Disrupted Corticosterone Pulsatile Patterns Attenuate Responsiveness to Glucocorticoid Signaling in Rat Brain

R. Angela Sarabdjitsingh, Sheena Isenia, Annelies Polman, Jona Mijalkovic, Servane Lachize, Nicole Datson, E. Ron de Kloet, and Onno C. Meijer

Division of Medical Pharmacology, Leiden/Amsterdam Centre for Drug Research/Leiden University Medical Centre, University of Leiden, 2300 RA Leiden, The Netherlands

Chronically elevated circulating glucocorticoid levels are although to enhance vulnerability to psychopathology. Here we hypothesized that such sustained glucocorticoid levels, disturbing corticosterone pulsatility, attenuate glucocorticoid receptor signaling and target gene responsiveness to an acute challenge in the rat brain. Rats were implanted with vehicle or 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 h 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 glucocorticoid receptor and attenuated mineralocorticoid receptor and glucocorticoid receptor translocation to the acute challenge. Interestingly, whereas target gene Gilz expression to the challenge was already attenuated by tonic daily average levels (40%), Sgk-1 was affected only after constant high corticosterone exposure (100%), indicating altered receptor responsiveness due to treatment. Washout of 100% corticosterone recovered all molecular markers (partial), whereas removal of the 40% corticosterone pellet still attenuated responsiveness to the challenge. We propose that corticosteroid pulsatility is crucial in maintaining normal responsiveness to glucocorticoids. Whereas 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 signaling as overt hypercorticism, suggesting an underlying mechanism sensitive to the pattern of hormone exposure. (Endocrinology 151: 1177–1186, 2010)
locate to the nucleus on ligand binding. In the nucleus the receptors modulate target gene expression via interactions with specific glucocorticoid-responsive elements in the regulatory region of glucocorticoid target genes or via protein-protein interactions with other transcription factors (17, 18). Nuclear translocation of GR is known to vary according to circadian fluctuations in corticosterone levels (19). Moreover, repeated rapid nuclear translocation of GR after iv corticosterone injections that mimic ultradian pulses was demonstrated in rats (20) whereas MR was continuously retained in the nucleus (21), implying that disturbances in glucocorticoid pulsatility would mainly affect GR.

It is now being recognized that access and binding of steroid receptors to regulatory elements in the genome (22), including GR, is highly dynamic (20, 23). The individual steps of the receptor signaling cascade (i.e. nuclear translocation, chromatin binding and modulation of target gene expression) together determine cellular and/or tissue sensitivity to glucocorticoids and efficacy (24). Still, little information is available on how rapidly fluctuating glucocorticoid levels in vivo affect receptor signaling, 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 signaling. Understanding the interplay between rapidly fluctuating glucocorticoid levels and receptor signaling therefore could greatly add 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 stress. Evidence from other hormonal systems suggests that the function of rapid ligand bursts is to maintain tissue sensitivity and receptor responsiveness (25). Therefore, we hypothesized that constant levels of glucocorticoids, thus loss of pulsatility, will similarly affect glucocorticoid signaling by changing the responsiveness of the GR and its proximal targets.

Glucocorticoid pulsatility was abolished by sc corticosterone pellet implantation thereby exogenously inducing constant levels of glucocorticoids (26–28). Consequently, we studied molecular markers for glucocorticoid signaling 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 normalized after reinstatement of glucocorticoid pulsatility. Here we find that the transient response in glucocorticoid signaling is attenuated during exposure to constant cort levels but that this apparent resistance is reversible.

