Acute stress enhances heterodimerization and binding of corticosteroid receptors at glucocorticoid target genes in the hippocampus

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A stressful event results in secretion of glucocorticoid hormones, which bind to mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) in the hippocampus to regulate cognitive and affective responses to the challenge. MRs are already highly occupied by low glucocorticoid levels under baseline conditions, whereas GRs only become substantially occupied by stress- or circadian-driven glucocorticoid levels. Currently, however, the binding of MRs and GRs to glucocorticoid-responsive elements (GREs) within hippocampal glucocorticoid target genes under such physiological conditions in vivo is unknown. We found that forced swim (FS) stress evoked increased hippocampal RNA expression levels of the glucocorticoid-responsive genes FK506-binding protein 5 (*Fkbp5*), Period 1 (*Per1*), and serum- and glucocorticoid-inducible kinase 1 (*Sgk1*). Chromatin immunoprecipitation (ChIP) analysis showed that this stressor caused substantial gene-dependent increases in GR binding and surprisingly, also MR binding to GREs within these genes. Different acute challenges, including novelty, restraint, and FS stress, produced distinct glucocorticoid responses but resulted in largely similar MR and GR binding to GREs. Sequential and tandem ChIP analyses showed that, after FS stress, MRs and GRs bind concomitantly to the same GRE sites within *Fkbp5* and *Per1* but not *Sgk1*. Thus, after stress, MRs and GRs seem to bind to GREs as homo- and/or heterodimers in a gene-dependent manner. MR binding to GREs at baseline seems to be restricted, whereas after stress, GR binding may facilitate cobinding of MR. This study reveals that the interaction of MRs and GRs with GREs within the genome constitutes an additional level of complexity in hippocampal glucocorticoid action beyond expectancies based on ligand–receptor interactions.

stress | mineralocorticoid receptor | glucocorticoid receptor | hippocampus | heterodimerization

Adrenal glucocorticoid hormones play a pivotal role in orchestrating adaptive responses to stressful challenges to maintain health and wellbeing. Acute surges in glucocorticoid secretion after stress are beneficial for the organism, whereas aberrant secretion, as a result of chronic stress or traumatic experiences, is damaging and increases susceptibility to mental disorders, such as major depression, anxiety, and posttraumatic stress disorder.

Over 40 y ago, McEwen et al. (1) discovered that glucocorticoids act through receptors located in the brain, primarily the hippocampus. In 1985, Reul and de Kloet (2) reported that these steroid hormones bind to two distinct types of receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), in this limbic brain region where these receptors are colocalized in neurons (3). Because of the extraordinary difference in binding affinity of MRs [K_d = 0.1–0.5 nM for binding corticosterone (CORT), the endogenous glucocorticoid of rats and mice] and GRs (K_d = 2–5 nM), there were marked differences in receptor occupancy between these receptors under baseline and stress conditions (2, 4). MRs are already >80% occupied with endogenous glucocorticoids under early morning (AM) baseline conditions, whereas GRs only become substantially occupied by elevated glucocorticoid levels, such as after stress and at the circadian peak of glucocorticoid secretion. These data gave rise to the concept that MRs exert tonic actions on brain, whereas GRs mediate the negative feedback and long-term cognitive changes evoked by glucocorticoids (2, 4, 5).

MR and GR are mainly intracellular receptors that act as ligand-dependent transcription factors. After binding of glucocorticoids, the receptors are translocated to the nucleus with the help of cochaperones and bind to specific glucocorticoid response elements (GREs) within the DNA of glucocorticoid-inducible genes to elicit transcriptional responses (6, 7). The molecular mechanisms underpinning the interaction of MRs and GRs with the genome have been primarily studied in vitro, predominantly using chromatin immunoprecipitation (ChIP), allowing the investigation of transcription factor binding to recognition sites within the genome. ChIP has been used to study the interaction of GR with GREs in glucocorticoid target genes in cell cultures in vitro and pharmacological studies in vivo (8, 9). Until now, however, the binding of MR and GR to GREs within glucocorticoid target genes under physiological conditions in hippocampus tissue in vivo has never been studied. Thus, currently, it is unknown how stressful challenges impact on MR and GR binding to GREs within the genome in vivo.

A long-standing question is whether, in addition to forming MR/MR and GR/GR homodimers, MRs and GRs also form MR/GR heterodimers and interact as such at the genomic level. In fact, the concept of heterodimer formation by these steroid receptors and their ability to bind DNA is based on studies in vitro and has not been shown in vivo. Work using cell cultures and cell-free approaches indicate that MR/GR heterodimers may form under conditions in vitro (10–12). In addition, Trapp et al. (10) found stronger DNA binding and gene transcriptional effects in vitro under conditions that favored MR/GR heterodimerization. These studies did not investigate MR/GR heterodimer binding to GREs within the genome. Moreover, evidence that MR/GR heterodimerization and

Significance

Glucocorticoid hormones are important mediators of the stress response and implicated in the etiology of stress-related psychiatric disorders. Glucocorticoids act via mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) in the hippocampus, resulting in altered transcription of target genes. Currently, however, little information is available about how they interact with the genome after stress in vivo. Here, we show that an acute stressful challenge results in an increased interaction of both MRs and GRs with their genomic recognition sites. Moreover, they may interact with these sites not just as homodimers but also, as heterodimers, the extent of which is highly gene-dependent. These findings provide insight into how MRs and GRs interact with the hippocampal genome after stress in vivo.

