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Membrane-Initiated Nuclear Trafficking of the Glucocorticoid Receptor in Hypothalamic Neurons

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ABSTRACT

Glucocorticoid binding to the intracellular glucocorticoid receptor (GR) stimulates the translocation of the GR from the cytosol to the nucleus, which leads to the transactivation or transrepression of gene transcription. However, multiple lines of evidence suggest that glucocorticoid signaling can also be initiated from the plasma membrane. Here, we provide evidence for membrane-initiated glucocorticoid signaling by a membrane-impermeant dexamethasone-bovine serum albumin (Dex-BSA) conjugate, which induced GR nuclear trafficking in hypothalamic neurons *in vitro* and *in vivo*. The GR nuclear translocation induced by a membrane-impermeant glucocorticoid suggests trafficking of an unliganded GR. The membrane-initiated GR trafficking was not blocked by inhibiting ERK MAPK, p38 MAPK, PKA, Akt, Src kinase, or calcium signaling, but was inhibited by Akt activation. Short-term exposure of hypothalamic neurons to dexamethasone (Dex) activated the glucocorticoid response element (GRE), suggesting transcriptional transactivation, whereas exposure to the Dex-BSA conjugate failed to activate the GRE, suggesting differential transcriptional activity of the liganded compared to the unliganded GR. Microarray analysis revealed divergent transcriptional regulation by Dex-BSA compared to Dex. Together, our data suggest that signaling from a putative membrane glucocorticoid receptor induces the trafficking of unliganded GR to the nucleus, which elicits a pattern of gene transcription that differs from that of the liganded receptor. The differential transcriptional signaling by liganded and unliganded receptors may contribute to the broad range of genetic regulation by glucocorticoids, and may help explain some of the different off-target actions of glucocorticoid drugs.

Keywords: glucocorticoid receptor, hypothalamus, membrane signaling, nuclear translocation, genomic, nongenomic

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INTRODUCTION

Canonical glucocorticoid transcriptional regulation is mediated by glucocorticoid binding to the intracellular glucocorticoid receptor (GR), translocation of the liganded GR complex to the nucleus, and binding of the GR to the glucocorticoid response element (GRE) or to other transcription factors [1]. Trafficking of the GR from the cytosol to the nucleus is the first step, following glucocorticoid binding, in the transcriptional regulatory cascade of the activated GR, and occurs within minutes of ligand binding [2]. In the absence of steroid, the unliganded GR is bound to chaperone proteins as part of a complex that is transcriptionally inactive. However, an equilibrium exists between the cytosolic and nuclear GR in the absence of ligand [3], indicating that shuttling of the GR between the cytosol and nucleus occurs in the absence of steroid, although the signal(s) that stimulate(s) the trafficking of unliganded GR and the transcriptional functionality of the unliganded GR are not well understood.

A GRα isoform, GRα-D, is expressed in neurons at varying levels at different developmental stages [4], and can be found in the nucleus of glucocorticoid-free cells and associated with GRE-containing promoters in the absence of glucocorticoid [5]. Although the cellular actions of unliganded GR in the nucleus are not fully understood, there is evidence from a mouse mammary cell line that unliganded GR is capable of both negatively and positively regulating gene transcription [6]. Some cellular conditions can induce nuclear localization of the unliganded GR, including heat shock [7] and shear stress [8]. Under shear stress conditions, such as those experienced by aortic endothelial cells, the unliganded GR has been reported to regulate gene transcription [8].

The rapid actions of glucocorticoids range from the production of endocannabinoid as a retrograde messenger [9], to regulation of ion channels [10,11], to inhibitory effects on immune cells [12–14]. Many different kinase signaling pathways have been implicated in the rapid actions of glucocorticoids mediated by putative membrane glucocorticoid receptors, including ERK-MAPK [9], SRC [15], protein kinase A [9,16,17], and protein kinase C [18].

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Here, we compared the membrane-impermeant glucocorticoid conjugate, dexamethasone-bovine serum albumin (Dex-BSA) with the membrane-permeant synthetic glucocorticoid dexamethasone (Dex) in its capacity to stimulate the nuclear translocation of GR and to induce transcriptional activity in hypothalamic cells *in vitro* and *in vivo*. We analyzed the Dex-BSA conjugate for its stability and purity in solution in a companion paper (Weiss et al., 2017).

EXPERIMENTAL

Cell Cultures:

mHypoE-N42 cell line (Cellutions Biosystems): Immortalized hypothalamic mHypoE-N42 (N42) cells [20] were plated at a density of 4.5×10^4 cells/well in 24-well plates (CORNING) containing 12 mm glass coverslips, size 0 (Carolina Biological, Burlington, NC), or in 96-well plates (Falcon, Tewksbury, MA) in 1X Dulbecco's Modified Eagle Medium (DMEM, MediaTech, Manassas, VA) with 1% Antibiotic Antimycotic Solution and 0.2% Plasmocin Prophylactic® (to protect against mycoplasma contamination, Invivogen, San Diego, CA) (N42 medium) supplemented with 5% fetal bovine serum (FBS, Atlas Biologicals) . Plates were incubated at 37˚C with 5% CO_2 until approximately 80% confluent. The N42 medium was then removed, cells were washed once with 1X D-phosphate buffered saline (PBS), and fresh N42 medium supplemented with 5% charcoal-stripped (CS) FBS was added for 16 hours at 37˚C with 5% CO_2 to ensure the removal of all exogenous hormones.

Primary Hypothalamic Cell Culture: Hypothalami were dissected from at least 5 male mouse pups (C57BL/6:129) on postnatal day 1 and placed in ice-cold sterile 1X Hanks' Buffered Salt Solution (HBSS) (GIBCO®, Pittsburgh, PA). Males were used to match our previous electrophysiological studies on rapid glucocorticoid actions in hypothalamic neurons. The tissue was digested in 3 ml of 1X 0.25% trypsin with 2.21 mM EDTA (CORNING, Manassas, VA) at

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37˚C for 15 minutes with gentle shaking. Neurobasal A (Invitrogen, Grand Island, NY) + 1% Glutamax (Invitrogen) supplemented with 2% B27 (Invitrogen), 5% FBS and 1% Antibiotic Antimycotic Solution (CORNING) (complete Neurobasal medium) was added. Cells were triturated with a series of three progressively smaller diameter fire-polished Pasteur pipets (Fisher Scientific, Pittsburgh, PA) until fully suspended. Supernatant was plated on poly-D-lysine-coated 12 mm glass coverslips, size 0, in 24-well plates or in PDL-coated 96-well plates and kept at 37˚C with 5% CO_2 overnight in complete Neurobasal medium plus 33% 1X DMEM with 5% FBS. Medium was removed and replaced at 2 days *in vitro* (DIV) with fresh complete Neurobasal medium, and cells were maintained at 37˚C with 5% CO_2 for 7 DIV. Half of the medium was removed and replaced with fresh complete Neurobasal medium every other day. 6 hours prior to hormone treatment, the medium was replaced with Neurobasal A supplemented with 1% Glutamax only.

Hormone Treatments: Inhibitors or activators were added to the media either 15 minutes prior to a 20-minute hormone treatment or 30 minutes prior to a 60-minute hormone treatment at 37˚C with 5% CO_2. All hormones and drugs were dissolved in dimethyl sulfoxide (DMSO) except for Dex-BSA (Steraloids, Newport, RI), which was dissolved in sterile water with 25% cyclodextrin (Sigma-Aldrich, St. Louis, MO) as a carrier to increase solubility. Dex and Dex-BSA were used at concentrations of 1 µM and 100 nM, respectively, unless otherwise stated. The Dex-BSA had a steroid-to-BSA molar ratio of 37:1. The number of Dex molecules in the Dex-BSA conjugate that are available for binding to a membrane receptor is unknown. We assumed that multiple Dex molecules per BSA were available for binding the membrane receptors. We selected the lowest effective concentration of Dex-BSA and used a 10-fold higher concentration of Dex for comparison. We determined empirically that the two compounds were of approximately equal efficacy (Fig. 1). For the filtrate experiments, Dex-BSA was dissolved in water at a stock concentration of 100 µM, then added to 5% charcoal-stripped DMEM with 5%

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CS FBS at 37°C for 20 minutes. The culture media was then filtered through a 10 kD Amicon filter (Millipore) at room temperature for 10 minutes at 14,000 rcf to obtain the Dex-BSA filtrate, which contained anything present in the Dex-BSA-treated media with a molecular weight <10 kD.