Materials and Methods

Animals

Male Sprague Dawley rats (Harlan, Leiden, The Netherlands) weighing approximately 250 g on the day of surgery were group housed on a 12-h light, 12-h dark cycle (lights on at 0700 h) 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 no. 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% cort pellets (40 or 100 mg corticosterone; MP Biomedicals, Solon, OH) and compared with 100 mg vehicle (cholesterol; Sigma-Aldrich, St. Louis, MO). Using isoflurane anesthesia, pellets were sc implanted in intact animals randomly assigned to either of the five treatment groups (n = 24): vehicle and 40, 100, 40 WO (washout), and 100% WO cort. Animals were weighed daily during the 7 d after pellet implantation. On experimental d 7, blood samples were collected via the tail vein in the morning (0800 h) and afternoon (1700 h) to verify flattening of circadian cort levels in plasma without anesthesia (29). Pilot studies indicated initial enhanced release of corticosterone from the pellet on the first 2 d after implantation and steady-state levels thereafter (28). As described in Results, cort levels were indeed constant the day before the corticosterone challenge. In addition, high-frequency blood sampling was used to verify flattened cort rhythms with higher resolution [10 min intervals (30)].

Early in the morning on postsurgery d 8 (0800–0900 h), animals from the WO groups were briefly anesthetized during which the pellet was rapidly removed (washout), whereas the animals from the other groups were sham operated. To ascertain normalization in responsiveness of most of the available GR molecules, the WO period was decided to be 24 h, according the half-life time of GR redistribution to the cytoplasm after hormone withdrawal [t_{1/2} = 8–9 h (31)]. After 24 h, animals from all five groups were challenged with a high dose of corticosterone (3 mg/kg ip corticosterone-HBC; Sigma-Aldrich). 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°C until further processing. Of each animal, one hemisphere of the brain was used for immunohistochemistry and in situ hybridization, whereas 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 RIA (MP Biomedicals Inc., Costa Mesa, CA.) according to the manufacturer’s instructions.

Protein sample preparation

Hippocampal total protein samples (n = 12) were obtained by homogenizing in ice-cold lysis buffer containing 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.15 M NaCl, 50 mM Tris (pH 7.5), and protease inhibitors (Complete Mini cocktail tablets; Roche, Woerden, The Netherlands). Protein concentrations were determined by a BCA protein assay (Pierce, Rockford, IL).

Western blotting and analysis

Western blot was performed as described before (21). Twenty-micromgram protein samples were loaded on 5–8% polyacrylamide gels and transferred to polyvinyl difluoride membranes (Amersham Biosciences, Uppsala, Sweden). Membranes were probed with either primary anti-GR antibody (M20; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-MR antibody (MR1-18 1D5, kindly supplied by Dr. Gomez-Sanchez, University of Mississippi Medical Center, Jackson, Mississippi (32)) and subsequently with antirabbit IgG-horseradish peroxidase (Santa Cruz Biotechnology) or antimouse IgG-horseradish peroxidase (Amersham Biosciences). Signal was detected using enhanced chemiluminescence reagent (ECLplus; Amersham Biosciences) and enhanced chemiluminescence hyperfilm (Amersham Biosciences). Membranes were also probed for α-tubulin as a loading control (Sigma-Aldrich).

Western blot bands were quantified by densitometry using an Epson perfection scanner (Epson Europe, Meerbusch, Germany) and ImageJ analysis software [National Institutes of Health (NIH); http://rsb.info.nih.gov/iij/]. OD of GR and MR bands were corrected for film background and normalized 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 visualize the subcellular distribution pattern of MR and GR immunoreactivity (IR) in hippocampal CA1 cells, immunohistochemistry was performed on 20-μm slices as described previously (33). Sections were incubated with primary GR (H300; Santa Cruz Biotechnology) and primary MR antibodies (MR1-18 1D5). After washing, sections were incubated with secondary antibodies AlexaFluor-A488 and AlexaFluor-A594 IgG (Molecular Probes, Leiden, The Netherlands). Nuclei were visualized with Hoechst 33258 (Molecular Probes), and slides were mounted with Aqua Polymount (Polysciences, Inc., Eppelheim, Germany). Controls included incubation of slides with equal amounts of normal rabbit and mouse IgG (Santa Cruz Biotechnology), swapping of secondary fluorescent labels, and incubation without any primary antibodies.

