Expression and function of nuclear receptor coregulators in brain: understanding the cell-specific effects of glucocorticoids

Laan, S. van der

Chapter V

Chromatin immunoprecipitation scanning identifies glucocorticoid receptor binding regions in the proximal promoter of a ubiquitously expressed glucocorticoid target gene in brain

S van der Laan, RA Sarabdjitsingh, MF Van Batenburg, SB Lachize, H Li, TF Dijkmans, E Vreugdenhil, ER de Kloet and OC Meijer

Abtract

While the actions of glucocorticoids on brain functions have been comprehensively studied, the underlying genomic mechanisms are poorly understood. Here we show that GILZ mRNA is strongly and ubiquitously induced in rat brain. To decipher the molecular mechanisms underlying these genomic effects, it is of interest to identify the regulatory sites in the promoter region. Alignment of the rat GILZ promoter with the well-characterized human promoter resulted in poor sequence homology. Consequently, we analyzed the rat 5'flanking sequence by Matrix REDUCE and identified two high-affinity glucocorticoid response elements (GRE) located 2kb upstream of the transcription start site. These findings were corroborated using the glucocorticoid receptor (GR) expressing Ns-1 PC12 rat cell-line. In these cells, dexamethasone treatment leads to a progressive increase of GILZ mRNA expression levels via a GR-dependent mechanism. Subsequently, using chromatin immunoprecipitation assays we show that the two high-affinity GREs are located within the GR-binding regions. Lastly, we demonstrate using multiple tissue in situ hybridisation a marked increase in mRNA expression levels in spleen, thymus, heart, lung, liver, muscle, testis, kidney, colon, ileum, as well as in brain and conclude that the GILZ gene can be used to study glucocorticoid effects in many additional rodent tissues.

1. Introduction

Glucocorticoid hormones modulate brain functioning and are considered essential for adaptation to stress. These hormones exert their actions in the mammalian brain by binding to intracellular glucocorticoid and mineralocorticoid receptors (GR and MR) in target cells (1;2). Upon hormone binding, the activated receptor translocates from the cytoplasm to the nucleus to modulate expression of target genes. In one important mode of action, the ligand-activated receptor scans the genome and interacts with specific response elements in the promoter region of target genes (3-5), termed glucocorticoid response elements (GREs). Once DNA-bound, the receptor coordinates the assembly of coregulator proteins and interacts with the basal transcription machinery to modulate gene expression. The type and concentration of the ligand, the architecture of the GREs and coregulator proteins present in the cell are all known to affect the transcriptional effects of the GR on target genes (6-8). Even within a single cell type GR can either activate or repress the expression of various target genes in this manner (9).

The GR is ubiquitously expressed in brain tissue and is known to be critically involved in the regulation of physiological processes such as neuronal excitability and cognitive functions (10;11). Despite the large number of glucocorticoid target genes identified in different cell lines and tissues, little is known about the physical association of the GR proteins within specific promoter regions in the brain. Previous studies comprehensively described binding of the GR to genome fragments containing GREs in cell lines, as well as in liver (12;13). Hitherto, the mechanisms underlying the genomic actions of glucocorticoids in brain are poorly understood. Therefore, it is of great interest to find a methodology to monitor GR chromatin occupancy in brain tissue. A recently developed method that examine protein-DNA interactions within the context of living cells, *i.e* chromatin immunoprecipitation (14;15), has not yet been performed to study DNA-binding by GR in brain. This approach would allow comprehensive analysis of the dynamics of binding of steroid receptors to the DNA, as well as studying the involvement of coregulator molecules as mediators of glucocorticoid effects in the brain (16).

Large-scale gene expression profiling of brain structures revealed to be challenging due to the high heterogeneity of the tissue samples and because gene expression is highly contextual (17;18). A large number of genes are expressed in brain and relatively small changes in expression levels have been reported after corticosterone treatment (19;20). Therefore, in order to further unravel the genomic actions of glucocorticoids in brain it is essential to find a robustly inducible glucocorticoid target gene that is expressed in rat brain.

In the present study, we sought for a ubiquitously expressed glucocorticoid target gene in brain. We show that the glucocorticoid-induced leucine zipper (GILZ) mRNA expression levels are strongly induced by corticosterone treatment in young adult rat brains. Due to poor sequence homology between the rat and human promoter, we screened the rat sequence for putative GREs using a position weight matrix. In addition, we found that GILZ mRNA expression levels are induced by glucocorticoids in rat Neuroscreen PC-12 cells (Ns-1 PC12) over time in a GR-dependent mechanism. Finally, using ChIP-assays we scanned the proximal promoter of the rat GILZ gene for GR binding, and provide evidence that the two putative GREs are localized within the GR-binding regions. We conclude that GILZ is induced via GR-binding to the regulatory sites in the proximal promoter of the rat gene. Finally, we propose that this promoter and its identified regulatory sites can indeed be used as a new molecular marker to monitor GR chromatin occupancy and transcriptional coregulator recruitment in brain.

2. Material & methods

2.1 Animals and tissue preparation

All animal experiments were performed in accordance with the European Communities Council Directive 86/609/EEC and with approval from the animal care committee of the Faculty of Medicine, Leiden University (UDEC numbers 04052 and 06055). Adult male Sprague-Dawley rats (300g; n=16) were obtained from Harlan (Leiden, The Netherlands) and adult male c57bl/6 mice (26g; n=16) from Janvier (France).

2.2 Multiple tissue in situ hybridisation in c57bl/6 mice

Sixteen adult male c57bl/6 mice were adrenalectomised under isoflurane anaethesia, single housed, and fed with oats containing 400 µg corticosterone or vehicle. Mice were decapitated 3 hours after oats administration, trunk blood was collected and organs were dissected and frozen on dry ice. Two mice were killed at 30 minutes after treatment to determine peak levels of hormone. Plasma corticosterone was determined by radio immuno assay (ICN Biomedicals, Costa Mesa, CA). Peak levels of plasma corticosterone 30 minutes after administration were 28±8 µg/dl. Since there can be a considerable degree of cell-specificity in the transcriptional response to glucocorticoids, we harvested multiple organs and evaluated expression and regulation of the mRNAs using multiple tissue in situ hybridisation. Tissues were cut at 16 µm in a cryostat and collected on poly-L-lysine coated slides for *in situ* hybridisation.