Immunocytochemistry: Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 15 minutes at room temperature. The fixative was removed and cells were washed in 1X D-PBS and then incubated in 10% Normal Goat Serum with 0.2% Triton X-100 in 1X PBS (blocking solution) for 1 hour at room temperature. Primary antibodies were diluted (Table 1) in blocking solution; cells were incubated in primary antibodies overnight at 4˚C. They were then washed in D-PBS and incubated in a secondary antibody diluted (Table 1) in the blocking solution for 45 minutes at room temperature. After a final series of washes, coverslips were mounted on glass slides (Fisher Scientific) using Vectashield with DAPI (Vector Labs). Fluorescence imaging of immunocytochemical staining was performed on an upright Olympus IX71 microscope with a Hamamatsu black and white camera and HCImage software (Hamamatsu, Hamamatsu City, Japan). Exposure times were set using cells incubated with secondary antibody only and kept consistent within experiments.

Image Analysis: The Cell Profiler image analysis software (www.cellprofiler.org) was used to measure the mean intensity of the fluorescence signal in the nucleus, the cytoplasmic region, and the whole cell [21]. The DAPI image was used to identify the nuclear compartment, from which the cytoplasmic region of interest was defined as a pre-set distance of 10 pixels from the nuclear border. In primary cell cultures, neurons were identified by their PSD-95 immunofluorescence under FITC filters. The nuclear outlines of the neuron were overlaid on the TXRED image to measure the fluorescence intensity of the protein of interest. Nuclear translocation was measured as a change in the ratio between the nuclear and cytoplasmic

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intensities of GR immunofluorescence, which was then normalized to the nuclear-to-cytoplasmic ratio in vehicle-treated cells.

Transfection: Transfection of cell lines was conducted at the time of plating, while cells were in suspension, using the Neon® Transfection System (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The specific transfection profile for N42 cells was 1600 mV, 10 ms, 2 pulses for a 100 µL transfection tip, at a density of $2x10^{-6}$ cells, which was then distributed evenly between 24 wells in a 96-well plate. Cells were co-transfected with 75 ng GRE-TK-Luc and 10 ng CMV-Renilla Luciferase (a gift from Dr. Greti Aguilera), and allowed to adhere to coverslips overnight. The media was then changed to N42 medium supplemented with 5% CS FBS for 16 hours. Cells were treated with hormone for 20 minutes; the hormones were then removed and fresh media was added for 24 hours. Cells were assayed for luciferase activity with the Dual-Glo Luciferase Assay System (Promega, Madison, WI) using a VICTOR luminometer and accompanying software (PerkinElmer, Waltham, MA).

GR-GFP transfection and live imaging: Transfection with GFP-tagged GR (a gift from Dr. Louis Muglia) was performed as described above. Following transfection, N42 hypothalamic cells were plated on 35 mm culture dishes with a No. 0 cover glass bottom, and allowed to adhere overnight. The culture media was changed to N42 media supplemented with 5% CS FBS 16 hours prior to imaging, which was performed on a Nikon A1+ laser confocal microscope (Nikon Instruments Inc, NY). Images were taken every 15 seconds with a 28 µm pinhole size to track GR transport from the cytosol to the nucleus. Dex or Dex-BSA was added after 5 minutes of baseline imaging. Average intensities within a nuclear region of interest were calculated using NIS-Elements analysis software (Nikon Instruments, Inc.), and used to quantify nuclear translocation.

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In-Cell Western: N42 Cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature in 96-well plates and then permeabilized with 1X D-PBS with 0.1% Triton X-100. The cells were incubated in Odyssey Blocking Buffer (in PBS) (LI-COR Biosystems) for 1 hour at room temperature and then incubated in primary antibodies diluted (Table 2) in Odyssey Blocking Buffer (PBS) overnight at 4℃ with gentle shaking. The cells were then washed in 1X D-PBS with 0.1% Tween20 and incubated in secondary antibody diluted (Table 2) in Odyssey Blocking Buffer (PBS) with 0.2% Tween20 for 1 hour at room temperature with gentle shaking. Plates were then imaged on an Odyssey CLx Imager (LI-COR Biosciences) and analyzed using Image Studio Lite Version 4.0.21 software (LI-COR Biosciences).

Microarray Analysis: For microarray analysis, three array groups were prepared: N42 cells were treated for 24 hours with vehicle, Dex (1 μM), or Dex-BSA (100 nM) and were tested with three biological replicates for each group. Total RNA from N42 cell cultures was extracted using the Aurum™ Total RNA Mini Kit (Bio-Rad, U.S.A). Mouse OneArray® microarray hybridization and data analysis were performed on the total RNA samples by Phalanx Biotech Group (Phalanx Biotech Group, San Diego, CA). Differentially expressed genes were those with a log2 (fold change) ≥ 0.5 and a $P < 0.05$.

***In Vivo* experiments:** Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) were adrenalectomized and implanted with bilateral cannulas aimed at the paraventricular nucleus of the hypothalamus (PVN), as previously described [22]. Males were used to match our previous electrophysiological studies on rapid glucocorticoid actions in hypothalamic neurons. Stereotaxic coordinates were 1.9 mm posterior and 0.5 mm lateral to bregma, with injection cannula tips placed 7.3 mm ventral to the dura mater. After cannula implantation, rats were allowed to recover for 7 days prior to injections, with access to 0.5 M NaCl and water. They then received bilateral 500 nl injections of Dex (25 pg), Dex-BSA (1.25 μg), or vehicle (0.9% saline).

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Thirty minutes after injections, the rats were killed by sodium pentobarbital overdose and perfused with 3.7% formaldehyde. Brains were immersed overnight in 30% sucrose and sectioned at 25 µm on a sliding microtome, and sections were stained for fluorescence immunohistochemistry. Primary antibodies used were rabbit anti-BSA (Rockland, Limerick, PA) at a 1:500 dilution and rabbit anti-GR (M-20, Santa Cruz Biotechnology, Dallas, TX) at a 1:500 dilution. Images were captured using an LSM 510 confocal microscope (Zeiss, Thornwood, NY).

Statistical Analysis: Data are presented as means ± SEM. All statistical analyses were performed using Prism version 5.04 (GraphPad Software, San Diego, CA). One-way analysis of variance (ANOVA) was used to compare three or more groups. Post-hoc multiple comparisons were performed using Bonferroni's analysis in most cases, or Tukey's analysis when comparing all possible combinations of data sets. Two-way ANOVA was used to compare multiple data sets over time.

RESULTS

Membrane-initiated glucocorticoid signaling induces GR nuclear translocation

Nuclear localization of the GR was measured in the N42 hypothalamic cell line following exposure to Dex, which crosses the plasma membrane, and to Dex-BSA, which is membrane-impermeant (Weiss et al., in review). Treatment with Dex and Dex-BSA for 20 minutes resulted in a concentration-dependent increase in the nuclear localization of the GR (Fig. 1a). Both Dex and Dex-BSA were sufficient to induce GR nuclear translocation at concentrations greater than 100 nM, and had similar effective concentrations at 1 µM and 100 nM, respectively. (Fig. 1b). Dex treatment resulted in a significant increase in the nuclear localization of the GR within 10 minutes, while Dex-BSA treatment required 20 minutes to significantly increase GR nuclear

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localization (Fig. 1c). GR translocation similar to that observed with Dex and Dex-BSA was also seen in response to exposure to the endogenous glucocorticoid corticosterone (1 µM) (data not shown).

To confirm that free Dex is not present in the Dex-BSA solution, we tested the effect of media treated with Dex-BSA at 100 nM for 20 minutes under experimental conditions (1X DMEM supplemented with 0.5% CS FBS at 37°C and 5% CO_2). Media filtered through a 10 kD Amicon filter, which should contain solutes with a mass <10 kD, including free Dex (mw = 392.461 g/mol), was applied to N42 cells for 20 minutes. The Dex-BSA filtrate did not induce GR nuclear localization (Fig. 2).

