Rapid hypothalamic-pituitary recovery after chronic glucocorticoid therapy enables strategies that prevent adrenal suppression in mice

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Abstract

Glucocorticoid-induced adrenal insufficiency (GIAI) can persist for months after cessation of chronic corticosteroid therapy, predisposing patients to life-threatening adrenal crises. The mechanisms underlying this prolonged suppression are poorly understood and have been primarily attributed to delayed recovery of hypothalamic-pituitary function (Graber *et al.,* 1965). We examined the timing of hypothalamic, pituitary, and adrenal (HPA) recovery after 8 weeks of dexamethasone (DEX) treatment in mice. During DEX exposure, HPA axis activity was fully suppressed. Within one week of withdrawal, *Crh* mRNA and plasma ACTH surprisingly rebounded above control levels, yet corticosterone (CORT) remained low for an additional seven weeks. DEX-treated adrenals were atrophic and contained large clusters of lipid-associated macrophages. Even after accounting for these macrophages, CORT secretion was disproportionately low relative to the size of the adrenocortical cell mass for at least two weeks after DEX withdrawal despite supraphysiologic ACTH stimulation. Thus, the adrenal is the primary site of post-withdrawal HPA axis dysfunction, involving both cortical atrophy and impaired steroidogenesis. We then assessed whether trophic stimulation of the adrenal using either a pharmacologic or genetic approach prevented GIAI. Co-treatment with DEX and daily cosyntropin, an ACTH analog, failed to prevent GIAI. However, mice with non-suppressible endogenous ACTH due to deletion of *Nr3c1* (the glucocorticoid receptor gene) in hypothalamic neurons maintained normal adrenal function and histology despite long-term DEX. Localizing the mechanisms of post-withdrawal GIAI to the adrenal may permit the development of strategies that prevent GIAI by delivering sustained trophic support to the adrenal during the period of glucocorticoid exposure.

Significance Statement

Glucocorticoids are among the most commonly prescribed anti-inflammatory agents, yet chronic exposure can cause sustained suppression of the hypothalamic-pituitary-adrenal (HPA) axis, predisposing patients to life-threatening adrenal crises. This glucocorticoid-induced adrenal insufficiency (GIAI) has been attributed primarily to delayed hypothalamic-pituitary recovery after treatment withdrawal, limiting the development of preventive strategies. Using a murine model of GIAI, we found that hypothalamic-pituitary function recovers rapidly after cessation of chronic glucocorticoids, whereas adrenal suppression persists for eight weeks. Continuous adrenal stimulation during glucocorticoid exposure, achieved through a targeted genetic approach, preserved adrenal function. These findings identify the adrenal as the principal site of post-withdrawal HPA axis dysfunction and suggest that therapies sustaining adrenal trophic signaling could prevent GIAI in patients requiring long-term glucocorticoid therapy.

Main Text

Introduction

Systemic glucocorticoids are prescribed to up to 3% of the population in developed countries for treatment of autoimmune and inflammatory diseases, malignancies, and to prevent allograft rejection[1,2]. Many additional patients are chronically treated with topical and inhaled formulations that can exert systemic effects even at moderate doses[3-5]. Like endogenous cortisol in humans and corticosterone in rodents, exogenous glucocorticoids engage the glucocorticoid receptor (GR) to suppress hypothalamic-pituitary-adrenal (HPA) axis activity through negative feedback. This feedback reduces the synthesis and secretion of corticotropin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus and adrenocorticotropic hormone (ACTH) from the anterior pituitary[6]. In the absence of ACTH, a key trophic factor for the adrenal cortex,

cortisol-producing cells of the zona fasciculata (zF) undergo both suppression of steroidogenesis and apoptotic cell loss[7,8].

Hypothalamic-pituitary-adrenal (HPA) axis suppression is typically transient in patients treated with short-term glucocorticoids. However, treatment exceeding four weeks can cause protracted adrenal insufficiency that can persist for up to a year after steroid withdrawal[9-11]. While awaiting HPA axis recovery, individuals are at risk for adrenal crisis with otherwise routine illnesses or trauma, which has been associated with longer hospitalizations, higher rates of ICU admissions, and even death in patients who are not promptly administered stress-dose steroids[12,13].

Despite the ubiquity of glucocorticoid use and the morbidity of glucocorticoid-induced adrenal insufficiency (GIAI), there are currently no available treatments to prevent or hasten recovery from adrenal suppression. This is due, in part, to the prevailing view that prolonged GIAI is mediated by delayed, post-withdrawal recovery of the hypothalamus and pituitary, which are relatively inaccessible therapeutic targets. Post-withdrawal central suppression was first described by Graber et al., who reported that ACTH levels were inappropriately low to normal for at least two months in patients withdrawn from chronic glucocorticoids despite release from negative feedback[14]. Since direct ACTH assays had not yet been developed, systemic ACTH levels in this study were interpolated from the corticosterone responses of hypophysectomized rats injected with extracted patient plasma[14,15]. Another study cited inadequate ACTH responses to CRH stimulation testing in patients withdrawn from long-term prednisone as evidence of post-withdrawal central suppression. However, testing was conducted 24 hours or two biological half-lives from the last prednisone dose, thus limiting differentiation between active, glucocorticoid-mediated negative feedback and genuine, post-withdrawal suppression[16,17]. Post-withdrawal central suppression has also been described after surgical cure of Cushing's syndrome, a state of endogenous glucocorticoid excess, though clinical studies have disagreed as to whether this is mediated by persistent hypothalamic or pituitary dysfunction[18-20].

Interestingly, patients with congenital adrenal hyperplasia, who also require chronic, suppressive glucocorticoid therapy, exhibit elevated levels of both ACTH and ACTH-dependent steroid precursors at their morning hydrocortisone trough[21,22]. This suggests that some patients have rapid normalization of hypothalamic-pituitary function after release from glucocorticoid feedback inhibition. The mammalian HPA axis is highly conserved evolutionarily, and all rodent studies have reported rapid restoration of hypothalamic-pituitary function after glucocorticoid treatment. Basal and stimulated ACTH levels (following lipopolysaccharide, arecoline, and CRH administration) were recovered within days of discontinuing 5 or 7 days of methylprednisolone or dexamethasone, respectively, in rats[23,24]. In mice treated with dexamethasone for 30 days, plasma ACTH and corticotrope *Pomc* mRNA levels were elevated above control levels by 1-week post-withdrawal[25]. In all studies, corticosterone (CORT) levels and/or steroidogenic pathway gene expression remained low 5-7 days after discontinuing steroids despite normalization of adrenal weights[23-25]. Importantly, no animal studies to date have examined HPA axis recovery after longer courses of glucocorticoids known to cause protracted adrenal suppression in humans.