2.3 Regulation and expression of GILZ in brains of Sprague-Dawley rats

To investigate GILZ responsiveness to corticosterone in brain, adrenalectomised male Sprague Dawley rats received a 3mg/kg intraperitoneal (i.p.) corticosterone injection or left untreated (n=8). Blood samples were taken from the tail vein by a small incision with a razorblade every 30 minutes after i.p. injection and collected in EDTA-coated tubes (Sarstedt). Plasma was obtained by centrifugation at 10000 x g rpm for 20 minutes at 4^{o}C and subsequently stored at –20^{o}C until assayed. Three hours after treatment all animals were sacrificed by decapitation. Tissues were cut at 20 µm in a cryostat and collected on poly-L-lysine coated slides. All in situ hybridisation experiments were performed as previously described (21). For RNA isolation and cDNA synthesis, the brains were dissected and the thalamus area was homogenized using the PRO200 homogenizer (Pro Scientific, Oxford , CT, USA). The samples were centrifuged at 13000 rpm 20 min. at 4^{o}C and subsequently stored at –20^{o}C until RNA extraction.

2.4 Oligonucleotides for in situ hybridisation and qPCR

A detailed description of oligonucleotides design for *in situ* hybridisation and qPCR is given in the Supplementary data (material and methods and table 1 & 2 in Supplementary data). Table S1: Sequence of primers designed for mRNA amplification by qPCR of target genes. Table S2: Sequence of primers used to ‘scan’ the proximal promoter of GILZ by by ChIP experiments (rattus norvegicus).

2.5 Tissue culture

Neuroscreen-1 PC12 (Cellomics ,Berkshire, UK) cells were grown and maintained in RPMI 1640, supplemented with 5% Foetal Bovine Serum, 10% Horse Serum, penicillin (20 U/mL) and streptomycin (20 ug/mL) (all Invitrogen Life Technologies, Breda, The Netherlands). To assess whether the rat GILZ gene is induced by GR, a day prior treatment, 1x10^{6} Ns-1 PC12 cells were seeded on collagen-coated (Roche) 100 mm dishes and treated

with ethanol (vehicle) or 10^{-7}M dexamethasone (DEX) for different time points. Next, total RNA was extracted using Trizol reagent (Invitrogen) and cDNA synthesis was performed as aforementioned.

2.6 Chromatin immunoprecipitation assays (ChIP)

Ns-1 PC12 cells were treated for 90 min. with either 10^{-7}M dexamethasone or ethanol. ChIP-assays were performed as described in detail in Supplementary data (figure of ChIP-assays in A549 cells). Fold-enrichment values were calculated as follow: fold enrichment for specific primer = $(GR^{DEX}/Igg^{DEX})/(GR^{EtOH}/Igg^{EtOH})$. Recovery is expressed as percentage of inputs.

2.7 Computational analysis

To identify potential GREs, we processed DNA sequence by Matrix REDUCE in which a position specific affinity representation of the Transfac position weight matrix M00205 (http://www.gene-regulation.com/cgi-bin/pub/databases/transfac/getTF.cgi?AC=m00205) was being used (Batenburg et al., unpublished). This representation is called a position specific affinity matrix, or PSAM (22). In the PSAM every nucleotide in the weight matrix has a value between 0 and 1. The product of the weights evaluated for a short segment associated with a PSAM at a location in the DNA, reflects the likelihood that the element at that locus is a potential GRE. The 3kb human GILZ proximal promoter DNA segment (Ensembl gene ID ENSG00000157514) and its orthologous sequence in rat (Ensembl gene ID ENSRNOG00000013786) were processed to identify potential GREs at various distances upstream TSS measured in nucleotide numbers.

2.8 Statistics

Data were subjected to Student's *t*-test pairwise comparisons. Significance was accepted at $P<0.05$. Results are presented as average ±SD.

3. Results

3.1 Distribution and regulation of a glucocorticoid-induced gene in brain

The aim of present study is to identify a candidate gene for understanding the genomic actions of glucocorticoids in brain. Because for chromatin immunoprecipitation, the amount of tissue is an important factor, we examined GILZ expression and regulation by corticosterone in rat brain. Representative GILZ Hybridisation autoradiographs are shown in Fig. 1. GILZ transcript was ubiquitously detected throughout the brain of adrenalectomised (ADX) animals. The hybridisation signal obtained with mismatch controls did not exceed film background. GILZ mRNA hybridisation signal was the highest in thalamic and hippocampal areas. In hippocampus, the signal was higher in the CA2 and CA3 pyramidal cell layers compared to the CA1. Hybridisation signal was also observed in areas associated with predominant presence of glial cells, such as the stratum radiatum, *i.e.* the border between the CA1 area and the dentate gyrus. In the thalamus, the ventral posteromedial thalamic and the subincertal nuclei showed the highest signal intensity. Cortical areas exhibited moderate homogenous hybridisation signal.

Secondly, to assess whether GILZ is induced by increasing glucocorticoid blood levels, we administered i.p. corticosterone injections to ADX animals. Corticosterone (CORT) plasma concentrations, as measured by radio-immunoassays, revealed to peak at 30 minutes and return to baseline as early as 90 minutes after corticosterone injections (Fig. 2A).