Primary hypothalamic cell cultures from postnatal day 1 C57BL/6 mice were also tested for GR nuclear translocation at 7 days *in vitro*. They were subjected to treatment with DMSO, Dex (1 µM), or Dex-BSA (100 nM) for 20 minutes. Neurons in the primary cultures were identified by immunostaining for PSD-95, a neuronal marker. Although GR immunofluorescence was localized to the nucleus and cytosol prior to treatment, both Dex and Dex-BSA treatments resulted in an increase in GR nuclear localization in these cells (Fig. 3).

The ability of Dex and Dex-BSA to induce nuclear translocation of the GR was also tested *in vivo* in the PVN of adrenalectomized rats. Dex (25 pg), Dex-BSA (1.25 µg), or saline was injected into the PVN of anesthetized rats. Dex and Dex-BSA, but not saline, caused a significant increase in the nuclear localization of the GR (Fig. 4A-C).

To further explore the differences in kinetics seen in GR immunostaining, we transfected N42 cells with a GFP-tagged GR and imaged the cultures under the confocal microscope in order to track the receptor in real time in living cells. Both Dex (1 µM) and Dex-BSA (100 nM) treatments caused nuclear translocation within 5 minutes (Fig. 5A). The relative change in nuclear intensities was ~45% lower in Dex-BSA- compared to Dex-treated cells at 5 minutes ($p < 0.001$) (Fig. 5B), but was no longer different at 25 minutes of hormone treatment ($p = 0.105$).

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Nonlinear regression confirmed a faster time constant for Dex-induced translocation (τ = 3.583 min) than for Dex-BSA-induced translocation (τ = 8.206 min). These data suggest that GR translocation due to Dex or Dex-BSA reaches a common saturation point, but that Dex-BSA-induced translocation occurs at a slower rate, consistent with the slower time course of GR translocation that we observed using immunostaining in fixed cultures.

Role of intracellular signaling pathways in membrane-initiated GR nuclear translocation

The difference in the kinetics of Dex- and Dex-BSA-induced GR nuclear translocation suggests that GR trafficking initiated by glucocorticoid signaling from the membrane uses a different signaling mechanism than that stimulated by intracellular glucocorticoid binding directly to the GR. Intracellular glucocorticoid-induced nuclear trafficking of the GR is dependent on the GR chaperone HSP90 [23]. To test the dependence of Dex-BSA-mediated GR nuclear translocation on HSP90, N42 cells were treated with the HSP90 inhibitor geldanamycin (100 μM) for 30 minutes prior to a 20-minute hormone treatment. Both Dex- and Dex-BSA-induced GR nuclear translocation were reduced following geldanamycin treatment (Fig. 6), suggesting a common dependence of the two signaling pathways on HSP90.

The membrane-initiated trafficking of an unliganded GR may involve signaling from the membrane to the GR via a kinase- or calcium-dependent mechanism. We have reported previously that rapid glucocorticoid-induced endocannabinoid release from hypothalamic neuroendocrine cells is dependent on G protein, PKA and PKC activities [24,25], as well as on ERK-MAP kinase, Src kinase, and intracellular calcium mobilization (unpublished observations). Here, we tested for the dependence of the membrane-initiated nuclear translocation on intracellular signaling pathways using kinase inhibitors and activators and a calcium chelator. We tested the pharmacological inhibition of ERK-MAP kinase with the MEK inhibitor PD 0325901 (50 nM, Tocris), of PKA with Rp-adenosine 3′,5′-cyclic monophosphorothioate

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(RpcAMPs) (50 μM, Sigma-Aldrich), of Src with PP2 (50 μM, Sigma-Aldrich), of p38 MAPK with SB 202190 (10 μM, Tocris), and of Akt with 10-DEBC・HCl (5 μM, Tocris), and the inhibition of intracellular calcium signaling with the membrane-permeant calcium chelator BAPTA-AM (50 μM, Sigma-Aldrich). None of these kinase or calcium signaling inhibitors had any effect on the Dex- or the Dex-BSA-induced increase in nuclear localization of GR (Fig. 7A). We also tested for PKC dependence of the Dex- and Dex-BSA-induced nuclear localization of GR with the PKC inhibitor Go6983 (10 μM, Tocris), but the autofluorescence of the inhibitor rendered the measurement of the GR immunofluorescence unreliable. We next tested for nuclear translocation of GR by pharmacological activation of PKA with 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP, 10 nM, Sigma-Aldrich), of p38 MAPK with anisomycin (10 μM, Sigma-Aldrich), and of Akt with SC79 (11 μM, Tocris), each of which failed to increase GR nuclear localization at the concentrations tested (data not shown). Finally, we tested the PKA, p38 MAPK, and Akt activators at the same concentrations in combination with Dex (1 μM) and Dex-BSA (100 nM); the PKA and p38 MAPK activators, 8-Br-cAMP and anisomycin, had no effect on the Dex- and Dex-BSA-induced increase in GR nuclear localization. Interestingly, however, the Akt activator SC79 (11 μM) blocked the Dex-BSA-induced, but not the Dex-induced, increase in GR nuclear localization (Fig. 7B, C).

In-cell westerns for phosphorylated ERK 1/2, Akt, and p38 MAPK were also performed on N42 cells treated with Dex or Dex-BSA for 10, 20, 30, 45, and 60 minutes to determine whether the glucocorticoids activated these kinases. There was no difference in the phosphorylation of any of the kinases tested at any of the time points (Fig.8).

Transcriptional regulation by Dex and Dex-BSA

We used a GRE-luciferase assay to determine the GRE-dependent transcriptional activity of Dex and Dex-BSA in the N42 cells. Dex (1 μM) or Dex-BSA (100 nM) was applied to

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cultures for 20 minutes and luciferase activity was measured to monitor GRE activation 24 hours later. The Dex exposure caused a robust increase, whereas the same exposure to Dex-BSA failed to cause a significant increase in luciferase activity (Fig. 9A).

The differential GRE activation observed with the GRE-luciferase assay suggested that the liganded and unliganded GR regulate gene transcription differently. We performed a preliminary microarray analysis on N42 cells to test this hypothesis. N42 cell cultures were treated with either Dex (1 μM) or Dex-BSA (100 nM) for 24 hours. As expected, 24 hours of Dex treatment resulted in the up- or down-regulation of more than 400 genes (Fig. 9B). Dex-BSA treatment also resulted in the regulation of hundreds of genes, although most of these were uniquely regulated by the Dex-BSA treatment and not by the Dex treatment (Fig. 9B), suggesting the differential transcriptional regulation by the unliganded GR compared to the liganded GR.

DISCUSSION

The main finding of this study is that glucocorticoid signaling from the membrane can induce the nuclear translocation of the GR, which suggests trafficking of an unliganded GR that is activated by glucocorticoid binding to a membrane-associated receptor. Corticosterone also caused GR nuclear translocation, indicating that the synthetic steroids acted in a similar manner as the endogenous steroid. The similar response observed in primary hypothalamic cell cultures *in vitro* and following steroid injection into the PVN *in vivo* provided evidence that the Dex- and Dex-BSA-induced nuclear translocation of GR observed in the N42 hypothalamic cell line also occurs in native hypothalamic neurons.

The Dex-BSA-induced GR translocation was slower than the Dex-induced GR translocation, which could provide insight into mechanisms of unliganded GR trafficking. For example, time might be required following glucocorticoid binding to the membrane for the GR to move to the membrane [26] to be exposed to the steroid signal. Thus, the GR may need to be

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trafficked to the membrane in order to receive a membrane-restricted signal, either from the ligand directly or from a nearby membrane receptor. Although not studied directly, we saw no evidence for trafficking of the GR to the membrane following glucocorticoid application. The Dex group of the Dex-BSA conjugate is not long enough to protrude through the plasma membrane lipid bilayer, so it would be necessary for some portion of the GR to enter the membrane in order for Dex-BSA to directly interact with the GR. Alternatively, Dex-BSA could bind to an unidentified membrane receptor, which could then relay a signal to the GR to trigger translocation. Either process may be slower than free Dex diffusing into the cell and binding to the GR. Another possible explanation for the difference in kinetics between the two steroids is that the number of Dex molecules in the Dex-BSA conjugate available to bind to the membrane receptor (the Dex-to-BSA ratio in the Dex-BSA conjugate was 37-to-1) was fewer than in the 1-μM Dex solution, which may have slowed the binding reaction.

