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Further evidence for a membrane receptor that binds glucocorticoids in the rodent hypothalamus

Jebun Nahar¹, Jennifer R. Rainville¹, Gary P. Dohanich^{2,3}, and Jeffrey G. Tasker^{1,3}

¹Department of Cell and Molecular Biology, Tulane University, New Orleans, LA 70118

²Department of Psychology, Tulane University, New Orleans, LA 70118

³Neuroscience Program, Tulane University, New Orleans, LA 70118

Abstract

In parallel with their well-characterized delayed genomic effects, steroid hormones exhibit rapid, non-genomic effects at molecular, cellular and behavioral levels. We have proposed a model of rapid, non-genomic glucocorticoid inhibition of hypothalamic neuroendocrine cells through a putative membrane-associated glucocorticoid receptor (GR). Here we tested for plasma membrane GR immunoreactivity and binding in the hypothalamic supraoptic and paraventricular nuclei. Selective cross-linking of membrane proteins with membrane-impermeant BS³ and subsequent Western blot analysis with a monoclonal GR antibody revealed a reduction in the intensities of a ~98 kDa immunoreactive band and a ~64 kDa band in the rat paraventricular and supraoptic nuclei, and of a 64 kDa band in hippocampal tissue, which suggested that these proteins are associated with the membrane. Saturation binding of [³H]-corticosterone and [³H]-dexamethasone in rat and mouse hypothalamic tissue revealed a K_d 4–24-fold lower and a B_{max} 4–7-fold lower for the membrane-associated GR compared to the intracellular GR, suggesting a lower affinity and abundance of the glucocorticoid binding sites in the membrane than in the cytosol. Together, these findings suggest the presence of a low-affinity, low-abundance membrane-associated GR in the hypothalamus that shares homology with the intracellular GR, and are consistent with physiological evidence of rapid, non-genomic glucocorticoid actions in hypothalamic neuroendocrine cells that are GR dependent.

Keywords

glucocorticoid receptor; corticosteroid; membrane cross-linking; hypothalamus; stress; HPA

Address for correspondence: Jeffrey Tasker, Department of Cell and Molecular Biology, 2000 Percival Stern, Tulane University, New Orleans, LA 70118; tasker@tulane.edu; tel: (504) 862-8726.

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Introduction

The study of rapid responses of steroid hormones predates the discovery of nuclear steroid receptors [1], however, it is only within the last 20 years or so that the rapid responses have begun to receive as much attention as the delayed responses. Glucocorticoid actions are mediated through both low-affinity corticosteroid type II receptors (glucocorticoid receptors, GRs) and high-affinity corticosteroid type I receptors (mineralocorticoid receptors) [2]. Like other steroid hormones, glucocorticoids are thought to access their cognate intracellular receptors by passive diffusion through the plasma membrane due to their lipophilicity, although there is evidence of facilitated transport of glucocorticoid across the membrane by a membrane transporter [3]. Binding to the cytosolic corticosteroid receptors forms a receptor-ligand complex that triggers the translocation of the receptor to the nucleus, where it binds to a hormone response element or interacts with other transcription factors to regulate gene transcription [4,5,6]. This genomic pathway is activated by nanomolar steroid concentrations to cause changes in gene expression and modulate protein levels some hours after stimulation with the steroid. On the other hand, in the rapid-response pathway, steroid hormone binds to putative receptors located on the membrane and activates second messenger systems to directly generate end biological responses or indirectly modulate delayed genomic responses within minutes. In addition to their short time scale, the rapid glucocorticoid signaling is insensitive to inhibitors of transcription and translation and is often, though not always, insensitive to blockade by the GR antagonist mifepristone (RU486), suggesting actions at a separate membrane GR [7–10]. A wide variety of rapid cellular and behavioral responses have been reported in several different species [11,12], indicating an evolutionarily conserved signaling mechanism [13,14].