Given these discrepancies in the existing clinical and preclinical literature, we developed a murine model of GIAI to define the locus and mechanisms of persistent HPA axis dysfunction following withdrawal of long-term glucocorticoids. After we discovered that adrenal dysfunction persists for months after steroid cessation despite rapid hypothalamic-pituitary recovery, we next tested whether sustained trophic stimulation of the adrenal during the period of glucocorticoid exposure could preserve steroidogenic function. Together, these studies identify the adrenal as

the principal site of post-withdrawal GIAI and highlight it as an accessible target for future preventative interventions.

Results

Mice treated with long-term dexamethasone (DEX) have protracted adrenal insufficiency despite early hypothalamic-pituitary recovery

We first established when hormonal function at each node of the HPA axis recovered following long-term DEX treatment. Adult, male, WT mice were treated with either continuous DEX or vehicle (DEX-naïve, or DN) in the drinking water for 8 weeks. Starting at the time of DEX withdrawal (8+0) and at 1-2 week intervals through 8+8 (where A weeks of treatment plus B weeks of withdrawal = A+B), separate cohorts of mice underwent serial cosyntropin stimulation and insulin-induced hypoglycemia (IIH) testing followed by necroscopy (Model 1, Fig. S1).

As expected, long-term DEX treatment suppressed *Crh* mRNA within the paraventricular nucleus of the hypothalamus (PVH; 28±5 in 8+0 vs. 156±32 arbitrary units [AU] in DEX-naïve (DN) control animals, $p<0.05$). However, *Crh* expression recovered to control levels 1 week after DEX was discontinued (8+1; 159±28 AU vs. DN, $p>0.9999$, Fig. 1*A*, *B*). Similarly, IIH-stimulated plasma ACTH concentrations were significantly suppressed during the period of glucocorticoid exposure (4.2±3.6 pg/mL at 8+0) but rebounded significantly above control levels by 1-week post-withdrawal (1707±85 pg/mL at 8+1 vs. 875±44 pg/mL). IIH-stimulated ACTH levels remained elevated through 4 weeks post-withdrawal and reached a peak two weeks after completion of DEX treatment (Fig. 1*C*). Despite this, both IIH- and cosyntropin-stimulated CORT levels remained significantly lower than DN controls until 8 weeks after DEX cessation (Fig. 1*D*, *E*). Thus, mice withdrawn from long-term DEX have delayed recovery of CORT secretion despite rapid restoration of hypothalamic-pituitary function.

Long-term DEX induces adrenocortical atrophy with accumulation of large macrophage clusters

Having found the adrenal to be the limiting site of post-withdrawal GIAI, we then assessed adrenal mass and histology at select timepoints after chronic DEX. At 8+0, adrenal mass was reduced by 60% compared to controls (1.0±0.041 mg vs. 2.5±0.063 mg in DN, $p<0.0001$, Fig. 2*A*). Although adrenals from DEX-exposed adrenals weighed significantly less than those from DN controls through 6 weeks post-withdrawal, divergent patterns of recovery were noted for adrenal mass vs. function. In particular, adrenal mass nearly doubled between 0 to 1 weeks post-withdrawal to 75% of controls (1.9±0.11 mg at 8+1) and remained relatively stable over the remaining recovery period. In contrast, CORT secretion was 75% lower than control values at 8+1 and more gradually recovered over the subsequent 7 weeks (Fig. S2*A*, *B*; raw data derived from Figs. 1*D*, *E* and 2*A*).

Hematoxylin and eosin staining revealed that DEX caused striking adrenocortical atrophy followed by gradual re-accumulation of cortical tissue in the 8 weeks post-withdrawal (Fig. 2*B*). Interestingly, DEX-exposed adrenals contained large, vacuolated structures with reduced nuclear density near the corticomedullary junction that persisted through 8 weeks post-withdrawal (Fig. 2*B*, magnification in 2*E*). These clusters were intensely autofluorescent, which was markedly attenuated with the addition of TrueBlack, a lipofuscin autofluorescence quencher[26] (Fig. S2*A*, *B*). ISH with a 5'-Cy5-Oligod(T)30 demonstrated similar, nuclear mRNA poly(A) content in these structures compared to surrounding cortical cells (Fig. S2*C*)[27,28]. The large, vacuolated, extranuclear space in these clusters was not strongly labeled with 568 NHS Ester (SE), a total protein stain (Fig S2*D*)[29,30]. Together, this suggests that the multinucleated clusters observed in DEX-exposed adrenals contain live, lipofuscin-rich, biologically active cells rather than acellular,

proteinaceous debris. These cells were negative for the steroidogenic enzyme *Cyp11b1* mRNA, a marker of zona fasciculata (zF) cells.

Immunostaining with the myeloid-specific marker, CD68, and macrophage-specific marker, F4/80, identified these cells as clusters of adrenal macrophages (AMs). Rare, mononuclear CD68- and F4/80-positive cells were visualized in the adrenal cortex (labeled with fluorescent 488-conjugated streptavidin[31]) of DN mice (Fig. 2*C*, *D*). Increased CD68- and F4/80-positive macrophages were present in the adrenal cortex after 2 weeks of DEX treatment (2+0). After 8 weeks of DEX (8+0), large AM clusters accumulated at the corticomedullary junction (Fig. 2*C*, *D*, magnified in 2*F*, *G*) and appeared to fragment and migrate into the adrenal medulla by 8 weeks post-withdrawal (8+8). AMs contained lipid detected by both oil red O and BODIPY staining but were negative for the lipid droplet associated proteins perilipin-1 and 2 (PLIN 1 and 2; Fig. S4*A*-*D*). AMs were also positive for CD11c and *Trem2* by IF and ISH, respectively, both of which are expressed by lipid-associated macrophages in the context of metabolic associated steatohepatitis and atherosclerosis (Fig. S4*E, F*)[32-35].

CORT secretion is disproportionately low relative to steroidogenic cell mass for at least 2 weeks post-DEX

As AMs likely contribute to adrenal mass but not biosynthetic function, we then asked specifically how the steroidogenic cell population contracted and recovered after chronic DEX treatment. To do this, we performed morphometric analysis on thick, immunostained adrenal sections, from which we selected the largest, equatorial one from each gland. Steroidogenic adrenocortical cells were labeled with 488-streptavidin[31] (green), and the medulla was identified by immunofluorescence for tyrosine hydroxylase (red). Sections were not treated with TrueBlack so that AMs could be identified by endogenous autofluorescence (cyan; Fig. 3*A*).