Confocal microscopy and image analysis

Nuclear translocation patterns were measured as previously described (33, 34). 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) on images (magnification, ×630, 155 × 155 μm) acquired with a Nikon confocal microscope (TE 2000-e; Amstelveen, The Netherlands). 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. Only cells that had a clear oval 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 hybridization

In situ hybridization was used to study expression patterns of glucocorticoid target genes Sgk-1 and Gilz in brain and was performed on 20-μm slices as previously published (35). 33Phosphorous end-labeled oligonucleotide probes (2 × 106 dpm) for Gilz (36) and Sgk-1 (37) were used. The signal was quantified from films exposed 6–8 d (X-OMAT AR; Kodak, Rochester, NY) using ImageJ 1.32j analysis software (NIH). Relative expression levels were determined and related to standard curves of 14C (RPA 504 microscales; Amersham, Diegem, Belgium), included to ensure that gray values were within the linear range between 0 and 255. Three hippocampal sections per animal (n = 8 per time point) were analyzed.

ChIP and quantitative RT-PCR (RT-qPCR)

ChIP to study Sgk-1 promoter occupancy by GR was adapted and modified from Van der Laan et al. (36). Fixed chromatin derived from the hippocampi of three animals was pooled and sheared, yielding fragments of 100–500 bp (20 pulses of 30 sec; Bioruptor; Diagenode, Liège, Belgium). 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, Düren, Germany), 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 the manufacturer’s instructions. Primers were designed around the glucocorticoid-responsive element of the rat Sgk-1 promoter (Sgk-1 forward, GGCTCTAATTTATGCGGAA; Sgk-1 reverse, CGGAATAAATCTCCTTGGC). Myoglobin was used as a negative control for GR chromatin occupancy: myoglobin forward, CCTCAGTGGCCAGCTT; myoglobin reverse, GCTTTGTCAAGTGAGAAA. PCR products were analyzed on an agarose gel to check the length of the amplicon. Immunoprecipitation with a nonspecific antibody (normal IgG) did not result in increased DNA recovery and was used to correct the GR immunoprecipitated samples (36, 38).

Statistical analysis

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

Controls for sc cort pellet treatment

To validate that sc cort pellet implantation effectively clamped circadian fluctuations at expected concentrations, morning and evening cort samples were taken 7 d after pellet implantation (n = 24). Vehicle animals showed typical circadian variation in cort levels (21.4 ± 2.7 and 171.7 ± 20.1 ng/ml; morning and evening levels, respectively). No circadian variation in morning and evening cort 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 postsurgery d 8 resulted in rapid decrease in cort levels (morning cort 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).

Cort pellet implantation led to a dose-dependent decrease in body and thymus weight [Fig. 1, A and 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 cort pellet implantation was effective. Pellet removal and 24 h of WO of exogenous cort did not result in recovery of any of the parameters measured (Fig. 1, dashed bars).

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

Western blot analysis for MR and GR was performed on hippocampal homogenate (Fig. 2). MR protein showed a trend toward decreased MR protein levels in the 100% WO cort group. MR protein levels were not affected by 40% cort pellet implantation or WO.

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 with vehicle [F(2,31) = 5.1; P < 0.01; Fig. 2B]. GR protein levels in the 40% WO cort group were still reduced after WO (0.74 ± 0.1), but in the 100% WO cort group, a recovery was noted because values were not different from vehicle-treated animals [0.88 ± 0.13; F(2,27) = 1.2; P = 0.33].

Next to the effect of cort pellet implantation, we studied whether there was an acute effect of the cort 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.