We have proposed a model of rapid glucocorticoid actions involved in the regulatory feedback of glucocorticoids on the mammalian hypothalamic-pituitary-adrenal axis in which glucocorticoids released during the stress response act in the hypothalamus to suppress the excitatory synaptic drive to corticotropin-releasing hormone (CRH) cells of the paraventricular nucleus (PVN) [15–18]. The rapid glucocorticoid effect in CRH neurons is mimicked by a membrane-impermeant glucocorticoid and is blocked by blocking postsynaptic G-protein activity and $G\alpha_s$ -driven PKA activation [17,19], which suggests it is dependent on a membrane receptor and G protein/protein kinase signaling in the CRH neurons. Unexpectedly, the rapid glucocorticoid actions in mouse PVN neurons were abolished by the conditional genetic deletion of GR, suggesting their dependence on the intracellular GR [20].

While it has yet to be established whether the membrane GR is a distinct membrane receptor or is the nuclear GR associated with the membrane [21–23], several studies have provided anatomical evidence for the localization of the nuclear GR at the membrane in different areas of the brain, including in the hypothalamus [32–34]. The purpose of this study was to further search for evidence of a membrane-associated GR in the rat and mouse hypothalamus by probing of cross-linked membrane proteins for GR immunoreactivity and radio-labeled glucocorticoid binding to hypothalamic membrane fractions.

Experimental

Animals

Male CD1 mice and Sprague Dawley rats 3–5 weeks of age were obtained from Harlan (Indianapolis, IN). Upon arrival at the animal facility, animals were placed on a 12:12 hour light–dark cycle with fresh food and water *ad libitum* for 1–2 weeks. They were then used for experiments at 6–8 weeks of age according to protocols approved by the Tulane University Institutional Animal Care and Use Committee and in accordance with US Public Health Service guidelines.

Reagents

Most reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless stated otherwise. [1,2,4,6,7-³H]-dexamethasone ([³H]-Dex; specific activity, 85–90 Ci/mmol; 1mCi = 37MBq) and [1,2,6,7-³H]-corticosterone ([³H]-Cort; specific activity, 75–79 Ci/mmol; 1mCi = 37MBq) were purchased from Amersham Biosciences (Pittsburgh, PA). Bis (sulfosuccinimidyl) suberate (BS³), a membrane-impermeant protein cross-linking agent [24], was purchased from Pierce (Rockford, IL).

Tissue Preparation

Mice were anesthetized with isoflurane (VetOne, Meridian, ID) inhalation in a closed chamber and decapitated using a rodent guillotine. For experiments that required tissue from more than one mouse, mice were kept in a separate room until the time of sacrifice and were sacrificed one at a time. The brains were removed and immediately immersed in 0–1°C oxygenated artificial cerebral spinal fluid (ACSF) composed of (in mM): 140 NaCl, 3 KCl, 1.3 MgSO₄, 1.4 NaH₂PO₄, 2.4 CaCl₂, 11 glucose, and 5 HEPES, with an osmolarity of 290–300 mOsm/l and a pH adjusted to 7.2–7.3 with NaOH. For western blot analyses, the hypothalamus was blocked and sectioned at a thickness of (500 μm?), and slices were trimmed to isolate the supraoptic nucleus (SON) and PVN. For saturation binding experiments, whole hypothalamic blocks, approximately 5 mm³, were used.

Rats were anesthetized with 50-mg/kg sodium pentobarbital, decapitated with a rodent guillotine and their brains were quickly removed and immersed in ice-cold, oxygenated artificial cerebral spinal fluid (aCSF) composed of (in mM): 140 NaCl, 3 KCl, 1.3 MgSO₄, 1.4 NaH₂PO₄, 2.4 CaCl₂, 11 glucose and 5 HEPES. The pH of the aCSF was adjusted to 7.2–7.4 with NaOH and the osmolarity was 290–300 mOsm/l. The hypothalamus was blocked and sectioned at 300 μm using a vibrating slicer (Vibratome) for Western blot analyses; slices were trimmed around the SON and PVN and pooled together. For saturation binding experiments, 8 whole hypothalamic blocks were pooled to prepare membrane fractions, and 4 whole hypothalamic blocks were pooled to prepare cytoplasmic fractions.