The trafficking of an unliganded GR by glucocorticoid actions at the membrane implicates the activation of a membrane receptor-initiated signaling mechanism. We found previously in *ex-vivo* brain slices that rapid glucocorticoid actions in hypothalamic neurons and neurons of the basolateral amygdala are dependent on G protein-coupled receptor and protein kinase activation [24,25,27,28]. Other studies have demonstrated that glucocorticoids can rapidly activate multiple kinase pathways in cultured cells, including both serine/threonine kinase and tyrosine kinase signaling pathways [29]. For example, glucocorticoids activate ERK 1/2 within 1 hour in an anterior pituitary cell line [30], and rapidly inhibit calcium influx into HT4 and PC12 cells via PKA and PKC activation, respectively [16]. In primary hippocampal neurons, glucocorticoids activate JNK and p38 MAP kinases [18], and inhibition of GR results in the reduction of Akt and ERK phosphorylation within 30 minutes [31]. In contrast to studies of other forms of rapid glucocorticoid signaling that showed kinase dependence (23-28), we did not find that the GR nuclear translocation induced by membrane glucocorticoid signaling was dependent on, or was activated by, any of a battery of kinases. It is possible that we missed inhibitory or

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activational effects of drugs tested at a single concentration, however, the concentrations of the kinase activators and inhibitors that we employed in these experiments have been demonstrated to be effective in other studies. The lack of kinase involvement in GR nuclear translocation suggests that either the membrane receptor or the signaling cascades engaged by the membrane receptor may be different for distinct rapid glucocorticoid actions.

Post-translational modifications of the GR can alter the transcriptional effects of GR activation [32], and may provide a mechanism for the interactions between cell signaling pathways and GR-mediated transcriptional activity. Dex-BSA treatment reportedly increased p38 MAPK activation in CD14-positive monocytes [19], and both pharmacological and siRNA-mediated inhibition of p38 MAPK increased GR nuclear localization [33], presenting the possibility that Dex-BSA-induced p38 activation could inhibit GR nuclear localization Consistent with this, GR phosphorylated at serine 232, a putative p38 MAPK site, was less transcriptionally active in rat primary cortical neuron cultures [34]. However, in leukemia cell lines [35] and in osteosarcoma and lung cancer cell lines [36], p38 MAPK phosphorylation of the GR at serine 211 (pGR S211, which is orthologous to serine 232 in the rat) increased GRE-mediated transcription, suggesting that the effects of phosphorylation are cell-type specific [37].

We did not find evidence for the induction of GR trafficking by the activation of any of several kinases or by calcium. Thus, we found no effect on GR nuclear translocation by activating PKA, Akt, MEK, p38 MAPK, or Src, and no effect on the Dex- or Dex-BSA-induced GR trafficking by inhibition of PKA, Akt, MEK, p38 MAPK, or Src, or by blocking intracellular calcium signaling. Similarly, we did not find evidence of phosphorylation or activation of PKA, Akt, MEK, p38 MAPK, or Src by Dex or Dex-BSA with an in-cell Western blot assay. While we tested for the dependence of glucocorticoid-induced GR trafficking on a battery of kinases, this list of kinases was not exhaustive, and only one concentration of the different kinase activators and inhibitors was tested, albeit a commonly used concentration. We did not test, for example, the dependence of GR translocation on PKC activity, as the PKC analogs we tested were

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autofluorescent, which interfered with the nuclear localization analysis. Thus, we cannot exclude the possibility that a kinase is responsible for signaling from the membrane to the intracellular GR to induce the translocation of the GR to the nucleus. Nevertheless, this raises the alternative possibility that the nuclear translocation of the unliganded GR is not kinase dependent and may be due either to a direct interaction of the GR with the membrane-restricted steroid or to another post-translational modification of the GR.

We found that Akt activation inhibited the nuclear translocation of GR induced by Dex-BSA, but not by Dex. Akt is part of the PI3 kinase signaling pathway, which can be activated at the plasma membrane by receptor tyrosine kinases and G-protein coupled receptors that respond to growth factors and cytokines [38]. Rapid Akt phosphorylation in response to glucocorticoids has been reported in human epithelial cells in the presence of a transcription inhibitor [39]. We were not able to detect any change in Akt phosphorylation in response to Dex or Dex-BSA treatment or an effect of Akt activation on Dex-mediated GR nuclear localization, which suggests that membrane glucocorticoid signaling may be regulated by the intracellular environment. Under conditions in which the PI3K pathway is active, such as in response to insulin exposure [40], hypothalamic neurons may respond differently to glucocorticoids.

The nuclear translocation of the GR by both Dex and Dex-BSA was blocked following inhibition of Hsp90. This suggests that the chaperone complex, of which HSP90 is a component and which traditionally retains the GR in the cytoplasm in the absence of ligand binding, is required for the successful nuclear transport of both the liganded and unliganded receptor.

The identity of the membrane receptor responsible for signaling to the GR is not known. GR immunoreactivity has been detected in the membrane in lymphoma [41] and breast cancer (MCF-7) cell lines [42], in transfected HEK 293T cells [19], in pituitary cell lines [26], and in native hypothalamic neuroendocrine cells [43], suggesting that the GR itself, or an isoform of the GR, may be the membrane receptor. The GR has also been detected in neuronal

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membranes in some studies using immunocytochemistry and electron microscopy [44,45], while other studies have suggested that standard immunocytochemical methods are not sensitive enough to detect low levels of GR in the plasma membrane [42]. Glucocorticoids bind to neuronal membranes with ~10-fold lower affinity than in the cytosol, suggesting that the membrane receptor is significantly lower in abundance than the cytosolic receptor [43,46], which may account for the difficulty of detection of the GR in the membrane. On the other hand, rapid actions via a membrane receptor have been shown in several, though not all, studies to have a different pharmacology than the intracellular GR (i.e., they are insensitive to the GR antagonist RU486) [24,28] and to be blocked by inhibiting G protein activity [25], which suggests that the membrane receptor may be distinct from the classical GR.

Dex treatment resulted in the expected activation of the GRE, and regulated the transcription of hundreds of genes in the N42 cell line. Although Dex-BSA did not significantly regulate transcription via the GRE based on our GRE-luciferase assay, our microarray results indicated that the unliganded GR is nevertheless capable of transcriptional regulation. Thus, multiple genes were upregulated and over two hundred genes were down-regulated in response to Dex-BSA treatment in N42 cells. Some of these genes may have been regulated in response to intracellular signaling pathways activated by Dex-BSA, but it is likely that the unliganded GR also regulated transcription following translocation to the nucleus. Interestingly, there was relatively little overlap between the genes regulated by Dex and those regulated by Dex-BSA, suggesting differential transcriptional regulation by the liganded and unliganded GR. These preliminary findings support the need for further investigation into the potential for membrane-initiated transcriptional regulation by the unliganded GR.

In conclusion, membrane-restricted Dex-BSA induced the nuclear translocation of GR in hypothalamic neurons to a degree that was comparable to that elicited by Dex, albeit with slower kinetics. Because free Dex does not dissociate from the Dex-BSA this suggests that glucocorticoid signaling from the membrane is capable of inducing the nuclear translocation of

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the unliganded GR. The molecular mechanism for this membrane-initiated trafficking of the unliganded GR is not known, although it appears to be independent of PKA, Akt, MEK, p38 MAPK, Src and calcium signaling, and it is blocked by Akt activation. Nuclear translocation is part of the classical GR signaling pathway, and is necessary for GRE regulation of transcription; however, we demonstrate here that non-classical glucocorticoid signaling via a membrane GR induces the nuclear translocation of an unliganded GR and subsequent transcriptional regulation that appears to be independent of activation of the GRE. This divergence in the transcriptional signaling of the unliganded and liganded GR may represent a mechanism for finely controlling transcriptional output by glucocorticoids under different physiological and/or cellular conditions.

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Figure Legends

Figure 1. Nuclear translocation of the GR by Dex and Dex-BSA. A) GR Immunofluorescence in N42 cells in control media (No Treatment) and media containing Dex and Dex-BSA. Nuclear localization of GR was seen following a 20-min treatment with Dex (10^{-6} M) and Dex-BSA (10^{-7} M). B) Dose-dependence of GR nuclear localization following a 20-min treatment with Dex and Dex-BSA. C) Time-course of GR nuclear localization with Dex and Dex-BSA. Dex achieved a maximal nuclear localization of GR within 10 min, whereas the Dex-BSA-mediated increase in GR nuclear localization reached a maximum at 20 min. Both persisted for over 90 min. **$p < 0.01$, two-way ANOVA.

