Rapid Nongenomic Glucocorticoid Actions in Male Mouse Hypothalamic Neuroendocrine Cells Are Dependent on the Nuclear Glucocorticoid Receptor

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Corticosteroids act classically via cognate nuclear receptors to regulate gene transcription; however, increasing evidence supports rapid, nontranscriptional corticosteroid actions via activation of membrane receptors. Using whole-cell patch clamp recordings in hypothalamic slices from male mouse genetic models, we tested for nongenomic glucocorticoid actions at glutamate and gamma aminobutyric acid (GABA) synapses in hypothalamic neuroendocrine cells, and for their dependence on the nuclear glucocorticoid receptor (GR). In enhanced green fluorescent protein-expressing CRH neurons of the paraventricular nucleus (PVN) and in magnocellular neurons of the PVN and supraoptic nucleus (SON), dexamethasone activated postsynaptic membrane-associated receptors and G protein signaling to elicit a rapid suppression of excitatory postsynaptic inputs, which was blocked by genetic deletion of type I cannabinoid receptors and a type I cannabinoid receptor antagonist. In magnocellular neurons, dexamethasone also elicited a rapid nitric oxide-dependent increase in inhibitory postsynaptic inputs. These data indicate a rapid, synapse-specific glucocorticoid-induced retrograde endocannabinoid signaling at glutamate synapses and nitric oxide signaling at GABA synapses. Unexpectedly, the rapid glucocorticoid effects on both excitatory and inhibitory synaptic transmission were lost with conditional deletion of GR in the PVN and SON in slices from a single minded-1-cre-directed conditional GR knockout mouse. Thus, the nongenomic glucocorticoid actions at glutamate and GABA synapses on PVN and SON neuroendocrine cells are dependent on the nuclear GR. The nuclear GR, therefore, is responsible for transducing the rapid steroid response at the membrane, or is either a critical component in the signaling cascade or regulates a critical component of the signaling cascade of a distinct membrane GR. (***Endocrinology* 156: 2831–2842, 2015)**

Increasing evidence both in peripheral tissues and in the central nervous system supports the concept of rapid, nongenomic actions of corticosteroids mediated by membrane-associated receptors. Electrophysiological and behavioral studies indicate the presence of rapid corticosteroid actions in a broad range of species and suggest, therefore, an evolutionarily conserved mechanism (1). Rapid corticosteroid effects have been demonstrated in a variety of neurons in electrophysiological studies, including in neurons from the hippocampal CA1 and CA3 regions (2, 3), the basolateral amygdala (4), the locus coeruleus (5), the reticular formation (5), the celiac ganglion

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in USA

Received March 26, 2015. Accepted June 5, 2015.
First Published Online June 10, 2015

For News & Views see page 2747; for related article see page 2843

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Abbreviations: aCSF, artificial cerebral spinal fluid; AM251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; CB, cannabinoid receptor; Dex, dexamethasone; Dex-BSA, Dex hemisuccinate-BSA; eGFP, enhanced green fluorescent protein; GABA, gamma aminobutyric acid; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal; L-NAME, Nω-nitro-L-arginine methyl ester hydrochloride; mEPSC, miniature excitatory postsynaptic current; mIPSC, miniature induced pluripotent stem cells; NO, nitric oxide; PVN, paraventricular nucleus; Sim1, single minded-1; SON, supraoptic nucleus; TTX, tetrodotoxin.

(6), the medulla (7, 8), the prefrontal cortex (9, 10), and the hypothalamus (11, 12). Within minutes of systemic administration of glucocorticoid in vivo, neuronal excitability and outputs are rapidly altered in hypothalamic neurons of the rat, cat, and rabbit (13–15) and in hindbrain neurons of the roughskin newt (7). Context-dependent behaviors are also regulated within minutes of glucocorticoid administration in a transcription-independent manner. The rapid behavioral responses include increased locomotor activity (16) and aggressive behavior in rats and mice (17), increased perch hopping in white crowned sparrows (18), and suppression of courtship behavior in newts (19). Membrane-impermeant glucocorticoids have the same rapid effects (6), whereas direct intracellular glucocorticoid application does not (20), and inhibiting G protein-dependent signaling mechanisms often blocks the rapid steroid actions (2), suggesting the involvement of a membrane-associated receptor and associated G protein signaling.

Glucocorticoids rapidly modulate excitatory and inhibitory synaptic transmission in neuroendocrine cells of the rat hypothalamic paraventricular nucleus (PVN) and supraoptic nucleus (SON) by activating membrane-associated receptors and stimulating the synapse-specific release of retrograde messengers (11, 21). Thus, glucocorticoids rapidly suppress glutamatergic excitatory synaptic inputs to both magnocellular and parvocellular neuroendocrine cells of the PVN by activating postsynaptic membrane receptors that stimulate the synthesis and retrograde release of the endocannabinoid 2-arachidonoylglycerol (11, 12, 22). Glucocorticoids also rapidly facilitate GABAergic synaptic inputs specifically to magnocellular neurons by triggering the retrograde release of nitric oxide (NO) (21). The glutamate synapse specificity of the glucocorticoid-induced endocannabinoid actions in magnocellular neurons is constrained by astrocytes (22). Glucocorticoid-induced endocannabinoid modulation of glutamate synapses is also seen in PVN preautonomic neurons, albeit via different receptor mechanisms (8).

Here, using an ex vivo brain-slice patch-clamp electrophysiological approach, we took advantage of several mouse genetic models to test for the type I cannabinoid (CB1) receptor and classical nuclear glucocorticoid receptor (GR) dependence of the rapid, synapse-specific actions of glucocorticoids at excitatory and inhibitory synapses on PVN magnocellular and parvocellular neurons, including CRH neurons. Our findings indicate that the rapid glucocorticoid regulation of synaptic transmission in PVN CRH neurons and in magnocellular neurons is dependent on the activation of the classical nuclear GR and are consistent with the GR dependence of fast glucocorticoid negative feedback regulation of the hypothalamic-pituitary-adrenal (HPA) axis in the PVN (38).

Materials and Methods

Animals

Male CD1 and C57BL/6 mice (3–4 wk) were supplied by Harlan. Homozygous founder CB1 knockout ($CB1^{-/-}$) mice (23–25) were obtained from the National Institute of Mental Health (kindly provided by Dr James Pickel) and bred to produce a colony maintained in the Tulane University vivarium. Pups were genotyped at 2–3 weeks of age from cDNA isolated from tail clips using as forward primer: 5′-GTACCATCACCACAGACCTCCTC-3′, reverse primer-knockout: 5′-AAGAACGAGATCAGCAGCCTCTGTT-3′, and reverse primer wild type: 5′-GGATTCAGAATCATGAAGCACTCCA-3′. The $CB1^{-/-}$ mice were bred on a C57BL/6 genetic background.

Conditional GR knockout mice with genetic deletion of GR in cells in the PVN and SON were generated by crossing heterozygous transgenic mice that carry a Cre recombinase allele under the control of the spatially restricted transcription factor single minded-1 (*Sim1-Cre*$^{+/-}$, kindly provided by Dr Bradford Lowell, Beth Israel Deaconess Medical Center, Boston, MA) (26) with mice carrying homozygous floxed GR exon 2 ($GR^{lox/lox}$) alleles on a C57BL/6 background ($Sim1^{GR-/-}$ mice) (27). The $Sim1^{GR-/-}$ mouse showed a 43% deletion of GR in the PVN in the original study in which this mouse was described by Muglia and coworkers (28); it showed an approximately 60% deletion of GR without any detectable neuron loss in PVN sections stained for GR and counterstained in adjacent sections and described in the companion article by Solomon et al (38). The $Sim1^{GR+/+}$ and $Sim1^{GR-/-}$ littermate mice were genotyped at 2–3 weeks of age using the primer sets: 5′-AATCAGAATTGCTCACTCACAA-3′ (GR 6452–73), 5′-CAGTGTTACTACTTCCAGTTC-3′ (GR 6670–50), 5′-TGCTATACGAAGT TATCAGTAC-3′ (LoxPForward), 5′-AAGTGCCTTCTCTACACCTG-3′ (Cre 1123–1004), and 5′-TGCTTATAACACCCTGTTACG-3′ (Cre 982–1002).