Membrane Protein Cross-Linking

Fresh hypothalamic slices from rats were minced on a tissue chopper and minced samples were treated with BS³ at a final concentration of 2 mM in 1 ml ice-cold ACSF. Stock solutions of BS³ were prepared in 5 mM sodium citrate, pH 5.0, and were diluted (1:100) just prior to addition of the tissue. Samples were incubated at 4°C for 30 min with gentle

mixing and the reaction was terminated with glycine at a final concentration of 100 mM. The samples were then centrifuged at 13,000 rpm for 10 min and the pellets were resuspended in 150 μ l ice-cold lysis buffer containing protease and phosphatase inhibitors (25 mM HEPES, 500 mM NaCl, 2 mM EDTA, 1 mM DTT, 1 mM phenylmethyl sulfonyl fluoride, 20 mM NaF, 0.1% Nonidet P-40 (v/v), and 1x protease inhibitor cocktail, pH 7.4). Tissue was homogenized rapidly by a sonic dismembrator (Fisher Scientific, Model 100) for 5 s and then spun at 14,000 RPM for 2.5 min. Supernatant was collected as whole-cell lysate and stored at -80°C prior to analysis. For the control group, all the steps were identical, except no BS³ was added [24]. All the steps were performed at 4°C . Protein concentrations were measured by the Lowry method [25].

Western Blot

Hypothalamic tissue samples from rats (95 μ g total protein/cell lysate) were loaded and electrophoresis was performed on a 12% gradient Tris-HCl gel (Bio-Rad, Hercules, CA) under reducing conditions. A high protein concentration was used in an attempt to reveal low levels of membrane GR protein in the surface protein aggregate. Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes for immunoblotting. PVDF membranes were washed in double-distilled H₂O and blocked with 1% goat serum and 5% nonfat dry milk in TBS-Tween 20 (TBS-T), pH 7.4, for 2 h at room temperature. The membranes were then incubated with a mouse monoclonal antibody directed against a short region of the DNA binding domain of GR (BuGR2, 1:200; Affinity Bioreagents) or a mouse monoclonal antibody directed against β -actin (1:1000, Sigma, Saint Louis, MO) overnight at 4°C . They were then washed 5 times with TBS-T, incubated for 1.5 h with a horseradish peroxidase-conjugated anti-mouse IgG and again washed with TBS-T. The membranes were then rinsed with double-distilled H₂O, immersed in chemiluminescence (ECL) detecting substrate (Amersham Biosciences, Piscataway, NJ) for 3 min, and exposed to HyperFilm ECL film.

Preparation of Neuronal Membrane and Cytoplasmic Fractions

Neuronal membranes were prepared from rat and mouse hypothalamic tissue according to methods described by Whittaker [26] and Orchinik and Murray [27], with slight modifications. Hypothalamic blocks were homogenized with a 2-ml dounce on ice in 5–6 volumes of membrane homogenization buffer (0.3 M Sucrose, 5 mM HEPES and 0.1% protease inhibitor cocktail, pH 7.4) or cytosolic homogenization buffer (10 mM Trizma-base, 1.5 mM Na₂-EDTA, 10% glycerol, 10 mM sodium molybdate, 2 mM DTT, 0.1% protease inhibitor cocktail, pH 7.4) for membrane and cytoplasmic fractions, respectively. For membrane fractions, the homogenate was centrifuged at 500 g for 10 min at 4°C to remove blood cells, nuclear debris, lipids and endogenous ligands. The supernatant containing the crude membrane fraction was ultracentrifuged at 60,000 g for 1 hour at 4°C (Beckman Instruments) and pellets were resuspended in fresh binding buffer (25 mM HEPES, 1 mM EGTA-free acid, 5 mM MgCl₂, 0.1% protease inhibitor cocktail, pH 7.4). For cytoplasmic fractions, samples were ultracentrifuged at 105,000 g for 1 hour and the supernatant was collected. Protein was measured by the Lowry method (Lowry et al., 1951). Samples were stored at -80°C until use.