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DNA binding is taking place under physiological conditions in vivo is presently lacking.

We investigated the interaction of MRs and GRs with GREs within the well-known glucocorticoid target genes FK506-binding protein 5 (*Fkbp5*), Period 1 (*Per1*), and serum- and glucocorticoid-inducible kinase 1 (*Sgk1*). These genes are involved in GR ligand binding affinity (13), circadian neuronal activity (14), and neuronal plasticity processes (15), respectively, and were transcriptionally activated after forced swim (FS) stress. Using ChIP, we found that, after stress, MRs and GRs bound transiently to GRE sites within these glucocorticoid target genes, albeit in a gene-dependent manner. For MR, the significant increase in DNA binding after stress was surprising given the high glucocorticoid occupancy of this receptor under baseline AM conditions, challenging the notion that high receptor occupancy would correlate with high DNA binding. Furthermore, as revealed by tandem ChIP, MRs and GRs bind concomitantly to the same GRE sites within *Fkbp5* and *Per1* but not *Sgk1* after stress, indicating that these steroid receptors, in addition to forming homodimers, indeed seem to bind to GREs as heterodimers. Thus, our study shows that, after stress, MRs and GRs may access the genome as homo- and/or heterodimers and in a gene-dependent manner.

Results

Acute Stress Increases Transcription of Glucocorticoid Target Genes Across All Hippocampal Subregions. A single 15-min FS challenge resulted in a significant, time-dependent increase in the transcription of the classic glucocorticoid-dependent genes *Fkbp5* (Fig. 1*A*), *Per1* (Fig. 1*C*), and *Sgk1* (Fig. 1*E*) in the dentate gyrus. Very similar patterns of transcriptional activation were found in the Cornu ammonis (CA) regions (Fig. 1 *B*, *D*, and *F*) and ventral hippocampus (Fig. S1). The time course of stress-induced gene transcription and the conversion time for splicing heteronuclear RNA (hnRNA) to mature messenger RNA (mRNA) were gene-specific, with *Fkbp5* peaking at 60 min (hnRNA) and significant increases in mRNA expression by 180 min, whereas RNAs of *Per1* and *Sgk1* peaked earlier (hnRNA, 30 min; mRNA, 60 min). The peaks in *Per1* and *Sgk1* hnRNA corresponded with the peak in plasma CORT after FS (30 min) (Fig. S2), but the *Fkbp5* hnRNA response was clearly delayed. Because the RNA responses were highly similar between the hippocampal subregions, we performed subsequent ChIP analyses on whole-hippocampus tissues.

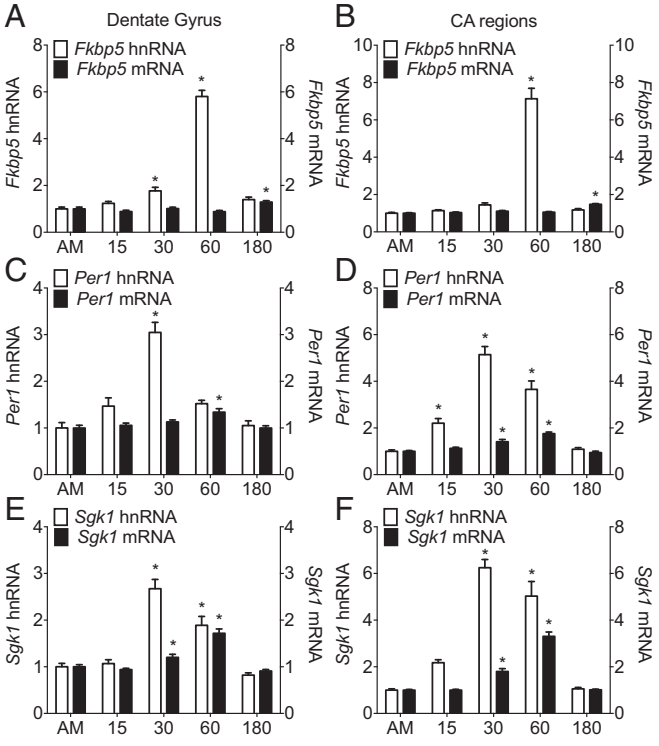

Fig. 1. hnRNA and mRNA expression of glucocorticoid-inducible genes in hippocampal subregions under baseline conditions and after stress. Rats were killed direct from home cage (~7:00 AM; AM baseline) or at 15, 30, 60, or 180 min after the start of FS (15 min, 25 °C water). The graphs show expression of *Fkbp5*, *Per1*, and *Sgk1* in the (*A*, *C*, and *E*, respectively) dorsal dentate gyrus or (*B*, *D*, and *F*, respectively) CA regions, and they are represented as mean fold change over baseline RNA levels (±SEM; $n = 7$–9 per group). Expressions of both hnRNA (white bars; left *y* axis) and mRNA (black bars; right *y* axis) are shown for individual genes. More information on statistical analyses in Figs. 1, 2, 3, 4, and 5 is in *SI Statistics Information to Figs. 1, 2, 3, 4, and 5*. *$P < 0.05$ compared with AM.

