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Research report

Discrimination between changes in glucocorticoid receptor expression and activation in rat brain using western blot analysis

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Abstract

These studies investigated autoregulation of glucocorticoid receptor (GR) protein expression and activation in rat brain using western blot methodology. By comparing GR immunoblot reactivity present in various tissue subcellular preparations we were able to discriminate between corticosterone-induced changes in GR activation or GR protein expression. Our cytosolic tissue preparation yielded a similar pattern of treatment effects on relative GR as measured by receptor binding assay or western blot. In both cases, short-term adrenalectomy (18 h) produced no change in cytosolic GR. On the other hand, long-term adrenalectomy (3–14 days) resulted in a large increase in cytosolic GR, whereas acute (1–2 h) treatment with high dose corticosterone produced a large decrease in cytosolic GR. Western blot measurement of GR levels in a nuclear extract or whole-cell extract from the same brains indicated that acute corticosterone treatment produced a large increase in nuclear GR and no change in whole-cell GR. Thus, all of the decrease in cytosolic GR observed after acute corticosterone treatment could be accounted for by receptor redistribution to the nuclear tissue fraction as opposed to rapid receptor protein downregulation. Long-term treatment of rats with adrenalectomy or high dose corticosterone produced a large increase and decrease, respectively, in whole-cell GR, indicating genuine changes in receptor protein expression. These studies indicate that in vivo regulation of GR protein expression in the rat brain can be studied using western blot analysis of a whole-cell tissue preparation. This procedure has an important advantage over receptor binding studies in that GR protein expression can be measured in adrenal-intact rats. These studies also support the validity of using cytosolic receptor binding assays to estimate relative changes in GR occupation/activation when appropriate comparison groups are included. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Since corticosterone is a systemic lipid soluble hormone, much of its selectivity and specificity of action in vivo is determined at the receptor expression level. Two closely related intracellular receptors for corticosterone have been characterized, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) [1,19]. These two receptors share nearly identical DNA binding domains, and similar hormone binding domains, but nevertheless differ in their affinity for corticosterone and in the cellular effects that they transduce [21]. Consequently, target tissues can vary widely in their responsiveness to corticosterone as a result of differential expression of MR and/or GR.

MR and GR have a number of interesting properties that stem from the fact that these receptors function as hormone activated transcription factors. For instance, these receptors are intracellular, they appear to have a differential intracellular distribution depending on activation state, and in many tissues/cell types they are autologously regulated in a negative manner [15,16,33]. These properties must also be taken into consideration when measuring the expression and function of MR and GR.

Characterization of the regulation and function of intracellular corticosterone receptors has been largely based on in vitro studies examining GR present in various cell lines. However, there is much interest in understanding GR activity and regulation in vivo. Alterations in GR function

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and expression level in the brain are believed to be important for: (1) adaptation and maladaptation to chronic stress [10,23,44]; (2) life-long neuroendocrine and neural developmental consequences of various neonatal experiences [27]; (3) neuroendocrine dysregulation and subsequent neurodegeneration associated with aging [44]; (4) self-administration and sensitization to drugs of abuse [35,37]; and (5) neuropathology associated with various psychological disorders, such as depression and post-traumatic stress disorder [20,22,49].

Most of the studies to date examining the impact of certain in vivo manipulations and conditions on GR protein levels have relied on receptor binding studies. More recently a number of studies have utilized in situ hybridization to assess GR RNA expression levels, however, a disparity between relative GR protein levels and steady state mRNA levels has frequently been noted [6,36,39]. A limitation of receptor binding studies for GR is that the presence of endogenous or exogenous glucocorticoids in the animal at the time of death interferes with the amount of GR that is measured ex-vivo in a radioligand binding assay [38]. As a working model of GR function has emerged from in vitro studies, this interference has become understood as resulting from the activation and nuclear translocation of the receptor upon binding steroid. Indeed, acute treatment of animals with radiolabelled corticosterone results in extensive retention of radioactivity in the nuclear tissue fraction, similar to that seen with estrogen [25]. Thus, when performing a receptor binding assay on a soluble tissue fraction (cytosol), the majority of receptors present in the cytosol may be those that were unoccupied and unactivated by ligand prior to tissue processing. However, unlike the situation with estrogen receptors [41], even if receptor binding assays are performed on an extract of the nuclear tissue fraction no significant binding of radioligand is typically observed [17,47]. Apparently the activated form of GR is unable to re-bind steroid under standard receptor binding assay conditions [7]. This result is consistent with recent GR model developments suggesting that the activated receptor has to re-associate with certain chaperone proteins, such as Hsp90 and Hsp70, in order to be in a conformation able to re-bind hormone [4].

Since the activated form of GR cannot be measured with a receptor binding assay, differences in measured (available) GR receptor level may reflect either differences in receptor protein expression or differences in receptor activation. With careful control of experimental condition, researchers have capitalized upon this limitation in order to assess both changes in GR expression and activation [28,38,47,48]. However, the appropriate experimental conditions for these assessments (e.g. adrenalectomy) are not always feasible. In addition, the validity of the assumptions underlying these measures (see Discussion) have not been systematically examined.

In this paper we explore another approach to studying GR protein expression and activation in vivo that does not have the same limitations of the receptor binding assay. We have used antibodies for GR and have combined their use with the semi-quantitative method of western blotting. Both GR-reactive antibodies used in these studies (BuGR2 and GR57) have previously been characterized to recognize the activated and unactivated form of GR, as well as the denatured form of GR present on immunoblots [8,45]. With the western blot procedure GR protein levels can be measured in both the cytosolic and nuclear tissue fraction. In addition, total cellular GR protein level can be measured with a whole-cell extraction procedure. Thus, we describe in this paper studies evaluating the acute and chronic effects of in vivo changes in glucocorticoid levels on cytosolic, nuclear and whole-cell GR measures in brain tissue as measured by western blot. For the cytosolic tissue fraction, GR levels were also measured by radioligand receptor binding assay. Thus, we were able to directly compare the relative effects of in vivo corticosterone manipulations on cytosolic GR as detected by western blot or receptor binding assay.

2. Materials and methods

2.1. Animals

All studies used adult male Sprague–Dawley rats obtained from commercial suppliers. Animals were housed 2–3 per cage in a temperature-controlled animal facility maintained on a 12 h light:dark cycle with lights on at 0700 h. Animals were given food and drinking water ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committee.

2.2. Adrenalectomy

Some rats were adrenalectomized in order to remove all detectable endogenous corticosterone. Rats were anesthetized with i.p. injection of a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg). Adrenals were removed through two dorsal-lateral incisions utilizing aseptic surgical technique. Completeness of adrenalectomy was verified by measuring with radioimmunoassay (see below) the amount of corticosterone present in a blood sample taken from the animal at the time of death. All adrenalectomized animals had undetectable plasma corticosterone levels (levels below 0.5 μg/100 ml, when assaying 20 μl of plasma). After adrenalectomy, rats were given 0.9% saline as their drinking water solution.

2.3. Corticosterone treatment

For chronic high-level corticosterone treatment rats were given subcutaneous corticosterone pellets. Each rat received four homemade pellets containing approximately 100 mg of corticosterone per pellet [30]. Rats were

anesthetized as described above and pellets were placed subcutaneously on the back of the rat. For acute high level corticosterone treatment rats were injected subcutaneously with corticosterone dissolved in propylene glycol (see Results for description of doses used in individual experiments). Control rats were injected with an equal volume of propylene glycol.

2.4. Corticosterone assay

Trunk blood was collected from animals at the time of death, and blood was centrifuged and the plasma saved at −20°C for subsequent analysis. Plasma corticosterone levels were measured by radioimmunoassay [46], with use of corticosterone antiserum (1:1700 dilution; antibody B3-163; Endocrine Sciences, Calabasas Hills, CA) and [1,2,6,7-^{3}H]-corticosterone (20 000 cpm; Amersham, Arlington Heights, IL).