[³H]-Corticosterone and [³H]-Dexamethasone Binding

All binding assays contained 100 µl of membrane or cytoplasmic extract (0.9 mg – 2.5 mg) in binding buffers in a final volume of 300 µl. A series of increasing concentrations of [³H]-corticosterone (Cort) was used for membrane fractions (1.875 nM – 80 nM) and cytoplasmic fractions (1.875 nM – 40 nM). For membrane binding, reactions were held for 2 h at room temperature, after which the reaction was terminated by rapid filtration of the membrane suspension through Whatman GF/C glass fiber filters using a cell harvester (Biomedical Research and Development Laboratories, Inc., MD, Gaithersburg, MD). Samples were washed twice with ~5ml of assay buffer for 10 s each. For the cytoplasmic fraction, reactions were carried out overnight at 4°C and then terminated by the addition of 500 µl of activated charcoal (1.25% Activated Norit A, 0.125% Dextran 70,000 in cytosolic buffer). Tubes were spun at 2000 g in a 4°C centrifuge for 10 min and the supernatant was collected in scintillation vials. The protein-bound ligand was counted in scintillation fluid (ScintiSafe™ Econo1, Perkin Elmer Life and analytical Sciences, Wellesley, MA) with a beta counter (Beckman Coulter, Beckman, Miami, FL) for 2 min. Concentrations of [³H]-Dex and [³H]-Cort up to 40 nM were used for cytoplasmic fractions and up to 80 nM for membrane fractions. All experimental data were analyzed using Graph Pad Prism software (Graph Pad, Inc., San Diego, CA). Specific binding was calculated by subtracting nonspecific binding (defined in the presence of unlabeled Cort, 10 µM for cytoplasmic and membrane fractions, or unlabeled Dex, 10 µM for cytoplasmic and 100 µM for membrane fractions) from the total binding. Curve fitting was generated with Graph Pad assuming a single binding site. K_d and B_{max} values were calculated using the equation $Y = B_{max} * X / (K_d + X)$. The Scatchard-Rosenthal plot was generated by transformation of saturation binding data. Both total and nonspecific binding were calculated from duplicate samples.

Results

Membrane Localization of GR

We used a membrane-impermeant covalent cross linker to selectively aggregate membrane surface proteins followed by Western blot analysis to detect GR immunoreactivity in the membrane. BS³ is a membrane-impermeant, water-soluble analog of N-hydroxysuccinimide ester that cross-links proteins in a nonspecific manner. Since BS³ is membrane impermeant, and since the tissue preparation does not disrupt the integrity of cell membranes, only membrane proteins with extracellular moieties should form cross-linked protein aggregates [24,28]. We applied the BS³ to rat PVN, SON, and hippocampus tissue samples and then performed Western blot analysis with a monoclonal antibody against the intracellular GR (BuGR2, Affinity Bioreagents). High concentrations of total protein (95 µg) from both BS³-treated and untreated samples were loaded to reveal low-abundance surface GR-immunoreactive protein. A faint GR-immunoreactive band near the gel loading site, representing a high-molecular weight protein aggregate, was detectable in the Western blots of BS³-treated tissue, but not in the untreated tissue samples of PVN, SON, and hippocampus (Fig. 1A–C). The GR immunoreactivity in the BS³ cross-linked tissue, but not in the BS³-untreated tissue, suggested the presence of a membrane surface protein recognized by the GR antibody. An immunoreactive band with a molecular weight of approximately 98 kDa, the molecular weight of GR, was present in both the BS³-treated and

untreated tissue samples. The 98 kDa band density was decreased in the BS³-treated samples in both the PVN and SON, suggesting the association of the intracellular GR with the membrane. However, there was no difference in the 98 kDa band density between treated and untreated samples of the hippocampus, despite the presence of a faint heavy band (Fig. 1C). That the BS³ cross linker was restricted to the extracellular space and failed to penetrate into the intracellular compartment was confirmed by probing in BS³-treated tissue with an antibody to actin, which is exclusively an intracellular protein; an actin-immunolabeled band was not detected in the heavy membrane protein aggregate (data not shown).