In order to target CRH neurons with genetic deletion of the GR for patch clamp recordings, the conditional $Sim1^{GR-/-}$ mice were crossed with mice that express enhanced green fluorescent protein (eGFP) in CRH neurons (strain: B6;FVB-Tg(Crh-EGFP)HS57Gsat/Mmucd, obtained from the Mutant Mouse Regional Resource Center at University of California Davis) to generate *CRH-eGFP*;$Sim1^{GR-/-}$ mice. The *CRH-eGFP* mouse has the eGFP reporter gene inserted immediately upstream of the coding sequence of the CRH gene (29). Mice were genotyped at 2–3 weeks of age using the primer sets: 5′-CTGTCTTGTCGTGGGTGTCCGAT-3′ (CRH F1), 5′-TAGCGGCTGAAGCACTGCA-3′ (eGFP R2), 5′-AATCAGAATTGCTCACTCACAA-3′ (GR 6452–73), 5′-CAGTGTTACTACTTCCAGTTC-3′ (GR 6670–50), 5′-TGCTATACGAAGT TATCAGTAC-3′ (LoxPForward), 5′-GCGGTCTGGCAGTAAAAACTATC-3′ (olMR 1084), 5′-GTGAAA CAGCATTGCTGTCACTT-3′ (olMR 1085), 5′-CTAGGCCACAGAATTGAAAGATCT-3′ (olMR 7338), and 5′-GTAGGTGGAAATTCTAGCATCATCC-3′ (olMR 7339). All mice were used at 5–8 weeks of age according to a protocol approved by the

Tulane University Institutional Animal Care and Use Committee and in accordance with US Public Health Service guidelines.

Brain slice preparation

Mice were anesthetized with isoflurane (VetOne) inhalation in a closed chamber and decapitated using a rodent guillotine. Their brains were removed and immediately immersed in 0–1°C oxygenated artificial cerebral spinal fluid (aCSF) composed of 140mM NaCl, 3mM KCl, 1.3mM $MgSO_4$, 1.4mM NaH_2PO_4, 2.4mM $CaCl_2$, 11mM glucose, and 5mM HEPES, with an osmolarity of 290–300 mOsm/L and a pH adjusted to 7.2–7.3 with NaOH. Hypothalamic blocks were prepared, and 3–4 coronal slices, 300–350 μm in thickness and containing the SON and/or the PVN, were sectioned and bisected along the midline. Slices were immediately transferred to a submersion storage chamber, where they were stored at room temperature in aCSF saturated with oxygen.

Electrophysiological recordings

Hypothalamic slices were transferred to a submersion recording chamber and continuously perfused with aCSF at room temperature. Magnocellular neurons in the PVN and SON were visually targeted under infrared-differential interference contrast illumination. In some cases, parvocellular CRH neurons from *CRH-eGFP* mice were first identified visually under epifluorescence illumination before switching to infrared-differential interference contrast visualization to target the neurons for recording. Whole-cell patch-clamp recordings were performed with a MultiClamp 700A amplifier (Molecular Devices) at a holding potential of −60 mV in the presence of tetrodotoxin (TTX) (1μM) to block sodium-dependent action potentials. Patch pipettes were fabricated on a horizontal puller (P97; Sutter Instruments) at a resistance of 3–5 MΩ. For recording glutamatergic miniature excitatory postsynaptic currents (mEPSCs), pipettes were filled with 120mM potassium gluconate, 10mM KCl, 1mM $MgCl_2$, 10mM HEPES, 1mM NaCl, 1mM $CaCl_2$, 10mM EGTA, 0.3mM Na-GTP, and 2mM Mg-ATP and had an osmolarity of 300 mOsmol and a pH adjusted to 7.2–7.3. For recording GABAergic miniature inhibitory postsynaptic currents (mIPSCs), a high-Cl^- intracellular solution was used, which contained 120mM CsCl, 2mM $MgCl_2$, 1mM $CaCl_2$, 11mM EGTA, 2mM ATP-Mg salt, and 30mM HEPES, and had an osmolarity of 300 mOsmol and a pH of 7.2–7.3.

Magnocellular neurons in the mouse PVN, like those in the rat PVN, display a prominent transient outward rectification that is not seen in PVN parvocellular neurons, and the presence or absence of this electrical fingerprint was used to confirm the identity of recorded cells in current-clamp recordings before switching to voltage clamp. Immediately after achieving the whole-cell configuration, all neurons recorded in the PVN were placed in current-clamp mode, hyperpolarized to approximately −85 mV, and brief current steps (from −50 to 50 pA in 10-pA intervals) were delivered to confirm the magnocellular or parvocellular identity of the recorded cells (30, 31). All recordings were subsequently switched to voltage-clamp mode to measure mEPSCs or miniature induced pluripotent stem cells (mIPSCs [in different neurons]), which were later analyzed in 3-minute epochs for changes in frequency, amplitude, and decay time using pClamp (Molecular Devices) and MiniAnalysis (Synaptosoft). Statistical comparisons were performed using the Student's paired *t* test for within-cell comparisons and the Student's unpaired *t* test for comparisons between groups. The one-way repeated measures ANOVA followed by the Student-Newman-Keuls test was used for multiple pairwise comparisons. $P < .05$ was considered significant.

Reagents

Most of the reagents used in this study were obtained from Sigma-Aldrich. A water-soluble form of dexamethasone (Dex) ((2-hydroxypropyl)-β-cyclodextrin-conjugated Dex) was used in these experiments. Dex hemisuccinate-bovine serum albumin (Dex-BSA) was obtained from Steraloids and dissolved in aCSF with 25% β-cyclodestrin (Sigma-Aldrich). Corticosterone was dissolved in ethanol before being added to the aCSF; final ethanol volume:volume ratio was 0.01%. N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251), Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), TTX, 6-cyano-7-nitroquinoxaline-2,3-dione, (±)-2-amino-5-phosphono-pentanoic acid, and bicuculline methiodide were obtained from Tocris.

Immunohistochemistry

GR immunohistochemistry

Mice were given an overdose ip injection of Fatal-plus solution (Vortech Pharmaceuticals, Ltd), after which they were exsanguinated with 0.9% saline and fixed transcardially with 3.7% formaldehyde in 0.1M potassium PBS (Fischer Scientific). After fixation, brains were removed, postfixed in 3.7% formaldehyde solution for 24 hours, stored in 30% sucrose in diethylpyrocarbonate-treated PBS at 4°C, and sectioned at 25 μm on a sliding microtome. The sections were rinsed in PBS, treated with 1% H_2O_2 to quench endogenous peroxidase, rerinsed in PBS, incubated for 1 hour at room temperature in blocking solution (1% Triton X-100 and 2% BSA [Sigma-Aldrich] in PBS), and then incubated overnight at 4°C in blocking solution containing the primary antibody against GR (M-20, 1:1000; Santa Cruz Biotechnology, Inc). Sections were then rinsed again in PBS, incubated for 1 hour in biotinylated antirabbit IgG (1:500; Vector Laboratories), rerinsed in PBS, incubated with Vectastain ABC (1:1000; Vector Laboratories), rinsed again in PBS, incubated with 0.4 mg/ml diaminobenzidine in 0.05% H_2O_2 in PBS, again rinsed in PBS, and mounted onto Fisher Super stick glass slides and cover slipped with DPX mounting medium.