Figure 2. Absence of free Dex in Dex-BSA preparation. Dex-BSA (100 nM) was maintained in solution at 37°C for 20 minutes, and was then filtered through a 10 kD Amicon filter (Millipore). Dex and Dex-BSA treatment induced an increase in the ratio of Nuclear-to-cytoplasmic GR immunofluorescence (Nuc : Cyto Intensity), but the Dex-BSA filtrate had no effect on GR localization. *, $p < 0.05$, **, $p < 0.01$ compared to vehicle or as indicated, one-way ANOVA with Tukey's's *post hoc* test.

Figure 3. Dex and Dex-BSA induce GR nuclear translocation in primary hypothalamic neurons. A) Representative images of GR immunofluorescence in primary hypothalamic neuronal cultures. Lower panel: Colorized images of GR immunofluorescence (red) in PSD-95-positive neurons (green) and DAPI nuclear stain (blue). Upper panel: High-magnification images of GR-positive neurons (yellow outline) from images below (arrows). Dex and Dex-BSA treatment for 20 min increased the nuclear staining and decreased the cytoplasmic staining for GR. B) Ratio of nuclear : cytoplasmic staining of GR. Data represent average nuclear : cytoplasmic GR immunofluorescence of individual neurons identified by PSD-95 staining (DMSO n=13, Dex n=9, Dex-BSA n=11). **, $p<0.01$, one-way ANOVA with Tukey's *post hoc* test compared to DMSO.

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Figure 4. Dex and Dex-BSA induce nuclear translocation of the GR *in vivo*. Glucocorticoid immunofluorescence in sections of the PVN from adrenalectomized adult male rat brains following in-vivo delivery into the PVN of vehicle (A), Dex (B), or Dex-BSA (C). Nuclear localization of the GR was seen after intra-PVN injection of Dex and Dex-BSA, but not vehicle. Images were taken under a 20x objective and a 63x objective (insets). 3V, third ventricle. Scale bar in inset in A also applies to insets in B and C.

Figure 5. Live-cell imaging of Dex- and Dex-BSA-induced nuclear translocation of GFP-labeled GR. A. Cells transfected with GR-GFP were imaged during application of Dex or Dex-BSA. Progressive GR-GFP nuclear translocation can be seen at 5 minutes and 25 minutes of steroid application. B. Nuclear fluorescence intensity normalized to baseline revealed a time-dependent increase in GFP intensity in the nucleus, which was more rapid with Dex application than with Dex-BSA application. Dex caused significantly more nuclear translocation than Dex-BSA at 5 minutes ($p<0.001$), but not at 25 minutes ($p=0.105$) of steroid application, one-way ANOVA with Bonferroni *post hoc* test.

Figure 6. HSP90 is required for Dex- and Dex-BSA-induced GR nuclear translocation. Nuclear : cytoplasmic intensity of GR after a 20-min exposure to Dex or Dex-BSA was increased compared to vehicle (DMSO), but was not significantly different from vehicle in N42 cells pre-treated for 15 min with the HSP90 inhibitor geldanamycin (Geld), compared to vehicle (DMSO + Geld), *, $p < 0.05$; **, $p < 0.01$, one-way ANOVA with Bonferroni *post hoc* test. Data represent the average of at least 3 independent experiments.

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Figure 7. Effect of kinase or intracellular calcium manipulation on GR nuclear translocation. A. GR nuclear localization by Dex and Dex-BSA was not significantly different from vehicle in N42 cells treated for 15 min with the following inhibitors: the MEK inhibitor PD 302591 (PD), PKA inhibitor RpcAMPs (RpcAMP), Src inhibitor PP2, p38 MAPK inhibitor SB 202190 (SB), Akt inhibitor 10-DEBC・HCl (DEBC), and the membrane-permeant calcium chelator BAPTA-AM (BAPTA). B. Akt activation with SC79 inhibited the Dex-BSA-induced GR nuclear localization in N42 cells. GR immunofluorescence is shown in red. C. Quantification of the intensity of the nuclear : cytoplasmic ratio in the presence and absence of the Akt activator SC79. SC79 had no effect on the increase in the nuclear : cytoplasmic GR ratio induced by Dex. However, the nuclear : cytoplasmic ratio following Dex-BSA treatment in the presence of SC79 was significantly less than following Dex-BSA treatment alone and did not differ from vehicle treatment. Data represent the mean of 3-13 independent experiments. *, $p < 0.05$, one-way ANOVA with Bonferroni *post hoc* test.

Figure 8. ERK, Akt and p38 MAPK activation do not change with Dex or Dex-BSA treatment. N42 cells were treated with vehicle for 60 minutes, or Dex or Dex-BSA for 10, 20, 30, 45, or 60 minutes. In-cell Westerns for A) phosphorylated ERK (T202/Y204)/total ERK (pERK/ERK), B) phosphorylated Akt (S473)/total Akt (pAkt/Akt), or C) phosphorylated p38 MAPK (T180/Y182)/total p38 MAPK (pp38MAPK/p38MAPK) were performed using the Odyssey system. There was no significant difference between Dex or Dex-BSA treatment and vehicle at any of the time points ($p > 0.05$, 2-way ANOVA).

Figure 9. Differential transcriptional effects of Dex and Dex-BSA. A. N42 cells transfected with GRE-TK-Luciferase for 24 hours were treated with Dex or Dex-BSA for 20 min. Hormone was

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removed and replaced with fresh media for 24 hours before lysis. **, $p < 0.01$; ns, $p=0.064$ compared to vehicle, two-tailed t-test; vehicles: DMSO and cyclodextrin in water (CD). B. Venn diagram for gene expression profiles in N42 cells following 24 hours of Dex and Dex-BSA treatment, from a Mouse OneArray® microarray (Phalanx Biotech Group) on 3 independent biological replicates. Genes reported show a ≥ 0.5-fold difference in expression ($p < 0.05$) compared to vehicle-treated cells.

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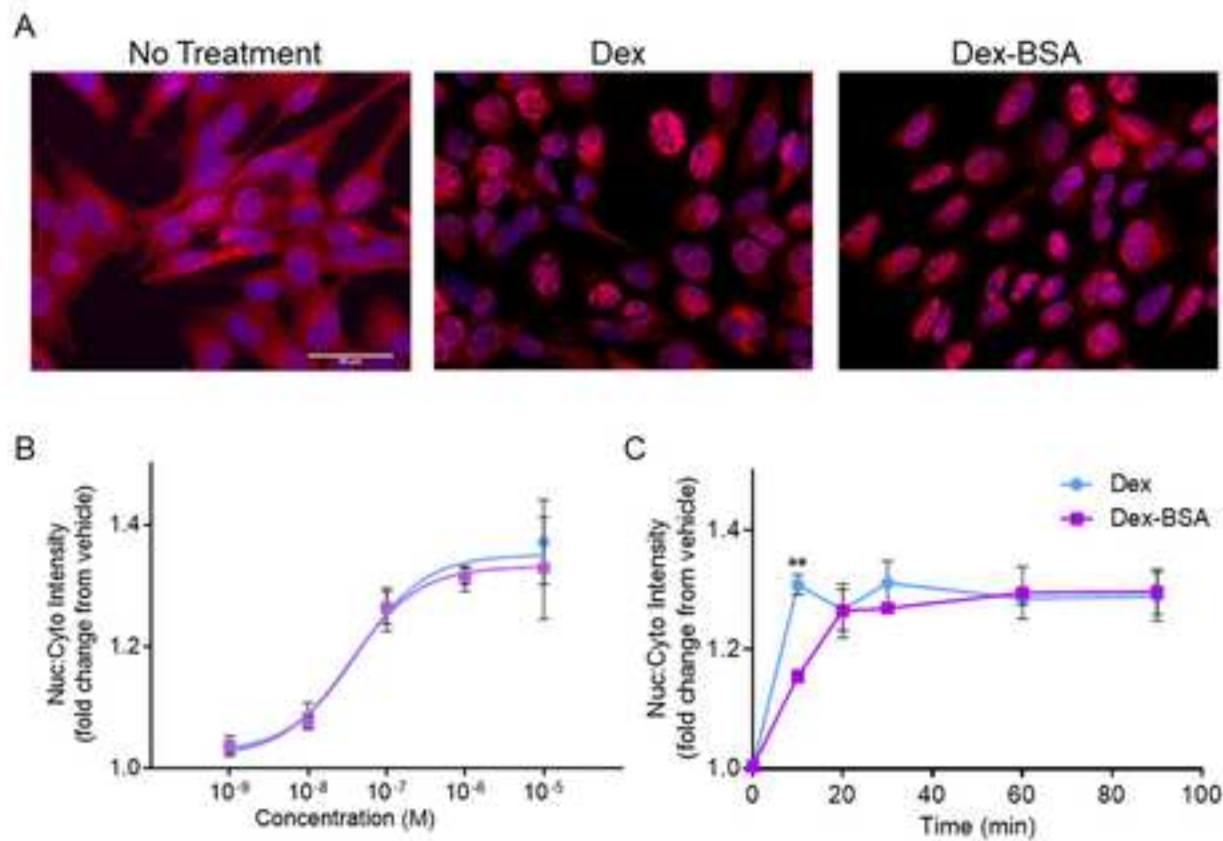