As observed with adrenal weights, pan-cortical area, which includes both streptavidin-positive and autofluorescent regions, decreased with DEX treatment (764±284 mm^2 in 8+0 vs. 1596±319.4 mm^2 in DN) but nearly normalized by 1-week post-withdrawal (1100±72 mm^2 in 8+1, Fig. 3*A*, *B*). Medullary (TH-positive) area was comparable at all time points, indicating that relatively equivalent, equatorial adrenal sections were used for morphometric analyses ($p=0.15$, Fig. 3*C*). Autofluorescent AM area increased with DEX treatment and remained stably elevated through 8 weeks post-withdrawal (Fig. 3*A*, *D*). Steroidogenic cell type area, computed by subtracting AMs (cyan autofluorescence) from the pan-cortical (green) area, markedly decreased with DEX treatment (272±47 mm^2 in 8+0 vs. 1286±153 mm^2 in DN, $p<0.001$) but more than tripled in the first week post-withdrawal (887±70mm^2; Fig. 3*A*, *E*).

To examine how adrenal function relates to adrenocortical cell mass, we calculated the ratio of cosyntropin-stimulated CORT secretion to the steroidogenic cell type area, normalized to DN controls (Fig. 3*F*). This ratio showed a nonsignificant decrease at the end of DEX treatment (0.60±0.14) but reached its lowest values at 1- and 2-weeks post-DEX (0.58±0.05 and 0.44±0.05, $p<0.05$ vs. DN). By 8+6, the ratio had returned to baseline (0.99±0.01) and was elevated relative to DN by 8+8 (2.0±0.18, $p<0.001$ vs DN). These findings indicate that, for at least 2 weeks after DEX withdrawal, CORT secretion is disproportionately low compared to the size of the non-macrophage adrenal cortex, even under supraphysiologic ACTH stimulation.

Immunofluorescence for CD31 revealed preserved vascularity in both DEX-treated (8+0) and withdrawn (8+1) adrenals (Fig. S5*A*). The zona glomerulosa, labeled by Dab2, thickened during chronic DEX exposure (45±2.6 µm at 8+0 vs. 36 ± 0.96 µm in DN) but returned to baseline by 8+1 (36±2.2 µm; Fig. S5B and C). Ki-67 staining demonstrated a robust increase in adrenocortical proliferation within one week of DEX withdrawal (17±1.5% of 8+1 cells vs. 2.0 ± 1.0% of DN cells; Fig. S5B and D), consistent with the known mitogenic effects of ACTH[36]. Together, these findings indicate that disproportionately low CORT secretion relative to steroidogenic cell type area cannot

be explained by impaired adrenal vascularity, zonation, or trophic signaling but instead reflects a selective defect in CORT biosynthesis.

Maintenance of endogenous ACTH secretion but not intermittent cosyntropin administration during the period of DEX exposures prevents GIAI

Having localized post-withdrawal GIAI to the adrenal, we next tested whether maintaining trophic stimulation during glucocorticoid exposure could prevent adrenal suppression. Wild-type mice were treated with DEX as above and received daily, intraperitoneal injections of cosyntropin (ACTH [1-24]; DEX+COS) for either 4 days (short-term) or 8 weeks (long-term). Long-term cohorts underwent either a) serial cosyntropin stimulation testing at 1–2-week intervals from 0-8 weeks after withdrawal, or b) necroscopy at 8+1 (Model 2, Fig. S1). Short-term DEX+COS treatment significantly but variably reduced DEX-induced adrenocortical apoptosis (5.7±1.7% vs. 11±0.8% in 4D DEX glands; Fig. S6*A*, *B*). In contrast, after 8 weeks of treatment (8+0), cosyntropin-stimulated CORT levels remained equally suppressed in DEX+COS and DEX-only mice (2.0±1.0 µg/dL vs. 2.0±0.61 µg/dL, p=0.99). Following withdrawal, CORT recovery was significantly delayed in DEX+COS mice (doubling time=5.2 weeks) compared to DEX-only mice (2.5 weeks; p=0.009, Fig. S6*C*). Morphometric analysis at 8+1 revealed comparable steroidogenic cell type area between DEX+COS and DEX-only adrenals (Fig. S6*D*, *G*) but reduced AM area in the DEX+COS group (Fig. S6*F*). AM morphology was also heterogeneous, ranging from scattered smaller cells throughout the zona fasciculata to typical aggregates at the corticomedullary junction (Fig. S6*D*). Thus, intermittent, pharmacologic ACTH stimulation during glucocorticoid exposure modestly blocks adrenocortical apoptosis and macrophage accumulation but is insufficient to sustain steroidogenic capacity or accelerate functional recovery.

We then tested whether sustained, endogenous ACTH signaling during the period of glucocorticoid exposure prevents GIAI (Model 3, Fig. S1). We created $Sim1^{Cre}$:$GR^{fl/fl}$ mice (HGRKO), which have reduced hypothalamic glucocorticoid receptor expression and exhibit constitutively elevated *Crh* mRNA content and plasma ACTH and corticosterone concentrations[37]. Cre-negative littermates (HGRWT) were used as controls. Mice were treated for either 4 days or 8 weeks with DEX. HPA axis activity was assessed pre-treatment and on chronic DEX (8+0) in the unstressed state, following physical restraint, and after cosyntropin stimulation (Fig. 4). On long-term DEX treatment (8+0), GR immunoreactivity was present in 94% of PVH neurons of HGRWT mice vs. 23% in HGRKO mice (Fig. S7*A*, *B*). *Crh* expression in the PVH was suppressed in these same DEX-treated HGRWT (49±12 AU) but not HGRKO animals (244±2.2 AU; Fig. S7*C, D*). GR was thus successfully deleted from the PVH, which conferred resistance DEX-induced feedback inhibition of *Crh* mRNA expression.

Apoptosis was uniformly rare in short-term DEX-treated HGRKO glands (1.7%±0.9 vs. X% DN, p=0.74; Fig. 4A, *B*). HGRKO mice had higher basal ACTH concentrations than HGRWT controls (470±76 vs. 104±55 pg/mL, $p<0.05$) that were not suppressed on long-term DEX (385±64 vs. 0±0 pg/mL; $p<0.01$; Fig. 4*C*). Pre-treatment basal and stimulated CORT levels were as expected higher in HGRKO than HGRWT mice (Fig. 4*D*). On long-term DEX, both basal, restraint- and cosyntropin-stimulated CORT were markedly suppressed in HGRWT vs. remaining high in HGRKO animals (basal: 1.5±0.98 vs. 14±1.6 µg/dL; restraint: 1.9±1.6 vs. 33±4.5 µg/dL; cosyntropin-stimulated: 1.1±0.62 vs. 33±3.9 µg/dL; $p<0.01$–0.001). Adrenal mass and morphology were normal in HGRKO animals, and these glands notably lacked the large clusters of AMs observed in HGRWT controls (Fig. 4E-I). Sustained, CRH-driven, endogenous ACTH signaling during long-term DEX treatment thus prevents GIAI and cortical atrophy.