Interestingly, along with the emergence of a faint heavy band in the BS³ tissue and the decrease in the 98 kDa band density, the density of a 64 kDa band also decreased in the BS³-treated samples compared to the samples from the untreated tissue (Fig. 1). This indicated a potential candidate for the membrane receptor protein complex with antigenic cross-reactivity with the intracellular GR. There was no difference in the densities of two other bands labeled by the GR antibody, a ~200-kDa band (Band 1) and a ~75-kDa band (Band 3), between untreated and BS³ cross-linked PVN, SON, and hippocampal samples, which suggested that the decrease in the 98-kDa and 64-kDa bands was not a non-specific effect of the BS³ treatment on GR immunoreactivity.

Glucocorticoid Binding to Membrane Fractions

Saturation binding experiments with [³H]-Cort and [³H]-Dex were performed in CD1 mouse and Sprague-Dawley rat hypothalamic tissues to compare the properties of glucocorticoid binding in membrane fractions with those in cytoplasmic fractions and to provide information on the relative abundance of the membrane glucocorticoid binding sites in both species. Cytoplasmic and membrane fractions were prepared separately and saturation binding was done using charcoal separation for cytoplasmic fractions and glass-fiber filter filtration separation with a cell harvester for membrane fractions. Using [³H]-Cort and [³H]-Dex as radioligands to investigate low-affinity membrane sites required high concentrations of radioligands, which increased non-specific interactions of the steroids with the plasma membrane. The relatively low affinity and low-abundance of the membrane glucocorticoid receptors made it necessary to conduct repeated runs (n=2) with increasing concentrations of radioligand. At each concentration, the data from two separate experiments on each fraction were normalized and compared, and values that were similar with repeated measures were considered reliable and disparate repeated measures were considered as reading errors and discarded (Figs. 2, 3). The K_d value for Cort was ~4-fold higher in membrane compared to cytoplasmic fractions from mice (Fig. 2A, Table 1), and 11-fold higher in membrane compared to cytoplasmic fractions from rats (Fig. 2B, Table 1). The B_{max} value for Cort was approximately 5-fold lower for glucocorticoid binding to the membrane fraction compared to the cytoplasmic fraction in both the rat and mouse hypothalamus (Table 1), indicating the presence of a low-affinity, low-abundance corticosteroid receptor on the cell membrane compared to the cytoplasm. The K_d value for Dex was 25-fold higher in membrane compared to cytoplasmic fractions from mice (Fig. 3A, Table 1), and ~21-fold higher in membrane compared to cytoplasmic fractions from rats (Fig. 3B, Table 1). Similar to Cort, the B_{max} value for Dex was approximately 5-fold lower for glucocorticoid binding to the membrane fraction compared to the cytoplasmic fraction in both the rat and mouse

hypothalamus, indicating the presence of a low-affinity, low-abundance glucocorticoid receptor on the cell membrane compared to the cytoplasm (Table 1). A low signal-to-noise ratio, with approximately 25% specific binding at the K_d , was observed and was attributed to the lipophilic nature of the steroids. Binding of Cort and Dex in membrane fractions approached asymptote at the higher concentrations, allowing the calculation of B_{max} values and indicating a saturable binding to a putative membrane receptor (Figs. 2,3).

Discussion

Our findings indicate the presence of a membrane-associated protein in the mouse and rat hypothalamus that is immunostained with a monoclonal antibody to GR and that binds corticosterone and dexamethasone. This provides biochemical evidence of a putative membrane-associated GR in the hypothalamic SON and PVN, as well as in the hippocampus, which supports our previous physiological evidence based on rapid, non-genomic actions of glucocorticoids [15,16,19].