CRH immunohistochemistry

To confirm eGFP expression in CRH neurons in the *CRH-eGFP* transgenic mice, 3 8-week-old *CRH-eGFP* mice were processed for CRH immunohistochemistry. Each mouse received a stereotaxic injection of colchicine (8 mg/mL, 1.5 μg/g body weight; Sigma-Aldrich) into the lateral ventricle under ketamine/xylazine anesthesia. Two days later, the mice were anesthetized with ketamine (100 mg/kg) and transcardially perfused with PBS followed by 4% paraformaldehyde in PBS. After fixation, the brains were removed and postfixed in 4% paraformaldehyde at 4°C overnight, and then submerged successively in 15% and 30% sucrose in PBS. The hypothalamus was blocked and coronal sections (30 μm) containing the PVN were cut on a cryostat. The sections were preincubated with a blocking solution containing 10% goat serum and 0.2% Triton X-100 in PBS for 1

hour and then incubated with an anti-CRH antibody (1:2000 diluted with the blocking solution, T4037; Peninsula Laboratories) at 4°C overnight. They were then incubated in DyLight 550 goat antirabbit IgG (1:1000, Thermo Scientific) at room temperature for 1 hour. Sections were then mounted, cover slipped, and imaged on a confocal microscope (Zeiss LSM510).

Results

Rapid glucocorticoid-induced suppression of synaptic excitation

We first performed whole-cell patch clamp recordings in slices of CD1 mouse hypothalamus to determine whether neuroendocrine cells of the outbred mouse PVN and SON respond to glucocorticoids in a manner similar to that seen in rat hypothalamic neurons (11, 21, 32). Magnocellular neurons were identified based on their location in the SON and PVN (33), on their relatively large size, and on their electrical properties; as in the rat (30, 34), magnocellular neurons in the mouse SON and PVN expressed a prominent transient outward rectification that caused a delay to action potential generation when depolarized from hyperpolarized membrane potentials. We used Dex as a GR agonist for most of these experiments because it has a higher affinity for GR over the mineralocorticoid receptor and is commonly used as a GR-selective agent. At a holding potential (V_H) = −60 mV and in the presence of TTX (1μM), bath perfusion of Dex (1μM, 10 min) had no effect on the holding current or on the input conductance of any of the neurons tested. However, Dex caused a significant decrease in the frequency of mEPSCs with an onset of 3–5 minutes and a peak effect at 5–7 minutes, from a baseline of 1.81 ± 0.40 to 0.78 ± 0.22 Hz (to 48.07 ± 8.7% of baseline; $P < .01$, $n = 10$) (Figure 1), measured over the last 3 minutes of the Dex application.

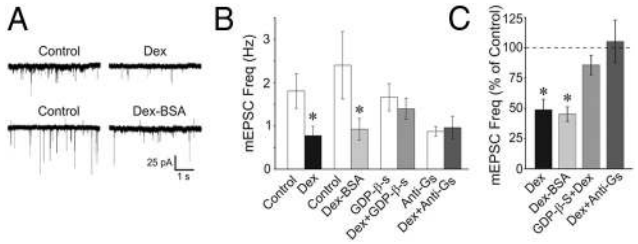

Figure 1. Glucocorticoid-induced suppression of synaptic excitation in mouse magnocellular neurons. A, Bath application of Dex (1μM) and the membrane-impermeant Dex-BSA (10μM) caused a decrease in the frequency of mEPSCs in 2 magnocellular neurons recorded in slices from wild-type CD1 mice. B, Summary data showing the mean mEPSC frequency over the 3 minutes before and at 8–10 minutes of application of Dex, Dex-BSA, and Dex after intracellular application of the G protein blocker GDP-β-S (500μM) or an antibody to Gα_s. C, Effects of Dex, Dex-BSA, and Dex in the presence of intracellular GDP-β-S and anti-G_S on mean mEPSC frequency expressed as a percent of baseline control values. *, $P < .05$.

Dex had no effect on the amplitude or decay kinetics of mEPSCs. The effect of Dex on mEPSC frequency had a rapid onset (3–5 min), suggesting a nongenomic mechanism of steroid action, but did not reverse with 30–60 minutes of washout of the steroid. The effect of Dex was similar in putative magnocellular neurons of both the SON and PVN, so data from SON and PVN magnocellular neurons were pooled in these and the next experiments.

To determine whether the rapid glucocorticoid effect on mEPSC frequency was mediated by activation of a membrane-associated GR, a membrane-impermeant Dex-BSA conjugate was tested. The Dex-BSA solution was filtered and the Dex-BSA eluted from the filter to remove any unconjugated Dex; the Dex-BSA was applied at a 10-fold higher concentration to account for an 8:1 Dex to BSA ratio. The Dex-BSA conjugate (10μM) retained the rapid Dex-induced effect, reducing the mEPSC frequency from 3.53 ± 1.30 to 1.69 ± 0.79 Hz (to 47.6 ± 5.6% of baseline frequency; $P < .05$, n = 6) (Figure 1), without affecting the mEPSC amplitude or decay kinetics. We next tested for the postsynaptic G protein dependence of the rapid glucocorticoid effect by blocking G protein signaling with intracellular application via the patch pipette of the G protein blocker GDP-β-S. Intracellular GDP-β-S application (0.5mM) inhibited the Dex-induced decrease in mEPSC frequency in 6 of 9 neurons tested (1.67 ± 0.31 vs 1.40 ± 0.24 Hz; $P = .19$, n = 6) (Figure 1). Additionally, intracellular application via the patch pipette of an antibody to the Gα_s G protein subunit (1:2000; EMD Bioscience) also blocked the Dex suppression of mEPSCs in 5 of 5 cells tested (0.78 ± 0.12 Hz baseline vs 0.85 ± 0.23 Hz in Dex; $P = .65$, n = 5) (Figure 1). The maintenance of the rapid Dex-induced suppression of mEPSCs by the membrane-impermeant Dex-BSA conjugate suggested a signaling mechanism involving a membrane-associated receptor. The blockade of the Dex suppression of mEPSCs by blocking postsynaptic G protein activity indicated that the glucocorticoid effect was mediated by a postsynaptic G protein-dependent mechanism and suggested the involvement of a retrograde messenger to modulate presynaptic glutamate release.