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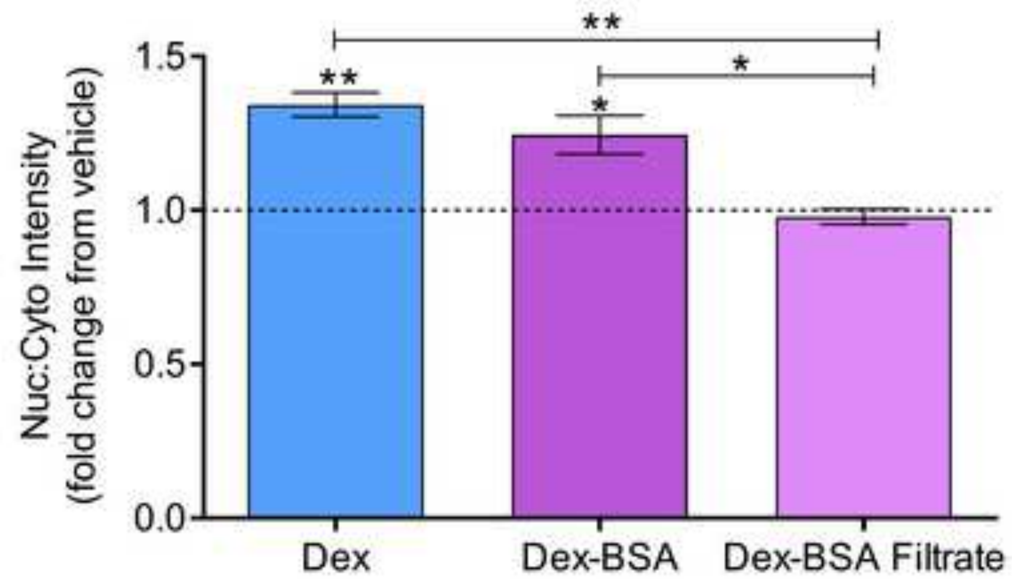

Nuc:Cyto Intensity
(fold change from vehicle)
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Dex
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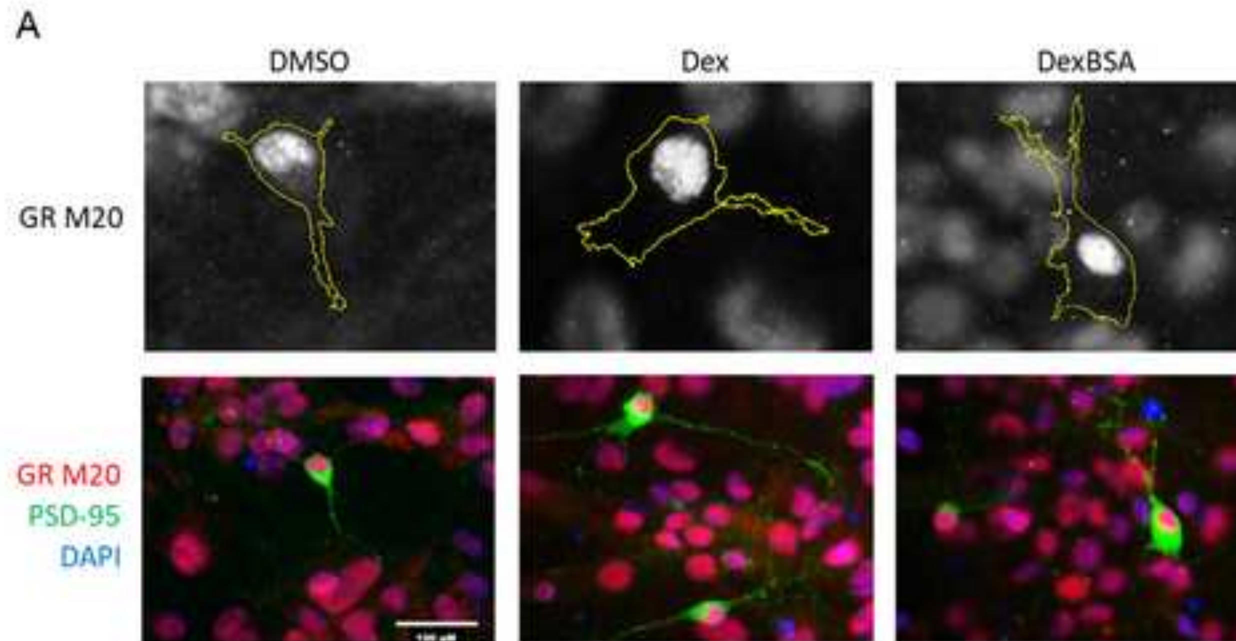

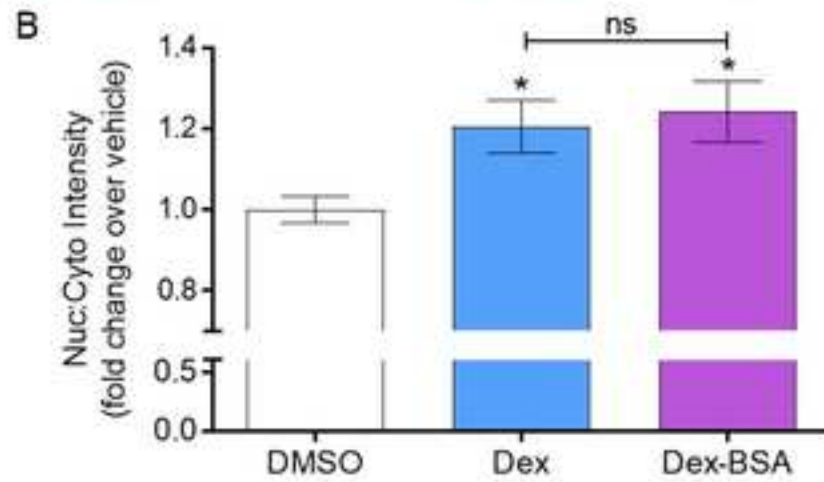

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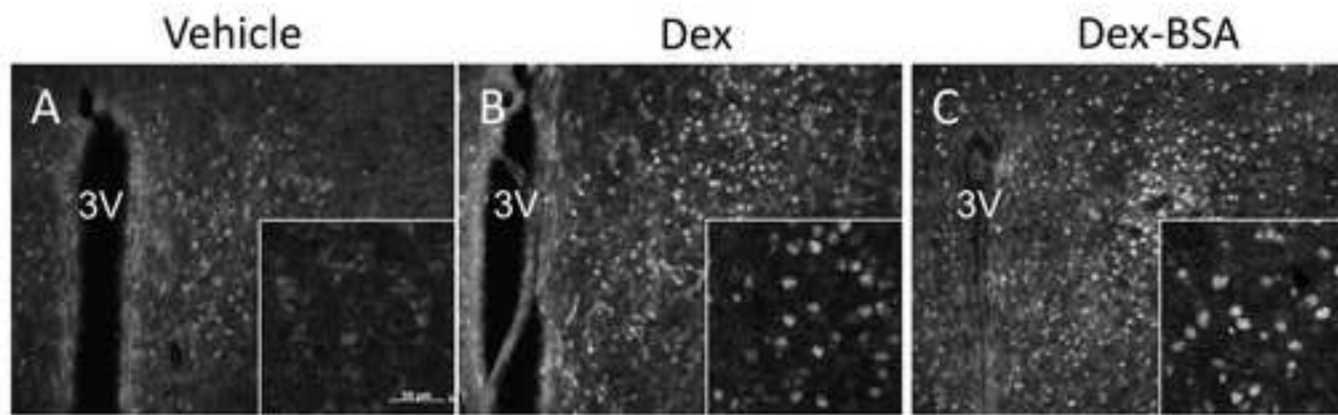

