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Abstract	Glucocorticoids are steroid hormones vital for organ system homeostasis and for the maintenance of essential biological processes. A significant part of these actions are mediated through glucocorticoid receptor (GR) that belongs to the nuclear receptor superfamily. To cover such variety of processes the different glucocorticoids act through different GR isoforms that are originated due to posttranscriptional and posttranslational mechanisms. For this reason when evaluating the levels of GRs we should preferentially determine protein levels instead of gene expression. Here, we describe the detection by Western blotting of the GR (α and β isoforms) protein, using macrodissected brain tissue.

Key words (separated by "-")	Glucocorticoid receptor - Brain - Protein levels - Western blot

Chapter 20

Detection of the Glucocorticoid Receptors in Brain Protein Extracts by SDS-PAGE

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Abstract

Glucocorticoids are steroid hormones vital for organ system homeostasis and for the maintenance of essential biological processes. A significant part of these actions are mediated through glucocorticoid receptor (GR) that belongs to the nuclear receptor superfamily. To cover such variety of processes the different glucocorticoids act through different GR isoforms that are originated due to posttranscriptional and post-translational mechanisms. For this reason when evaluating the levels of GRs we should preferentially determine protein levels instead of gene expression. Here, we describe the detection by Western blotting of the GR (α and β isoforms) protein, using macrodissected brain tissue.

Key words Glucocorticoid receptor, Brain, Protein levels, Western blot

1 Introduction

Glucocorticoids are steroid hormones that are regulated in a circadian and stress-associated manner to maintain various functions of the different body systems [1, 2]. These hormones regulate essential biological processes including growth, inflammation, metabolism, apoptosis, and behavior. Most of these actions are mediated at the genome level through the action of two distinct receptors: the high-affinity mineralocorticoid receptor (MR) and the low-affinity glucocorticoid receptor (GR) [1]; herein, we will describe the detection of GR given its ubiquitous distribution throughout the body (including the central nervous system cells), and because of the availability of good antibodies for its identification. In the absence of glucocorticoids, GR is located in the cytoplasm bound to chaperone proteins such as heat shock protein 90 [3]. Glucocorticoids are lipophilic molecules and they can easily cross the cell membrane. Then, intracellularly they bind to GR that undergoes a conformational change, with the consequent GR translocation to the nucleus. In the nucleus the ligand-bound GR recognizes and binds to specific DNA sequences, known as

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glucocorticoid-response elements (GREs), which are usually located in the promoter region of target genes [4]. Once it binds to these DNA regions GR modulates gene expression mainly through activation and/or repression mechanisms. The diversity observed in GR signaling comes from the fact that different genes may contain, or not, GREs, and multiple receptor isoforms can be generated by alternative splicing and alternative translation initiation [2]. Also of relevance are the multiple posttranslational modifications that GR can suffer such as phosphorylation, acetylation, ubiquitination, and SUMOylation with small ubiquitin-related modifier proteins that can alter the function of this transcription factor [5]. One of the most relevant posttranscriptional alterations in GR synthesis is the alternative splicing that occurs in exon 9 (GR is the product of a single gene, NR3C1, that contains nine exons both in humans and in rodents). This alternative splicing results in two isoforms of the GR: GRα and GRβ, which are identical through amino acid 727 but differ in their C terminal [5]. The GRα isoform binds to glucocorticoids, translocates to the nucleus, and recruits coregulators to exert transcriptional effects. However, the GRβ isoform resides constitutively in the nucleus and acts as a natural dominant negative inhibitor of the GRα isoform [2]. Here we will describe a Western blot method to quantify GR in rodents using an antibody that recognizes both isoforms [GRα (more abundant) and GRβ], since it is an affinity-purified polyclonal antibody raised against a peptide mapping at the N-terminus of GR of mouse origin. The first step in the Western blotting procedure is, after protein extraction and quantification, using mixtures of solubilized proteins under denaturing conditions [in the presence of detergent, sodium-dodecyl-sulfate (SDS), and reducing agent (β-mercaptoethanol)] to separate the macromolecules based on their molecular weights using a polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the separated molecules are transferred or blotted onto a second matrix, generally a nitrocellulose or polyvinylidene-difluoride (PVDF) membrane. Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. After blocking, the membrane is incubated with a primary antibody that will recognize and bind the antigen. A labeled secondary antibody that is directed against the primary antibody is then used. Labels include biotin, fluorescent probes such as fluorescein or rhodamine, and enzyme conjugates such as horseradish peroxidase or alkaline phosphatase. An appropriate substrate is then added to the enzyme and together they produce a detectable product such as a chromogenic precipitate on the membrane for colorimetric detection. The most sensitive detection methods use a chemiluminescent substrate that, when combined with the enzyme, produces light as a by-product. The light output can be captured using film, a CCD camera, or a phosphoimager that is designed for chemiluminescent detection.