CB1 dependence of rapid glucocorticoid suppression of glutamate release

We next investigated whether, as in rat neuroendocrine cells (11), the rapid glucocorticoid suppression of glutamate release in mouse SON and PVN neurons is mediated by the retrograde release of an endocannabinoid and presynaptic CB1 receptor activation. In magnocellular neurons recorded in slices from wild-type C57BL/6 mice, bath application of Dex (1μM) in the presence of TTX reduced

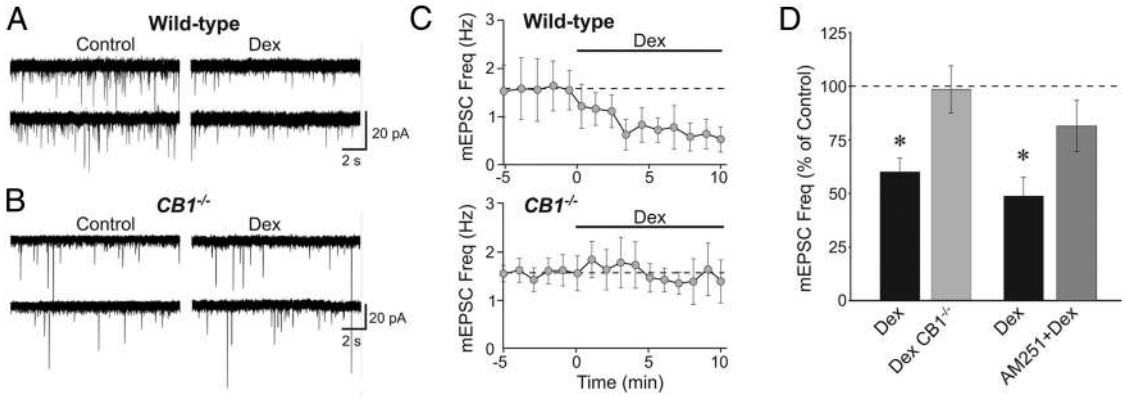

Figure 2. Glucocorticoid-induced suppression of synaptic excitation is blocked by inhibiting CB1 receptors. A, Raw traces of recorded mEPSCs in a magnocellular neuron from a wild-type C57BL/6 mouse before (control) and during bath application of Dex (1μM). Dex caused a decrease in the mEPSC frequency. B, Raw traces of recorded mEPSCs in a magnocellular neuron from a CB1 receptor knockout mouse (*CB1*$^{-/-}$) before (control) and during bath application of Dex (1μM). Dex failed to cause a decrease in the mEPSC frequency in the neuron from the *CB1*$^{-/-}$ mouse. C, top, Average (1-min bins) of the mEPSC frequency as a function of time in magnocellular neurons (n = 5) from wild-type C57BL/6 mice. Dex application (1μM, horizontal bar) caused a rapid decrease in the frequency of mEPSCs. Bottom, Average of the mEPSC frequency as a function of time in magnocellular neurons (n = 6) from *CB1*$^{-/-}$ mice. Dex did not cause a decrease in mEPSC frequency in neurons from CB1 knockout mice. D, Mean mEPSC frequency expressed as a percentage of baseline averaged over the last 3 minutes of a 10-minute Dex application in magnocellular neurons from wild-type C57BL/6 mice (Dex, n = 5) and CB1 knockout mice (Dex *CB1*$^{-/-}$, n = 6), and in slices from CD1 mice in the absence (Dex, n = 10) and the presence of the CB1 receptor antagonist AM251 (1μM, n = 6); *, $P < .05$.

the mEPSC frequency from 1.18 ± 0.07 to 0.69 ± 0.07 Hz (to 60.0 ± 6.5% of baseline; $P < .01$, n = 5) (Figure 2), without altering mEPSC amplitude or decay time. In magnocellular neurons in slices taken from *CB1*$^{-/-}$ mice (on a C57BL/6 background), bath application of Dex (1μM) failed to significantly decrease mEPSC frequency (1.80 ± 0.39 Hz baseline vs 1.73 ± 0.34 Hz in Dex; $P = .71$, n = 7) (Figure 2). Similarly, the rapid reduction in mEPSC frequency elicited by Dex (1μM) in magnocellular neurons from wild-type CD1 mice was blocked by a 10-minute preapplication of the CB1 receptor antagonist AM251 (1μM) ($P = .19$, n = 6) (Figure 2).

Rapid glucocorticoid-induced facilitation of synaptic inhibition

Glucocorticoids also induce NO-mediated facilitation of GABA inputs to magnocellular neuroendocrine cells in rat hypothalamic slices by activating a membrane-associated GR (21). Here, we tested for a rapid glucocorticoid-induced, NO-dependent facilitation of GABA release in magnocellular neurons in mouse hypothalamic slices from wild-type C57BL/6 mice. Bath application of Dex (1μM) caused an increase in mIPSC frequency in magnocellular neurons, from 2.00 ± 0.43 to 2.38 ± 0.53 Hz (to 117.0 ± 3.0% of baseline; $P < .05$, n = 7) (Figure 3). Preapplication of the NO synthase inhibitor L-NAME (50μM) had no effect on basal mIPSC frequency, but blocked the Dex-induced increase in mIPSC frequency (2.11 ± 0.44 vs 1.98 ± 0.27 Hz; $P = .33$, n = 6) (Figure 3), suggesting that the glucocorticoid-induced facilitation of GABA synaptic inputs to magnocellular neurons in mice, as in rats, is mediated by the retrograde release of NO.

Loss of rapid glucocorticoid synaptic modulation in GR knockout mice

Conditional GR knockout mice, in which the GR was deleted specifically in neurons of the PVN and SON, were generated by crossing mice with LoxP sites flanking exon

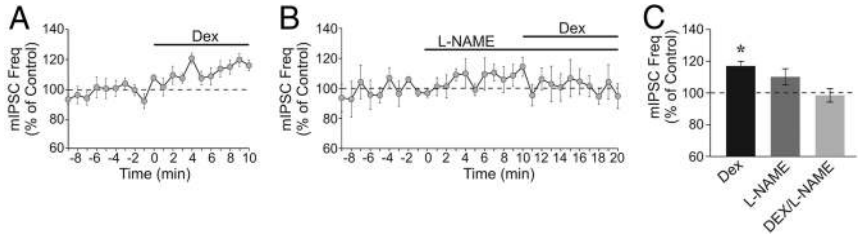

Figure 3. Glucocorticoid-induced facilitation of synaptic inhibition is blocked by inhibiting NO synthesis. A, Average (1-min bins) of the normalized mIPSC frequency as a function of time in magnocellular neurons (n = 7) from wild-type C57BL/6 mice. Dex (1μM) caused a rapid increase in the frequency of mIPSCs. B, The Dex-induced increase in mIPSC frequency in magnocellular neurons was blocked by preapplication of the NO synthase inhibitor L-NAME (50μM, n = 6). C, Average percent change in mean mIPSC frequency compared with baseline (3 min before drug) after 7–10 minutes of Dex application (1μM, n = 7), L-NAME application (50μM, n = 6), and Dex coapplication 10 minutes after the introduction of L-NAME (n = 6). *, $P < .05$.