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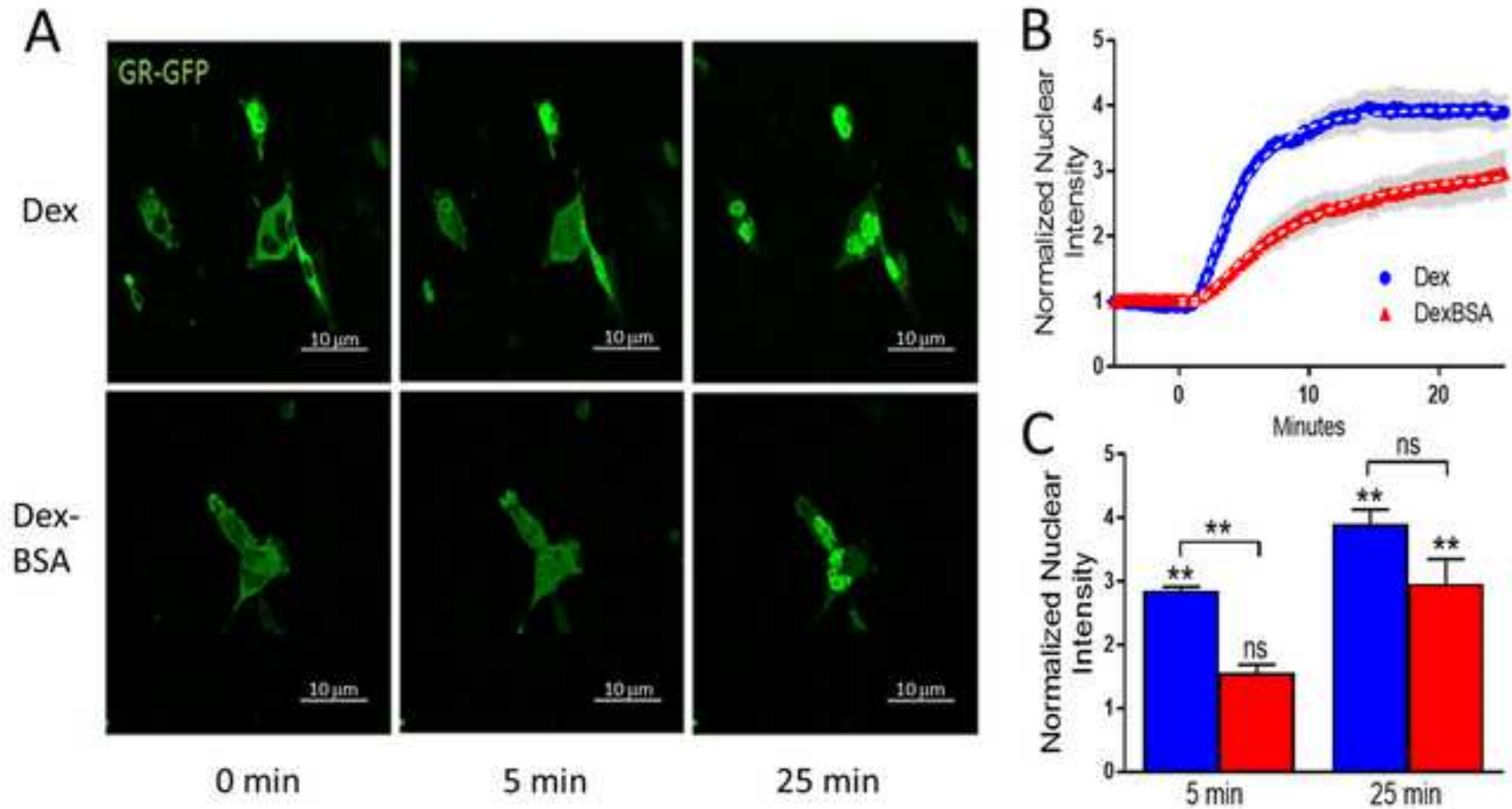
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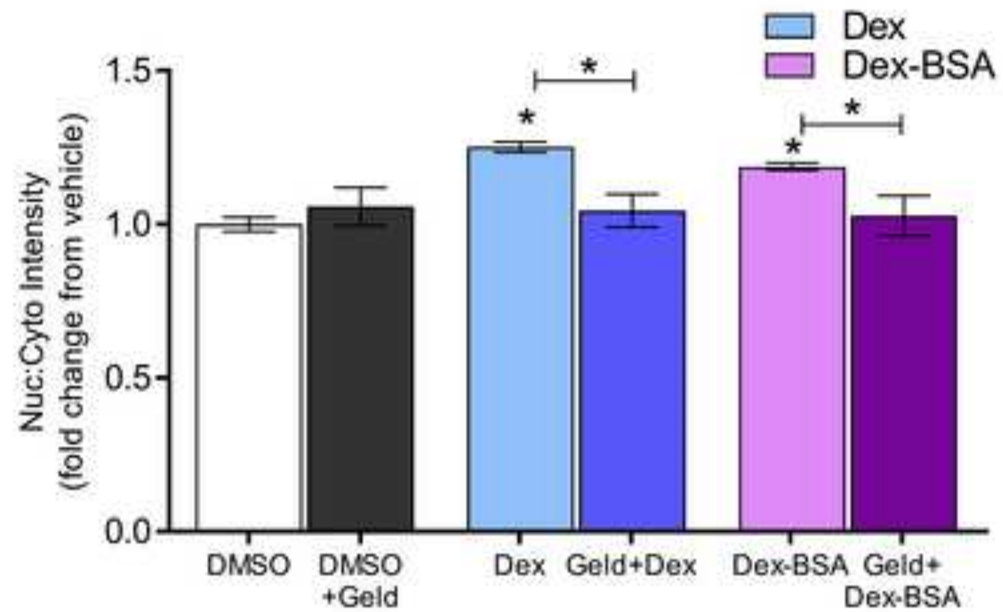

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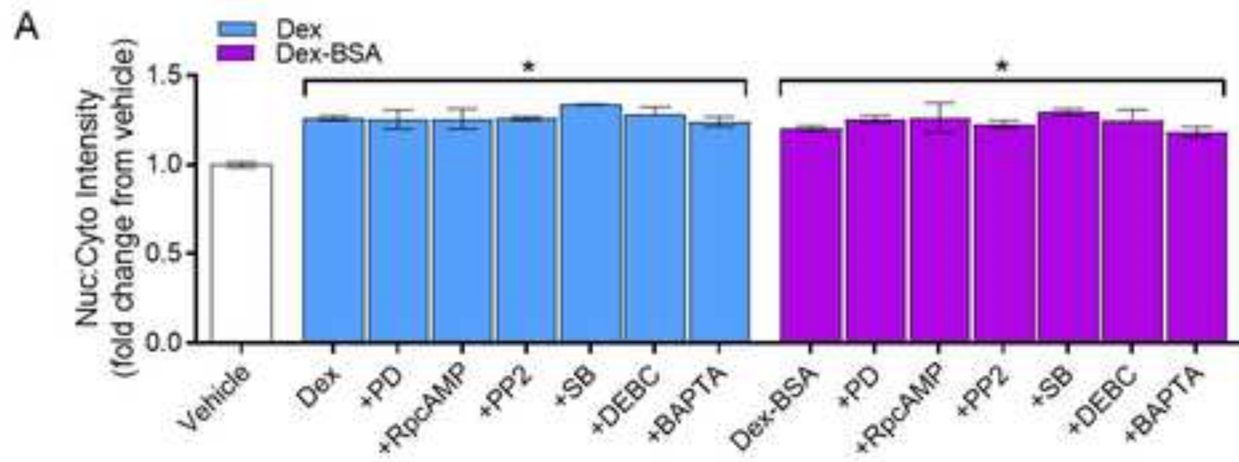