2.5. Tissue collection

All animals were killed in the morning under no-stress conditions by rapid decapitation. Trunk blood was collected for subsequent corticosterone measurement and brains were removed and brain regions dissected within 5 min of death. Hypothalamic tissue consisted of a block of tissue overlying the mammillary bodies. Cortex was taken from dorsal-posterior brain regions surrounding the hippocampus and consisted primarily of parietal cortex. Dissected brain tissue was then either placed in cold buffer (4°C) and homogenized within 2 h after collection or rapidly frozen in tissue wells placed directly on dry ice, and stored at −70°C for subsequent processing. Similar results were obtained if the tissue had been frozen first or was processed unfrozen within hours after collection (data not shown).

2.6. Glucocorticoid receptor binding assay

Glucocorticoid receptor binding levels in tissue cytosolic preparations were measured using the procedure previously described [47]. Briefly, individual tissues were homogenized with motor driven pestle in a buffer solution comprised of 10 mM Tris, 1 mM EDTA, 10% glycerin, 20 mM molybdic acid, and 1 mM dithiothreitol at a pH of 7.4 at 4°C. The homogenate was then centrifuged (105 000 g) for 30 min in an ultracentrifuge. The supernatant (cytosol) was incubated for 18–24 h in the presence of [6,7-^{3}H]-dexamethasone (15 nM; New England Nuclear, Boston, MA)±non-radioactive competitor. GR binding was determined from the amount of total radioligand binding that was displaced by the selective GR ligand RU28362 (0.5 μM; gift from Roussell-Uclaf) [9]. Bound [6,7-^{3}H]-dexamethasone was separated from unbound steroid by gel filtration using mini Sephadex (LH-20; Amersham Pharmacia, Piscataway, NJ) columns. The resulting eluate was mixed with scintillation cocktail and the radioactivity was measured using a scintillation counter. Protein concentrations for each cytosolic sample were determined using a Bradford assay [5]; bovine serum albumin (fraction V; Sigma, St. Louis, MO) was used for the protein standard. The cytosolic protein levels ranged from 0.5 to 1.5 mg/ml.

2.7. Tissue preparation for western blotting

For cytosolic and nuclear extract preparations brain tissue from individual animals were homogenized with a hand-held 7 ml dounce glass-on-glass tissue grinder (Wheaton, Millville, NJ) and tissue was homogenized in 0.5 ml buffer/100 mg tissue. Western blot homogenization buffer consisted of 50 mM Tris buffer (pH 7.2, 4°C) containing 6 mM $MgCl_2$, 1 mM EDTA, 10% (wt/vol) sucrose, 1 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine, 5 μg/ml leupeptin, 1 μg/ml of pepstatin A, 1 μg/ml antipain, 1 μg/ml aprotinin and 1 μg/ml of soybean trypsin inhibitor. Homogenate was then centrifuged for 5 min at low speed (2000 g) in a refrigerated centrifuge. The resulting supernatant and pellet were further processed to generate cytosol and nuclear extract, respectively. For cytosol preparation supernatant was then centrifuged (105 000 *g*) at high speed for 30 min in an ultracentrifuge. The final supernatant was used as the cytosolic tissue fraction. For nuclear extract preparation the pellet from low speed centrifugation of homogenate was washed twice by resuspension in 0.5 ml of homogenization buffer followed by additional low speed centrifugation (5 min, 2000 *g*). The washed pellet was then resuspended in 0.25 ml of homogenization buffer containing 0.5 M NaCl. The tissue salt suspension was then incubated for 1 h in an ice bath with frequent vortexing. After incubation the tissue salt suspension was centrifuged (8000 *g*) for 10 min at 4°C. The final supernatant was used as the tissue nuclear extract.

For whole-cell preparations, brain tissue from individual animals were either homogenized with a motor driven pestle or sonicated. Tissue was homogenized in 1.0 ml buffer/100 mg tissue. For tissue homogenization with motor driven pestle, western blot homogenization buffer (see above) was used with the addition of 0.5% sodium dodecyl sulfate (SDS). For sonication, western blot homogenization buffer without SDS was used. In both cases the homogenate or sonicate was ultracentrifuged (105 000 *g*) for 30 min and the supernatant was used as whole-cell extract. Similar results were obtained if tissue was homogenized in the presence of 0.5% SDS, 1.0% SDS or sonicated without SDS (data not shown).

2.8. Western blotting procedure

Samples were adjusted to a final protein concentration of 1.5 mg/ml (DC protein assay, Bio-Rad, Hercules, CA). Supernatants were mixed with Laemmli's sample buffer

and boiled for 5 min. Samples (30 μg) were loaded onto 8% bis-acrylamide gels and separated by SDS polyacrylamide gel electrophoresis. Separated proteins were electrophoretically transferred from gels to PVDF membranes (Immobilon-P; Millipore, Marlborough, MA). Blots were blocked for 30 min in a Tris-buffered saline solution with 0.1% Tween-20 (TBST) and 5% Carnation instant dried milk. Membranes were incubated (4°C) overnight with either the monoclonal mouse antibody, BuGR2 (0.025 μg/ml TBST; Affinity BioReagents, Golden, CO), or the polyclonal rabbit antibody, GR57 (0.25 μg/ml TBST; Affinity BioReagents). BuGR2 was raised against purified rat liver GR and its epitope has been mapped onto a portion of GR adjacent to the hinge region towards the amino-terminus end of the protein [45]. GR57 was raised against a 22 amino acid peptide sequence taken from the human GR and located in the regulatory region of GR (aa 346–367) [8]. Previous characterization indicates that BuGR2 and GR57 recognize both the unactivated and activated form of the undenatured as well as denatured receptor [8,45]. Immunopositive bands were visualized by a chemiluminescent method (ECL, Amersham). The optical density of GR reactive bands visible on light-sensitive film (ECL film, Amersham) was measured with an image analysis system (NIH Image software). For each experiment samples were run in duplicate. In cases where there were more samples than would fit on a single gel, treatment groups were counterbalanced across gels and all resulting blots were processed in parallel and exposed to the same sheet of light-sensitive film. Exposure times were adjusted so that the darkest bands did not saturate the film. Background optical density levels were taken for each image of a blot and were subtracted from the optical density obtained for each individual immunoreactive band.

2.9. Statistics

Experiments consisting of more than two treatment groups were analyzed by one-way analysis of variance (ANOVA) followed by either Tukey's or Fisher's Least Significant Difference post-hoc test. Experiments with only two treatment groups were analyzed by between-groups Student's *t*-test. A separate analysis was performed for each brain region examined. A 0.05 alpha-level was chosen a priori. All data in the text and figures is presented as the mean±S.E.M.

3. Results

3.1. Validation of antibody specificity for GR on western blot of hippocampal cytosol

Hippocampi ($n=2$) were dissected from: (1) adrenal-intact rats; (2) 14 day adrenalectomized rats; and (3) 14 day adrenalectomized rats injected with a high dose of corticosterone (15 mg/kg s.c.) 2 h prior to death. Tissue cytosol was prepared and western blot for GR immunoreactivity performed according to Methods. Both GR-reactive antibodies (BuGR2 and GR57) recognized predominantly a protein band at 96.6±0.8 kD (molecular weight approximation derived from linear regression using coomassie pre-stained molecular weight standards, $n=9$ separate immunoblots; Fig. 1). The mobility of this predominant band agrees well with the theoretical molecular weight of rat GR-94 kD [31]. Further confirmation that the 97 kD band represents GR is provided by the robust treatment effects evident as an increase in GR-immunoreactivity after long-term adrenalectomy and decrease in GR-immunoreactivity after acute corticosterone treatment. The GR57 antibody also produced a prominent immunoreactive band at 119.1±1.1 kD (Fig. 1B). This higher molecular weight band was not evident with the BuGR2