2 of the GR gene (35, 36) with mice expressing Cre recombinase under the control of the Sim1 promoter (26). Sim1 is a transcription factor expressed preferentially in PVN and SON neurons of the hypothalamus (26). This allows for the site-specific knockout of GR in the PVN and SON using the Cre-lox approach. Quantification of the GR immunoreactive neurons in the PVN was performed in the $Sim1^{GR+/+}$ control mice (n = 8) and $Sim1^{GR-/-}$ mice (n = 9). These analyses are described in detail in the companion article by Solomon et al (38). This revealed a 60% reduction in GR expression in the PVN with no overall loss of PVN neurons. Because the rapid glucocorticoid modulation of synaptic transmission in the rat hypothalamus is not blocked by the classical GR antagonist RU486 and is dependent on a membrane-associated receptor and G protein-dependent signaling mechanism (11, 21, 32), we postulated that the genetic deletion of GR would have little or no effect on the rapid glucocorticoid actions. To test this hypothesis, we first recorded mEPSCs and mIPSCs in magnocellular neurons from $Sim1^{GR-/-}$ mice and tested for rapid glucocorticoid effects on synaptic glutamate and GABA release. Littermate mice homozygous for floxed GR, but negative for Cre recombinase, were used as controls ($Sim1^{GR+/+}$ mice). The knockdown of the GR expression in the PVN was confirmed immunohistochemically using a monoclonal antibody to GR (Figure 4A). In recordings of mEPSCs (with bicuculline methiodide in the medium to block $GABA_A$ receptors), bath application of Dex (1μM) caused a rapid decrease in mEPSC frequency in magnocellular neuroendocrine cells from $Sim1^{GR+/+}$ control mice (1.09 ± 0.19 to 0.94 ± 0.19 Hz; $P < .01$, n = 8) (Figure 4). This 15% decrease in mEPSC frequency in magnocellular neurons from $Sim1^{GR+/+}$ mice was less robust than the 52% and 40% decreases seen in magnocellular neurons from wild-type CD1 and C57Bl/6 mice, respectively, suggesting that the LoxP sites flanking the GR may have an inhibitory effect on the rapid glucocorticoid modulation of glutamate release. Nevertheless, the significant Dex-induced decrease in mEPSC frequency seen in magnocellular neurons from the $Sim1^{GR+/+}$ mice was lost in magnocellular neurons recorded in slices from the $Sim1^{GR-/-}$ mice (1.90 ± 0.32 vs 2.08 ± 0.32 Hz; $P = .36$, n = 8) (Figure 4). The loss of the rapid Dex effect on mEPSC frequency with genetic knockdown of GR suggested that the rapid glucocorticoid-induced, endocannabinoid-dependent suppression of glutamate release requires the expression of the classical nuclear GR.

In recordings of mIPSCs (with ionotropic glutamate receptors blocked with 6,7-dinitroquinoxaline-2,3-dione and (±)-2-amino-5-phosphono-pentanoic acid), bath application of Dex (1μM) caused an increase in the mIPSC frequency in magnocellular neurons from $Sim1^{GR+/+}$ control mice (from 2.36 ± 0.89 to 2.63 ± 0.95 Hz; $P < .05$, n = 8) (Figure 5, A, B, and D). This 11% increase in mIPSCs was also somewhat less robust than the 17% increase seen in magnocellular neurons from wild-type C57Bl/6 mice, again suggesting a possible negative regulation by the GR exon 2-flanking LoxP sites of the region of the GR involved in rapid glucocorticoid signaling. The rapid glucocorticoid-induced increase in mIPSC frequency was abolished in cells from slices from the $Sim1^{GR-/-}$ mice (1.87 ± 0.31 vs 1.74 ± 0.26; $P = .40$, n = 12) (Figure 5, A, C, and D). This indicated that the rapid glucocorticoid-induced NO facilitation of presynaptic GABA release is also dependent on the expression of the classical nuclear GR in PVN magnocellular neurons.

Finally, we tested the rapid glucocorticoid effect for GR dependence specifically in CRH neurons in slices from *CRH-eGFP;*$Sim1^{GR-/-}$ mice, which expressed both eGFP specifically in CRH neurons and the Sim1-driven conditional knockout of GR (Figure 6). eGFP expression in the PVN of *CRH-eGFP* mice was exclusively in CRH neurons, as revealed by CRH immunofluorescence performed in hypothalamic sections from colchicine-treated *CRH-eGFP* mice (Figure 6A). Because PVN parvocellular neuroendocrine cells express Sim1, we expected to see a similar loss of the rapid glucocorticoid effect in the CRH neurons. We limited our experiments to testing for the GR dependence of the rapid glucocorticoid effect on mEPSCs, and not mIPSCs, because our previous studies in the rat had shown a rapid glucocorticoid suppression of synaptic excitation, but no effect on synaptic inhibition, in PVN parvocellular neurons (17, 18). Here, we tested the endogenous glucocorticoid, corticosterone, for a rapid effect and for the GR dependence of the rapid effect on glutamate release. This allowed us to confirm the rapid effect of the endogenous ligand at excitatory synapses on CRH neurons. Bath application of corticosterone (2μM) caused a rapid decrease in the frequency of mEPSCs in control neurons from *CRH-eGFP;*$Sim1^{GR+/+}$ mice (ie, with floxed GR, no Cre) from 1.80 ± 0.31 to 1.48 ± 0.27 Hz (by 18%; $P < .05$, n = 8). The corticosterone-induced suppression of mEPSC frequency was lost in CRH neurons in slices from *CRH-eGFP;*$Sim1^{GR-/-}$ mice (1.20 ± 0.16 vs 1.17 ± 0.17 Hz; $P = .91$, n = 8) (Figure 6).

Discussion

Glucocorticoids secreted in response to stress stimulation of the HPA axis in the male rat feed back onto the brain to suppress HPA axis activation via a rapid, nongenomic mechanism involving endocannabinoid suppression of ex-