FS Transiently Increases MR and GR Binding to GREs Within Hippocampal Glucocorticoid Target Genes. Presently, it is unknown whether stress-induced transcriptional activation of glucocorticoid-dependent genes involves physical interaction of MRs and GRs with GREs within these genes. Using ChIP, we studied MR and GR binding to GREs within *Fkbp5* (GRE2), *Per1*, and *Sgk1* (Fig. S3 shows the within-gene location of targeted GREs) in hippocampal chromatin from rats killed under early morning baseline conditions (AM), at various time points after FS stress, or under late afternoon baseline conditions (PM) (Fig. 2). FS stress resulted in a highly significant, transient increase in corticosteroid receptor binding to all glucocorticoid target genes investigated (Fig. 2) that largely paralleled the changes in plasma CORT levels (Fig. S2). The peak in MR and GR binding (at 30 min) coincided with (*Per1* and *Sgk1*) or preceded (*Fkbp5*) the increases in hnRNA levels after stress (Fig. 1). In contrast to GR (Fig. 2 *B*, *D*, and *F*), MR binding to GREs was already near-maximal at 15 min (Fig. 2 *A*, *C*, and *E*). After stress, MR binding to GREs increased gene dependently between 1.5- (*Sgk1*) and 6-fold (*Fkbp5*) (Fig. 2), which was surprising given that MR occupancy by endogenous glucocorticoids is already very high (>80%) under baseline AM conditions (2, 4). Thus, in case of MR, high receptor occupancy does not predict or guarantee high GRE binding. Occupancy of GRs by endogenous glucocorticoids after stress was shown to follow the course of the plasma glucocorticoid concentration (2, 4). Fig. 2 *B*, *D*, and *F* shows that the binding of GRs to the different GREs was highly responsive to stress and also followed the pattern of plasma glucocorticoid levels (Fig. S2) and GR occupancy levels (2, 4). The magnitude of the stress-evoked enhancement in binding of MR and GR to GREs was highly gene-dependent, with highest increments found in *Fkbp5* GRE2 and *Per1* GRE and smaller increases found in *Sgk1* GRE. These observations indicate that, under both baseline and stress conditions, accessibility of GRE sites within glucocorticoid target genes seems to be different within hippocampal cells. Comparison of MR and GR binding between baseline AM and PM presents a clear circadian variation in the interaction of both receptors with the target gene GREs, except for MR binding to the *Sgk1* GRE, which failed to reach statistical significance (Fig. 2). These observations show that rises in MR and GR binding can occur in response to circadian-driven increases in circulating glucocorticoids (Fig. S2), independent of stress.

Comparison of Different Stressors Regarding MR and GR Binding to Glucocorticoid Target Genes. Subsequently, we investigated whether the degree of MR and GR binding to GREs depends on the severity of the stressor. The plasma CORT levels after novelty [novel environment (NE)], restraint (RS), and FS stress were ~230, 670, and 1,160 ng/mL, respectively (16) (Fig. S2) (baseline AM levels: ~10 ng/mL). All stressors caused substantial increases in MR and GR binding to all target gene GREs investigated (Fig. 3). Overall, the magnitude of the binding responses was gene-dependent, with *Fkbp5* (GRE2) and *Per1* showing much higher responses than *Sgk1*. Remarkably, however, although these stressors are well-known to produce distinct glucocorticoid responses, binding of MRs and GRs to a GRE within a particular gene was very similar. For instance, NE and FS stress evoke very different plasma glucocorticoid levels; nevertheless, binding of MRs and GRs to GREs was not different