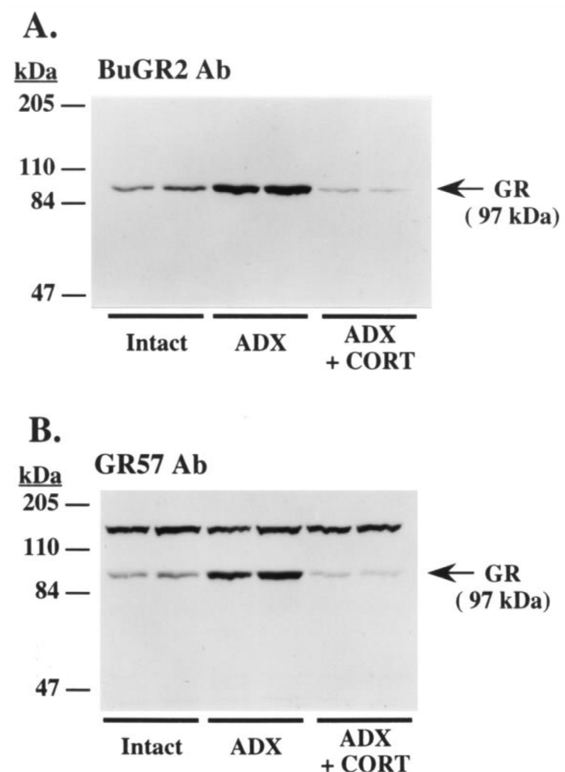

Fig. 1. Western blot images showing specific immunoreactivity of two different antibodies for cytosolic GR protein isolated from hippocampus. Cytosol samples from individual rats were subjected to SDS–PAGE and proteins were transferred to PVDF membranes. Migration of molecular weight markers are indicated on the left of each blot. Samples were taken from adrenal-intact rats (Intact, $n=2$), long-term (14 day) adrenalectomized rats (ADX, $n=2$) or long-term adrenalectomized rats treated acutely (2 h) with corticosterone (ADX+CORT, $n=2$). Blots were visualized by treatment with chemiluminescent reagents followed by exposure to light-sensitive film. (A) Blot probed with the GR-reactive monoclonal antibody BuGR2. Cytosol samples yielded a single prominent band at approximately 97 kDa. (B) Blot probed with the GR-reactive polyclonal antibody GR57. Cytosol samples yielded two prominent bands, however, only the approximately 97 kDa band showed treatment dependent changes in amount of immunoreactivity.

antibody and its immunoreactive intensity did not change systematically with treatment group. Nevertheless, both the 97 kD and 119 kD bands were not present on parallel blots probed with GR57 in the presence of antigen (0.25 μg/ml of immunization peptide; Affinity BioReagents PEP-001; data not shown). No prominent bands were present on parallel blots in which isotype control antibody (0.025 μg/ml of combined mouse IgG2a and IgG2b; Sigma) was substituted for BuGR2 (data not shown).

3.2. Validation of semi-quantification of GR immunoreactive bands

In order to validate the semi-quantitative relationship between the amount of antigen and the optical density on light-sensitive film produced by GR-immunoreactivity, the concentration of cytosol protein examined by western blot was systematically varied. In the initial evaluation a wide range of total protein concentrations was examined (Fig. 2). Pooled hippocampal cytosol (1–95 μg/lane) from 3 day adrenalectomized rats was examined with western blot using BuGR2 as primary antibody. The optical densities corresponding to GR-specific immunoreactive bands were plotted against protein concentration. The detection limit for a specific GR-immunoreactive signal was around 10 μg of total hippocampal cytosolic protein concentration. The relationship between protein concentration and OD appeared very linear for proteins ranging from 10 to 50 μg, with some leveling off in ODs between 50 and 95 μg, due primarily to film saturation (gray levels >220). To examine in more detail the relationship between sample GR concentration and measured immunoreactivity, another protein concentration vs. optical density curve was performed using a narrow range of 7 cytosolic concentrations in the apparent linear range (15–30 μg/lane). For this analysis, cytosol was prepared from thymus tissue, a GR enriched tissue source [32,39]. Various concentrations of cytosol were generated by dilution with homogenization buffer. After cytosol dilution, varying amounts of bovine serum albumin (Sigma) was added to each diluted fraction so that the total amount of protein added to each lane was constant (30 μg). The relationship between total cytosolic protein added to each lane, and the optical density of the corresponding GR-immunoreactive band was very linear when probing the blot with either BuGR2 (r=0.989; Fig. 2C), or GR57 (r=0.957; data not shown).

3.3. Effect of in vivo glucocorticoid manipulations on cytosolic GR: comparison between western blot analysis and cytosolic receptor binding assay

The first experiment comparing cytosolic GR levels as determined by western blot and receptor binding assay examined 3 treatment groups (n=4–5): (1) adrenal-intact rats; (2) 5 day adrenalectomized rats; and (3) adrenal-intact rats killed 2 h after injection with high dose

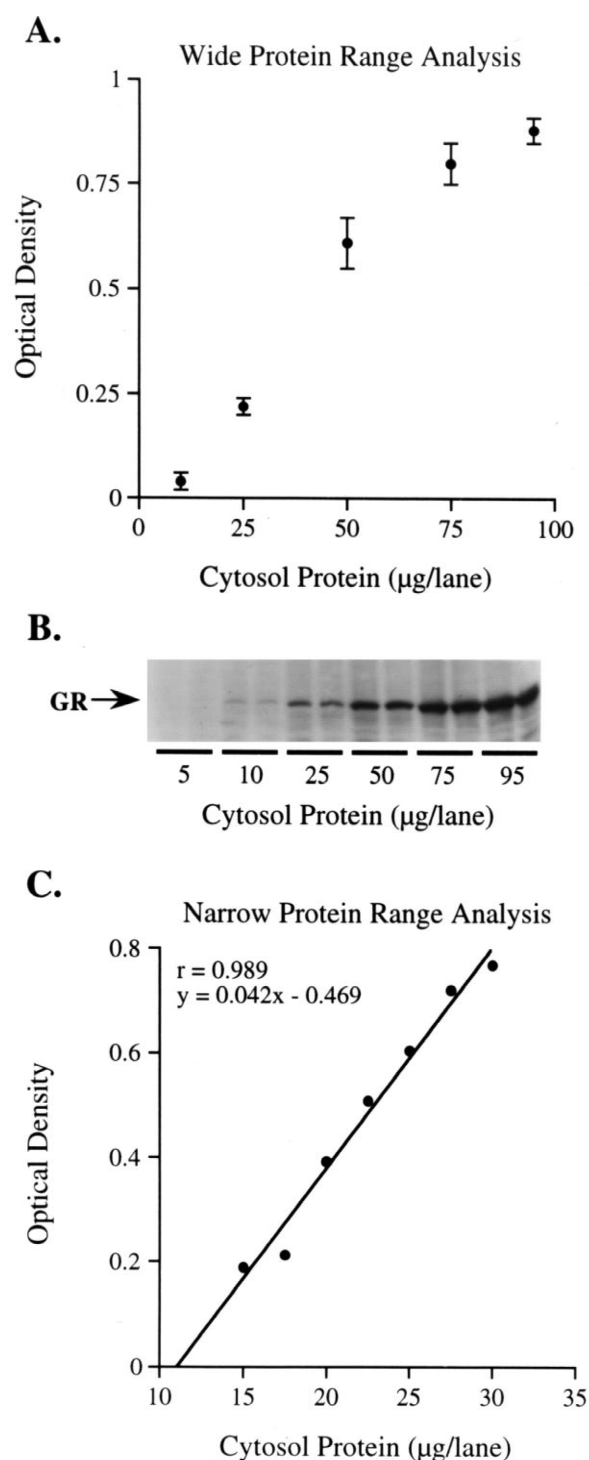

Fig. 2. Relationship between sample protein concentration and optical density corresponding to GR immunoreactivity. (A) A wide range of protein concentrations (1–95 μg/lane) from pooled hippocampal cytosol were examined by western blot. The plot shows the mean±S.E.M. optical density on light-sensitive film that corresponds to GR-specific immunoreactivity for each sample dilution (n=4). (B) Sample of an immunoblot used to generate the plot in panel A. (C) A narrow range of protein concentrations (15–30 μg/lane) from pooled thymus cytosol were examined by western blot. The plot shows the mean optical density on light-sensitive film that corresponds to GR-specific immunoreactivity for each sample dilution (n=2).

corticosterone (15 mg/kg s.c.). Hippocampal tissue from each rat was divided in half for GR measurement by western blot and receptor binding assay (see Methods). There was an overall significant effect of treatment on GR level as determined by both western blotting ($F[2,11]=45.7$, $P<0.001$) and receptor binding ($F[2,11]=124.2$, $P<0.001$). Post-hoc tests indicated that long-term adrenalectomy produced a significant increase in cytosolic GR, as determined by both assay methods (Fig. 3). Acute high dose corticosterone treatment produced a significant decrease in cytosolic GR, as determined by both assay methods. There was good agreement between both methods of GR measurement in the proportional treatment changes. Plasma corticosterone levels were undetectable in adrenalectomized rats, were barely detectable in the adrenal-intact rats (0.9±0.4 μg/100 ml), and were supraphysiological in the corticosterone injected rats (339.8±25.1 μg/100 ml).