Discussion

Using a murine model of GIAI, we found that hypothalamic-pituitary function rapidly recovered after cessation of chronic glucocorticoids, whereas adrenal suppression persisted for

up to 8 weeks. DEX-exposed adrenals exhibited marked cortical atrophy and infiltration of large, lipid-laden macrophages. Even after accounting for these macrophages, CORT production was disproportionately low relative to the size of the adrenal cortex, suggesting that post-withdrawal GIAI involves both adrenocortical cell loss and steroidogenic or secretory dysfunction. The provision of continuous, CRH-driven, endogenous ACTH signaling to the adrenal during long-term DEX treatment preserved adrenal size, morphology, and function. These results identify the adrenal as the principal site of ongoing suppression following withdrawal from chronic glucocorticoids and point to sustained trophic stimulation of the gland as a promising therapeutic strategy to prevent GIAI.

Consistent with our findings, prior rodent studies have shown that hypothalamic-pituitary function recovers within one week of discontinuing short- to moderate-term glucocorticoid treatment (5-30 days)[23-25]. We extended treatment to a duration known to cause protracted GIAI in humans, making the rapid central recovery observed in our model particularly striking. Notably, the 1965 study that established the paradigm of post-withdrawal central suppression included only six patients with exogenous glucocorticoid exposure. Because ACTH could not be directly measured, circulating levels were inferred from corticosterone responses in hypophysectomized rats injected with patient plasma compared to three standard ACTH doses or saline[14,15]. Another frequently cited study assessed HPA axis function just 24 hours (1-2 biological half lives) after the final glucocorticoid dose[16]. ACTH suppression in this context could therefore reflect direct, glucocorticoid-mediated feedback inhibition on the hypothalamus and pituitary rather than delayed central axis recovery. Interestingly, patients with congenital adrenal hyperplasia, who require chronic, suppressive glucocorticoid therapy, exhibit elevated levels of ACTH and ACTH-dependent precursors at their morning hydrocortisone troughs, as would be expected with rapid normalization of hypothalamic-pituitary function[21,22]. Together, the methodological limitations of earlier clinical studies and our finding of rapid hypothalamic-pituitary recovery in mice underscore the need to reexamine the paradigm of post-withdrawal central suppression using modern, well-timed ACTH assays and adequately powered cohorts of glucocorticoid-treated patients.

The brief or absent post-withdrawal central suppression found in our study encouraged us to investigate mechanisms of GIAI at the adrenal level. Since ACTH is a well-established trophic factor for the zF[7,8], the time required for cellular regeneration from DEX-induced cortical atrophy is an intuitive explanation for persistently low CORT secretion after glucocorticoid withdrawal despite supraphysiologic ACTH stimulation. The adult zona fasciculata is considered a “modestly proliferative” tissue with a turnover rate of 120 days in mice[38,39]. While this is longer than the 8 weeks required for functional recovery in our study, more rapid renewal would be expected in states of increased ACTH stimulation, as occurs after DEX withdrawal[40]. Interestingly, we found that adrenal mass recovered more rapidly than steroidogenic function. This is consistent with prior studies that demonstrated that CORT levels and steroidogenic pathway gene expression were low one week after discontinuing short- to moderate-term glucocorticoids despite normalization of adrenal weights[23-25]. Using quantitative morphometry, we further demonstrated that this dissociation between adrenal size and function persisted even after accounting for the DEX-induced expansion of large, lipid-laden adrenal macrophages. Together, these findings suggest that post-withdrawal GIAI reflects not only adrenocortical cell loss but also a persistent defect in CORT biosynthesis or secretion, the mechanisms of which should be a focus of future studies.

While Wylie and Kerr noted accumulation of apoptotic bodies “within phagosomes of ‘histiocytes’” after short-term glucocorticoid treatment[7], macrophage accumulation has not been reported after long-term glucocorticoid therapy. Large AMs in our model share multiple features with adrenal macrophages described in acute and chronic stress states. They express classical macrophage markers, such as CD68, F4/80 and Cd11c, and are intensely autofluorescent due to lipid accumulation[34,41-43]. Male murine adrenal macrophages are also long-lived[42], consistent with our continued detection of AMs at least 8 weeks after DEX withdrawal. The AMs in the present study most strikingly differ from typical adrenal macrophages in both their large size and apparent multinucleation. Morphologically similar adrenal macrophages have been described in aged, male

Znrf3 knockout mice, which undergo early adrenocortical hyperplasia followed by senescence[44]. It is therefore plausible that adrenal macrophages undergo expansion, fusion, and multinucleation when the demand for phagocytic clearance is high, as has been reported in adipose, ovary and bone tissues[45-47]. Interestingly, Xu et al. reported that adrenal macrophages in atherogenic conditions restrict glucocorticoid production via Trem2-dependent paracrine suppression of steroidogenic acute regulatory protein (StAR), the rate limiting enzyme in CORT biosynthesis, in neighboring zona fasciculata cells[34]. Since AMs in our model strongly express *Trem2,* further work is needed to determine if they contribute to GIAI via a similar paracrine signaling mechanism.

Most importantly, our finding of rapid hypothalamic-pituitary recovery after glucocorticoid cessation has enabled us to begin development of new strategies to prevent GIAI targeted to the adrenal. We demonstrated that sustained, CRH-driven ACTH signaling but not intermittent cosyntropin treatment during chronic glucocorticoid treatment prevents GIAI. The differential efficacy of our genetic and pharmacologic strategies may reflect the short half-life of cosyntropin or a requirement for physiologic ACTH dynamics to maintain adrenal steroidogenic capacity[48-50]. We could not assess ultradian ACTH rhythms in HGRKO mice because of blood volume constraints and the stress associated with serial sampling, but corticosterone secretion in these animals follows a typical circadian pattern[37]. Moreover, continuous CRH infusion restores circadian ACTH and CORT rhythms in CRH knockout mice[51]. Consistent with our findings in mice, limited clinical data suggests that CRH-dependent ACTH signaling but not intermittent, pharmacologic treatment with ACTH may protect against GIAI. Patients with ectopic CRH-producing tumors, though rare, recover HPA axis function more rapidly after tumor resection than those with ACTH-secreting adenomas[52]. In contrast, combination therapy with intramuscular ACTH and glucocorticoids produced more severe HPA axis suppression in 18 patients with rheumatologic disease compared to those treated with glucocorticoids alone. Together, these findings identify sustained, potentially CRH-driven, tropic stimulation of the adrenal as a promising strategy to prevent GIAI. Future translational efforts should define the characteristics of ACTH signaling that are required to preserve adrenal function during chronic glucocorticoid therapy.