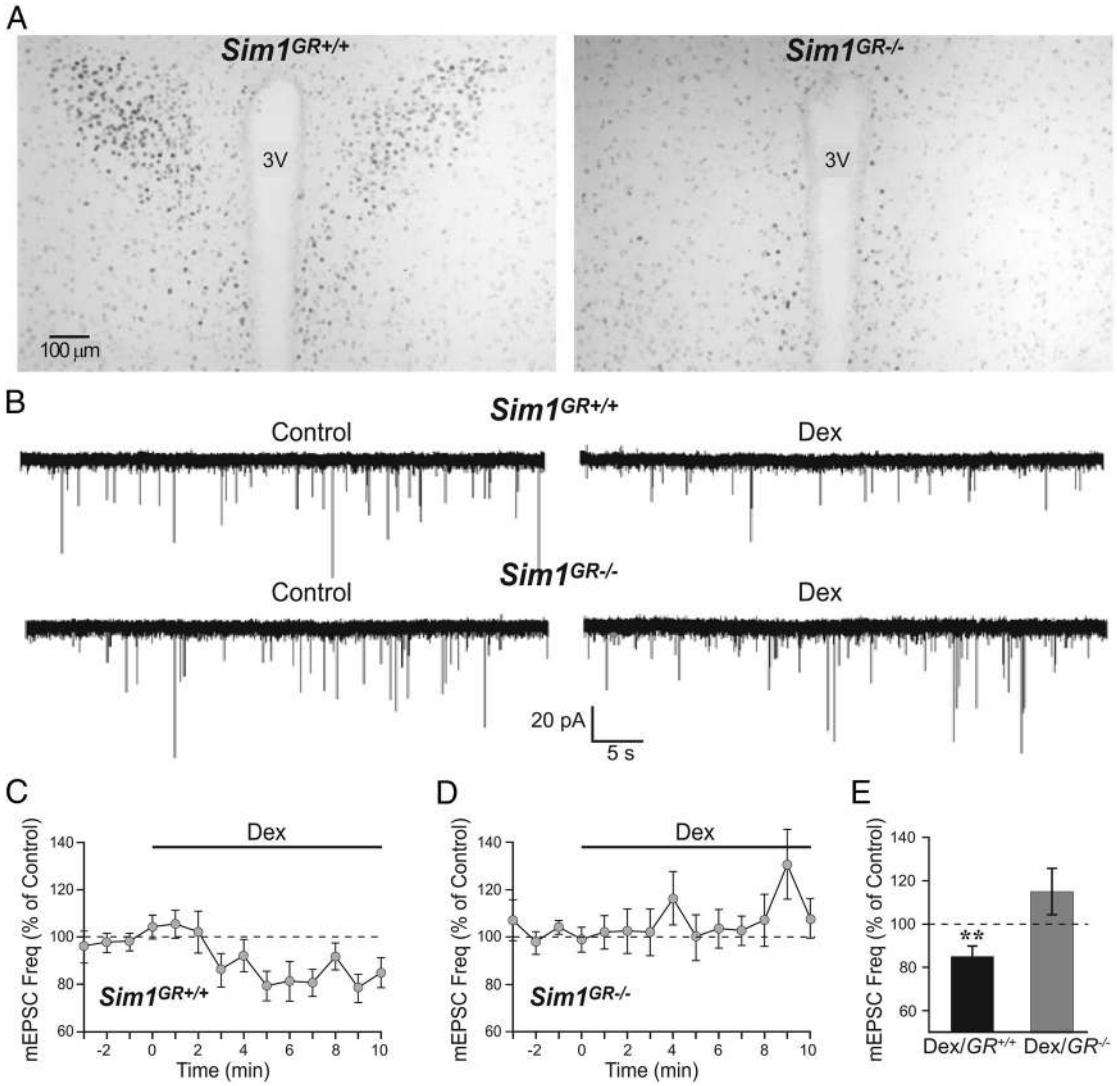

Figure 4. Rapid glucocorticoid suppression of synaptic excitation is absent in magnocellular neurons from conditional GR knockout mice. A, Immunohistochemical labeling of the GR in the area of the PVN in sections from a *Sim1*$^{GR+/+}$ control mouse and a *Sim1*$^{GR-/-}$ mouse in which the GR was deleted in Sim1-expressing PVN neurons. 3V, third ventricle. B, Raw traces of mEPSCs recorded in magnocellular neurons at baseline (control) and after 10 minutes of bath application of Dex (1 μM) in slices from a control floxed GR mouse (*Sim1*$^{GR+/+}$) and a conditional GR knockout mouse (*Sim1*$^{GR-/-}$). Dex induced a decrease in the frequency of mEPSCs in the neuron from the control mouse but not in the neuron from the conditional GR knockout mouse. C, Average (1-min bins) of the mean normalized mEPSC frequencies over time from magnocellular neurons (n = 8) recorded in slices from control mice (*Sim1*$^{GR+/+}$). D, Average (1-min bins) of the mean normalized mEPSC frequencies over time from magnocellular neurons (n = 8) recorded in slices from GR knockout mice (*Sim1*$^{GR-/-}$). E, Average percent change in mean mEPSC frequency after 7–10 minutes in 1 μM Dex in magnocellular neurons from control (*Sim1*$^{GR+/+}$) and GR knockout (*Sim1*$^{GR-/-}$) mice. The Dex-induced suppression of synaptic excitation was absent in the magnocellular neurons from conditional GR knockout mice. **, $P < .01$.

citatory synaptic inputs to CRH neurons (11, 37). Glucocorticoids also rapidly modulate both excitatory and inhibitory synaptic inputs to magnocellular neurons in the male rat via nongenomic, synapse-specific actions of endocannabinoids and NO at glutamate and GABA synapses, respectively (21, 22). Here, we found a rapid modulation of excitatory and inhibitory synaptic transmission by glucocorticoid activation of a membrane-associated GR and a G protein-dependent signaling mechanism in neuroendocrine cells of the male mouse hypothalamus. Dex induced a suppression of synaptic excitation in both parvocellular CRH and magnocellular neuroendocrine cells and an enhancement of synaptic inhibition in magnocellular neurons of the PVN and SON. We found similar rapid Dex effects in both CD1 and C57BL/6 strains of mouse.

The rapid glucocorticoid suppression of synaptic excitation was blocked by a CB1 receptor antagonist and by the genetic deletion of CB1 receptors, indicating a critical role for the retrograde endocannabinoid modulation of glutamate synapses. The rapid glucocorticoid facilitation of synaptic inhibition in magnocellular neurons was

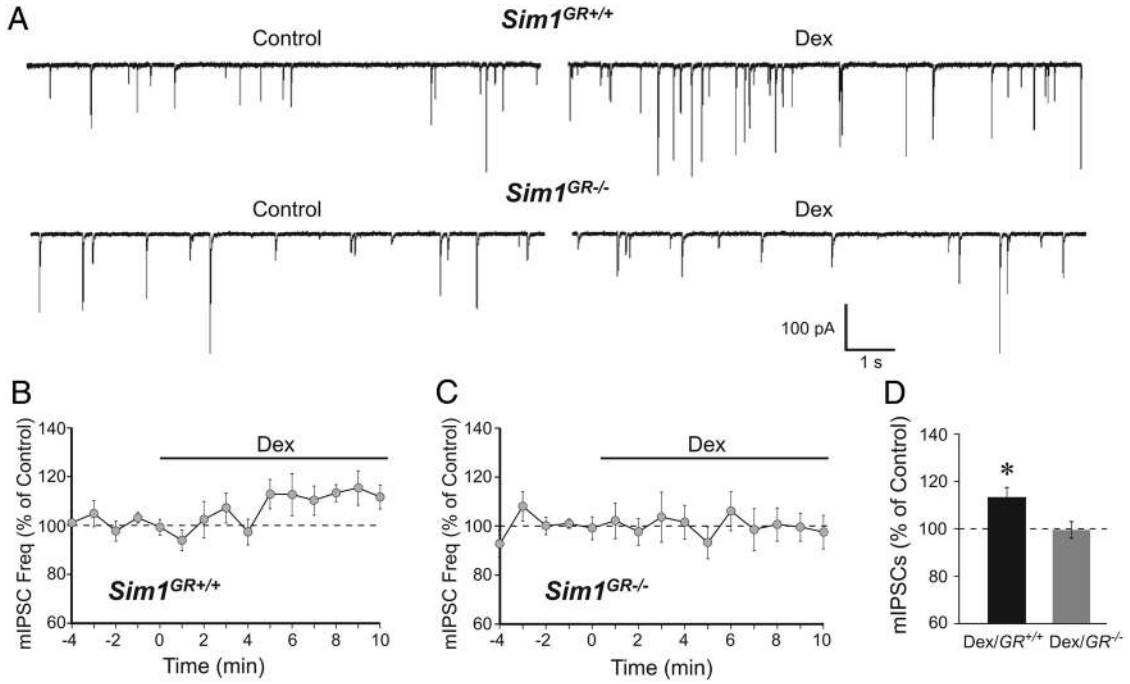

Figure 5. Rapid glucocorticoid facilitation of synaptic inhibition is absent in magnocellular neurons from conditional GR knockout mice. A, Raw traces of mIPSCs recorded in magnocellular neurons in slices from a floxed GR mouse (top, $Sim1^{GR+/+}$) and a conditional GR knockout mouse (bottom, $Sim1^{GR-/-}$). Dex (1μM) caused an increase in the frequency of mIPSCs in the neuron from the $Sim1^{GR+/+}$ mouse compared with baseline (control) but had no effect on the mIPSC frequency in the neuron from the $Sim1^{GR-/-}$ mouse. B, Mean normalized mIPSC frequencies (1-min bins) recorded over time in magnocellular neurons (n = 8) in slices from $Sim1^{GR+/+}$ control mice. C, Mean normalized mIPSC frequencies recorded over time (1-min bins) in magnocellular neurons (n = 12) in slices from $Sim1^{GR-/-}$ mice. D, Average percent change in mean mIPSC frequency after 7–10 minutes of Dex application (1μM) in cells from floxed control mice ($Sim1^{GR+/+}$, n = 8) and conditional GR knockout mice ($Sim1^{GR-/-}$, n = 12). *, $P < .05$.

blocked by inhibiting NO synthesis, indicating a primary role for retrograde NO modulation of GABA synapses in these cells. Therefore, the synapse-specific glucocorticoid modulation of glutamate release via endocannabinoid actions and GABA release via NO actions in neuroendocrine cells of the male mouse PVN and SON is similar to what we have reported previously in male rat neuroendocrine cells (12, 20, 21). Our findings indicate that the rapid glucocorticoid actions at membrane-associated receptors in hypothalamic parvocellular CRH and magnocellular neurons are conserved between the mouse and the rat. Although these rapid glucocorticoid actions were observed here and previously in hypothalamic tissue from 5- to 8-week-old animals, it is likely that they are not exclusive to the adolescent stage of development, because rapid glucocorticoid-induced endocannabinoid release in vivo was reported in the PVN of adult rats (39), and we previously found an endocannabinoid-dependent rapid suppression of HPA activation in vivo by intra-PVN glucocorticoid application in adult rats (36).