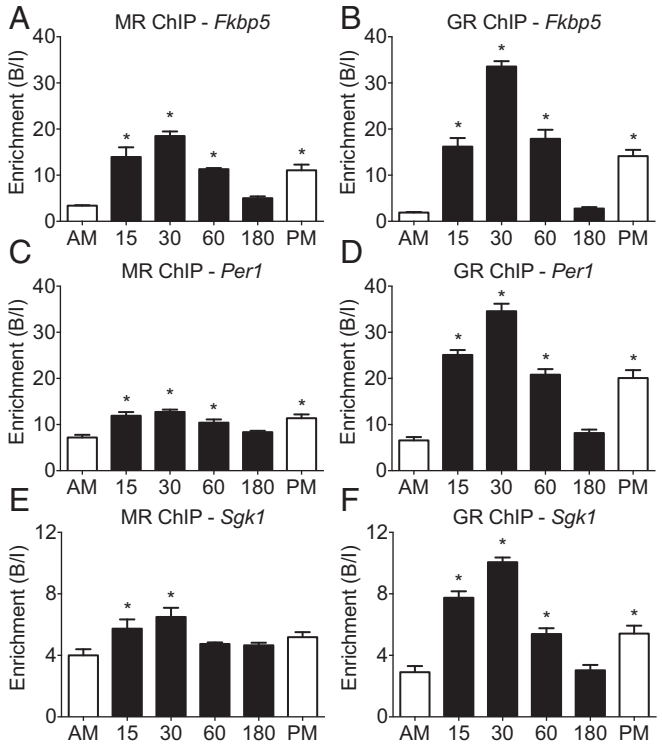

Fig. 2. MR and GR binding to GREs within glucocorticoid-inducible genes in the hippocampus under baseline conditions and after stress. Rats were killed under AM (~7:00 AM) or PM (~5:00 PM) conditions or at 15, 30, 60, or 180 min after the start of FS (15 min, 25 °C water). The graphs show enrichment [bound/input (B/I); mean ± SEM; $n = 3$–4] at GREs within (*A* and *B*) *Fkbp5*, (*C* and *D*) *Per1*, and (*E* and *F*) *Sgk1* after MR and GR ChIP on hippocampal chromatin, respectively. $^{*}P < 0.05$ compared with AM.

between these stressors (Fig. 3). RS, generating lower plasma glucocorticoid responses than FS, resulted in significantly higher GR binding to *Fkbp5* GRE2 and *Per1* GRE (Fig. 3*B*). Thus, MR and GR binding to GREs after stress is virtually an on/off switch possibly controlled by other factors in addition to glucocorticoids.

Selective MR and GR Binding to GREs Within Intron 5 of the *Fkbp5* Gene. GR regulation of the *Fkbp5* gene occurs predominantly via interaction with intronic GREs (17). Previously, a study in vitro has shown that the intron 5 GRE2 site (Fig. S3) is crucial for glucocorticoid stimulation of *Fkbp5* gene transcription, whereas the GRE1 site within this intron was inactive (17). In Figs. 2 and 3, we presented significant increases in MR and GR binding to the *Fkbp5* GRE2 site after FS. In Fig. S4, we compared receptor binding to *Fkbp5* GRE1 and GRE2 at AM and 30 min after FS. In contrast to GRE2, there was no significant increase in MR or GR binding to GRE1 after FS, which corresponds with reports (17) that this site is not actively involved in transducing glucocorticoid effects on *Fkbp5* transcription.

MR and GR Interaction at GREs Within Glucocorticoid Target Genes. Our results show that both MRs and GRs bind to GREs within target genes after stress and at the circadian peak of glucocorticoid secretion. It is unclear, however, whether the receptors bind to separate GREs, thus strictly as homodimers, or whether they can bind concomitantly at the same GRE site as heterodimers. Although there are indications from cell culture and cell-free studies (10–12) that MRs and GRs may interact with GREs as heterodimers, direct evidence that this may be occurring at the chromatin level under physiological conditions in vivo is lacking. To resolve this question, we adopted a serial ChIP approach. We reasoned that, if MR and GR interact at the same GREs within a given gene, then immunoprecipitation (IP) of one receptor would lead to relative depletion of the other receptor. Fig. 4 shows that, if ChIP was conducted after FS stress for either receptor first followed by a second ChIP for the other receptor on the unbound fraction, then significantly less binding for this receptor at *Fkbp5* GRE2 was measured after the second ChIP. This result indicates that ChIP for MR leads to a reduced ChIP outcome for GR and vice versa, providing indirect evidence that MRs and GRs are binding concomitantly to the same GREs. This phenomenon was only observed after FS and not observed in the AM and PM samples. A similar result was found regarding GR binding to the *Per1* and *Sgk1* GREs conducted after MR ChIPs (Fig. S5 *B* and *D*) but not found for MR binding to these GREs after GR ChIP (Fig. S5 *A* and *C*), possibly because the stress-induced increases in MR binding to the *Per1* and *Sgk1* GREs are lower in magnitude than the rise in binding to the *Fkbp5* GRE2 (Fig. 2). This experiment provides indirect evidence that MR and GR may interact in part at the same GRE sites within glucocorticoid target genes after stress.

Evidence Supporting MR/GR Heterodimerization at GREs Within Glucocorticoid Target Genes. To provide direct evidence for concomitant binding of MR and GR to the same GRE sites within glucocorticoid target genes, we applied a tandem ChIP protocol. We conducted an MR ChIP or a GR ChIP on hippocampal chromatin of AM or FS (30 min) rats, and subsequently, we re-chromatin immunoprecipitated the eluted immunoprecipitated chromatin with an antibody against the same (ChIP and re-ChIP samples: MR→MR and GR→GR) or the other receptor (MR→GR and GR→MR). This procedure was followed by quantitative PCR (qPCR) analysis of GREs within *Fkbp5* (GRE2) (Fig. 5), *Per1*, and *Sgk1* (Fig. S6). Based on the different tandem ChIP combinations, the MR→GR and GR→MR tandem ChIP selectively revealed concomitantly bound MR and GR (indicating MR/GR heterodimer formation) at the qPCR-targeted GRE. The MR→MR and the GR→GR combinations determine the binding of the respective homodimers (MR/MR and GR/GR, respectively) plus the binding of MR/GR heterodimers. Accordingly, the difference between the MR→MR or the GR→GR combination and the parallel MR→GR or GR→MR combinations would provide an estimate for the contribution of the respective MR/MR and GR/GR homodimers to the ChIP result. These tandem ChIPs provide tangible but not absolute evidence for MR/GR heterodimerization, because MR and GR cooccupancy of GREs could possibly be occurring in conjunction with other proteins.