As a potential limitation, this study only included male mice to minimize biological variability across a broad, exploratory survey of HPA axis recovery. While there are no reported sex differences in the risk, severity, or duration of GIAI in humans[10,53-55], HPA axis responsiveness, rates of adrenocortical turnover, and adrenal macrophage populations are all sexually dimorphic in rodents[44,56-62]. Future studies will specifically investigate sex differences in the mechanisms of post-withdrawal GIAI and time to HPA axis recovery in this preclinical model. Additionally, sample sizes were relatively modest to permit interrogation of HPA axis function at multiple timepoints after chronic glucocorticoid treatment. Since we interrogated HPA axis function in the stimulated state, as is done to clinically diagnose adrenal insufficiency, key analyses were all still adequately powered. Nonetheless, subsequent mechanistic studies will include larger cohorts of animals now that biologically relevant timepoints have been identified.

In conclusion, our findings demonstrate that post-withdrawal GIAI arises primarily from adrenal dysfunction rather than delayed hypothalamic-pituitary recovery. This prolonged adrenal suppression, driven by both adrenocortical cell loss and impaired steroidogenesis, is prevented by maintaining sustained trophic stimulation of the adrenal during glucocorticoid exposure. These results establish the adrenal as the principal locus of post-withdrawal GIAI and an accessible target for therapeutic intervention. Pharmacologic strategies that reproduce physiologic adrenal stimulation could prevent GIAI, thereby reducing the morbidity associated with one of the most widely prescribed drug classes while preserving their therapeutic efficacy.

Materials and Methods

Animals

All animal protocols were in accordance with the National Institutes of Health guidelines and were approved by the Animal Care and Use Committee of Boston Children's Hospital (Protocol 00001983). Mice were housed on a 12-hour/12-hour light-dark cycle (lights on at 7 am) with ad libitum access to rodent chow and water unless otherwise indicated. Mice used for studies were all aged either 8 weeks at the time of DEX initiation (long-term studies) or 8-16 weeks (DEX-naïve controls or short-term studies).

Wild-type (WT) mice: C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were maintained by breeding within our population.

Sim1^{Cre}:GR$^{fl/fl}$ mice (HGRKO): Sim1^{Cre} mice (generous gift of Brad Lowell, Beth Israel Lahey Medical Center, Boston, MA) were bred to floxed GR exon 3 mice (B6.Cg-*Nr3c1$^{tm1.1Jda}$*/J, Catalog #021021, The Jackson Laboratory, Bar Harbor, ME), both maintained on an inbred C57BL/6J background[37,63]. Littermates that were Cre negative but had GR exon 3 floxed alleles were used as controls. Genotyping was performed as per Friedman *et al*[64] using the primers and conditions detailed in Table SI.

Pharmacologic treatments: Mice were treated with either water-soluble dexamethasone (Sigma-Aldrich 2915) at a concentration of 3.6 mcg/mL (~20 mcg/day assuming an average water intake of 5.8 mL/day[65]) or no-drug control via drinking water for either 4 days (short-term) or 8 weeks (long-term studies). When specified, mice were co-treated with 10 mcg/kg cosyntropin, (synthetic ACTH 1-24, Sandoz, NDC 0781-3440-95), dissolved at 10 mcg/mL in 0.9% saline. Cosyntropin was administered once daily by intraperitoneal injection at 16:30 to align with the murine, circadian ACTH peak, for either 4 days or 8 weeks, as above.

Hormonal assays: All testing was performed, as specified, on separate days so that no mouse was bled more than once on a given day. Plasma for measurement of corticosterone and ACTH was obtained by rapid retroorbital phlebotomy without anesthesia into EDTA-coated capillary tubes with a total time from first handling the animal to completion of bleeding not exceeding 30 seconds. Basal/unstimulated phlebotomy was performed at 16:30 in a ventilated hood in the same room in which the animals were housed. Cosyntropin stimulation[51], insulin-induced hypoglycemia testing (1 U/kg dose)[66], and physical restraint (20 minutes)[66] were all performed as previously published. Blood was collected on ice, and plasma was separated by centrifugation at 1500 RPM at 4°C and stored at -80°C until assay. Plasma concentrations of corticosterone (ALPCO, 55-CORMS-E01) and ACTH (21-ACTHU-E01) were measured by ELISA per manufacturer instructions. For the ACTH assay, plasma was diluted 1:10 with ACTH 0 calibrator (ALPCO; 11-C-270-10-G25). The lower limit of detection is 0.061 mcg/dL and 0.22 pg/mL for the CORT and ACTH assays, respectively.

Necroscopy and tissue preparation: Animals were euthanized by rapid decapitation without anesthesia followed by necroscopy for isolation of hypothalami and adrenals. Coronal slabs flanking the hypothalamus were prepared with a surgical blade and then fixed in 10% formalin overnight before transfer to 70% ethanol while awaiting paraffin embedding. Adrenals were trimmed of surrounding fat tissue, rinsed in phosphate-buffered saline (PBS), and weighed before overnight fixation in 10% neutral buffered formalin.

Single-molecule RNA in situ hybridization

Three 5 µm-thick coronal brain sections were mounted onto each slide, and every other slide was stained with hematoxylin and visualized with brightfield microscopy (Nikon E800). The paraventricular nucleus was identified histologically based on its characteristic "butterfly-shaped" appearance in coronal sections, which was used to anchor selection of 2 unstained slides immediately rostral and caudal to this structure for analysis. Single-molecule RNA in situ hybridization was performed using a catalog Mus musculus *Crh* probe (ACD #316091) and 2.5 HD RED detection kit (ACD #316091) following manufacturer's protocol. Slides were counter stained with 50% Gill's Hematoxylin II and mounted with EcoMount. Positive control *Ppib (*ACD

#313911) and negative control *dapB (*ACD #310043) were used in every experiment to ensure sample quality. Brightfield images at 40x magnification were generated with an Olympus VS120 Virtual Slide Scanner and then analyzed with semi-quantitative densitometry using the Weka Segmentation plugin for Fiji ImageJ per ACDBio instructions[67]. Analysis was restricted to freehand sections encompassing the characteristic "butterfly" morphology of PVH cell bodies, and CRH expression, reported as arbitrary units (AU) was quantified by dividing the area of the *Crh* densitometric signal (μm^2) by the total area of this region (mm^2).