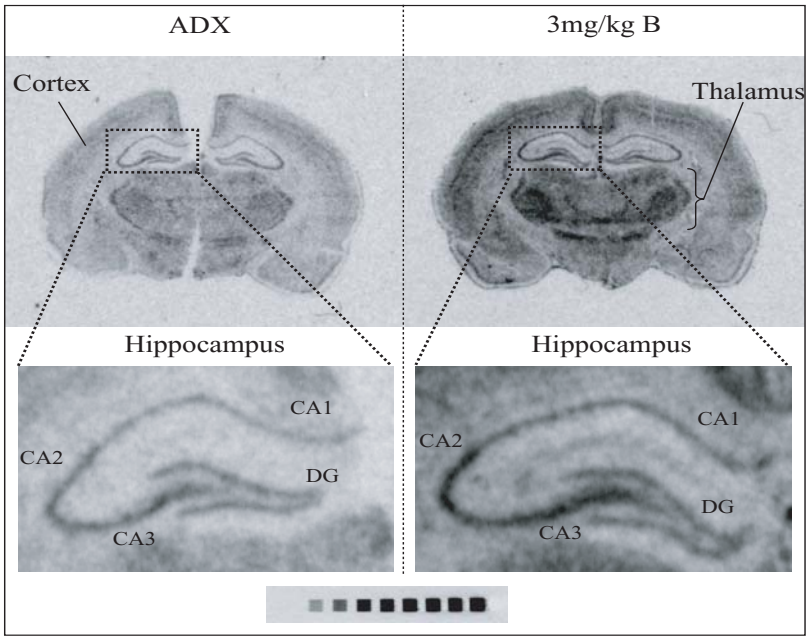

Fig. 1: Neuroanatomical distribution and regulation of GILZ in Sprague-Dawley rats. Representative autoradiographs of GILZ hybridisation signal in untreated and 3mg/kg corticosterone treated ADX animals. GILZ is ubiquitously expressed in rat brain. Following corticosterone treatment GILZ is induced in all hippocampal subfields, cortex and thalamic nuclei. CA: Cornu Ammonis; DG: dentate gyrus; Sub: subincertal nucleus; VPM: ventral posteromedial thalamic nucleus.

GILZ transcript was detected 3 hours after injections (Fig. 1). Overall, GILZ hybridisation signal was higher in all areas studied including thalamus and hippocampus. In hippocampus, treatment did significantly increase GILZ mRNA expression levels in all hippocampal subfields with the highest increase in the pyramidal cell layer of the CA1 area.

To corroborate these findings we tested by means of qPCR the GILZ mRNA contents of thalamic homogenates from ADX animals with and without corticosterone treatment. To control for specificity, the mRNA expression levels of a control gene that is not regulated by glucocorticoids, *i.e.* the potassium voltage-gated channel Kv3.2, were measured in both groups. As shown in Fig. 2B, corticosterone injections in adrenalectomised animals (filled bars) resulted in a 2.4 fold-increase in GILZ mRNA expression levels compared to control animals (open bars). In sum, GILZ is ubiquitously expressed throughout the rat brain and importantly strongly induced by corticosterone treatment. To further study GILZ regulation by glucocorticoids in brain, it is of importance to characterize the regulatory sites in the promoter region of the gene.

3.2 Matrix-based prediction of GREs in rat promoter sequence

Previously, the promoter sequence of the human GILZ gene has been topic of several studies. So far, several functional GREs have been identified in the human promoter (23; 9). A schematic representation of the human GILZ promoter with the precise location of the known response elements is given in Fig. 3A. Alignment of the human and rat promoter sequences resulted in a very low homology; 57% sequence homology in the promoter region

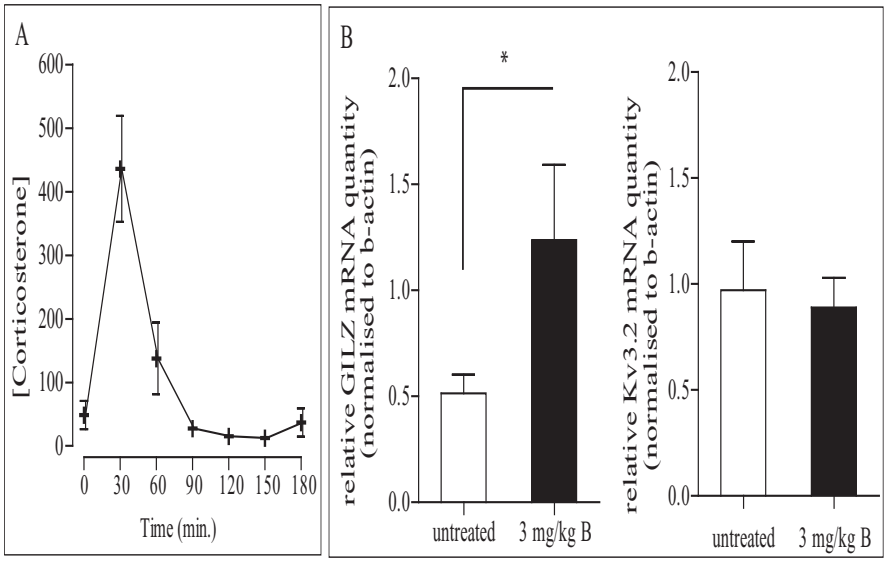

Fig. 2: (A) Plasma corticosterone concentrations at different time points after intraperitoneal injections with 3mg/kg corticosterone in ADX Sprague-Dawley rats. Data are expressed as averages (n=3 ± SD). (B) GILZ and Kv3.2 mRNA levels in thalamic homogenates of untreated (open bars) or corticosterone treated (filled bars) animals. All data were normalized against beta-actin expression levels. Asterisk indicates significantly different from untreated animals.

in contrast to 84% in e.g. exon 3. All previously identified functional GREs in human appeared to be absent or only partially present in the rat sequence. Therefore, to localize putative GREs in the promoter of the rat gene, we screened the genomic sequence using a position-weight matrix (Fig. 3B).First, we validated the matrix by applying it to the human sequence (Fig. 3C). Two GREs were detected; the first GRE located at -1958/-1944 bp (GGAACCcaaTGTTCT) and the second GRE at -2418/-2404 bp (TTAACAgaaTGTTCT) upstream of the transcription start site (TSS). Interestingly, both GREs have an identical second half-site that precisely overlap with the consensus GRE used in the position-weight matrix and known to be essential for DNA binding and transcriptional activity of the GR (24). Using this matrix-based GRE prediction method, we show that within the 3kb sequence upstream TSS of the rat GILZ gene, two putative high-affinity GREs are localized (Fig. 3D). The first one, containing a second half site that precisely overlap with the above mentioned consensus sequence, was found at position -2370/-2355 bp upstream TSS (TCTATActtTGTTCT). The second putative GRE was found at position -1873/-1858 (GGAACCtaaTGTTCC). To substantiate these findings, we further studied the GILZ promoter in the GR-expressing Neuroscreen-1 PC12 (Ns-1 PC12) rat cell-line.