Rapid glucocorticoid actions are dependent on the nuclear GR

Unexpectedly, the rapid glucocorticoid effects on both glutamatergic and GABAergic synaptic transmission were abolished in slices from mice in which the classical nuclear GR was deleted in a tissue-specific manner. Thus, Dex failed to decrease the mEPSC frequency or increase the mIPSC frequency in magnocellular neurons from $Sim1^{GR-/-}$ mice, and failed to decrease mEPSC frequency in CRH neurons from *CRH-eGFP;* $Sim1^{GR-/-}$ mice. This suggests that the rapid glucocorticoid effects mediated by a membrane-associated GR in PVN and SON neuroendocrine cells depend on the functional expression of the classical nuclear GR, this despite the membrane localization of the receptor and the G protein dependence of the rapid glucocorticoid effects. Our qualitative immunohistochemical data suggested a robust loss of GR (see Figure 4), and a loss of about 60% of the GR expression in PVN, with no loss of PVN neurons, was reported by Herman and coworkers using the same mouse (38). The original study of the Sim1-GR knockout model by Muglia and coworkers reported a 43% loss of GR in the PVN (28). The complete absence of the rapid glucocorticoid effects in slices from these mice reveals the loss of a critical component in the rapid glucocorticoid signaling mechanism with the deletion of exon 2 of the GR. The rapid glucocorticoid effects in neurons from the $Sim1^{GR+/+}$ mice, used as controls in the GR knockout experiments, were less robust

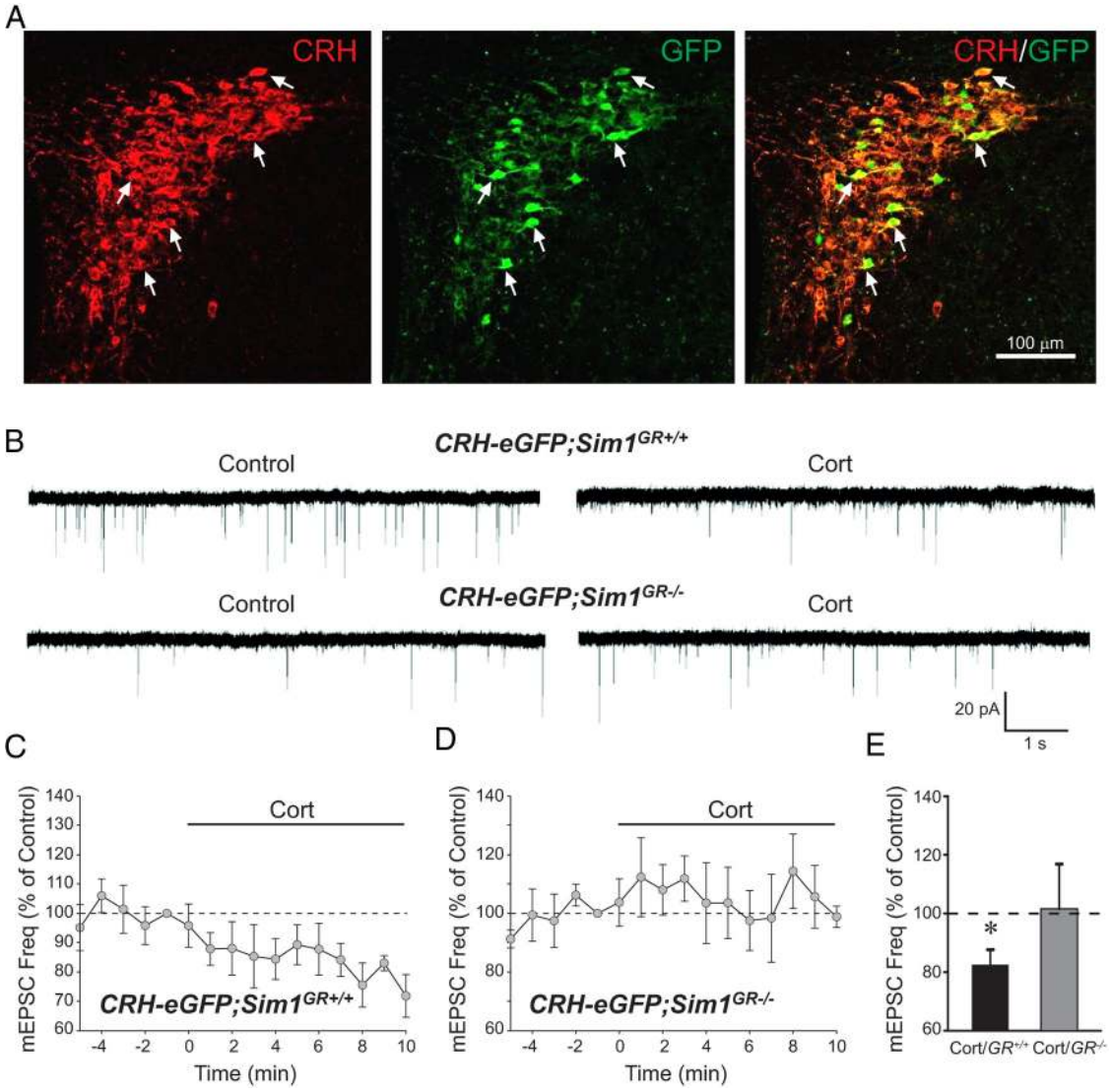

Figure 6. Rapid glucocorticoid facilitation of synaptic excitation is absent in CRH-eGFP neurons from conditional GR knockout mice. A, CRH immunostaining in PVN of CRH-eGFP mice. Left, CRH immunofluorescence in the PVN viewed under red filters after in vivo colchicine pretreatment. Middle, eGFP fluorescence in the same section viewed under green filters. Right, Overlay of the 2 images showing eGFP and CRH coexpressing neurons in the PVN labeled orange-yellow, a few of which are designated by the arrows. B, Raw traces of mEPSCs recorded in an eGFP-expressing CRH neuron from a floxed GR control mouse (top, *CRH-eGFP;Sim1*$^{GR+/+}$) and in an eGFP-expressing CRH neuron from a conditional GR knockout mouse (bottom, *CRH-eGFP;Sim1*$^{GR-/-}$). Corticosterone (2μM) elicited a decrease in the frequency of mEPSCs compared with baseline (control) in the neuron from the floxed GR mouse (*CRH-eGFP;Sim1*$^{GR+/+}$) but not in the conditional GR knockout mouse (*CRH-eGFP;Sim1*$^{GR-/-}$). C, Mean normalized mEPSC frequencies (1-min bins) recorded in CRH-eGFP neurons (n = 8) as a function of time in slices from floxed GR control mice (*CRH-eGFP;Sim1*$^{GR+/+}$). D, Mean normalized mEPSC frequencies (1-min bins) recorded in CRH-GFP neurons (n = 8) as a function of time in slices from conditional GR knockout mice (*CRH-eGFP;Sim1*$^{GR-/-}$). E, Average percent change in mean mEPSC frequency after 8–10 minutes of Cort application (2μM) in CRH-eGFP neurons recorded in slices from floxed GR control mice (Cort/$GR^{+/+}$, n = 8) and conditional GR knockout mice (Cort/$GR^{-/-}$, n = 8). *, $P < .05$.

than those recorded in slices from wild-type CD1 and C57BL/6 mice, which may be due to a deleterious effect of the LoxP fragments flanking exon 2 of the GR gene.