Presence of the Glucocorticoid Receptor in the Membrane

We used a monoclonal antibody against the classical GR (BuGR2) to probe for a membrane-associated GR. This antibody had been used previously to detect glucocorticoid receptor-like immunoreactivity in membranes of human CCRF-CEM acute lymphoblastic leukemia cells [29,30] and S-49 mouse lymphoma cells [31], and in rat liver [32] using light microscopy. Using electron microscopy, GR immunoreactive profiles were also visualized with the BuGR2 antibody on the membrane surface of neuronal processes in sections of rat hypothalamus and amygdala [33–35]. We used a BS^3 protein cross-linking technique to separate covalently cross-linked surface receptors from uncross-linked intracellular receptor proteins according to their different molecular weight [24,36]. Western blot analysis using total protein prepared from BS^3 -treated rat hypothalamic and hippocampal tissue showed low-density immunolabeling of a high-molecular weight protein band in the BS^3 -crosslinked preparation, but not in the BS^3 -untreated tissue, indicating the presence of a GR antigen on the cell surface. A ~98 kDa band, which corresponds to the molecular weight of the intracellular glucocorticoid receptor, was decreased in the cross-linked samples from the PVN and SON compared to the untreated samples, suggesting a possible association of the full length intracellular GR with the plasma membrane in the hypothalamus. There was no significant difference in the 98 kDa bands between untreated and cross-linked hippocampal samples, which may indicate that membrane localization of the full-length GR is reduced or absent in the hippocampus. Additionally, a ~64 kDa immunoreactive band was detected in both hypothalamic and hippocampal samples, and was decreased in the cross-linked groups from the PVN and SON of the hypothalamus, as well as in cross-linked hippocampal samples. This unknown GR-immunoreactive protein presents another candidate for a membrane-localized GR. The Western blot analysis with the BuGR2 antibody revealed multiple immunoreactive bands, the identity or specificity of which is not known. However, only the 98 kDa and 64 kDa bands, and not the other two prominent bands at approximately 200 kDa and 75 kDa, changed in density following the cross linking of membrane proteins, suggesting that the proteins labeled in the other bands are not associated with the membrane. Thus, these data indicate that the monoclonal antibody against the intracellular

glucocorticoid receptor detects both cytosolic and membrane glucocorticoid receptor-like proteins, and suggest that, in addition to their similar ligand binding domains, the membrane glucocorticoid receptor and the intracellular glucocorticoid receptor exhibit common antibody recognition sites. The ~64 kDa protein revealed in our Western blot analysis was similar in size to a 63-kDa putative membrane GR isolated from newt neuronal membranes using affinity chromatography [37].

Distinct Binding Properties of Cytosolic vs. Membrane Glucocorticoid Receptors

Corticosterone bound to hypothalamic membrane with a relatively low affinity ($K_d = 32.18$ nM and 30.75 nM for [3 H]-Cort binding to mouse and rat hypothalamic tissue, respectively). In our experiments, Cort binding in membrane fractions displayed affinities that were ~4-fold (mouse) and 11-fold (rat) lower than those measured in cytosolic fractions, in which we found K_d values for Cort of 8 nM and 2.8 nM in mouse and rat tissue, respectively. The B_{max} values for the membrane fraction represent roughly a 5-fold reduction in membrane receptor abundance in hypothalamic tissue from both mouse and rat. Hypothalamic membranes displayed a comparably low affinity for Dex ($K_d = 38.8$ nM and 42.2 nM for [3 H]-Dex binding to mouse and rat hypothalamic tissue, respectively), with a ~25-fold (mouse) and 21-fold (rat) lower binding affinity compared to the cytoplasmic fractions. The B_{max} values for Dex binding to the membrane fraction showed a similar 5-fold reduction in membrane receptor abundance in hypothalamic tissue from both mouse and rat. The putative membrane GR in both mouse and rat hypothalamic tissue, therefore, has a relatively low affinity and is expressed in low abundance compared to the cytosolic receptor. These K_d values indicate a somewhat higher affinity of the hypothalamic membrane receptors than that reported in rat synaptic plasma membrane fractions ($K_d \approx 100$ nM) [38–40], which may be due to a higher affinity for GR in the hypothalamus than in whole-brain synaptosomal fractions. Synaptic membrane fractions in those studies also showed a significantly lower affinity of glucocorticoid binding than cytoplasmic fractions. Only approximately 25% specific binding was found at the K_d for the membrane receptor, which we attribute to the lipophilic nature of the steroid radioligands and the relatively low abundance of the membrane steroid receptors.