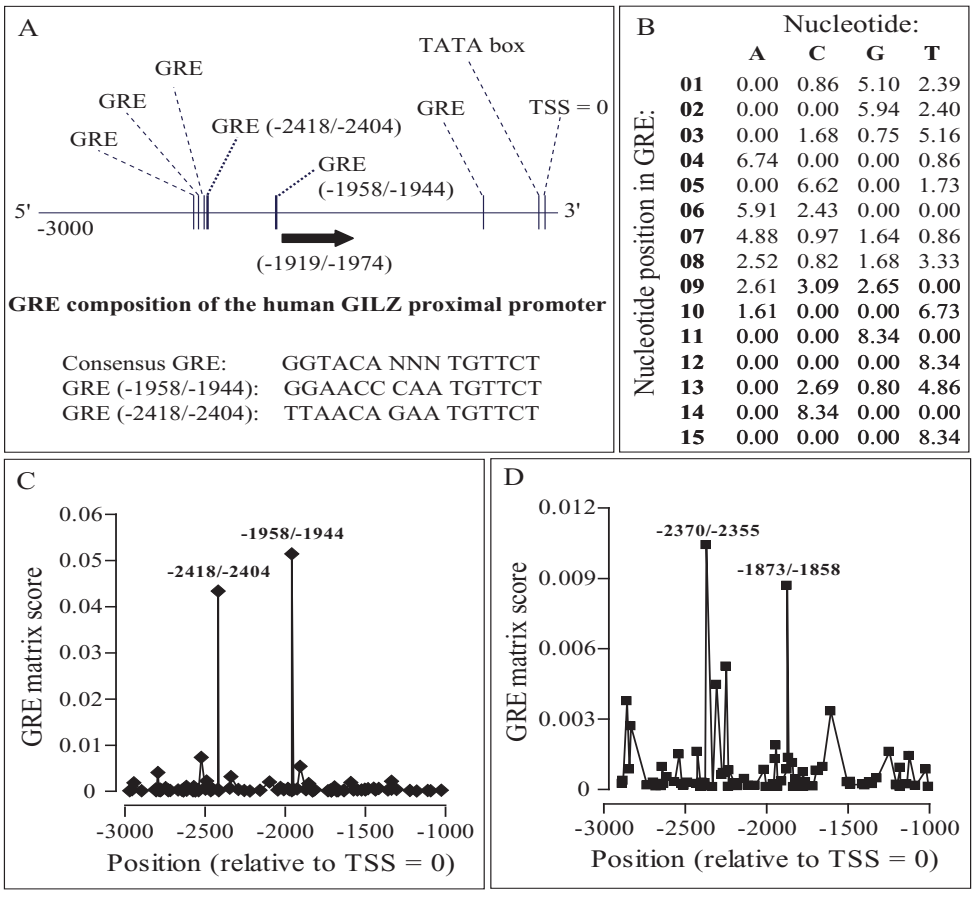

Fig. 3: (A) Schematic representation of the human GILZ promoter and location of the putative GREs relative to the transcription start site (TSS). (B) Position specific affinity matrix (Transfac M00205). Every nucleotide in the weight matrix has a value between 0 and 1. (C) The 3kb human GILZ promoter DNA segment and its orthologous sequence in rat (D) were evaluated using the position specific affinity matrix on potential GREs. The scores represent the products of the weights and reflect the likelihood that the element at that locus is a potential GRE.

3.3 GILZ expression and regulation in rat Ns-1 PC12 cells

In order to gain insight in the molecular interactions between GR and DNA in rat brain, it is necessary to validate the putative GREs in the promoter region of GILZ in a rat cell-line. For this purpose we tested GILZ responsiveness to glucocorticoid treatment in the GR-expressing rat pheochromocytoma Ns-1 PC12 cells. A time course of GILZ mRNA expression levels after dexamethasone (DEX) treatment was performed. As can be seen in Fig. 4, a progressive increase in expression level was found over a period of 6 hours. GILZ mRNA expression levels were significantly induced as early as 2 hours following DEX treatment (4x higher compared to vehicle treated cells), suggesting that promoter occupancy by GR is likely to occur within 2 hours following treatment. Importantly, we have found that GR expression is indispensable for DEX to induce the expression of GILZ mRNA in these cells.

Transfection of a specific shRNA construct directed against the GR (95% knock-down of

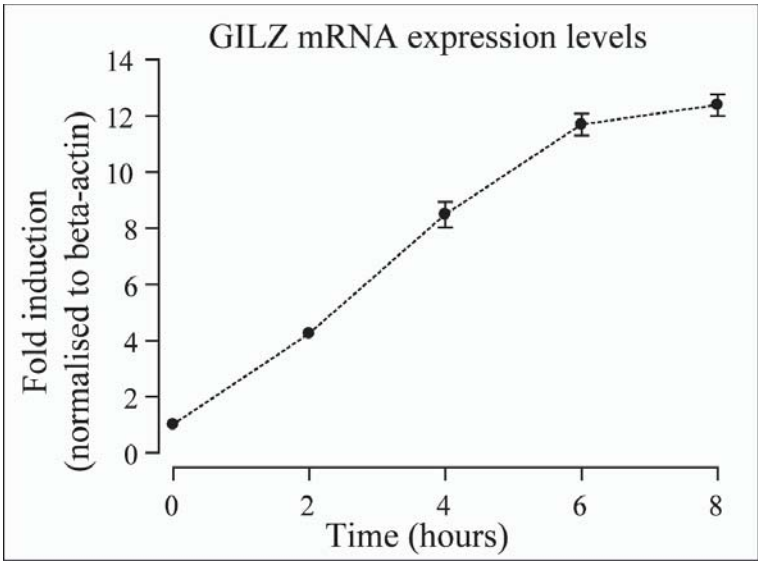

Fig. 4: Time course of GILZ mRNA expression levels in endogenously GR-expressing rat Neuroscreen-1 PC12 (Ns-1 PC12) cells treated with 10-7M DEX. All data were normalized against beta-actin expression levels. GILZ mRNA expression level progressively increases over a period of at least 6 hours of 10-7M DEX treatment. All data are expressed as averages (n=4 ± SD).

the GR protein (8)) resulted in a complete loss of DEX-induced stimulation of GILZ mRNA expression (Dijkmans TF *et al. in preparation*). In sum, DEX treatment induces in a GR-dependent mechanism the expression of GILZ mRNA.