Cort pellet implantation attenuates GR translocation in CA1 pyramidal cells

To study a different aspect of the receptor protein, we visualized cort-induced MR and GR translocation patterns to the nucleus, rather than total protein hippocampal levels (Fig. 2), using immunohistochemistry and confocal imaging (Fig. 3). MR IR in CA1 pyramidal cells of the rat hippocampus was mostly localized in the nucleus before the cort challenge in all conditions (Fig. 3, A 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 min [Fig. 3A; F(2,16) = 3.6; P = 0.049]; no MR translocation was observed in the other treatment groups. WO of cort 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. 3, B 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 min after the acute injection (Fig. 3B). The 100% cort pellet increased basal levels of nuclear GR significantly \[ F(2,19) = 8.7; P < 0.01 \], but no additional nuclear GR localization was observed after the cort challenge. WO of cort levels did not result in recovery of GR nuclear translocation in the 40% WO group. However, a trend toward recovery in GR translocation \( P = 0.1 \) was found in the 100% WO group (Fig. 3B).

**Cort pellet implantation affects expression of glucocorticoid target genes Gilz and Sgk-1**

To monitor primary receptor responsiveness, we studied the expression profiles of two primary glucocorticoid-responsive genes: Gilz (36, 39) and Sgk-1 (37) in the rat hippocampal area.

**Gilz expression in rat hippocampus**

In vehicle pellet animals, low basal expression of Gilz was observed in the CA1 pyramidal cell layer, whereas 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 cort challenge (Fig. 4B). Although pellet removal and WO of exogenous cort decreased baseline levels back to vehicle, the pattern of gene expression did not fully recover, whereas the kinetics of the response in the 100% WO group seemed changed: a trend toward recovery was found for the 100% cort WO group at 60 and 180 min 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 cort 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 (37). 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 cort in the 40% cort pellet implantation group \[ F(2,19) = 46.9; P < 0.001 \] was identical with the vehicle pellet group \[ F(2,20) = 185.5; P < 0.001 \]. WO of exogenous cort 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).
Sgk-1 promoter occupancy by GR in rat hippocampus 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 ChIP for the Sgk-1 gene. Because 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 because levels were already increased before injection. Again, we observed elevated basal levels compared with vehicle (P < 0.01), which was still slightly elevated after washout. Washout of cort 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 locus of the myoglobin 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.

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 supraphysiological 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 desensitization 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 (1, 2, 11, 13). In line with previous studies that have shown flattening of circadian corticosterone rhythms (26–28), we effectively clamped corticosterone levels around the daily average or at levels...
seen during chronic stress [40 and 100% cort pellet, respectively (30)]. This allowed us to directly compare the impact of a corticosterone challenge on molecular targets in the context of either constant or fluctuating hormone patterns.

Typically, sc cort pellet implantation results in dose-dependent decreases in body, thymus, and adrenal gland weight (27, 40). This decrease was also present in the 40% cort group, although 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 cort because 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.

One hundred percent cort pellet treatment

MR and GR are both regulated by glucocorticoids (16). In the present data, the dose-dependent down-regulation of hippocampal GR, rather than MR protein (40), was more pronounced after constant cort 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 (14, 28) because 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 cort (3 mg/kg ip) 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 (33, 41, 42). Even though the target

FIG. 4. Gilz mRNA expression profiles in rat hippocampus. A, In situ hybridization was used to visualize Gilz expression in vehicle-treated animals 0, 60, and 180 min after the cort challenge (n = 8 per time point). Values were calibrated against a C14 gray-scale ladder. Quantification of relative OD (ROD) in CA1 pyramidal cells (B) show transient responses in vehicle-treated 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.