The properties of membrane receptors for glucocorticoids appear to vary across species. The affinity and abundance of membrane receptors are linked to the circulating levels of glucocorticoids characteristic of each species. Amphibian and avian species have lower levels of circulating glucocorticoids, and express higher-affinity, higher-abundance membrane receptors for these steroids [11,41–43] than mammalian species, which have higher levels of circulating glucocorticoids and express lower-affinity, lower-abundance membrane receptors [38,39,44]. Saturation binding experiments in newts with [3 H]-Cort revealed a membrane binding site with a K_d of 0.51 nM [11], indicating about a 10-fold higher affinity than the mouse/rat membrane binding site. The low affinity of glucocorticoid binding to mouse/rat hypothalamic membranes compared to brain tissue may be because mammals have a 50- to 100-fold higher circulating level of corticosterone compared to salamanders, such that less steroid binding may be necessary to produce a similar magnitude of effect. Specific [3 H]-Cort binding in neuronal membranes in the newt was found to be about 85% of total binding, which is consistent with the higher membrane binding affinity in

the newt brain [11]. Also consistent with lower-affinity glucocorticoid receptors in mammalian cell membranes, membrane binding sites for Dex in rat liver were shown to have a K_d value of approximately 400 nM [45], and studies using S-49 mouse lymphoma cells and human CCRF-CEM acute lymphoblastic leukemia cells [29,30] revealed a glucocorticoid K_d value of 239 nM for the membrane fraction compared to 19.5 nM for the cytosolic fraction [46]. Our findings here of K_d values of 30–42 nM suggest an approximately 10-fold higher affinity of glucocorticoid receptors in hypothalamic cell membranes.

Conclusions

Together, our findings suggest the presence of a membrane-associated GR in hypothalamic membranes that is recognized by a monoclonal antibody raised against the DNA binding domain of the classical GR, but that has a lower abundance and binding affinity for Cort or Dex than the intracellular GR. The membrane GR appears, therefore, to share homology with the intracellular GR, which is further suggested by the surface protein cross linking experiments that showed reductions in the levels of both the 98 kDa protein, or putative intracellular GR, in samples of PVN and SON, and a 64 kDa protein recognized by the antibody in samples of PVN, SON, and hippocampus. This also suggests that the membrane-associated GR may be a product of the GR gene, which has been proposed in studies in different immortalized cell lines [8,47]. That the membrane GR and cytosolic GR are products of the same gene is also consistent with our finding of the loss of rapid glucocorticoid effects in hypothalamic neurons with deletion of the GR gene [20], though further studies are required to show this definitively.

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Highlights

- Evidence is presented for a membrane glucocorticoid receptor.
- Glucocorticoid receptor immunoreactive bands are membrane-associated.
- Glucocorticoids bind to rodent hypothalamic membrane fraction.