Our results show that the extent of putative homodimer and heterodimer formation at GREs under AM and stress conditions was highly gene-dependent (Fig. 5 and Fig. S6). Regarding *Fkbp5* GRE2, under AM conditions, it appeared that there was higher MR/MR homodimer binding than MR/GR or GR/GR binding,

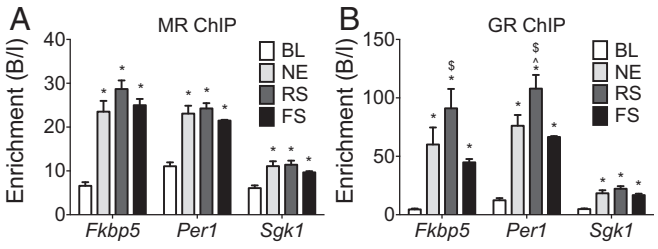

Fig. 3. Effects of different stressors on MR and GR binding to glucocorticoid target genes in the hippocampus. Rats were killed either under baseline AM conditions (BL) or 30 min after stress onset. The graphs show mean enrichment [bound/input (B/I); ±SEM; $n = 8$ for baseline group; $n = 4$ for stress groups] at GREs within glucocorticoid target genes after (*A*) MR or (*B*) GR ChIP on hippocampal chromatin. $^{*}P < 0.05$ compared with the respective BL group; $^{\wedge}P < 0.05$ compared with the respective NE group; $^{\$}P < 0.05$ compared with the respective FS group.

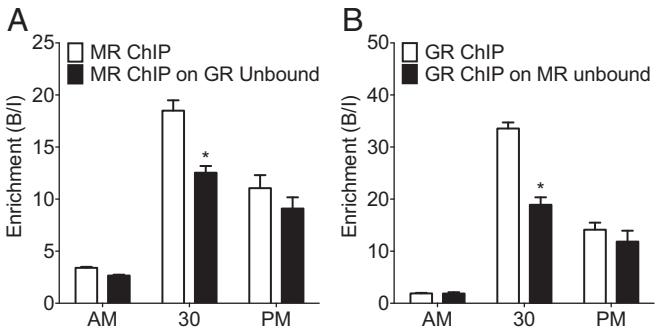

Fig. 4. Comparison of MR and GR ChIP on original vs. GR/MR unbound hippocampal chromatin at the *Fkbp5* GRE2 under baseline conditions and after stress. Rats were killed under AM or PM conditions or at 30 min after the start of FS (15 min, 25 °C water). The graphs show mean enrichment [bound/input (B/I); ±SEM; n = 3–4 per group] at *Fkbp5* GRE2 after (*A*) MR ChIP on original chromatin (white bars) and on the unbound fraction of chromatin after GR ChIP (black bars) or (*B*) GR ChIP on original chromatin (white bars) and on the unbound fraction of chromatin after MR ChIP (black bars). *$P < 0.05$ compared with the respective ChIP at the same time point.

but differences were not statistically significant (Fig. 5). FS resulted in a significant increase in the binding of putative MR/GR heterodimers to *Fkbp5* GRE2 as revealed by both MR→GR and GR→MR tandem ChIPs (Fig. 5). Because there was no significant difference between the MR→MR and the MR→GR results, it is likely that MRs participate in binding to this GRE only together with GRs as heterodimers and not as MR/MR homodimers (Fig. 5*A*). After stress, the substantial difference between the GR→GR and the GR→MR results (Fig. 5*B*) indicates that, in addition to forming heterodimers with MRs, GRs also bound significantly as GR/GR homodimers.

Under baseline conditions and after FS stress, the MR→GR ChIP result was significantly lower than the MR→MR result at *Per1* GRE, indicating that, under these conditions, corticosteroid receptors may be binding as both MR/MR homodimers and MR/GR heterodimers (Fig. S6*A*). In addition, the rise in GR→GR binding after stress at *Per1* GRE was highly significant, but the increase in GR→MR binding failed to reach statistical significance (Fig. S6*B*). In conjunction, these tandem ChIP data suggest that, after stress, MRs and GRs bind to the *Per1* GRE largely as GR/GR homodimers and to a lesser extent, as MR/MR homodimers and MR/GR heterodimers (Fig. S6 *A* and *B*). At the *Sgk1* GRE, FS increased both MR→MR and GR→GR binding (Fig. S6 *C* and *D*). Given that FS-induced binding in MR→GR and GR→MR failed to reach statistical significance, it is likely that this gene is regulated predominantly by homodimers (Fig. S6 *C* and *D*).