3.4 GR-binding regions in the rat promoter

To identify the specific genomic regions occupied by GR in the rat GILZ promoter, we designed 14 primer pairs for 'scanning' the region up to 5kb upstream of the transcription start site (TSS) of the gene (see table 2 in Supplementary data). All primer pairs were separated by approximately 500bp, *i.e.* the average length of the genomic fragments resulting from sonication. A preliminary scan of the promoter with all 14 primer pairs resulted in a fold-enrichment >3 for primer pairs 6, 7, 8 and 14 using two different GR-specific antibodies (Fig. 5A-B). Signal intensity was the highest around primer pair 7, suggesting the presence of a GRE in this GR binding region. Although both antibodies resulted in a highly similar GR binding profile over the 5kb genomic stretch, the H300 antibody was used for subsequent ChIP experiments because of the higher obtained sensitivity. To validate the preliminary ChIP-scan, we concentrated on GR-binding in the genomic region targeted by primer pair 7. Expectedly, DEX treatment resulted in a significant and marked increase in recovery indicating that this region contains GR-binding elements (Fig. 5C). Importantly, the two high-affinity GREs predicted by computational analysis are located precisely within the GR-binding region identified by ChIP-scan. The region targeted by primer pair 7 is located exactly in between the two predicted GREs, reflected by the highest fold enrichment observed with the ChIP-scan using this primer pair.

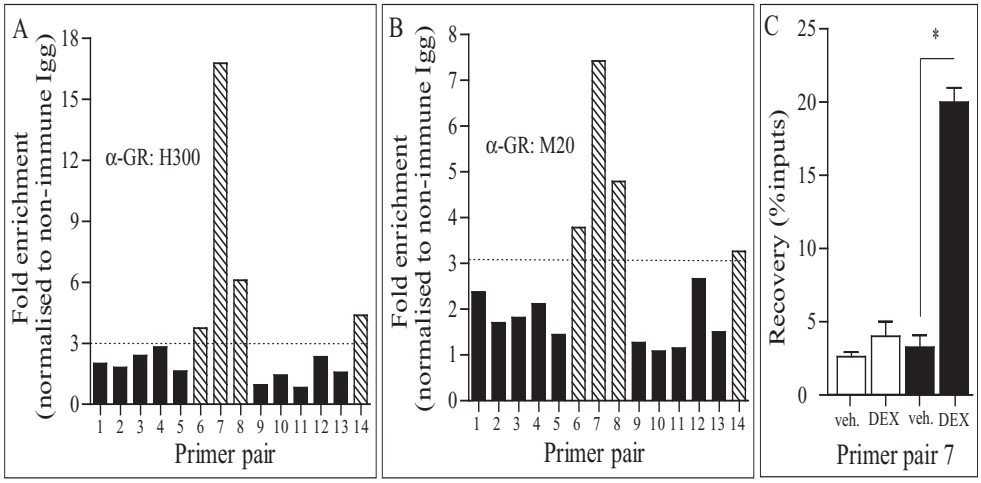

Fig. 5: ChIP-scanning of the promoter region of the rat GILZ gene. (A) H-300 and (B) M20 GR-specific antibodies result in highly similar GR binding profiles in the promoter region of GILZ. The fold enrichment values for the amplified regions were determined by normalizing to the normal rabbit Igg value. The data represent specific enrichment in DEX-treated cells divided ethanol-treated cells. Hatched bars indicate the primer pairs which were enriched >3-fold. (C) Recovery expressed as percentage of the inputs after immunoprecipitation with the H-300 GR-specific antibody and the Igg-control. 10-7M DEX treatment results in a significant higher recovery compared to the ethanol treated cells of the region amplified by primer pair 7. Data are expressed as averages (n=3 ± SD). Asterisk indicates significantly different to ethanol treated group.

3.5 Multiple tissue in situ hybridisation in adult male c57bl/6 mice

In order to assess its use as a marker for GR and MR activity in a more comprehensive manner, we performed GILZ mRNA in situ hybridisation on available multiple mouse tissues harvested 3 hours after a single dose of corticosterone. GILZ mRNA was found to be induced in spleen, thymus, heart, lung, liver, muscle, testis, kidney, colon, ileum and importantly in brain (Fig. 6). GILZ mRNA expression levels were induced by corticosterone in all tissues studied, although differences in the extent of induction were observed. For example, in liver the induction is much higher than observed in testis.

4. Discussion

The glucocorticoid receptor is ubiquitously expressed in brain tissue and is known to be critically involved in the regulation of neuronal excitability and cognitive functions via GRE binding (10). In present study, we sought for a robustly induced glucocorticoid target gene in brain and described the regulatory sites in its promoter region. GILZ was found to be ubiquitously expressed in brain tissue of young adult male rats and strongly induced by corticosterone i.p. injections. Because, the promoter sequence of the gene in rat has not yet been studied, we identified by computational analysis two putative GREs located approximately 2kb upstream of the transcription start site (TSS). Furthermore, using Ns-1 PC12 rat cells we show that GILZ is induced by DEX treatment via a GR-dependent mechanism. Importantly, we provide evidence that the two putative high-affinity GREs identified by computational analysis are located exactly within the GR-binding region of the promoter. We conclude that the transcriptional activity of the rat proximal promoter of GILZ is induced by GR via DNA binding. In sum, we propose that the promoter of the GILZ gene is suitable for studying GR-binding to DNA in specific brain structures using ChIP-assays.

