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

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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 $BS^3$ 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 [$^3$H]-corticosterone and [$^3$H]-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.



## 1. 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 [4–6]. 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

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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 cross-linked membrane proteins for GR immunoreactivity and by radio-labeled glucocorticoid binding to hypothalamic membrane fractions.

## 2. Experimental

### 2.1. 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 h 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.

### 2.2. Reagents

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

### 2.3. 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 $MgSO_4$, 1.4 $NaH_2PO_4$, 2.4 $CaCl_2$, 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 $mm^3$, were used.

Rats were anesthetized with 50-mg/kg sodium pentobarbital ip, decapitated with a rodent guillotine and their brains were quickly removed and immersed in ice-cold, oxygenated (ACSF composed of (in mM): 140 NaCl, 3 KCl, 1.3 $MgSO_4$, 1.4 $NaH_2PO_4$, 2.4 $CaCl_2$, 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.

### 2.4. Membrane protein cross-linking

Fresh hypothalamic slices from rats were minced on a tissue chopper and minced samples were treated with $BS^3$ at a final concentration of 2 mM in 1 ml ice-cold ACSF. Stock solutions of $BS^3$ were prepared in 5 mM sodium citrate, pH 5.0, and were diluted 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 μl 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 1× 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 $BS^3$ was added [24]. All the steps were performed at 4 °C. Protein concentrations were measured by the Lowry method [25].

### 2.5. Western blot

Hypothalamic tissue samples from rats (95 μg 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 $H_2O$ 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 $H_2O$, immersed in chemiluminescence (ECL) detecting substrate (Amersham Biosciences, Piscataway, NJ) for 3 min, and exposed to HyperFilm ECL film.

### 2.6. 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 $Na_2$-EDTA, 10% glycerol, 10 mM sodium molybdate,