Discussion

This study shows that, in the hippocampus, an acute stressful challenge transiently increases MR and GR binding to GREs within the glucocorticoid target genes *Fkbp5*, *Per1*, and *Sgk1* and enhances transcription of these genes. Surprisingly, despite the high occupancy level of MRs under baseline conditions (2, 4), a relatively low binding of this receptor to GREs was observed under these conditions. Different stressors, although evoking different glucocorticoid peak levels, resulted in largely similar increases in MR and GR binding to GREs within these genes. Overall, we observed that the interaction of MRs and GRs with GREs under the various conditions investigated was highly gene-dependent. Sequential and tandem ChIP analyses showed that, after stress, MRs and GRs may bind as homodimers as well as heterodimers to *Fkbp5* and *Per1* GREs, whereas *Sgk1* GRE appeared only to be bound by the respective homodimers. These data show that the interaction of GRs with the genome seems to be a reflection of circulating glucocorticoid levels and expected receptor occupancy levels, whereas MRs' genomic interaction may be restricted under baseline AM conditions and/or depend on cobinding with GRs. Together, these results reveal gene-dependent and receptor-specific modes of interactions with the genome, which cannot be predicted solely on the basis of hormone concentrations and receptor occupancy levels.

FS caused a significant increase in RNA expression of *Fkbp5*, *Per1*, and *Sgk1*, which is consistent with their well-known responsiveness to glucocorticoids (17–20). The hnRNA levels for *Per1* and *Sgk1* peaked at 30 min, whereas *Fkbp5* hnRNA levels reached their maximal levels later at 60 min. The mRNA expression levels after stress followed the hnRNA responses with a delay of at least 30 min in all genes, indicative of time required for the splicing process. Whereas GREs in the *Per1* and *Sgk1* genes are located in their proximal promoter region, in the *Fkbp5* gene, the transcriptionally active GREs are located within intronic sequences (6, 17–19, 21, 22). In the rat, GRE2 within intron 5 has been identified as being particularly important for glucocorticoid-induced *Fkbp5* transcription (17). Receptor-bound GREs within introns of the *Fkbp5* gene are thought to stimulate gene transcription through chromatin remodeling including loop formation that allows the direct interaction of the intronic region with the transcriptional start site (23, 24). Such a process is anticipated to require more time than the direct transactivational stimulation originating from promoter-located GREs, like in *Per1* and *Sgk1*, which may explain the differences in the time course of the stress-induced hnRNA (and mRNA) responses, despite similar time courses of MR and GR binding to these GREs.

Although corticosteroid receptor interaction with glucocorticoid target genes has been studied under pharmacological conditions [e.g., glucocorticoid injections in ADX rats (9)], the interaction of MRs and GRs with such genes in the hippocampus under glucocorticoid-relevant physiological conditions has not been studied to date. Under baseline conditions, MR and GR binding levels at GREs were relatively low in the early morning but rose significantly during the day, reaching significantly elevated levels in late afternoon, except for MR binding at the *Sgk1* GRE. An acute FS challenge evoked a substantial rise in receptor binding to GREs, with MRs reaching near-maximal level at 15 min and GR binding peaking at 30 min. These peak binding levels superseded the respective levels observed at baseline PM. The receptor binding profiles at baseline and after stress largely followed the circulating CORT levels. Regarding GR binding to GREs, this result may have been expected given that studies had shown that GR occupancy by endogenous glucocorticoids critically depends on circulating hormone concentrations (2, 4). The peak in GR binding at 30 min after stress concurs with the peak in stress-induced plasma glucocorticoid levels, but in view of recent

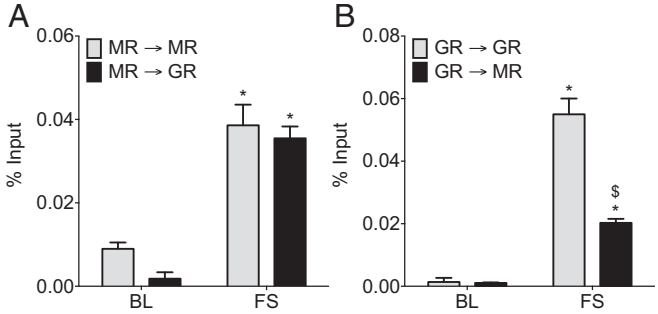

Fig. 5. Tandem ChIP for MR and GR binding to *Fkbp5* GRE2 in the hippocampus under baseline conditions and after stress. Rats were killed either under AM conditions [baseline (BL)] or 30 min after the start of FS stress. Graphs show percentage input (mean ± SEM; n = 3 per group) at *Fkbp5* GRE2 after (*A*) MR ChIP immediately followed by MR, GR, or IgG (negative control) binding to the MR bound chromatin or (*B*) GR ChIP followed by GR, MR, or IgG binding to the GR bound chromatin. The IgG levels (percentage input) were deducted from the MR and GR bound data. *$P < 0.05$ compared with the respective BL ChIP; $^{\$}P < 0.05$ compared with GR→GR FS ChIP.

findings on stress-induced free CORT levels (16), this finding was unexpected. Recent microdialysis studies in vivo have shown that, after FS stress, the peak in free CORT in the hippocampus is delayed 20–30 min compared with the plasma hormone response (16, 25). Thus, because the free CORT concentration is the critical parameter for hormone–receptor interaction, maximal GR binding to GREs after stress would be expected to occur at 60 min rather than at 30 min. At 60 min poststress, however, GR (and MR) binding levels were substantially lower than at 30 min. Thus, GR's interaction with GREs (and MR's interaction as well) is only partly determined by glucocorticoid levels, indicating the on and off status regarding GREs is actively regulated by additional molecular factors (26).