Total mRNA and protein staining

For quantifying total mRNA *in situ*, FFPE sections were deparaffinized and antigen-retrieved in buffer TE (pH=9). We then adapted the protocol in Farny *et al.* and Hurt *et al.* to label polyA-capped RNAs on FFPE sections using Cy5-conjugated polyDT (26-4430-02, GeneLink)[27,28]. Total protein *in situ* was measured using the N-Hydroxysuccinimide (NHS) ester method; NHS esters react with primary amines and well-approximate total protein mass[29,30]. Briefly, FFPE sections were deparaffinized and antigen-retrieved in buffer TE (pH=9). Slides were stained with NHS-Alexa 568 dye conjugates (Thermo Scientific A20003) in PBS for 30 minutes and then washed 5X in PBS.

Paraffin section immunofluorescence

After fixation, adrenals were oriented with their broadest surface parallel to the cutting plane in 4% low-melting-temperature SeaPlaque agarose (Lonza, #50101). Agarose blocks were then dehydrated in ethanol, xylene, and embedded in paraffin blocks. Paraffin sections were cut at 5 µm thickness, and immunofluorescence was performed as described in Leng *et al.*[68] with the antigen retrieval conditions and antibody concentrations detailed in Table S2. When indicated, Streptavidin, Alexa Fluor 488 Conjugate (Invitrogen, #S112233) was added to the secondary antibody solution at a final concentration of 1:500. In all experiments, sections were incubated in TrueBlack Lipofuscin Autofluorescence Quencher (Biotium, #23007) in 70% EtOH at RT for 3 minutes and rinsed three times in PBS prior to mounting with Prolong Gold Antifade Mountant with DAPI (Invitrogen, #P36931). Images were acquired with either a Nikon upright Eclipse 90i microscope with a 20x/0.75 Plan-Apochromat objective or an Olympus VS120 Virtual Slide Microscope using a 20x objective and adjusted for brightness and contrast in ImageJ.

Floating section immunofluorescence.

After fixation, adrenals were oriented with their broadest surface parallel to the cutting plane in 4% low-melting-temperature SeaPlaque agarose (Lonza, #50101) and sectioned at a thickness of 100 µm with a vibratome. Individual slices were separated by free floating in PBS. Sections were wet mounted in PBS on Superfrost Plus slides (Fisher Scientific, 12-550-15) and imaged with a 4x objective on a Nikon E800 brightfield microscope. The area of each section was measured in ImageJ, and the largest section was selected for subsequent immunostaining. Floating section immunofluorescence was then performed as per Leng *et al*[68] with the addition of Streptavidin, Alexa Fluor 488 Conjugate (1:500, Invitrogen, #S112233) to the secondary antibody solution. Images were acquired with a Nikon Ti2 microscope with a Yokogawa CSUW1 confocal unit, a Nikon Plan Apo Lambda 20x objective lens and a Zyla 4.2 PLUS sCMOS camera (ANDOR). All images were taken with identical laser strength, gain and exposure settings for the 405nm (used to capture autofluorescence), 488nm, and 647nm channels. Each image was acquired using the Large Image function in the NIS elements software. This function allowed us to take images in a 4x4 grid with 15% overlap, allowing the images to be stitched together. Brightness and contrast were adjusted in ImageJ.

Image analysis

Fluorescent images were analyzed using QuPath (version 0.4.3), an open-source software for digital pathology.

Positive Cell Detection: QuPath's cell detection feature was employed to label cells as either Ki-67 or TUNEL positive based on co-localization of the 647 channel signal with the DAPI nuclear signal. Images were opened in fluorescence mode, and regions of interest (ROI) were manually defined to include only the adrenal cortex based on morphologic characteristics. ROI were further restricted to the zF for Ki-67 analysis, as defined by DAB2-negative cortical cells. The "cell detection" feature was then run specifying DAPI as the detection channel and the 647 nm channel nuclear (Ki-67 analysis) or cellular (TUNEL analysis) mean as the score compartment. Nuclear size and intensity threshold parameters for both were adjusted and inspected visually to ensure accurate positive cell detection. Any cell exhibiting signal overlap between the DAPI and 647 nm channels above the specified thresholds was classified as either TUNEL or Ki-67 positive and reported as a percentage among total cells.

Morphometry: Morphometric analysis was performed on thick, floating section immunofluorescent specimens to quantify the area of either the adrenal cortex (Streptavidin staining, 488 nm channel), medulla (tyrosine hydroxylase immunostaining, 647 nm channel), or adrenal macrophages (autofluorescence, 408 nm channel) based on signal intensity across multiple channels using the "create thresholder" function in QuPath. Images were opened in fluorescence mode, and brightness/contrast of individual channels were adjusted for optimal signal visualization. Regions of interest were manually defined to include the entire adrenal slice but not surrounding adipose tissue. For each channel, QuPath's intensity thresholding was applied to ensure accurate delineation of stained or autofluorescent areas, as confirmed by visual inspection. As DEX treatment has been shown to attenuate streptavidin staining in the murine zF, we applied a lower threshold in 8+0 adrenals to ensure detection of all residual adrenocortical tissue[31]. The area of tissue with a signal above this threshold for each channel was classified as positive and reported in square microns.

Statistics

Two-tailed Student's t tests were performed to compare significance among two groups. Comparisons done beyond 2 groups were performed by ANOVA, and Bonferroni correction was applied for post-hoc, pairwise multiple comparison testing. Nonlinear regression using an exponential growth equation was used to model stimulated CORT recovery (dependent variable) over time (independent variable), and doubling time was computed as ln(2)/K where K is the rate constant. When two exponential growth models were compared, the extra-sum-of-squares test was employed to determine whether the exponential equations provided significantly different fits to the data. All statistical analyses were performed in GraphPad Prism v.10.3.1. Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com. *P* values below 0.05 were considered statistically significant.

Study approval

All animal experiments were approved by the Boston Children's Hospital Institutional Animal Care and Use Committee (Protocol Number 00001983).

Data availability

All raw data associated with findings presented in this manuscript are included in the Supporting Data Values file and are available from the corresponding author upon reasonable request.