FIG. 5. Sgk-1 mRNA expression profiles in rat hippocampus. A, In situ hybridization was used to visualize Sgk-1 expression in vehicle-treated animals 0, 60, and 180 min after the cort challenge (n = 8 per time point). Values were calibrated against a C14 gray-scale ladder. Quantification of relative OD (ROD) in white matter corpus callosum (B) and CA1 pyramidal cells (C) show transient increases in response to the challenge in vehicle and 40% cort-treated animals but not in 100% cort animals. D, No significant effect of time or treatment was found in the CA3 pyramidal cell layer. *, P < 0.001; $, P < 0.01; #, P < 0.05.
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FIG. 6. ChIP and RT-qPCR to represent SGK-1 promoter occupancy by GR for vehicle and 100% cort pellet animals (three hippocampi pooled per time point). In the vehicle animals, the cort challenge resulted in a transient increase in promoter occupancy by GR 60 min after, whereas this response was attenuated in the 100% cort animals. Basal levels were increased compared with vehicle ($P<0.01$). WO of corticosterone shows a recovery in the transient response to the challenge. *, $P<0.001$.

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 adrenalectomized animals (36), we did not observe any change in transcriptional output after constant or acute cort treatment. Thus, our data show that both the 40 and 100% pellets in particular affected GR signaling.

GR homologous down-regulation after chronic high glucocorticoid treatment has been observed before (40, 43, 44). Despite 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 cort levels on the brain mediated via GR. For instance, feedback sensitivity of the hypothalamic-pituitary-adrenal axis after stress is attenuated after chronic treatment with cort (27) through occupancy of GR (45). Also, CA1 pyramidal cells seem to lose their potential to normalize enhanced activity after stress (46), e.g., as seen from the risk of calcium overloading (47) and attenuation of functional 5-hydroxytryptamine responses by developing resistance to GR-mediated enhancement of membrane hyperpolarization (48). 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 (49).

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

Forty percent cort pellet treatment

The results from the present study show that 40% cort pellet treatment, which results in much lower, physiologically relevant constant cort levels, also has tonic effects. Previous studies have shown attenuated stress responsiveness (27, 28) and suppressed expression levels of hippocampal 5-hydroxytryptamine receptor 1A receptor (28). These effects were interpreted as mediated via MR (35, 45). In our study some additional GR-mediated effects occurred, e.g., thymus involution and a trend toward 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 because 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 cort exposure differs from vehicle animals (30). We propose that the pattern of cort exposure is therefore a major determinant in glucocorticoid signaling 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 normalization in GR responsiveness after WO of constant 100% cort 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, whereas the transcriptional profiles of target genes were affected gene specifically. Whereas induction of Gilz expression was already attenuated by 40% cort pellet implantation, Sgk-1 expression in the hippocampal area was sustained only after exposure to high levels of cort (100% cort). We therefore conclude that receptor translocation is certainly not rate limiting for all signaling 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 (49, 50). 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 (51).

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 GH elicits significant sexual dimorphic effects on gene expression (52). Similarly, modulation of episodic release of GnRH influences the secretory patterns of LH and FSH and prevents receptor desensitization (53, 54). These studies imply that ultradian hormone signaling enables systems to maintain responsiveness and prevent receptor desensitization and is supported by our findings. Manipulation of the temporal aspect is already a successfully used approach in clinical therapy for instance for GHs (55) and estrogens (56). Linking the knowledge about ultradian glucocorticoid pulsatility and receptor signaling could therefore greatly contribute to clinical therapy.

In the present study, we provide evidence that frequency encoding by rapid glucocorticoid signaling is critical in functionally maintaining receptor responsiveness and preventing desensitization 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 signaling is lost. We therefore propose that the molecular response of target tissues induced by a corticosterone challenge is a result of the dynamic interplay between the corticosterone exposure regimen (pulsatile or continuous) and steroid receptor signaling. The findings of this study suggest that pulsatile glucocorticoid release is required to maintain the normal resilience in glucocorticoid responsiveness.

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Address all correspondence and requests for reprints to: R. Angela Sarabdjitsingh, Division of Medical Pharmacology, Leiden/Amsterdam Centre for Drug Research/Leiden University Medical Centre, University of Leiden, Einsteinweg 55, P.O. Box 9502, 2300 RA Leiden, The Netherlands. E-mail: r.a.sarabdjitsingh@lacdr.leidenuniv.nl.

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