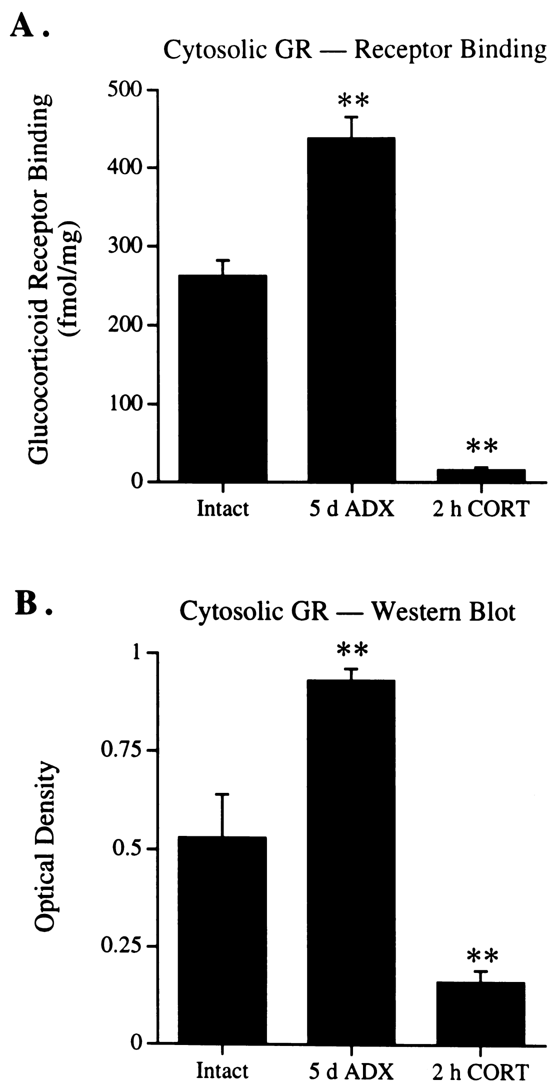

Fig. 3. Receptor binding (panel A) vs. western blot (panel B) comparison of relative GR level (mean±S.E.M.) in hippocampal cytosol from adrenal-intact rats (Intact, $n=4$), 5 day adrenalectomized rats (5 d ADX, $n=5$), or rats treated for 2 h with high dose corticosterone (2 h CORT, $n=5$). The western blot was probed with the GR-reactive antibody BuGR2. $^{**}P<0.005$ vs. adrenal-intact group, Tukey's post-hoc test.

A second experiment was conducted which also compared cytosolic GR levels as determined by western blot and receptor binding assay. For this second study four treatment group were examined ($n=5$). Three of the treatment groups were identical to the three described in the previous experiment. The fourth treatment group consisted of short-term (18 h) adrenalectomized rats. In addition, this second experiment compared treatment effects on GR levels in hippocampal, cortical and hypothalamic (western blot only) tissue (Fig. 4). There was an overall significant effect of treatment on cortical ($F[3,16]=8.8$, $P<0.005$) and hypothalamic ($F[3,16]=5.5$, $P<0.01$) GR as assessed by the western blotting method. Due to an error in tissue processing, only 3 hippocampal samples were analyzed by western blot for each treatment group, and overall group differences did not reach statistical significance ($F[3,8]=2.2$, $P=0.17$). For the receptor binding measure there was also an overall treatment effect in both tissues examined — cortex ($F[3,16]=436.2$, $P<0.001$ and hippocampus ($F[3,16]=123.6$, $P<0.001$). Similar to the previous experiment, post-hoc analysis indicated that long-term adrenalectomy produced a significant increase in cytosolic GR levels and acute corticosterone treatment produced a decrease in cytosolic GR levels. Short-term adrenalectomy did not produce a significant change in cytosolic GR levels. The treatment effects were similar for each brain region examined. As seen in the previous experiment, the pattern of treatment effects on cytosolic GR was similar for both assay methods, although in this experiment the magnitude of treatment effects was somewhat less as determined by western blot compared to receptor binding. Plasma corticosterone levels of each treatment group were very similar to those obtained in the previous experiment. Thus, plasma corticosterone levels were undetectable in both groups of adrenalectomized rats, were low in the adrenal-intact rats (mean±S.E.M.= 2.2±0.4 μg/100 ml), and were greater than the assay maximum (>100 μg/100 ml) in each of the corticosterone injected rats.

3.4. Effect of acute corticosterone treatment on GR cellular distribution: western blot analysis of cytosol vs. nuclear extract

The dramatic decrease in cytosolic GR levels produced by acute corticosterone treatment may reflect either a rapid downregulation of total cellular GR protein expression and/or a selective loss of GR from the cytosolic tissue fraction. To test for these possibilities we used western blot analysis to examine GR levels in both the cytosolic and nuclear tissue fraction (see Methods) of rats that had been

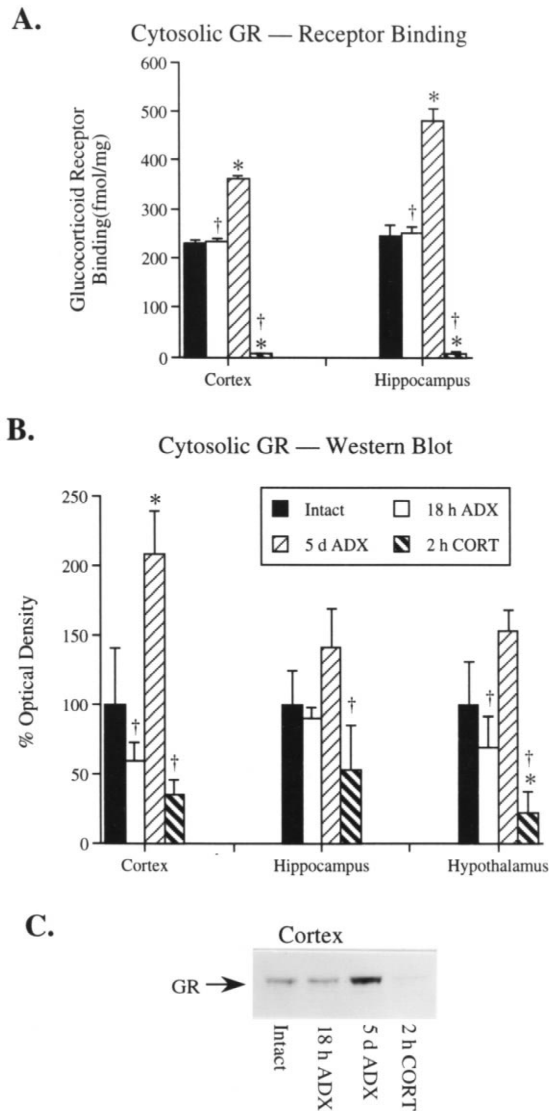

Fig. 4. Tissue comparison of receptor binding (panel A) and western blot (panel B and C) analysis of relative GR level (mean±S.E.M.) in cytosol from adrenal-intact rats (Intact, n=5), 18 h adrenalectomized rats (18 h ADX, n=5), 5 day adrenalectomized rats (5 d ADX, n=5), or rats treated for 2 h with high dose corticosterone (2 h CORT, n=5). Only 3 hippocampal samples from each treatment group were analyzed by western blot. The western blots were probed with the GR-reactive antibody GR57. $^{*}P<0.05$ vs. adrenal-intact group, Fisher's least significant difference (FLSD) test. $^{\dagger}P<0.05$ vs. 5 day adrenalectomized group, FLSD test. (C) Sample of a western blot showing GR-immunoreactivity in cortex cytosol used to generate data in panel B.