A companion study by Solomon et al indicates that fast feedback inhibition of the HPA axis during acute stress is also lost in the male *Sim1*$^{GR-/-}$ mouse (38), which suggests that the nuclear GR is necessary for the rapid glucocorticoid feedback inhibition of the HPA axis in male mice. Because fast feedback inhibition of the HPA axis is mediated in part by an endocannabinoid-dependent mechanism in the PVN (37), it is likely that the GR-dependent fast feedback in male mice is mediated by the rapid glucocorticoid actions in CRH neurons described here.

The loss of the rapid glucocorticoid response with knockdown of the nuclear GR suggests the possibility that the membrane-associated GR may be a product of the gene that encodes the nuclear GR. The G protein dependence of

the rapid glucocorticoid actions, in this case, would be mediated by the interaction of the downstream signaling of the membrane GR with a distinct G protein signaling mechanism. This would be similar, then, to the proposed signaling mechanism of a rapid action of estrogen in the hippocampus, in which the estrogen receptor-α is thought to be located at the membrane and to signal in a G protein-dependent manner via interactions with metabotropic glutamate receptors (40, 41). On the other hand, the membrane GR may signal via the release into the extracellular space of an intermediate autocrine messenger, which then activates a G protein-coupled receptor, similar to the proposed mechanism of membrane estrogen receptor-α interactions with a receptor tyrosine kinase, IGF-1 receptor, in breast cancer cells to induce epidermal growth factor release, activation of the epidermal growth factor receptor, and downstream activation of MAPK (42). Finally, the membrane GR may, in fact, be a distinct G protein-coupled receptor, produced by a different gene, and the rapid glucocorticoid actions depend on the nuclear GR for downstream signaling to endocannabinoid and NO synthesis, either because the nuclear GR regulates the membrane GR expression/function and/or its signaling cascade, or because the nuclear GR itself is integrated into the membrane receptor's downstream signaling pathway. This latter possibility would suggest that signaling to the nucleus by the nuclear GR may also require the activation of the membrane GR (43).

Rapid glucocorticoid actions in other brain areas

The rapid glucocorticoid modulation of excitatory and inhibitory synaptic transmission in hypothalamic neuroendocrine cells shows certain similarities and dissimilarities with rapid corticosteroid actions described in the hippocampus, basolateral amygdala, prefrontal cortex, and nucleus of the solitary tract. Although rapid corticosteroid effects in each of these brain regions are mediated by nongenomic steroid actions, the mechanisms and outcomes of the rapid corticosteroid actions are quite varied. Thus, rather than inhibiting glutamate release via a retrograde endocannabinoid effect, corticosterone elicits a rapid facilitation of glutamate release onto CA1 pyramidal neurons, dentate granule cells (3, 44), and principal neurons of the basolateral amygdala (4) via the activation of presynaptic mineralocorticoid receptors. However, although the effect is reversible in the hippocampus, it is nonreversible in the basolateral amygdala, as in the PVN, but the maintenance of the effect in the basolateral amygdala, unlike in the PVN, is transcription dependent and is reversed by a second exposure to glucocorticoid, which causes a rapid endocannabinoid-dependent suppression of glutamate release (4). This latter effect of a second corticosterone exposure is similar to the rapid glucocorticoid-induced endocannabinoid effect in hypothalamic glutamatergic circuits (Refs. 11, 12 and current findings). There are also rapid glucocorticoid effects on inhibitory synaptic circuits in the hippocampus, prefrontal cortex and amygdala. In hippocampal CA1 neurons, as in hypothalamic magnocellular neurons, glucocorticoids rapidly stimulate an NO-mediated increase in inhibitory synaptic inputs, but, unlike in hypothalamic neurons, this is mediated by an increase in spiking in the presynaptic GABA neurons. The rapid glucocorticoid actions at inhibitory synapses in the medial prefrontal cortex differ in distinct circuits, causing an endocannabinoid-mediated suppression of GABA release onto layer V pyramidal neurons of the prelimbic region (9) and an NO-induced, spike-dependent facilitation of GABA release onto layer II/III pyramidal neurons (10). We have preliminary evidence for a rapid glucocorticoid-induced suppression of GABA inhibition in basolateral amygdala principal neurons that, like at PVN excitatory synapses, is mediated by a retrograde endocannabinoid inhibition of release (Di and Tasker, unpublished observation; see also Ref. 45). Thus, the mechanisms of rapid glucocorticoid synaptic modulation involve retrograde endocannabinoid and NO signaling, however, subtle differences in these mechanisms are found at different synapses in stress neural circuits.

The data presented here support the involvement of a postsynaptic membrane-associated GR in the acute effects of corticosteroids in mouse neuroendocrine cells, which is similar to the putative membrane GR mechanism described in rat neuroendocrine cell populations (20, 21, 32). The membrane GR shows properties that are distinct from the nuclear GR by: 1) its membrane localization, 2) its rapid response, 3) its G protein dependence, and 4) its synapse-specific induction of retrograde messengers. However, the dependence of the rapid glucocorticoid actions on the expression of the nuclear GR suggests the possibility that the membrane receptor may be an isoform of the nuclear GR located at the membrane, which, if borne out, will impact future preclinical and clinical studies focused on the membrane receptor as a therapeutic target. Glucocorticoids are among the most heavily prescribed drugs within the Western medical pharmacopoeia and constitute central components in the treatments of various hematological malignancies, autoimmune disorders, and inflammatory diseases, as well as in immunosuppressive treatments. The characterization of the membrane GR will provide an opportunity for detailed systemic research into its molecular nature and significance, and is critical for the design of targeted therapies that distinguish between the divergent modes of glucocorticoid signaling and avoid detrimental side effects.

Acknowledgments

We thank Dr Audrey Seasholtz, Dr Simon Evans, and Dr Robert Thompson for graciously welcoming us into their labs at the University of Michigan during a difficult time after hurricane Katrina and for their generosity of spirit and expert technical guidance in the initial radioligand binding and molecular studies from which the current study sprang. We also thank Dr James Pickel of the National Institute of Mental Health for providing the CB1 receptor knockout mice and Dr Bradford Lowell of the Beth Israel Deaconess Medical Center for providing the Sim1-Cre mice. We thank Dr Matia Solomon for providing immunohistochemical controls of GR knockdown. The CRH-eGFP mouse founders were provided by the National Institutes of Health-supported Mutant Mouse Regional Resource Center of the University of California, Davis.

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This work was supported by National Institute of Mental Health Grants R01 MH066958 (to J.G.T.), R01MH079010 (to L.J.M.), and R01 MH MH069860 (to J.P.H.); the Tulane University Research Enhancement Fund; and the Catherine and Hunter Pierson Endowed Chair in Neuroscience.

Disclosure Summary: The authors have nothing to disclose.

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