1989, Spencer et al. 1991). In spite of down-regulation, GR still exerted tonic effects, e.g. as is seen from the constant high occupancy of the Sgk-1 promoter. Many studies described such sustained effects of chronic high corticosterone levels on the brain mediated via GR. For instance, feedback sensitivity of the HPA axis after stress is attenuated after chronic treatment with corticosterone through occupancy of GR (Akana et al. 1992, Bradbury et al. 1994). Also, CA1 pyramidal cells seem to lose their potential to normalise enhanced activity after stress (Joels et al. 2007), e.g. as seen from the risk of calcium overloading (Karst & Joels 2007) and attenuation of functional 5-HT responses by developing resistance to GR-mediated enhancement of membrane hyperpolarisation (Karten et al. 1999). We observed sustained effects of GR on expression of target genes Sgk-1 and Gilz in a gene and cell type dependent manner. This is in line with recent data that show complex kinetic gene response profiles under constant glucocorticoid conditions which can be transient but also tonic (John et al. 2009).

The acute effects of corticosterone that are superimposed on the 100% corticosterone treatment were attenuated, e.g. GR nuclear translocation and Sgk-1 promoter occupancy after the cort challenge. The very high circulating levels of corticosterone are very likely to saturate GR and render it non-responsive to additional hormone. This likely creates a non-favourable situation in which the normal flexibility in glucocorticoid signalling is lost.

40% corticosterone pellet treatment

The results from the present study show that 40% cort pellet treatment, which results in much lower, physiologically relevant constant corticosterone levels, also has tonic effects. Previous studies have shown attenuated stress responsiveness (Akana et al. 1992, Meijer et al. 1997) and suppressed expression levels of hippocampal 5-HT1A receptor (Meijer et al.

1997). These effects were interpreted as mediated via MR (Bradbury et al. 1994, Meijer et al. 2000). In our study some additional GR mediated effects occurred, e.g. thymus involution and a trend towards down-regulation of hippocampal GR protein levels. However, 40% cort treatment did not change baseline expression of our rather sensitive expression markers. A lack of GR responsiveness became evident after the acute challenge as no additional increase in GR nuclear translocation and target gene expression was observed. This suggests that the functionality of glucocorticoid pulsatility becomes apparent when the system is challenged and requires a rapid onset and termination of tissue responses. In the 40% cort group the pattern, but not so much the total amount, of corticosterone exposure differs from vehicle animals (RA Sarabdjitsingh 2009). We propose that the pattern of corticosterone exposure is therefore a major determinant in glucocorticoid signalling and that changes in this pattern have at least as many consequences for cellular responses than overt hypercorticism (100% cort). This is also strengthened by the normalisation in GR responsiveness after washout of constant 100% corticosterone levels but not in the 40% cort group. This suggests that tonic effects of glucocorticoids can be reversible and are modulated by an underlying mechanism that is sensitive to changes in the normal pulsatile pattern.

The use of 40% cort pellet resulted in aberrant GR translocation while the transcriptional profiles of target genes were affected gene-specifically. While induction of Gilz expression was already attenuated by 40% cort pellet implantation, Sgk-1 expression in the hippocampal area was only sustained after exposure to high levels of corticosterone (100% cort). We therefore conclude that receptor translocation is certainly not rate-limiting for all signalling pathways. The molecular response to glucocorticoids via GR involves many complex regulatory actions and cannot therefore simply be explained by the duration and presence of the hormone. These gene-specific effects may be related to differences in the potency of GR at target genes, resulting in different transcriptional output patterns (Meijer 2006, John et al. 2009). In addition, rapid receptor dynamics together with the repeated cycling of chaperones and cofactors continuously change the local environment to which promoters are exposed and underline the time and region -dependency of receptor effectiveness (Biddie & Hager 2009).

Conclusion

Frequency encoding of intercellular signals is a well accepted mode of communication in mammalian systems including the endocrine system. For instance, rapidly fluctuating levels of growth hormone elicits significant sexual dimorphic effects on gene expression (Waxman et al. 1995). Similarly, modulation of episodic release of GnRH influences the secretory patterns of LH and FSH and prevents receptor desensitisation (Belchetz et al. 1978, Wildt et al. 1981). These studies imply that ultradian hormone signalling enables systems to maintain responsiveness and prevent receptor desensitisation and is supported by our

findings. Manipulation of the temporal aspect is already a successfully used approach in clinical therapy for instance for growth hormones (Amato et al. 2000) and estrogens (Shoupe 2001). Linking the knowledge about ultradian glucocorticoid pulsatility and receptor signalling could therefore greatly contribute to clinical therapy.

In the present study, we provide evidence that frequency encoding by rapid glucocorticoid signalling is critical in functionally maintaining receptor responsiveness and preventing desensitisation of some targets. We clearly demonstrate that the ability of the receptor to respond to glucocorticoids as defined by receptor translocation and binding to its proximal targets is attenuated when pulsatile corticosterone signalling is lost. We therefore propose that the molecular response of target tissues induced by a corticosterone challenge is a resultant of the dynamic interplay between the corticosterone exposure regime (pulsatile or continuous) and steroid receptor signalling. The findings of this study suggest that pulsatile glucocorticoid release is required to maintain the normal 'resilience' in glucocorticoid responsiveness.

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