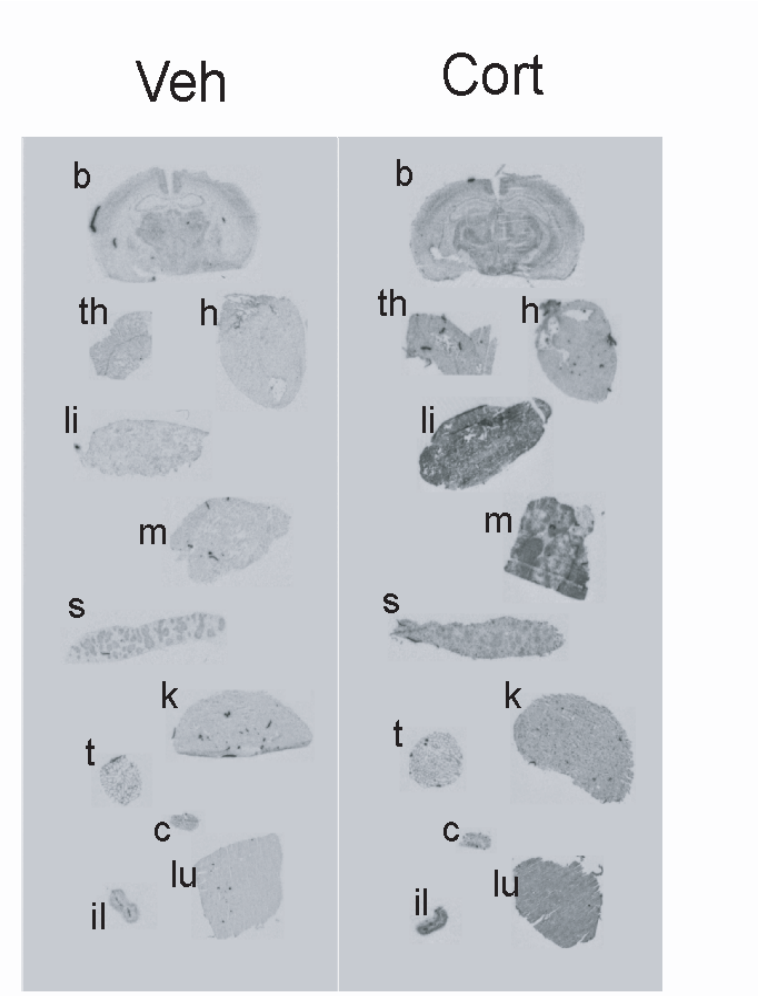

Fig. 6: Distribution and regulation of GILZ in adrenalectomised adult male c57bl/6 mice fed with oats containing 400 µg corticosterone or vehicle. All animals were sacrificed 3 hours after oats administration Legende: b(rain), th(ymus), h(eart), li(ver), m(uscle), s(pleen), k(idney), t(estis), c(olon), il(eum), lu(ng).

A recent study suggests that the precise binding sequences at individual GREs found in responsive genes are strongly conserved throughout evolution (25). Paradoxically, we found that the homology between human and rat proximal promoter sequence was low. The GREs previously described in human differed in nucleotide composition from the GREs identified in present study; additionally, their relative spacing as well as the distance to the transcription start site is somewhat different. Nevertheless, GILZ mRNA expression is induced by glucocorticoid treatment both in human (26) and rat tissue likely through a GR binding region located at approximately 2kb upstream TSS. Specificity of the ChIP-scanning was confirmed by using two different GR-specific antibodies. Immunoprecipitation reactions with both antibodies resulted in highly similar binding profiles showing the highest enrichment surrounding primer pair 7 (Fig 3). A common characteristic of most GRE sequences described

in both species is the presence of the conserved TGTTCT half-site known to be essential for GR-binding to DNA . Additional reporter assays with point-mutations in the GREs would give insights in the relative importance of these GREs. Importantly, in the human A549 cells, GR-binding to DNA was shown to be strongly correlated (nearly 90%) with genes that are glucocorticoid responsive. Taken together, identification of a GR-binding region in the proximal promoter of the rat GILZ gene is sufficient to monitor the action of GR and its associated proteins.

Among the four recently characterized GILZ isoforms, the 137 amino acid long variant with a molecular mass of 17kDa, termed GILZ1, is the originally identified glucocorticoid induced protein in humans (27). The genomic organization of the GILZ gene has only been studied in mouse and human and revealed the presence of two promoter regions. A 5' distant TATA-less promoter containing a CpG island and Sp1 sites, separated by approximately 55kb with the GRE-containing proximal promoter (28). Because no GREs were identified in this TATA-less distant promoter, it is likely that the glucocorticoid-responsiveness of the gene is exclusively mediated by the promoter sequence described in the present study.

Issues that can now be investigated include the relative occupation of GRE containing promoters by mineralocorticoid and glucocorticoid receptors (MR and GR) as a function of circulating steroid levels, and recruitment of coactivator proteins that mediate the effects of steroid receptor activation. GILZ has been shown to be induced by aldosterone via MR in kidney cells (29). In brain, the MR is mainly expressed in limbic structures, such as the hippocampus, and its activity plays an important role in mediating the effects of corticosterone on hippocampal excitability, cognitive function and hypothalamus-pituitary-adrenal axis axis activity (30). Our data show that in the CA3 neurons of the hippocampus, GILZ is induced by corticosterone treatment. Because these neurons express minimal amount of GR and high amount of MR (31;32), we suggest that the CORT-induced mRNA levels in CA3 are possibly meditated by the MR. Therefore, the current assay could readily be adapted to study MR chromatin occupancy in brain.

The neuroanatomical distribution of GILZ mRNA in the current study (in rat) was found to be in part similar to the recently mapped expression in mouse (33). Among the differences observed, the most compelling is found in the hippocampal subfields. We show a marked difference in basal expression in the hippocampal subfields of the Sprague-Dawley rats. The strong hybridisation signal observed in the CA2 and CA3 subfields clearly differed from the much lower signal in the CA1. In the C57BL/6J mice Yachi *et al.* reported a homogenous distribution of GILZ mRNA expression over all hippocampal subfields. Additionally, regulation of GILZ mRNA expression levels by glucocorticoids was also studied in these C57BL/6J mice. A single water-immersion stress significantly increased GILZ mRNA expression in the medial prefrontal cortex (mPFC) and hippocampus in by approximately 2 fold, as measured by qPCR. This effect required the presence of the adrenal glands (33) and therefore glucocorticoids. In line with these results, in Sprague-Dawley rats we measured a 2.4 fold-increase in thalamic homogenates of corticosterone treated animals. The combined results of the experiments in rats and mice suggest that an increase in corticosterone is both necessary and sufficient for the induction of GILZ after stress.