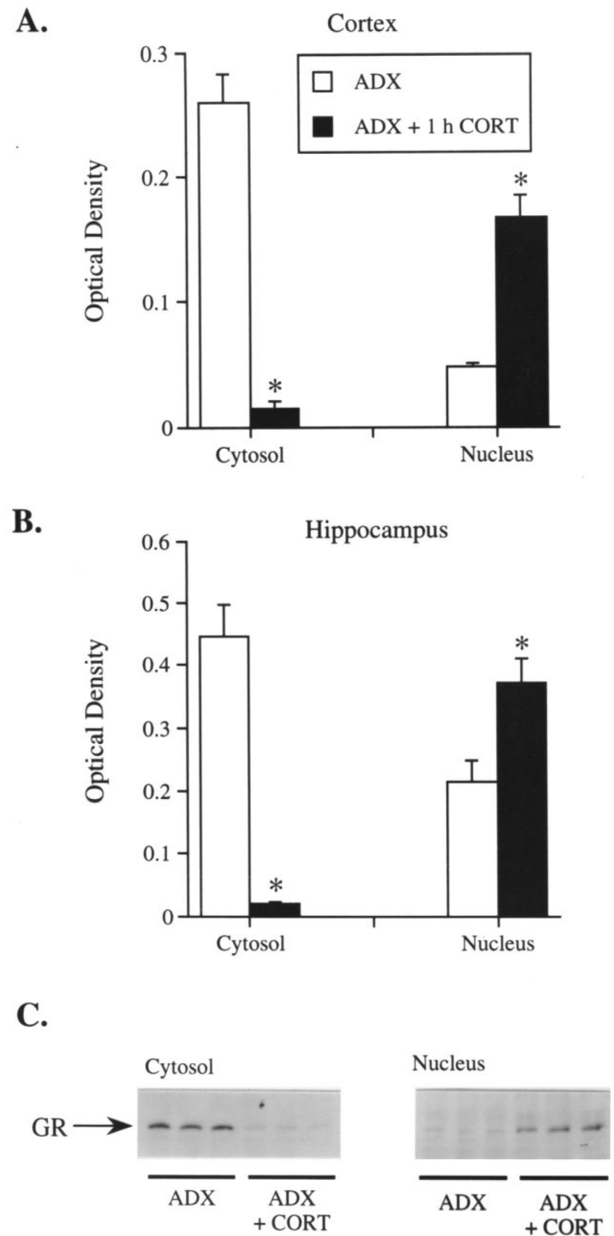

Fig. 5. Effect of acute corticosterone treatment on GR-immunoreactivity (mean±S.E.M.) in the cytosolic or nuclear tissue fraction. Tissue was from 3 day adrenalectomized rats injected 1 h before death with either corticosterone (ADX+1 h CORT, n=4) or vehicle (ADX, n=4). Relative GR-immunoreactivity on immunoblots (probed with BuGR2 antibody) was examined on samples prepared from either cortex (panel A) or hippocampus (panel B). $^{*}P<0.05$ vs. vehicle treated group for the same tissue fraction, Student's t-test. (C) Sample of a western blot showing GR-immunoreactivity in cortex cytosolic or nuclear tissue fraction used to generate data in panel A.

adrenalectomized for 3 days and then treated with either vehicle (1 ml/kg propylene glycol) or corticosterone (15 mg/kg s.c.) 1 h before death (n=4). Acute corticosterone treatment produced a large decrease in cytosolic GR immunoreactivity and a concurrent large increase in nuclear extract GR immunoreactivity (Fig. 5). A similar result was observed in both cortical and hippocampal tissue.

3.5. Effect of acute corticosterone treatment on total cellular GR levels: western blot analysis of whole-cell extract

Although the previous analysis indicates that acute

corticosterone treatment produced a large redistribution of GR from the cytosolic to the nuclear tissue fraction, the analysis does not rule out the possibility that total cellular GR levels also declined to some extent following acute corticosterone treatment. To test this possibility, using cortical and hippocampal tissue from the same animals described in the previous experiment, we examined GR protein levels in a whole-cell extract (see Methods). Regardless of the whole-cell extract method (tissue sonication or homogenization in the presence of 0.5% SDS) we observed no difference in GR-immunoreactivity levels of adrenalectomized rats treated with vehicle or corticosterone 1 h before death (Fig. 6).

3.6. Effect of long-term adrenalectomy or long-term high dose corticosterone treatment on total cellular GR levels

To demonstrate that changes in total cellular GR protein expression can be detected with western blot we examined GR levels in rats that received long-term treatments expected to produce either GR upregulation or downregulation. Cortical and hippocampal tissue from three groups of rats were examined (n=4): (1) adrenal-intact rats; (2) 5 day adrenalectomized rats; and (3) adrenal-intact+5 day high dose corticosterone (s.c. corticosterone pellets) treated rats. There was an overall significant treatment effect on cortical GR (F[2,9]=20.4) and hippocampal GR (F[2,9]=28.2, P<0.001). Post-hoc analysis indicated that long-term adrenalectomy produced a significant increase in total cellular GR immunoreactivity, and long-term high dose corticosterone treatment produced a significant decrease in total cellular GR immunoreactivity (Fig. 7).

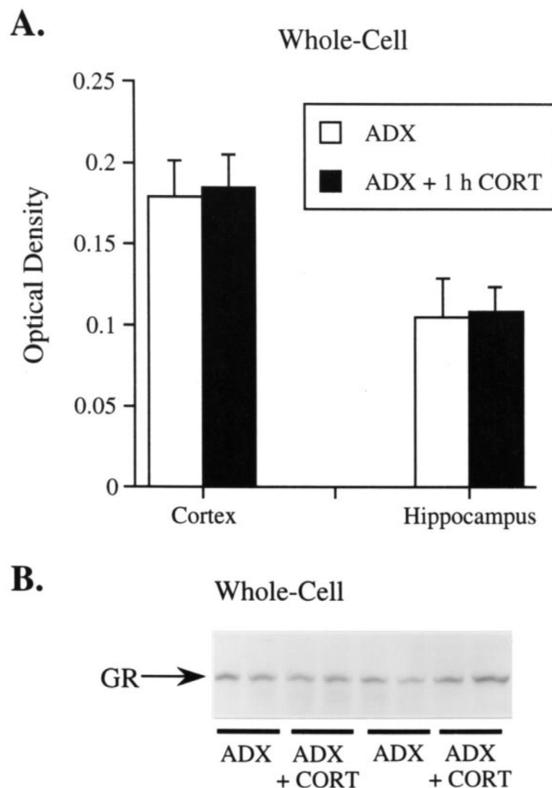

Fig. 6. Effect of acute corticosterone treatment on GR-immunoreactivity in the whole-cell tissue fraction. Tissue was from the same animals as is presented in Fig. 5. (A) Relative optical density of GR-immunoreactivity (probed with BuGR2 antibody) for each treatment group (mean±S.E.M.). (B) Sample of a western blot showing GR-immunoreactivity in cortex whole-cell tissue fraction used to generate data in panel A.