Alternatively, fluorescent-tagged antibodies can be used, which are directly detected with the help of a fluorescence imaging system. Whatever system is used, the intensity of the signal should correlate with the abundance of the antigen on the membrane when normalized for a housekeeping protein.

2 Materials

All solutions are prepared using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. C57BL/6 male mice at 8- to 9-week of age (Charles River) are used and mice brain regions are collected by macrodissection, using a light microscope (SZX7, Olympus).

2.1 Protein Extraction and Denaturation Buffers

1. Radio-Immunoprecipitation Assay (RIPA) buffer (*see* **Note 1**): 50 mM Tris–HCl, pH 8.0, 250 mM sodium chloride (NaCl), 2 mM EDTA, 10 % Glycerol, 1 Complete Protease Inhibitor Cocktail Tablet (Roche Diagnostics), and inhibitor phosphatases 1 and 2 cocktail (Sigma-Aldrich).
   Prepare:
   - 1 M of Tris–HCl, pH 8.0: weight 30.3 g Trizma Base and add 150 mL of water, adjust pH to 8.0, and adjust with water until 250 mL.
   - 3 M NaCl: weight 5.84 g of NaCl and add 100 mL of water.
   - 0.5 M Ethylene-diamine-tetraacetic acid (EDTA), pH 8.0: weight 186.1 g of EDTA and add 700 mL of water, adjust pH to 8.0 with NaOH 10 M (~50 mL), and complete with water until 1 L.
   - Dissolve one tablet of Complete Protease Inhibitor Cocktail in 2 mL of water.

   For 20 mL of RIPA buffer, use 1 mL of 1 M Tris–HCl, pH 8.0, 0.2 mL of 3 M NaCl, 40 µL of 0.5 M EDTA, 2.3 mL Glycerol (87 %), 500 µL proteases inhibitor, 200 µL cocktail inhibitor phosphatases 1 and 2. Add water until 20 mL. Store at −20 °C. Before use, add 1 mM phenyl-methanesulfonyl-fluoride (PMSF). A PMSF solution at 100 mM can be prepared by weighting 0.7 g of PMSF in 40 mL ethanol 100 % (*see* **Note 2**). Add 200 µL of this solution to the 20 mL of RIPA solution.
2. For quantification: use Quick Start™ Bradford 1× Dye Reagent (Bio-rad, Hercules, CA, USA) following the manufacturer instructions.
3. SDS denaturating/lysis buffer (5×): 0.3 M Tris–HCl, pH 6.8, 10 % SDS (*see* **Note 3**), 25 % β-mercaptoethanol, 0.1 % bromophenol blue, 45 % glycerol. For 10 mL use 6 mL of 0.5 M

Tris–HCl, pH 6.8 (*see* Subheading 2.2), 1 g of SDS, 2.5 mL of β-mercaptoethanol, 0.01 g of bromophenol blue, and 4.5 mL glycerol 100 %. Aliquot and store at −20 °C.