Although the function of GILZ in brain is currently not known, it was originally identified as a dexamethasone-induced gene in murine thymocytes and contains a leucine zipper which is characteristic for DNA-binding transcriptional regulators (27). The expression of GILZ selectively protects T cells from apoptosis induced by anti-CD3 monoclonal antibody treatment. Furthermore, in the context of the immunosuppressive effects of glucocorticoids,

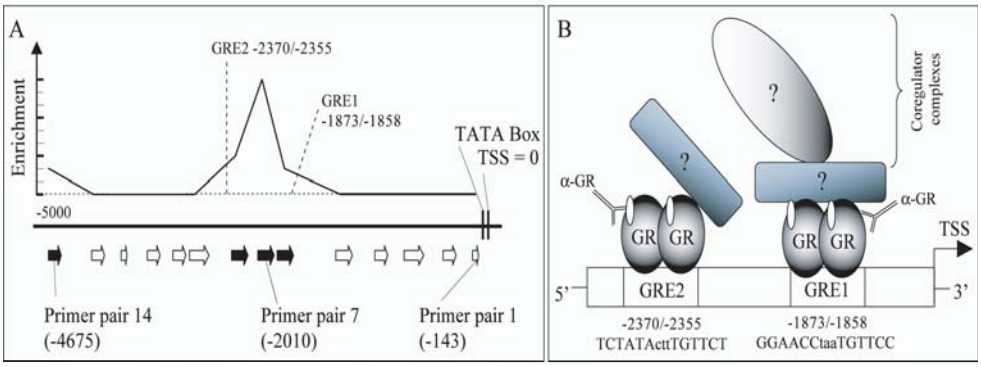

Fig. 7: (A) Schematic representation of the putative GREs and their locus relative to TSS in the rat promoter of the GILZ gene. Primer pair 7 is located exactly in between the two GREs and of all the scanning region was the most enriched. (B) Issues that can be addressed using ChIP-assays on specific brain structures, i.e. hippocampus. The GILZ promoter can be used to study coregulator recruitment by GR on the GILZ promoter in brain.

GILZ was found to inhibit NF- κB nuclear translocation and DNA-binding and to potently suppress AP1-driven transcription (34;35). Through these mechanisms GILZ protein may affect a large number of cellular processes, and regulates NF- κB and AP1-directed gene expression.

Recently it was found that GR-binding invariably takes place at nuclease-accessible sites (36). In addition, the authors showed that these sites are highly cell-specific, implicating that the chromatin organization is a critical determinant for tissue-selective receptor function. We found that GILZ expression and regulation by corticosterone is rather ubiquitous, although the extent of regulation is tissue-specific in adult male c57bl/6 mice (Fig. 6). This likely reflects these cell-specific nuclease-accessible sites and the chromatin landscape. Consequently, we hypothesize that GRE accessibility depends on the chromatin organization in the cell-type and might explain why some GREs were found not to be active in human cells (9).

The type of transcriptional coregulator present in cells is also known to determine the genomic effects of ligand-activated GR (8). Nuclear receptor coregulators are proteins that mediate the transcriptional activity of the GR and may explain cell-specific effects of glucocorticoids action in brain (37). These proteins are enzymatically active proteins that reorganize the chromatin environment and form docking platforms for recruitment of other coregulator proteins (38). We previously described the expression of various transcriptional coregulator proteins in brain, but their recruitment by GR has exclusively been addressed *in vitro* (16;21;39;40). Taken together, the methodological approach described in present study allows the analysis of the coregulator proteins role in the GR-mediated effects within specific brain structures. These experiments are likely to provide valuable information on the mechanism underlying the genomic effects of glucocorticoids (Fig. 7). In this study we have shown that GILZ is transcriptionally induced by corticosterone in many distinct regions in brain of young adult Sprague-Dawley rats. We have also located the GR binding region within the proximal promoter of the gene and identified two putative GREs. Taken together, we conclude that GR and MR chromatin occupancy in specific brain structures can be assessed by ChIP-assays using the GILZ promoter. This approach that examines protein-DNA interactions in intact cells will certainly provide a strong basis to further study essential aspects of glucocorticoid action in brain.

5. Acknowledgments

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6. Supplemetary data

6.1 Oligonucleotide for in situ hybridization

A 45-mer oligonucleotide probe encoding rat GILZ mRNA was 3' end labelled with α [^{35}S] deoxyadenosine triphosphate (GE Healthcare Europe Gmbh, Diegem, Belgium) using deoxynucleotidyl transferase (Promega Benelux, Leiden, The Netherlands). A mismatch control containing 8 evenly spaced mismatches was used as a control for hybridization specificity.

The sequence of the GILZ perfect match oligoprobe is:
TGTTAGGTGTAAAGTTCTCCACATGAGATGACGCTTGGGGAGCCA

and the sequence of the GILZ mismatch oligoprobe is:
GGTTAGGGGTAAATTTCTCAACATGCGATGAAGCTTGTGGAGCCT

6.2 Oligonucleotide primers for qPCR

Primer sequence design was performed by the Primer3 software (Rozen S and Skaletsky H. 2000). Primer pair sequences for GILZ, beta-actin and Kv3.2 mRNA expression are listed in Table S1 (supplementary data).

Gene ID	Forward	reverse
GILZ (rat)	agcgtggtggccctagacaaca	caaccagctcacgaatctgctcctt
Beta-actin (rat)	tgaccgagcgtggctaca	cagcttctctttaatgtcacgca
Kv3.2 (rat)	ctctgtaatttgcagcaaaacca	agcttcaagaaatgcccacaa

Table S1: Sequence of primers designed for mRNA amplification by qPCR of target genes.

Specificity of amplification of each primer pair was controlled by BLAST search against the rat genome. Expression levels of each gene of interest were normalized against beta-actin expression levels in tissue of interest, i.e. rat thalamus or Ns-1 PC12 material. The qPCR oligonucleotide primer pairs for ChIP-scanning were designed to amplify ± 100 bp fragments and were separated by ± 500 bp upstream (5'). Primer pair 1 (pp1) is the first pair upstream transcription start site (TSS = 0 bp). Given the average size of the sheared DNA fragments (±800bp), the 14 primer pairs should effectively scan the promoter for GR occupancy in a 5kb stretch upstream of the TSS. Primer pair sequences for promoter scanning are listed in Table S2 .