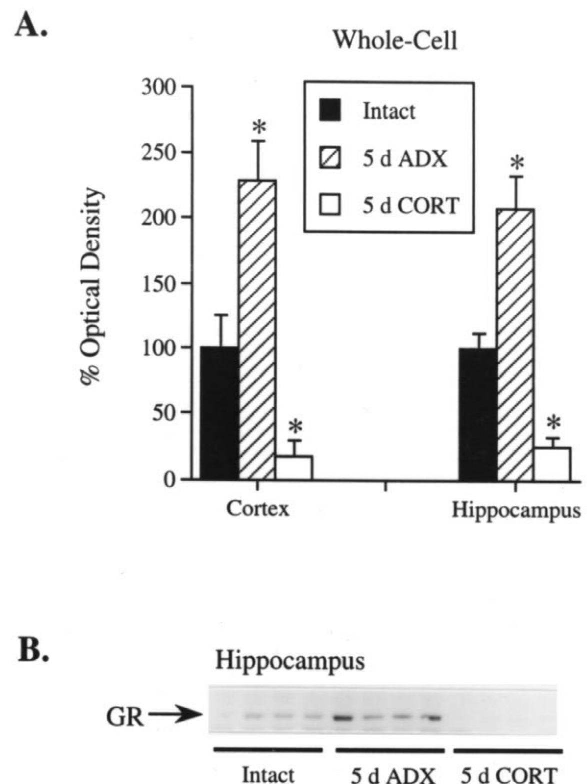

Fig. 7. Effect of long-term manipulation of corticosterone levels on GR-immunoreactivity in the cortical and hippocampal whole-cell tissue fraction. Tissue was from adrenal-intact rats (Intact, n=4), 5 day adrenalectomized rats (5 d ADX, n=4), or adrenal-intact rats with 5 days of high dose corticosterone treatment (5 d CORT, n=4). (A) Relative optical density of GR-immunoreactivity (probed with BuGR2 antibody) for each treatment group (mean±S.E.M.). *P<0.05 vs. adrenal-intact group, FLSD test. (B) Sample of a western blot showing GR-immunoreactivity in hippocampal whole-cell tissue fraction used to generate data in panel A.

4. Discussion

These studies illustrate that the western blot method can be used to measure relative levels of GR in rat brain tissue. We found that if tissue was homogenized under conditions that minimize nuclear disruption (hand-held dounce homogenization), a very similar pattern of corticosteroid manipulation treatment effects on cytosolic GR was observed as detected by either a receptor binding assay or by western blot. With both procedures we saw a substantial

increase in GR binding or immunoreactivity after long-term adrenalectomy (3–14 days) but not after 18 h of adrenalectomy when compared to adrenal-intact rats with low circulating corticosterone levels at the time of sacrifice. In addition, both assay procedures produced a large decrease in GR after acute (1–2 h) corticosterone treatment of adrenal-intact or adrenalectomized rats. O'Donnell et al. [34], when examining the cytosolic tissue fraction of rat brain, also found that the western blot method provided a reliable measure of relative differences in cytosolic GR level. Importantly, we have found that with different tissue preparation methods we can measure not only the relative amounts of GR present in the cytosol, but also in the nuclear or whole-cell tissue fraction. Thus, we can discriminate between conditions that produce a change in the proportion of activated GR from conditions producing a change in GR protein expression. This is an important advance since receptor binding assays for GR appear to be able to only measure the cytosolic/unactivated fraction of receptors.

4.1. Determination of corticosterone-induced GR activation

A number of studies have examined the effects of acute and chronic manipulation of corticosteroid levels in the rat on ex-vivo cytosolic GR levels as measured by a receptor binding assay [28,38,43,47,48]. The large increase in GR binding levels seen in this study after long-term adrenalectomy is typical and is believed to reflect an upregulation of GR protein expression [24]. On the other hand, the large decrease in cytosolic GR binding levels observed after acute elevation of glucocorticoids is believed to reflect activation of GR rather than a rapid downregulation of GR protein expression [7,38]. This conclusion is based on the indirect evidence that the activated form of GR cannot be measured in a cytosolic receptor binding assay [7]. Moreover, the conclusion is based on the assumption that a significant proportion of GR does not downregulate within several hours. However, receptor binding studies cannot rule out the possibility that acute glucocorticoid treatment not only activates GR, but also produces some rapid downregulation of GR.

Our western blot studies indicate that all of the decrease in cytosolic GR observed after acute corticosterone treatment can be accounted for by activation of GR. Examination of specific cellular compartments revealed that while acute corticosterone treatment produced a decrease in cytosolic GR there was a concurrent increase in GR levels in a high-salt nuclear extract (nuclear fraction). Although this finding confirms that GR had redistributed between the two measurement compartments, it does not preclude the possibility that some receptor downregulation had occurred in addition to tissue compartment redistribution. However, results from the whole-cell assay (tissue homogenization in the presence of SDS or tissue sonication) clearly demonstrate that 1–2 h of high dose corticosterone treatment was not sufficient to produce a downregulation of GR protein expression. These results are consistent with western blot studies of GR in various cell lines which also find that acute glucocorticoid treatment of cells produces a redistribution of GR from the cytosolic to nuclear tissue fraction without producing a change in total cellular GR content [2,18,29,42]. Apparently the general functional properties of GR that have been characterized in vitro are also representative of GR function in rat neural tissue.

The present studies indicate that the decrease in cytosolic GR binding level after acute corticosterone treatment can be largely accounted for by the absence of activated GR in the cytosolic tissue fraction. However, we have some indication from these studies that there was a portion of GR present in the cytosol that was detected by western blot but not by receptor binding. For our acute GR activation studies we administered a very high dose of corticosterone which we expected would activate nearly all of GR. Consistent with this expectation, very little detectable GR was observed (approximately 5% of vehicle-injected adrenal-intact control levels) in samples from acute corticosterone treated rats as measured by the receptor binding assay. On the other hand, although this same acute corticosterone treatment produced a substantial reduction in GR as detected by western blot, there was a greater level of GR detected (approximately 30–50% of control levels) than with the receptor binding assay. Thus, there may be some nuclear contamination of the cytosolic tissue fraction. Whereas contamination of the cytosol with activated GR would go undetected in the receptor binding assay, the western blot would be able to detect these activated receptors. O'Donnell et al. [34] observed even a greater discrepancy between the acute effect of corticosterone on cytosolic GR as measured by receptor binding or western blot. However, for those studies the more tissue disruptive method of sonication was used to generate a soluble tissue fraction. In our studies, we found that homogenization of tissue in the presence of SDS or sonication of tissue produced what we refer to as a whole-cell tissue preparation. With our whole cell preparation we did not observe an effect of acute corticosterone treatment on GR-immunoreactivity. Apparently, different proportions of activated GR can be present in a cytosolic tissue preparation depending on the method of tissue disruption. By comparing samples from animals with or without acute corticosterone treatment one can gain an indication of the extent to which the tissue processing procedure generates a whole-cell preparation versus a cytosolic preparation that lacks contamination from the nuclear compartment.

4.2. Estimate of GR activation with receptor binding assay vs. western blot

These results suggest that the binding assay may be the preferred method for estimating acute activation of GR

since the procedure is not vulnerable to nuclear contamination of the cytosolic tissue fraction. We and others have used the receptor binding assay to study relative level of GR activation in vivo under various hormonal conditions [12,38,40,47,48]. For estimates of receptor activation we have measured the amount of cytosolic GR binding present in adrenal-intact rats (available GR) and compared that to GR binding levels in overnight adrenalectomized rats (total GR). These estimates, however, are dependent on the assumptions that: (1) the receptor binding assay only measures unactivated receptors; (2) total cellular GR levels are measured in the cytosol of adrenalectomized rats; and (3) there is no upregulation of GR protein levels with overnight adrenalectomy. The results of the studies in this paper suggest that for the most part these assumptions are valid. Acute increases in corticosterone causes a decrease in GR receptor binding levels and this is due to both the extensive loss of activated receptors from the cytosolic tissue fraction and the inability of any residual activated receptors in the cytosol to participate in the ex-vivo receptor binding assay. Thus, the first assumption appears to be well supported. We have some evidence that the second assumption may not be completely accurate. When examining GR levels in the nuclear extract from adrenalectomized rats we observed some low level GR-immunoreactivity, especially in the hippocampus. Thus, some GR may be present in the nuclear fraction of tissue prepared from adrenalectomized rats. Consequently the receptor binding assay may somewhat underestimate total cellular GR levels. The third assumption appears to be well supported by our present studies. Neither the receptor binding assay or the western blot procedure detected a significant increase in cytosolic GR within 18 h of adrenalectomy compared to adrenal-intact rats. It is important to note that for these studies the adrenal-intact rats were killed under no-stress conditions at the trough of their circadian cycle of corticosterone secretion. Thus, these results not only suggest that there was very little upregulation of GR within 18 h after adrenalectomy, but also that very little GR was occupied and activated by the barely detectable levels of corticosterone present in adrenal-intact rats.