Figure 1. Association of GR immunoreactivity with membrane proteins following BS³ cross-linking

Western blot analysis of rat hypothalamus and hippocampal lysates with a monoclonal mouse anti-GR (BuGR2, Affinity Bioreagent) following BS³ cross-linking of membrane proteins. Following loading of 95 µg total protein, the GR antibody revealed multiple immunoreactive bands. A heavy molecular weight band near the loading wells was present in BS³-treated tissue, but absent in the untreated tissue, from PVN, SON and hippocampus. Each lane contains PVN, SON, or hippocampal tissue collected from a single rat (n=4 rats/treatment); bar graphs represent mean optical density measures ± SEM. (A) In the PVN, immunoreactive bands at ~98 kDa (IR Band 2) and 64 kDa (IR Band 4) were decreased in the BS³-crosslinked samples compared to untreated samples (Student's *t* test, $p=0.0065$ and 0.036 , respectively). (B) A similar change in band density was observed in the SON, with decreases in both the 98-kDa (IR Band 2, $p=0.0028$) and 64-kDa (IR Band 4, $p=0.0068$) bands in the BS³-crosslinked samples. (C) In the hippocampus, there was a decrease in density of the 64-kDa band (IR Band 4) between the BS³-cross-linked group and untreated group ($p=0.0002$), but no change in the 98-kDa band ($p=0.70$). There was no significant difference in the other immunoreactive bands between untreated and crosslinked samples in

any of the brain regions. β -actin was used as a loading control for all the samples. *, $p < 0.05$; **, $p < 0.01$.

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Figure 2. Saturation binding of [^3H]-corticosterone to mouse and rat hypothalamic membrane and cytoplasmic fractions

[^3H]-corticosterone concentrations up to 40 nM for cytoplasmic and 80 nM for membrane fractions were used. Specific binding was calculated by subtracting nonspecific binding (defined in the presence of 10 μM Cort for both membrane and cytoplasmic fractions) from the total binding (data not shown). (A) Binding of [^3H]-Cort to mouse membrane (left) and cytoplasmic (right) fractions. The K_d values are shown in the insets. (B) Binding of [^3H]-Cort to rat membrane (left) and cytoplasmic (right) fractions. The K_d values are shown in the insets. Data are representative of two independent experiments. Both total and nonspecific binding were calculated from duplicate points.

Figure 3. Saturation binding of $[^3\text{H}]$ -dexamethasone to mouse and rat hypothalamic membrane and cytoplasmic fractions

$[^3\text{H}]$ -dexamethasone concentrations up to 40 nM for cytoplasmic and 80 nM for membrane fractions were used. Specific binding was calculated by subtracting nonspecific binding (defined in the presence of 10 μM Dex for membrane fractions and 100 μM Dex for cytoplasmic fractions) from the total binding (data not shown). (A) Binding of $[^3\text{H}]$ -Cort to mouse membrane (left) and cytoplasmic (right) fractions. The K_d values are shown in the insets. (B) Binding of $[^3\text{H}]$ -Cort to rat membrane (left) and cytoplasmic (right) fractions. The K_d values are shown in the insets. Data are representative of two independent experiments. Both total and nonspecific binding were calculated from duplicate points.

Summary of saturation binding with ^3H -Dex and ^3H -Cort: K_d and B_{max} values were calculated assuming one-site binding (hyperbola): $Y = B_{\text{max}} \cdot X / (K_d + X)$

Table 1

Radioisotope	Tissue	Conc ⁿ of cold ligand	K_d (nM)	Standard Error	B_{max} (fmol/mg)	Standard Error
^3H -Dex	Rat hypothalamic cytoplasm	10 μM	2.03	0.30	56.03	1.92
^3H -Dex	Rat hypothalamic membrane	100 μM	42.22	3.95	11.97	0.60
^3H -Dex	Mouse hypothalamic cytoplasm	10 μM	1.56	0.22	115.9	4.04
^3H -Dex	Mouse hypothalamic membrane	100 μM	38.80	9.66	21.45	2.70
^3H -Cort	Rat hypothalamic cytoplasm	10 μM	2.76	0.30	79.32	2.48
^3H -Cort	Rat hypothalamic membrane	10 μM	30.75	9.10	12.59	1.70
^3H -Cort	Mouse hypothalamic cytoplasm	10 μM	7.64	0.61	141.6	4.07
^3H -Cort	Mouse hypothalamic membrane	10 μM	32.18	6.61	30.08	2.83