Acknowledgments

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Figures and Tables

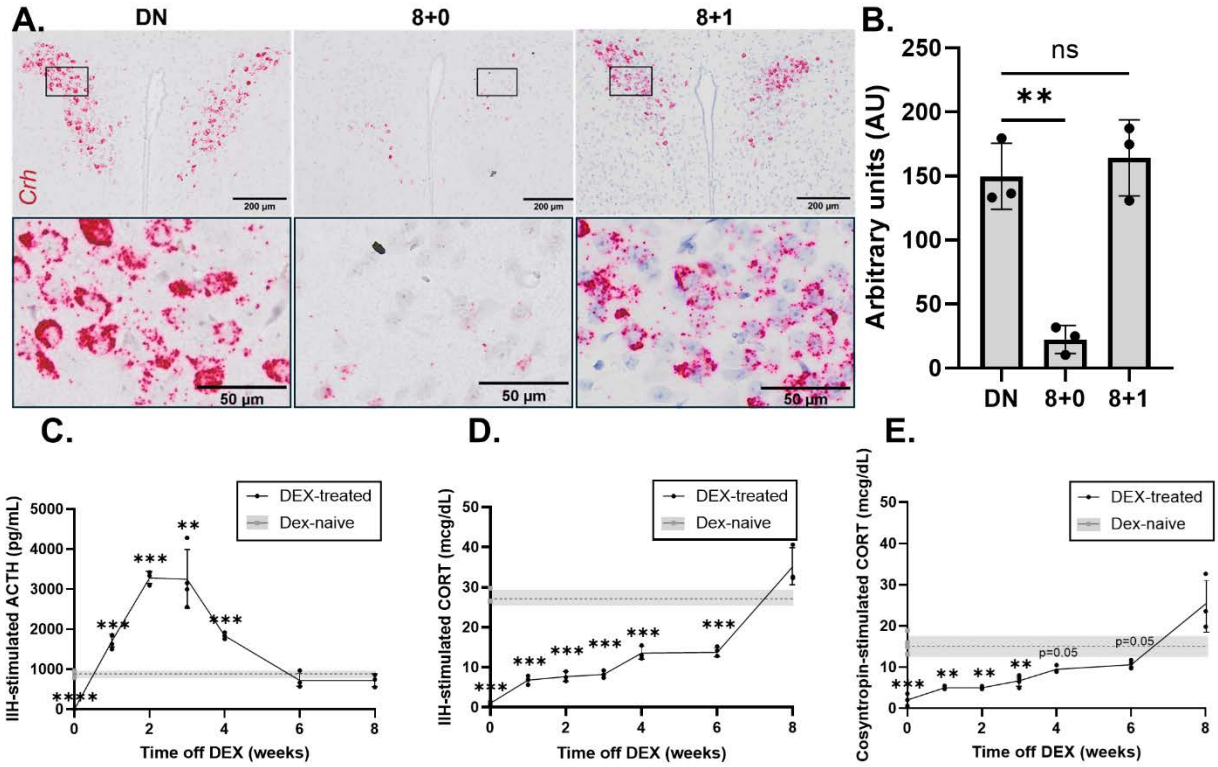

Figure 1. **Corticosterone (CORT) secretion is suppressed for up to 8 weeks after discontinuing long-term DEX despite rapid hypothalamic-pituitary recovery.** A) *In situ* hybridization (RNAScope) for basal *Crh* (red) in the paraventricular nucleus (PVH) of the hypothalamus in DEX-naïve (DN) animals and those treated with 8 weeks DEX both immediately prior to (8+0) and 1-week post-withdrawal (8+1; top row, scale bar=200 microns). Nuclei are labeled by hematoxylin (purple). Magnification of region within the black box is shown directly below (bottom row, scale bar=50 microns). B) Quantification of *Crh* mRNA expression from RNAScope images of the PVH from DN, 8+0, and 8+1 animals (n=3). p<0.001 by 1-way ANOVA. Recovery of insulin-stimulated plasma C) ACTH and D) CORT and E) cosyntropin-stimulated CORT levels 0-8 weeks after withdrawal of an 8-week DEX course. Data shown as individual mice with mean (connecting line) ± SEM (error bars). Grey bar (mean± SEM) indicates the reference range for adult, male, wildtype, untreated (DEX-naïve or DN) controls (n=4-8). * denotes p<0.05, ** p<0.01, *** p<0.001, and ****p<0.0001 vs. DN.