There may be some circumstances, however, when there are not only acute changes in GR activation but also rapid changes in GR expression level, such as has been noted in several cell lines (see below). Parallel measure of cytosolic receptor binding and whole-cell GR by western blot is a strategy that we have recently used to insure that changes in GR receptor binding level reflect changes solely in receptor activation [11].

4.3. Determination of chronic corticosterone-induced GR downregulation

Our results indicate that western blot analysis of GR level present in a whole-cell preparation provides a measure of relative tissue level of GR protein expression. Thus, using our whole-cell preparation we observed a decrease in GR-immunoreactivity only after chronic treatment of rats with high levels of corticosterone. This decrease in GR-immunoreactivity represents an overall cellular downregulation of GR protein expression. Previous studies of in vivo regulation of GR protein levels have had to rely on receptor binding assays. For those studies, all endogenous or exogenous glucocorticoid had to first be cleared from the animal's system before death so that there would be no confusion between changes in receptor activation and changes in receptor protein expression. Such receptor binding based studies have several limitations. First, some upregulation of GR may occur between the time of adrenalectomy and death, and this receptor upregulation may vary across treatment groups. Although in this study we observed very little upregulation of GR within 18 h after adrenalectomy, there may be other conditions in which GR exhibits a greater rate of upregulation. Eldredge et al. [14] found a diminished degree of GR upregulation, as measured by receptor binding, after adrenalectomy in aged rats compared to young rats. Although that study only examined GR upregulation subsequent to 24 h of adrenalectomy, it illustrates the point that the rate or extent of GR upregulation can vary between different comparison groups. Secondly, receptor binding studies do not allow for study of short-term changes in GR. Typically for receptor binding studies animals are adrenalectomized 16–24 h prior to death in order to allow for complete clearance of endogenous glucocorticoids. Although we didn't observe a decrease in whole-cell GR protein levels 2 h after bolus injection of corticosterone, there is the prospect that GR may downregulate in less than 16 h after either exogenous corticosterone treatment or some other manipulation. In various cell lines a substantial (50%) and fairly rapid (5–12 h) downregulation of GR after agonist treatment has been observed [3,13,26]. Third, there may be some conditions that produce a downregulation of GR that reverses over the course of 16–24 h of subsequent adrenalectomy. In such cases a treatment-induced downregulation of GR would be undetected.

4.4. Final summary

Overall, these studies indicate that in vivo changes in GR protein expression in the rat brain can be effectively measured using the western blot procedure. This approach has substantial advantages over measuring GR expression with receptor binding assays since it does not depend on prior adrenalectomy and the accompanying confounds. This study importantly confirms the conclusions drawn from receptor binding studies indicating that there is no upregulation of GR within 18 h after adrenalectomy, whereas a substantial upregulation occurs by 5 days after adrenalectomy. Moreover, this study demonstrates that a high dose bolus injection of corticosterone does not

produce a significant downregulation of GR protein levels within 2 h. In addition, this study helps to cross validate the ability of receptor binding studies to estimate relative level of GR activation/occupation as long as appropriate comparison groups are utilized. Further detailed investigation of the in vivo regulation of GR protein expression and function will be important given the wide-ranging regulatory effects that glucocorticoids have on brain function. Utilizing a combination of both receptor binding and western blot measures of GR may be especially effective for these investigations.