2.2 SDS Polyacrylamide Gel Materials and Solutions

1. Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8, weigh 181.7 g Tris, and transfer to a 1 L flask. Add water to a volume of 900 mL. Mix and adjust pH with HCl. Complete to 1 L with water. Store at 4 °C.
2. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8. Weigh 60.6 g Tris and prepare a 1 L solution, as in the previous step. Store at 4 °C.
3. 40 % acrylamide and bis-acrylamide solution 19:1 (Bio-Rad; *see* **Note 4**). Store at 4 °C.
4. SDS 10 %: weigh 5 g of SDS to 50 mL of water. Store at room temperature (*see* **Note 5**).
5. *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED; Bio-Rad). Store at room temperature (*see* **Note 6**).
6. Ammonium persulfate (APS) 10 %: weigh 1 g of APS and dissolve in 10 mL of water (*see* **Note 7**).
7. SDS-PAGE running buffer: 0.025 M Tris–HCl, pH 8.3, 0.192 M glycine, 0.1 % SDS. Prepare 10× native buffer (0.25 M Tris, 1.92 M glycine, 1 % SDS), for that weigh 30 g Tris, 144 g glycine, and 10 g SDS, mix, and make it to 1 L with water (*see* **Note 3**). Dilute 100 mL of 10× native buffer to 900 mL with water.
8. Protein markers (Page Ruler™ Plus Prestained Protein Ladder, Fermentas, Thermo Scientific).

2.3 Immunoblotting Solutions

1. Western blot transfer buffer: 0.025 M Tris, 0.192 M glycine, and 20 % methanol. Prepare 10× transfer buffer (0.25 M Tris, 1.92 M glycine). Weigh 30 g Tris and 144 g glycine to make it to 1 L with water and mix until dissolution of the powder. Dilute 100 mL of 10× transfer buffer to 700 mL with water and add 200 mL of methanol (*see* **Note 8**).
2. Phosphate buffered saline (PBS; 10×): 1.5 M NaCl, 0.1 M Tris–HCl, pH 7.4. Add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate dibasic (Na_2HPO_4), and 2.4 g potassium phosphate monobasic (KH_2PO_4), dissolve with 800 mL of water, adjust pH to 7.4, and fill with water until 1 L.
3. PBS-0.2 % T: dissolve 2 mL of Tween-20 in 1 L of PBS. Store at 4 °C.
4. Blocking solution (5 % milk in PBS-T): weigh 5 g of milk in 100 mL of PBS-T and store at 4 °C.

5. Antibody diluent solution (2.5 % milk in PBS-T): weigh 0.25 g of milk in 10 mL of PBS-T and store at 4 °C.
6. Clarity™ Western ECL Substrate (Bio-Rad).

2.4 Western Blot Antibodies

1. Primary antibody: mouse anti-GR (M-20; Santa Cruz Biotechnology). This antibody is recommended for detection of mouse and rat GRα and GRβ. Cross-reactivity with human GR is, however, indicated by the manufacturer.
2. Secondary antibody: anti-rabbit IgG-HRP (SC-2301; Santa Cruz Biotechnology).

2.5 Immunoblotting Materials

1. Mini-PROTEAN Tetra Cell Electrophoresis Module (Bio-Rad).
2. Mini Trans-Blot cell (Bio-Rad).
3. Nitrocellulose membranes (Amersham, GE Healthcare).
4. Whatman no.3 filter.

3 Methods

Unless otherwise specified, carry out all procedures at room temperature.

3.1 Preparation of the Gel

1. Set the casting frames (clamp two glass plates in the casting frames) on the casting stands (*see* **Note 9**).
2. Prepare the running gel: 12.5 mL of 40 % acrylamide solution, 12.5 mL 1.5 M Tris–HCl, pH 8.8, 500 µL SDS 10 %, complete with water until 50 mL. From this mixture and for each mL of solution use 10 µL of APS 10 % and 1 µL of TEMED for polymerization. This is a 10 % acrylamide gel that will allow a good resolution of the GR protein, which migrates with an apparent molecular weight of 95 kDa. The volume will depend on the protein apparatus that is available in the laboratory.
3. Swirl the solution gently but thoroughly.
4. Pipet appropriate amount of separating gel solution (listed above) into the gap between the glass plates.
5. To make the top of the separating gel horizontal, fill the gap until the top of the glasses with water (isopropanol can also be used).
6. Wait for 20–30 min to let it gelate (*see* **Note 10**).
7. Prepare the stacking gel: 5 mL of 40 % acrylamide solution, 12.6 mL of 0.5 M Tris–HCl, pH 6.8, 500 µL SDS 10 % water until 50 mL. From this mixture and for each milliliter of solution use 10 µL of APS 10 % and 1 µL of TEMED for polymerization. This is a 4 % acrylamide gel.