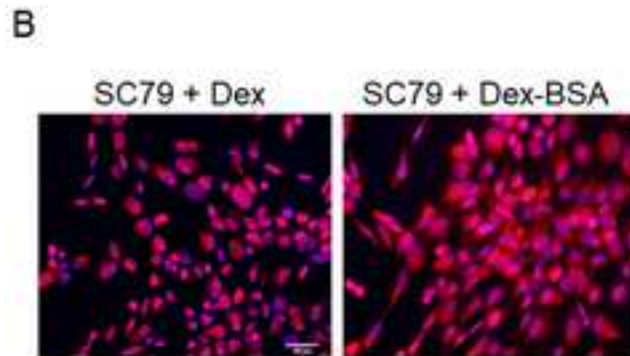

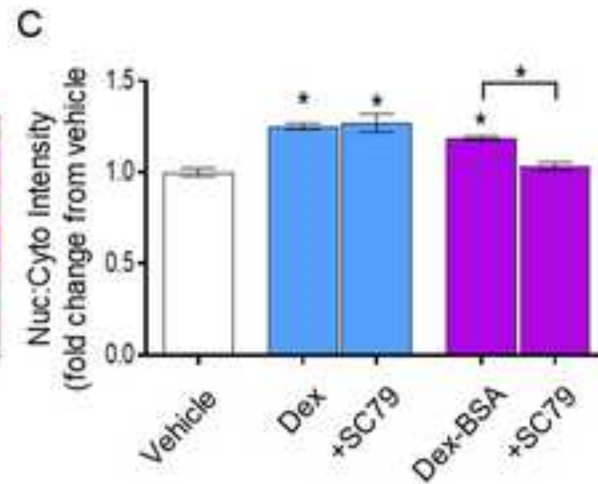

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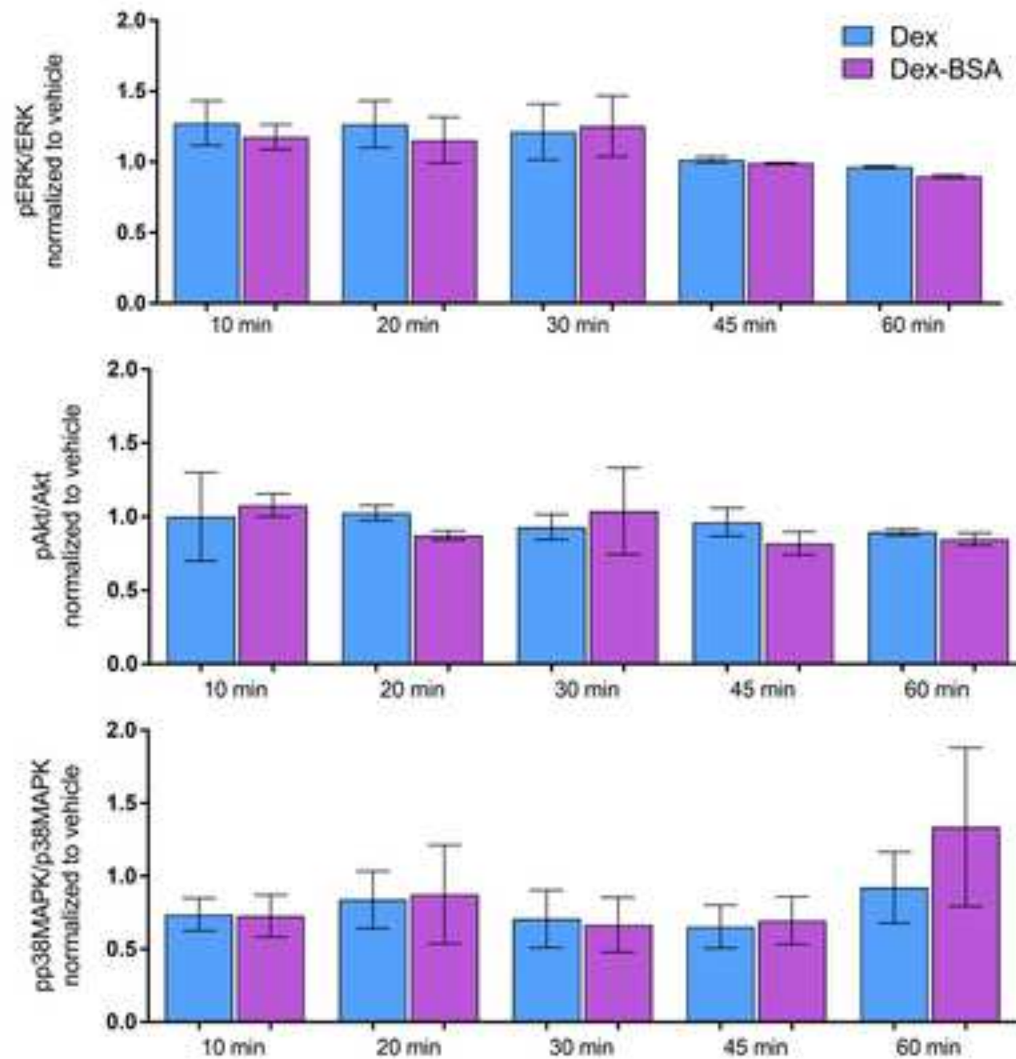

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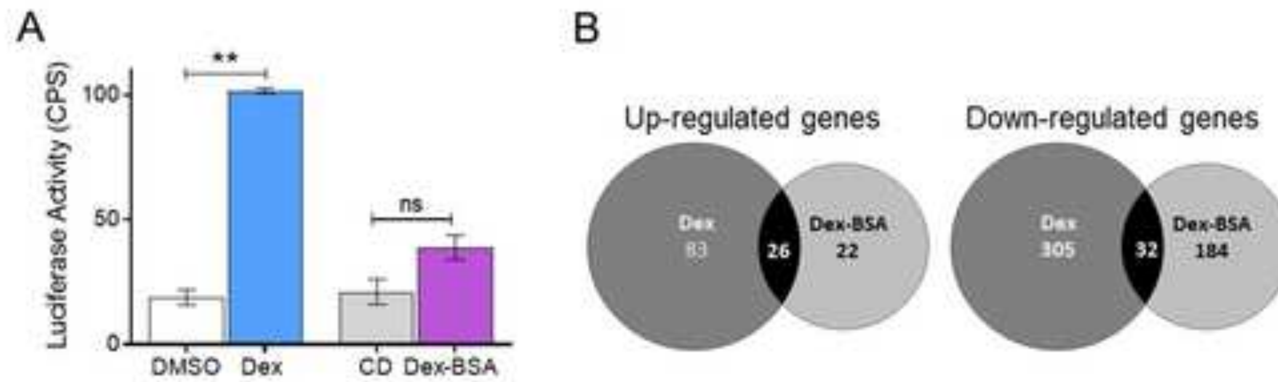

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Table 1. Antibody Dilutions (N42 and primary hypothalamic cell cultures)

Antibody Name/Target	Source	Host Species	Dilution
GR (M-20)	Santa Cruz Biotechnology, Inc.	Rabbit	1:300
PSD-95	NeuroMab	Mouse	1:1000
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Dylight 594 conjugate	Thermo Fisher Scientific	Goat	1:750
Goat anti-Mouse IgG (H+L) Secondary Antibody, Dylight 488 conjugate	Thermo Fisher Scientific	Goat	1:750

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Table 2. Antibody Dilutions for In-Cell Westerns™

Antibody Name/Target	Source	Host Species	Dilution
anti-Phospho$^{T202/Y204}$ ERK	Phosphosolutions®	Rabbit	1:100
pan ERK	BD Biosciences San Jose, CA	Mouse	1:300
Phospho-Akt (Ser473) (D9W9U)	Cell Signaling Technology®	Mouse	1:100
Akt Antibody	Cell Signaling Technology®	Rabbit	1:200
Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP®	Cell Signaling Technology®	Rabbit	1:800
P38α/β (A-12)	Santa Cruz Biotechnology, Inc.	Mouse	1:100
IRDye 680RD Goat anti-Mouse IgG	LI-COR Biosystems	Goat	1:800
IRDye 800CW Goat anti-Rabbit IgG	LI-COR Biosystems	Goat	1:800

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