The binding profile of MRs to GREs under baseline and stress conditions is remarkable, because Reul and de Kloet (2) reported 30 y ago that hippocampal MRs are at least 80% occupied with endogenous glucocorticoids under all physiological conditions studied (4) and that occupancy levels only dropped after adrenalectomy (4). Therefore, we had expected MR binding to GREs to be relatively high under baseline AM conditions, with only small increases after stress. Our results, however, show a different picture: relatively low binding at baseline AM and substantial increases after stress and at baseline PM (except *Sgk1* GRE). Thus, high occupancy of MR does not translate into high binding to GREs. One reason may be that, under baseline AM conditions, MR binding to GREs is restricted because of an action of a steroid receptor corepressor like death-associated protein (DAXX), which after stress, is expunged and/or exchanged for a steroid receptor coactivator like Fas-associated factor 1 (FAF-1). DAXX and FAF-1 are hippocampal proteins that have been shown to modulate MR transcriptional activity in hippocampal cells in vitro (27). Alternatively, MR binding to GREs may be weak as supported by early transfection studies in vitro (28). Trapp et al. (10) showed that DNA binding of MR was low in monkey kidney COS-1 cells solely transfected with MR (compared with GR) but could be increased dramatically when both receptors were transfected together. Furthermore, transcriptional activity of cotransfected receptors was higher than that of separately transfected receptors (10). Thus, MR binding in the absence of activated GRs, such as is the case under baseline AM conditions, is weak, which changes considerably after GRs become activated because of stress-induced glucocorticoid production. In other words, MRs seem to require GRs for substantial binding to GREs to occur. This notion is consistent with MRs and GRs heterodimerizing after stress.

In absolute terms, MR and GR binding to GREs within *Fkbp5* and *Per1* after stress was overall substantially higher than receptor binding observed to *Sgk1* GRE. Because results are from the same ChIP DNA samples, these binding profiles are directly comparable. The observed differences in receptor binding were consistent across the different stressors. Because each hippocampal cell contains two copies of each gene (if located on autosomal chromosomes), theoretically, MR and GR binding to GREs could be similar, but this situation is clearly not the case. Possibly, there is less availability of the *Sgk1* GRE for binding compared with the *Fkbp5* GRE2 and *Per1* GRE, because in a significant number of hippocampal cells, the gene may be located within inactive, condensed chromatin. In situ hybridization analysis suggests that *Sgk1* mRNA is indeed primarily expressed in hippocampal pyramidal neurons, whereas *Fkbp5* and *Per1* mRNA is ubiquitously expressed in hippocampal neurons (29). Alternatively, there may be differences at the GRE level in terms of its nucleotide sequence as well as involvement of local modulators (e.g., steroid receptor coregulators) affecting the affinity and stability of receptor–GRE interactions. Our work points to a role of other molecular mechanisms in vivo in addition to GRE nucleotide sequence, because although both GRE1 and GRE2 within the *Fkbp5* present ideal nucleotide consensus sequences, MR and GR binding to these GREs is dramatically different. Whereas GRE2 shows significant increases in MR and GR binding after FS, GRE1 shows no significant change in binding. Our findings correspond with transcriptional analyses in vitro showing that GRE2 is a transactivationally active site, whereas GRE1 is not (17) and may be explained by distinct accessibility of GRE1 vs. GRE2 as a result of epigenetic and other molecular (e.g., steroid receptor coregulators) mechanisms (23, 24, 26). These observations are consistent with our notion that MR and GR binding to GREs within the genome is highly controlled at the cellular level as well as the single-gene level.

To investigate stressor specificity and the role of different levels of stress-induced glucocorticoid levels, we compared the effects of FS with those of NE and RS. FS and RS are strong stressors, resulting in high glucocorticoid responses, whereas NE is regarded as a mild psychological stressor, leading to moderate increases in plasma hormone levels (16). MR binding to GREs was very similar after the different stressors, albeit with consistent intergene differences. Apparently, the mechanisms triggering MR binding to GREs are independent of the extent of stress-induced glucocorticoid responses and other stressor-specific mediators. The independence of glucocorticoid responses is not surprising, because MRs are already highly occupied at baseline AM glucocorticoid levels (2, 4). Our findings regarding GR binding to GREs were, however, surprising, because despite the substantial difference in glucocorticoid responses between stressors, the interactions of GRs with GREs were largely similar. These observations underscore that the glucocorticoid response is not an all-determining factor in the genomic action of GRs (and MRs). It seems that additional stressor-specific factors are involved in determining GR binding to GREs in hippocampal cells, including signaling pathways, epigenetic factors, and local modulators. An additional factor may be the duration of the stressful experience: the RS (and NE) experience lasted the full 30 min until death, whereas FS lasted 15 min, after which the rats were returned to their home cages for the remaining 15 min. Therefore, the shorter-lasting FS challenge may have allowed rats to shut down the stress response, resulting in lower GR binding levels. Elucidation of the factors determining GR (and MR) interaction with the genome should be an intriguing challenge for future research.