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References

[1] J.L. Arriza, C. Weinberger, G. Cerelli, T.M. Glaser, B.L. Handelin, D.E. Housman, R.M. Evans, Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor, Science 237 (1987) 268–275.
[2] C.A. Beck, P.A. Estes, B.J. Bona, C.A. Muro-Cacho, S.K. Nordeen, D.P. Edwards, The steroid antagonist RU486 exerts different effects on the glucocorticoid and progesterone receptors, Endocrinology 133 (1993) 728–740.
[3] D.L. Bellingham, M. Sar, J.A. Cidlowski, Ligand-dependent down-regulation of stably transfected human glucocorticoid receptors is associated with the loss of functional glucocorticoid responsiveness, Mol. Endocrinol. 6 (1992) 2090–2102.
[4] S.P. Bohen, K.R. Yamamoto, Modulation of steroid receptor signal transduction by heat shock proteins, in: The Biology of Heat Shock Proteins and Molecular Chaperones, Cold Spring Harbor Laboratory Press, 1994, pp. 313–334.
[5] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 27 (1976) 248–254.
[6] H.M. Chao, P.H. Choo, B.S. McEwen, Glucocorticoid and mineralocorticoid receptor mRNA expression in rat brain, Neuroendocrinology 50 (1989) 365–371.
[7] Y.C. Chou, W.G. Luttge, Activated type II receptors in brain cannot rebind glucocorticoids: relationship to progesterone's antiglucocorticoid actions, Brain Res. 440 (1988) 67–68.
[8] J.A. Cidlowski, D.L. Bellingham, F.E. Powell-Oliver, D.B. Lubahn, M. Sar, Novel antipeptide antibodies to the human glucocorticoid receptor: recognition of multiple receptor forms in vitro and distinct localization of cytoplasmic and nuclear receptors, Mol. Endo. 4 (1990) 1427–1437.
[9] H. Coirini, A.M. Magarinos, A.F. De Nicola, T.C. Rainbow, B.S. McEwen, Further studies of brain aldosterone binding sites employing new mineralocorticoid and glucocorticoid receptor markers in vitro, Brain Res. 361 (1985) 212–216.
[10] E.R. de Kloet, E. Vredugdenhil, M.S. Oitzl, M. Joels, Brain corticosteroid receptor balance in health and disease, Endocr. Rev. 19 (1998) 269–301.
[11] T. Deak, K.T. Nguyen, C.S. Cotter, M. Fleshner, L.R. Watkins, S.F. Maier, R.L. Spencer, Long term changes in mineralocorticoid and glucocorticoid receptor occupancy following exposure to an acute stressor, Brain Res. 847 (1999) 211–220.
[12] F.S. Dhabhar, A.H. Miller, B.S. McEwen, R.L. Spencer, Differential activation of adrenal-steroid receptors in neural and immune tissues of Sprague Dawley, Fischer 344 and Lewis Rats, J. Neuroimmunology 56 (1995) 77–90.
[13] Y. Dong, L. Poellinger, J.-A. Gustafsson, S. Okret, Regulation of glucocorticoid receptor expression: evidence for transcriptional and posttranslational mechanisms, Mol. Endo. 2 (1988) 1256–1264.
[14] J.C. Eldridge, D.G. Fleenor, D.S. Kerr, P.W. Landfield, Impaired up-regulation of type II corticosteroid receptors in hippocampus of aged rats, Brain Res. 478 (1989) 248–256.
[15] R.M. Evans, The steroid and thyroid hormone receptor superfamily, Science 240 (1988) 889–895.
[16] J.-A. Gustafsson, J. Carlstedt-Duke, L. Poellinger, S. Okret, A.-C. Wikstrom, M. Bronnegard, M. Gillner, Y. Dong, K. Fuxe, A. Cintra, A. Harfstrand, L. Agnati, Biochemistry, molecular biology, and physiology of the glucocorticoid receptor, Endocr. Rev. 8 (1987) 185–234.
[17] W.J. Hendry, R. Hakkak, R.W. Harrison, An analysis of autologous glucocorticoid receptor protein regulation in AtT-20 cells also reveals differential specificity of the BuGR2 monoclonal antibody, Biochim.Biophys. Acta 1178 (1993) 176–188.
[18] W. Hoeck, S. Rucsoni, B. Groner, Down-regulation and phosphorylation of glucocorticoid receptors in cultured cells, J. Biol. Chem. 264 (1989) 14396–14402.
[19] S.M. Hollenberg, C. Weinberger, E.S. Ong, G. Cerelli, A. Oro, R. Lebo, E.B. Thompson, M.G. Rosenfeld, R.M. Evans, Primary structure and expression of a functional human glucocorticoid receptor cDNA, Nature 318 (1985) 635–641.
[20] F. Holsboer, N. Barden, Antidepressants and hypothalamic-pituitary-adrenocortical regulation, Endocr. Rev. 17 (1996) 187–205.
[21] S.S. Lim-Tio, M.-C. Keightley, P.J. Fuller, Determinants of specificity of transactivation by the mineralocorticoid or glucocorticoid receptor, Endocrinology 138 (1997) 2537–2543.
[22] J.F. Lopez, D.T. Chalmers, K.Y. Little, S.J. Watson, Regulation of serotonin$_{1A}$, glucocorticoid, and mineralocorticoid receptor in rat and human hippocampus: implications for the neurobiology of depression, Biol. Psychiatry 43 (1998) 547–573.
[23] S. Makino, M.A. Smith, P.W. Gold, Increased expression of corticotropin-releasing hormone and vasopressin messenger ribonucleic acid (mRNA) in the hypothalamic paraventricular nucleus during repeated stress: association with reduction in glucocorticoid receptor mRNA levels, Endocrinology 136 (1995) 3299–3309.
[24] B.S. McEwen, G. Wallach, C. Magnus, Corticosterone binding to hippocampus: immediate and delayed influences of the absence of adrenal secretion, Brain Res. 70 (1974) 321–334.
[25] B.S. McEwen, J.M. Weiss, L.S. Schwartz, Retention of corticosterone by cell nuclei from brain regions of adrenalectomized rats, Brain Res. 17 (1970) 471–482.
[26] W.R. McIntyre, H.H. Samuels, Triamcinolone acetonide regulates glucocorticoid-receptor levels by decreasing the half-life of the activated nuclear-receptor form, J. Biol. Chem. 260 (1985) 418–427.
[27] M.J. Meaney, D.H. Aitken, C. van Berkel, S. Bhatnagar, R.M. Sapolsky, Effect of neonatal handling on age-related impairments associated with the hippocampus, Science 239 (1988) 766–768.
[28] M.J. Meaney, V. Viau, D.H. Aitken, S. Bhatnagar, Stress-induced occupancy and translocation of hippocampal glucocorticoid receptors, Brain Res. 445 (1988) 198–203.
[29] A.S. Meyer, T.J. Schmidt, Differential effects of agonists and antagonists on autoregulation of glucocorticoid receptors in a rat colonic adenocarcinoma cell line, J. Steroid Biochem. Molec. Biol. 62 (1997) 97–105.
[30] J.S. Meyer, D.J. Micco, B.S. Stephenson, L.C. Krey, B.S. McEwen, Subcutaneous implantation method for chronic glucocorticoid replacement therapy, Physiol. Behav. 22 (1979) 867–870.
[31] R. Miesfeld, S. Okret, A.-C. Wikstrom, O. Wrange, J.-A. Gustafsson, K.R. Yamamoto, Characterization of a steroid hormone receptor

gene and mRNA in wild-type and mutant cells, Nature 312 (1984) 779–781.
[32] A.H. Miller, R.L. Spencer, M. Stein, B.S. McEwen, Adrenal steroid receptor binding in spleen and thymus after stress or dexamethasone, Am. J. Physiol. 259 (1990) E405–E412.
[33] R.H. Oakley, J.A. Cidlowski, Homologous down regulation of the glucocorticoid receptor: the molecular machinery, Crit. Rev. Eukaryotic Gene Expression 3 (1993) 63–88.
[34] D. O'Donnell, D. Francis, S. Weaver, M.J. Meaney, Effects of adrenalectomy and corticosterone placement on glucocorticoid receptor levels in rat brain tissue: a comparison between western blotting and receptor binding assays, Brain Res. 687 (1995) 133–142.
[35] J. Ortiz, J.L. de Caprio, T.A. Kosten, E.J. Nestler, Strain-selective effects of corticosterone on locomotor sensitization to cocaine and on levels of tyrosine hydroxylase and glucocorticoid receptor in the ventral tegmental area, Neuroscience 67 (1995) 383–397.
[36] A. Peiffer, N. Barden, M.J. Meaney, Age-related changes in glucocorticoid receptor binding and mRNA levels in the rat brain and pituitary, Neurobiol. Aging 12 (1991) 475–479.
[37] P.V. Piazza, M. Le Moal, Glucocorticoids as a biological substrate of reward: physiological and pathophysiological implications, Brain Res. Rev. 25 (1997) 359–372.
[38] J.M.H.M. Reul, E.R. de Kloet, Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation, Endocrinology 117 (1985) 2505–2511.
[39] J.M.H.M. Reul, P.T. Pearce, J.W. Funder, Z.S. Krozowski, Type I and Type II corticosteroid receptor gene expression in the rat: effect of adrenalectomy and dexamethasone administration, Mol. Endo. 3 (1989) 1674–1680.
[40] J.M.H.M. Reul, F.R. van den Bosch, E.R. de Kloet, Relative occupation of type-I and type-II corticosteroid receptors in rat brain following stress and dexamethasone treatment: functional implications, J. Endocr. 115 (1987) 459–467.
[41] E.J. Roy, B.S. McEwen, An exchange assay for estrogen receptors in cell nuclei of the adult rat brain, Steroids 30 (1977) 657–669.
[42] E.R. Sanchez, Heat shock induces translocation to the nucleus of the unliganded glucocorticoid receptor, J. Biol. Chem. 267 (1992) 17–20.
[43] R.M. Sapolsky, L.C. Krey, B.S. McEwen, Stress down-regulates corticosterone receptors in a site-specific manner in the brain, Endocrinology 114 (1984) 287–292.
[44] R.M. Sapolsky, L.C. Krey, B.S. McEwen, The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis, Endocr. Rev. 7 (1986) 284–301.
[45] W.K. Shea, J.W. Cowens, M.M. Ip, New site-directed polyclonal antibody maps n-terminus of occluded region of the non-transformed glucocorticoid receptor oligomer to within BUGR epitope, J. Steroid Biochem. Molec. Biol. 39 (1991) 433–447.
[46] R.L. Spencer, P.J. Kim, B.A. Kalman, M.A. Cole, Evidence for mineralocorticoid receptor facilitation of glucocorticoid receptor-dependent regulation of hypothalamic-pituitary-adrenal axis activity, Endo. 139 (1998) 2718–2726.
[47] R.L. Spencer, E.A. Young, P.H. Choo, B.S. McEwen, Adrenal steroid type I and type II receptor binding: estimates of in vivo receptor number, occupancy, and activation with varying level of steroid, Brain Res. 514 (1990) 37–48.
[48] R.L. Spencer, A.H. Miller, H. Moday, M. Stein, B.S. McEwen, Diurnal differences in basal and acute stress levels of type I and type II adrenal steroid receptor activation in neural and immune tissues, Endocrinology 133 (1993) 1941–1950.
[49] R. Yehuda, M.T. Lowy, S.M. Southwick, D. Shaffer, E.L. Giller Jr., Lymphocyte glucocorticoid receptor number in posttraumatic stress disorder, Am. J. Psychiatry 148 (1991) 499–504.