Name (primer pair)	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon (bp)	Amplified region (primers included)
pp1	aggttcagggaggggatgtggtt	cgtcaggggccatgcaaataagt	73	-143
pp2	gactgaggggttagtcggctgga	aagctctttgggtgccagtcagc	116	-423
pp3	gggagcagtctctgttgccactc	tctggaacccactcaacccatctc	175	-701
pp4	aaagaggggaggaagggctgga	gtgagctaatctcgcagccatcg	94	-969
pp5	ggcatgtccctcttggtgtcgat	tgacacataaactgcgcctcagga	145	-1287
pp6	cctctcgttctgggttggttggt	aggtggcatctgagccttgaggt	164	-1860
pp7	cctcctttgctgataagaggtcccagt	accaaccaacccagaacgagagg	173	-2010
pp8	aatggtctgaaatgggcttatgcaa	ggccttgacctcttcttggcttt	173	-2267
pp9	gcggcaggggcagataatagat	aaggaggagcgagtggtctcgaa	200	-2664
pp10	tcttcccaccatctcccttggaa	tgccgcctagagctttcttggtc	121	-2779
pp11	ggtacagccagcgcaatgtcaac	ggattgcccacaagactgcactg	142	-3085
pp12	ccactgatgtttgctggcatcct	caggtaaggaggaagaacgggtga	62	-3372
pp13	tgccctgaactcacagaacccttc	gcccctactcactggcatcctca	169	-3846
pp14	ggttgcattctcccaacccaaac	gctaggcccttcactggctttca	190	-4675

Table S2: Sequence of primers used to 'scan' the proximal promoter of GILZ by by ChIP experiments (rattus norvegicus).

6.3 Chromatin immunoprecipitation assays (ChIP).

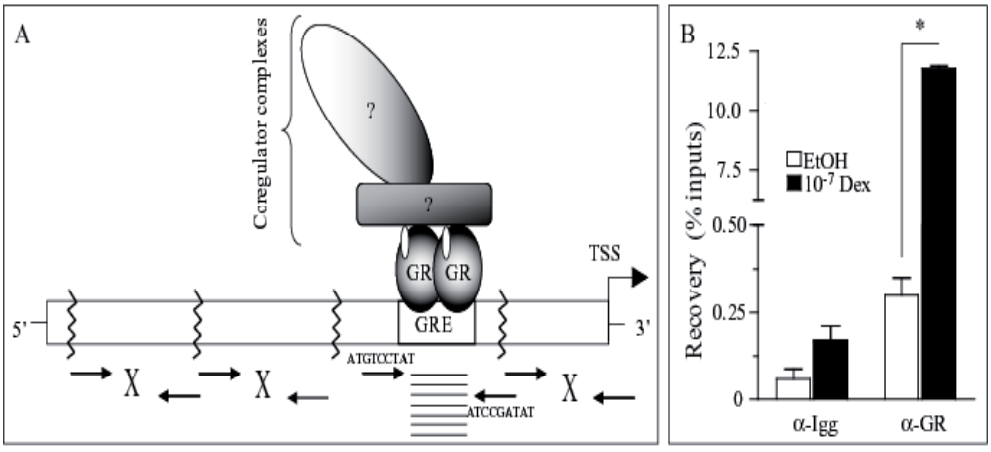

Figure S1: (A) Schematic representation of the principle of chromatin immunoprecipitation assays. (B) Optimization of the ChIP-procedure using the human A549 cells and the previously described GR binding region in the GILZ promoter.. Data are expressed as averages (n=3 ±SD). Asterisk indicates significantly different to ethanol treated group.

Ns-1 PC12 cells were treated for 90 min. with either 10-7M dexamethasone or ethanol. Following treatment, cells were formaldehyde cross-linked (1% formaldehyde concentration) for 10 minutes and incubated with glycine to stop the cross-linking reaction. Cells were rinsed twice, scraped in 2ml 1xPBS with protease inhibitors (Roche), collected in 1.5ml eppendorf tubes and centrifuged at 2000rpm for 5 min. at 4°C. Pellets were rinsed and resuspended in 300µl RIPA lysis buffer (0.1% SDS, 1% DOC, 150mM NaCL, 10mM Tris

pH 8.0, 2mM EDTA, 1mM NaVO3, 1%NP-40, β-glycerolphophate and Na-butyrate). Next, samples were sheared using a Branson Sonifier 250 fitted with a 3mm microtip in 8x10-sec. bursts followed by 1 min. of cooling on ice. The procedure resulted in DNA fragment sizes of 0.3-1.5 kb. Pre-coated 50% slurry Protein A beads (Pierce) in 1×RIPA Buffer containing 20μg/ml sheared salmon sperm DNA and 20mg BSA were added to the pre-cleared samples and incubated with rotation for 60 min. at 4°C. Next, samples were centrifuged at 2000 rpm for 2 minutes and the supernatant was transferred to fresh 1.5 ml eppendorf tubes. Either 6μg of GR-specific H300 (Santa Cruz sc-8992) or M20 (Santa Cruz sc-1004) or normal rabbit Igg (Santa Cruz sc-2027) antibody were added and incubated with rotation overnight at 4°C. Pre-coated Protein A beads (50% slurry) were added and incubated with rotation for 2 hours at 4°C. Beads were centrifuged at 2000 rpm for 2 minutes and washed extensively with following ice-cold buffers: 1x with low salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 150mM NaCl and 20mM Tris-HCl pH 8.0), 4x with high salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 500mM NaCl and 20mM Tris-HCl pH 8.0), 1x with LiCl wash buffer (0.25M LiCl, 1% NP-40, 1% Na DOC, 1mM EDTA and 10mM Tris-HCl pH 8.0) and 2x with TE pH 8.0. Following the last washing step, samples were centrifuged at 2000 rpm for 5 minutes and eluted twice with 250μl elution buffer (1% SDS, 0.1 M NaHCO3) with rotation for 10 min. at room temperature. Samples were reverse cross-linked by incubation at 65°C overnight with proteinase K. DNA was phenol-CHCl3 extracted once and ethanol precipitated in presence of glycogen. Pellets were resuspended in 20 μl TE and assayed by qPCR.

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