To study if MR and GR are acting separately (as homodimers) to stimulate transcription of glucocorticoid target genes or possibly, together in a complex, we initially performed a serial ChIP first with one antibody (anti-MR or anti-GR) and then, rechromatin immunoprecipitated the unbound fraction using the opposite receptor antibody (either MR or GR). The rationale was that, if GR and MR were interacting at the same GRE, IP of one DNA-bound receptor with the first antibody would also result in IP of the other receptor into the bound fraction and deplete it from the unbound fraction. A subsequent ChIP for the "other" receptor on the unbound fraction from the first ChIP would recover less target DNA compared with the amount recovered in the original ChIP. If, however, MR and GR were not bound to the same DNA strand but instead, bound to the same GRE location but on different strands, recovered DNA (covering this GRE site) would be comparable between ChIPs performed on both the original chromatin and the unbound fraction of opposite receptor ChIP. We found that MR binding to GREs after stress was substantially reduced if GRs had been removed from the sample by IP previously and vice versa. These effects were most clear for the *Fkbp5* GRE2, most likely because this GRE presented the largest stress-induced MR binding response. Cross-receptor depletion was only observed in hippocampal chromatin samples from stressed rats but was not observed in chromatin from baseline PM animals, ruling out that depletion is the result of an assay artifact and indicating that MRs and GRs interact concomitantly with GREs within glucocorticoid target genes specifically after stress. We used a more direct approach to investigate MR and GR interaction at GREs in vivo using MR→GR and GR→MR tandem ChIPs, which only immunoprecipitate GRE DNA bound to both MR and GR at the same time. The results show that, after stress, MRs and GRs bind concomitantly to the same GRE sites within *Fkbp5* and *Per1* genes, possibly through the formation of heterodimers. Previous co-immunoprecipitation (co-IP) experiments in a cell-free system have shown that MRs and GRs can

heterodimerize in solution (11); our work provides evidence to support that this can occur at the DNA template in vivo. Moreover, after stress, MRs and GRs may bind to GREs as heterodimers as well as homodimers but with striking gene-dependent differences. After stress, there appeared to be a strong recruitment of MR/GR heterodimers at the *Fkbp5* GRE2, less at the *Per1* GRE, and very low recruitment at the *Sgk1* GRE. Thus, local chromatin status possibly defined by epigenetic factors in conjunction with coregulatory factors may determine the level of recruitment as well as preference for homo- vs. heterodimer binding. MR and GR cotransfection studies indicated that formation of the MR/GR heterodimer results in stronger GRE binding and greater reporter gene responses than shown by the respective homodimers (10). Presently, the gene transcriptional significance of MR/GR heterodimer formation is unclear. Our findings allow for the study of the significance of heterodimer formation for gene transcriptional responses using pharmacological approaches as well as high-throughput sequencing methods.

Based on receptor occupancy studies, 30 y ago, de Kloet and Reul (2,5) proposed a concept on the role of MRs and GRs in the effects of glucocorticoids on the brain. In view of its constant high occupancy, it was thought that MRs exert a tonic influence on hippocampus function, including neuronal excitability, hypothalamic–pituitary–adrenal (HPA) axis activity, sympathetic outflow, and cognitive behavior (2, 5, 30). GRs only became significantly occupied by elevated glucocorticoid levels and were thought to exert negative feedback action on HPA axis activity and facilitate memory formation of stressful events (2, 5, 31). In view of our data, this concept may require adjustments. The interaction of MRs and GRs with GREs under baseline and stress conditions and its gene transcriptional consequences seem much more complex than originally thought. The terminology tonic and feedback fall short in view of the multitude of mechanisms controlling the interaction of these steroid receptors with GRE sites, including the highly diverse molecular processes governing accessibility of such sites within different genes and different cells and moreover, their distinct, gene-specific way to interact with GREs, probably as homodimers and heterodimers. This complexity may further grow after MR and GR binding has been conducted across the entire genome by ChIP sequencing. This work has provided the basis to continue elucidating the critical question of how glucocorticoids affect brain function. The answer to this question may hold the key to resolving stress-related disorders.

Materials and Methods

Animals and Stress Procedures. Male Wistar rats (150–175 g) were purchased from Harlan and group-housed. Rats were forced to swim for 15 min in 25 °C water or subjected to RS or NE or left undisturbed (32). Rats were killed under baseline conditions or at the indicated times after stress (shown in the figures). All animal procedures were approved by the University of Bristol Ethical Committee and the Home Office of the United Kingdom (Animal Scientific Procedures Act, 1986, UK).

Tissue Preparation. After decapitation, the entire hippocampus was dissected, or the dentate gyrus and CA regions were microdissected from the dorsal hippocampus. Tissues were snap-frozen in liquid N_2 and stored at −80 °C.

ChIP, RNA Analysis, and qPCR. Hippocampal chromatin preparation (Fig. S7), ChIP, and RNA extraction were performed using published methods (33). Analysis by qPCR was conducted using primer and probe sets listed in Dataset S1 (34). Control experiments validated our ChIP method (Fig. S8).

Statistical Analysis. Data were analyzed by ANOVA and appropriate posthoc tests. More information on materials and methods is in *SI Materials and Methods*.

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