8. Discard the water or the isopropanol.
9. Pipette in stacking gel until it overflows.
10. Insert the well-forming comb without trapping air under the teeth. Wait for 20–30 min to let it polymerized.
11. Make sure a complete polymerization of the stacking gel occurred and remove the comb. Take the glass plates out of the casting frame and set them in the running tank. Put the running buffer (electrophoresis buffer) into the inner chamber and keep pouring after overflow until the buffer surface reaches the required level in the outer chamber.

3.2 Preparation of the Samples

C57BL/6 male mice (8- to 9-week of age) are utilized. Mice are anesthetized with ketamine hydrochloride (150 mg/kg) plus medetomidine (0.3 mg/kg). Upon transcardiac perfusion with saline buffer, brain regions are collected by macrodissection using a conventional light microscopy. Samples are immediately frozen in dry ice and stored at −80 °C. Use chemical inhibitors and controlled temperature is strongly suggested during the below described steps to minimize the activity of proteases and other enzymes that may modify the protein composition of the sample.

1. Add RIPA buffer to the brain samples (*see* **Note 11**) and pass through a 20 G needle until all the tissue is disrupted (*see* **Note 12**).
2. Sonicate the sample on ice five times, for 2 s every time (*see* **Note 13**).
3. Centrifuge at 13,000 rpm during 10 min at 4 °C. [AU1]
4. Remove the supernatant for a new centrifugation, as in **step 3**.
5. Perform a protein assay to determine the protein concentration of the supernatant that should be 5–10 μg/μL.
6. Mix 25 μg of total protein of each sample with SDS denaturating/lysis buffer (loading buffer). The maximum volume that is possible to load in the well depends on the comb used.
7. Heat them in boiling water for 5–10 min.
8. Load the protein marker to the first well and then load prepared samples into the other wells, making sure not to overflow. Then close the chamber with the cover and connect the anodes.
9. Set an appropriate volt, usually 100 V, and run the electrophoresis.
10. As for the total running time, since we are analyzing a protein that is approximately 95 kDa, stop SDS-PAGE running when a good resolution of the proteins with higher molecular weight in the protein marker used is visible.

3.3 Immunoblot

We use the wet transfer unit (Mini Trans-Blot cell) to perform the transference of the proteins from the gel to the membrane. Using this electrophoretic transfer system, the gel and membrane sandwich is entirely submerged in transfer buffer within a buffer tank. A nonconducting cassette holds the membrane in close contact with the gel and the cassette assembly is placed in the tank between the electrodes, transverse to the electrical field, and submerged under conducting transfer buffer.

1. Place the transfer tank onto a magnetic stir-plate.
2. Add enough transfer buffer to the tank to fill it approximately halfway, add a stirbar, and begin stirring. This will help to maintain uniform conductivity and temperature during electrophoretic transfer.
3. Set up the cooling system for the tank transfer system.
4. Immediately following SDS-PAGE separate the gel plates with the help of a spatula or similar tool. Remove the stacking gel.
5. Insert the gel carefully in a box with deionized water to remove traces of SDS-PAGE running buffer and then let it equilibrate in Western blot transfer buffer.
6. Let the membrane equilibrate for 5 min in western blot transfer buffer. [AU2]
7. Embed the six sections of Whatman no. 3 filter paper in the Western blot transfer buffer.
8. Soak a fiber pad in transfer buffer and place it on top of the white side of the cassette.
9. Place on the top of the fiber pad three sections of embedded Whatman no. 3 filter paper and remove the bubbles. On top put the membrane without the formation of bubbles (*see* **Note 14**).
10. Place the gel on the top of the membrane followed by three or more embedded Whatman no. 3 filter paper. Remove the bubbles (*see* **Note 14**).
11. Soak a fiber pad in transfer buffer and place it on top of the filter paper.
12. Close the cassette and lock it, insert it into the tank. Make sure the black cassette plate faces the black electrode plate.
13. Add transfer buffer to the tank until the tank is filled and place the lid on top, checking that the color-coded cables on the lid are attached to the electrode cards of the same color.
14. Connect the cables to the power supply and set at 100 V/350 mA and run for 1 h.
15. Upon completion of the run, remove the cassettes and disassemble the gel and membrane sandwich. Rinse the membrane briefly in water to ensure that no residual gel pieces stay adherent to the membrane.

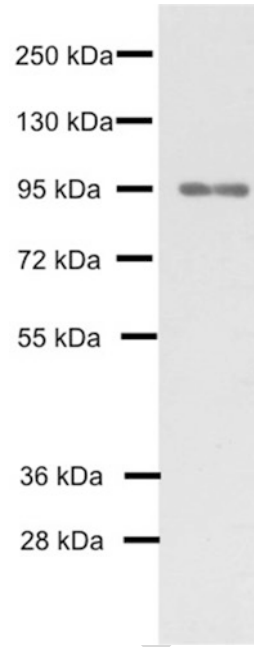

Fig. 1 The figure shows the immunoblot of protein extracted from mouse hippocampus with anti-GR Ab. One 95 kDa band is detected. It corresponds to GRα, the most abundant isoform

16. Incubate for 5 min in Ponceau S dye (*see* **Note 15**).
17. Wash in water until the Ponceau S staining disappears from the membrane.
18. Block the membranes with blocking solution for 1 h.
19. Wash 3 × 10 min in PBS-T.
20. Incubate with diluted (1:1,000 in PBS-T) anti-GR antibody and incubate overnight at 4 °C with shaking.
21. Wash 3 × 10 min in PBS-T.
22. Incubate with secondary antibody (diluted 1:10,000 in PBS-T) for 1 h at RT.
23. Wash 3 × 10 min in PBS-T.
24. Develop the blot using Clarity™ Western ECL Substrate and incubate for 5 min, following manufacture instructions.
25. Expose the membrane to X-ray film during approximately 3–5 min (*see* **Note 16**).
26. Check the molecular weight of the band putting the film on the top of the membrane and checking the position of the band relatively to the protein marker (Fig. 1) (*see* **Note 17**).

4 Notes

1. RIPA buffer enables efficient cell lysis and protein solubilization while avoiding protein degradation and interference with the proteins' immune reactivity and biological activity.
2. Store the PMSF solution at −20 °C protected from the light.
3. Care should be taken to weight SDS since it is toxic; individual protection mask should be used.
4. Due to the Acrylamide/Bis solution toxicity we advice to use the commercially available ones.
5. SDS can precipitate if stored at 4 °C or if the room temperature is too low, if this happens dissolve it by warming if needed.
6. Due to its pungent smell when pipetting TEMED you should use individual protection mask. If stored at 4 °C, its pungent smell is reduced.
7. When aliquots of APS are stored at −20 °C for long periods, the correct gel polymerization might be compromised.
8. Care should be taken to add methanol since it is toxic; it should be done in a hood. Use only high-quality, analytical grade methanol. Impure methanol can cause increased transfer buffer conductivity and poor transfer. Avoid adding methanol directly to the 10× buffer, since it precipitates its ingredients.
9. All glass plates should be clean and dry.
10. Various factors affect the properties of the resulting gel: higher concentration of APS and TEMED will lead to a faster polymerization, but on the other hand, to a lower stability and elasticity. The optical temperature for gelation is 23–25 °C. Low temperature will lead to turbid, porous, and inelastic gels.
11. A suggestion of the RIPA volume that should be used for different brain regions is indicated in Table 1.

Table 1
Volume of RIPA for each mouse brain region to obtain approximately 14 ng of protein

Brain region	RIPA volume (μL)
Brainstem	400
Cerebellum	300
Hypothalamus	150
Hippocampus	300
Cortex	600
Substantianigra	30

12. Mechanical cell lysis usually generates heat. Use cooling where required to avoid overheating the sample.
13. Pause between sonication steps to avoid overheating.
14. A 10 mL pipette can be used to remove by rolling out the air bubbles from the gel-membrane sandwich prior to placing in the transfer cassette.
15. It is recommended to perform Ponceau S staining before proceeding for the immune-blot to control the transference efficiency and the efficient running of the samples in the gel. If the efficiency was low or if the protein running in the acrylamide gel was abnormal or if there are bubbles in the membrane, do not proceed with the immunoblot.
16. Exposure time to the X-ray film will also depend on the expression level of GR in the sample.
17. Predicted band size: 87 kDa; observed band size: 95 kDa.

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Queries	Details Required	Author's Response
AU1	Please provide g-force value for 13,000 rpm.	
AU2	Please check if edit to the sentence "Let the membrane equilibrate …" is appropriate.	