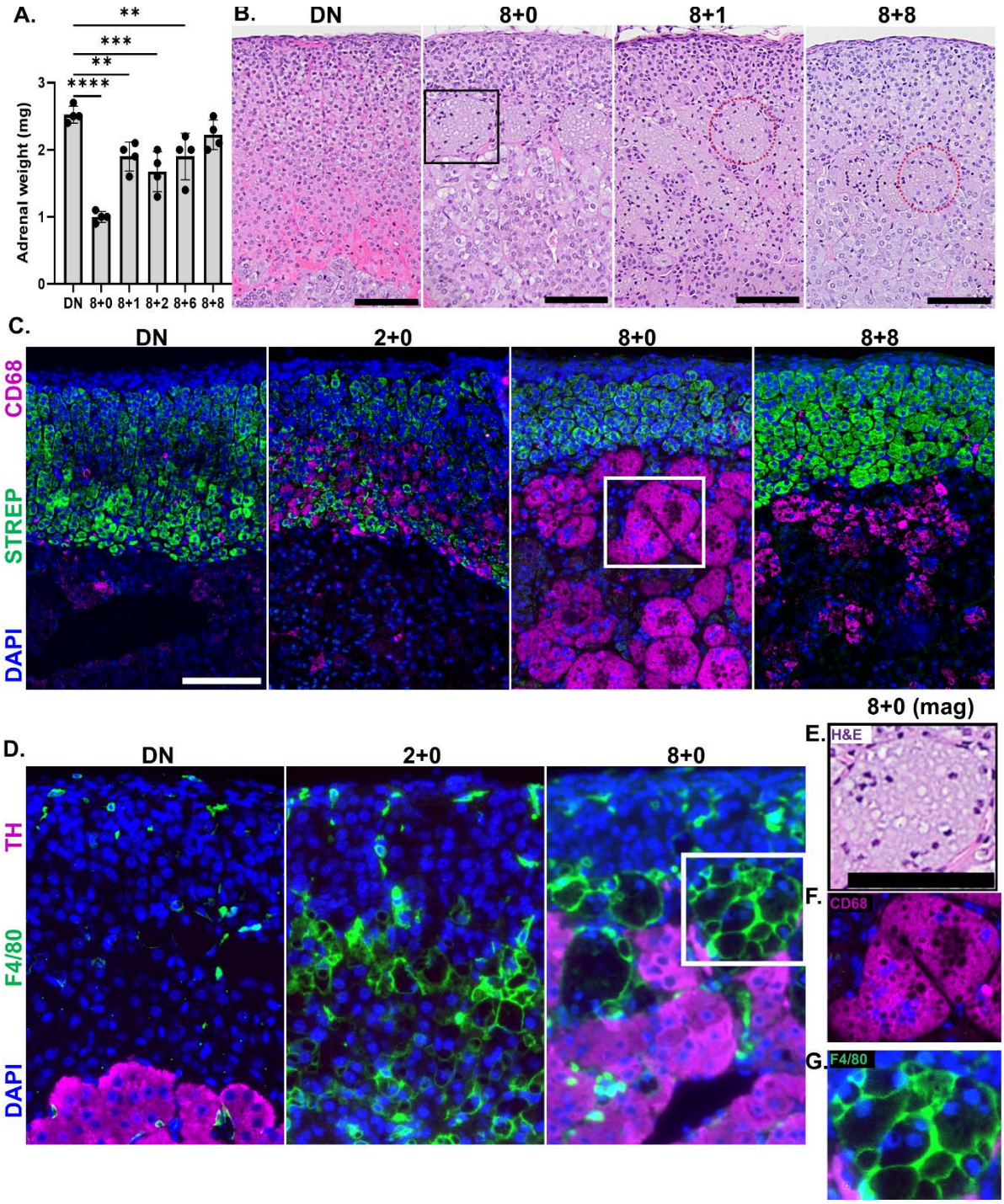

Figure 2. Long-term DEX induces adrenocortical atrophy with accumulation of giant macrophage clusters that persist for at least 8 weeks post-withdrawal. A) DEX-treated adrenals weigh significantly less than DN controls through 6 weeks post-withdrawal. P<0.0001 by one-way ANOVA; * indicates p<0.05, ** p<0.01, *** p<0.001, and ****p<0.0001 vs. DN. B) Hematoxylin and eosin staining reveals large, poorly defined, vacuolated structures with decreased nuclear density at the corticomedullary junction in DEX-exposed adrenals that persist through 8-weeks post-withdrawal. Representative structures (outlined with red, dashed circles) correspond with clusters of cells that are positive for the classical macrophage markers C) CD68

and D) F4/80. C) Immunofluorescence for CD68 (magenta) with DAPI (blue) and 488-streptavidin (green) counterstains, the latter of which labels all biotin-rich cells of the adrenal cortex. Representative images shown for DN adrenals, those on DEX for 2-8 weeks (e.g. 2+0 to 8+0), as well as one (8+1) and eight (8+8) weeks after an 8-week DEX course. D) Immunofluorescence for the mouse macrophage marker F4/80 (green) and medullary marker tyrosine hydroxylase (TH; magenta) in DN and DEX-exposed adrenals (2+0 and 8+0). Magnification of representative macrophages in 8+0 glands from E) H&E and immunostaining for F) CD68 and G) F4/80. All staining performed with TrueBlack lipofuscin autofluorescence quencher (C, D, F, and G).

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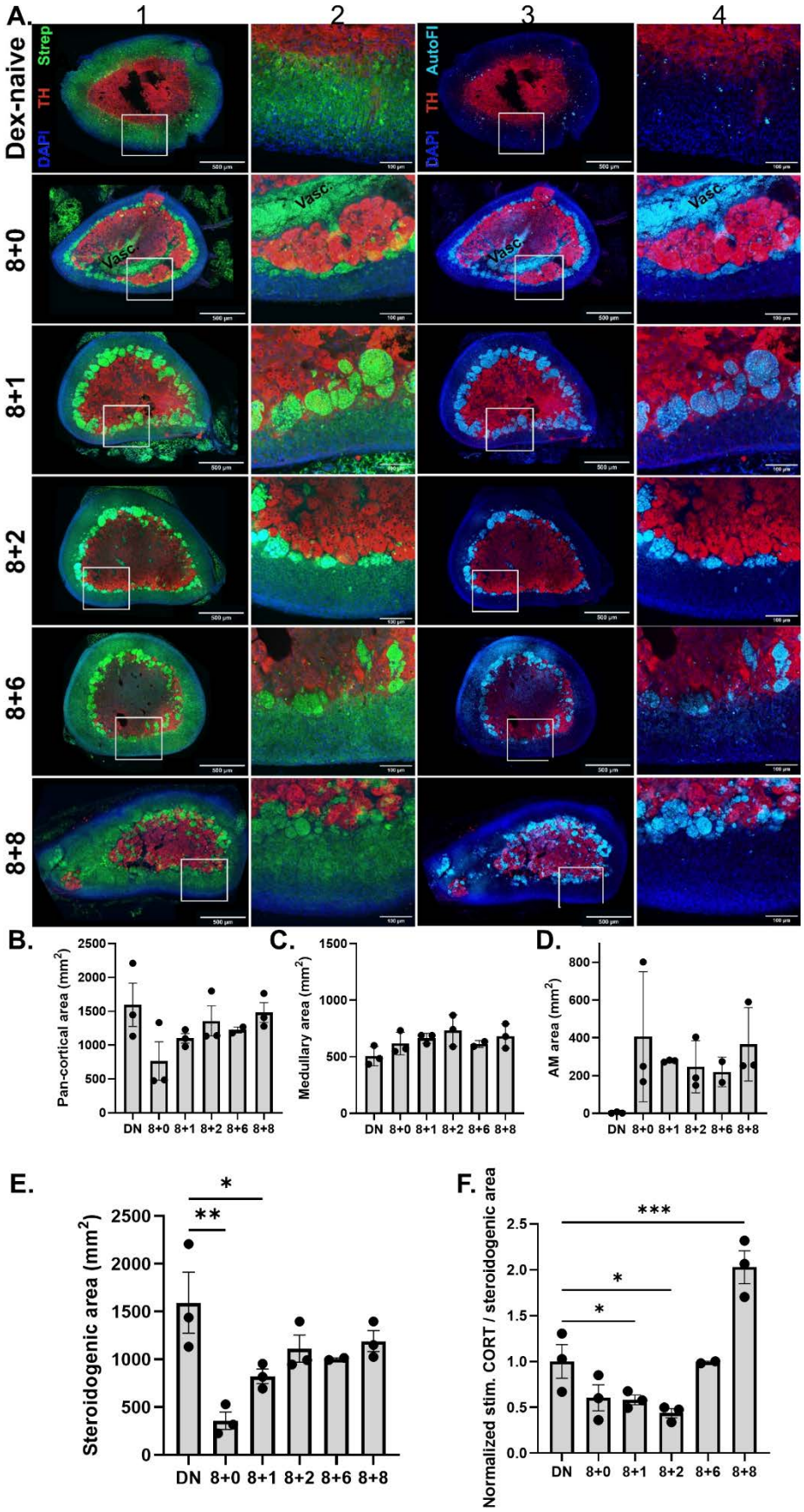
A.
1
2
3
4
Dex-naive
8+0
8+1
8+2
8+6
8+8
DAPI TH Strep
DAPI TH AutoFl
Vasc.
B.
Pan-cortical area (mm²)
C.
Medullary area (mm²)
D.
AM area (mm²)
DN 8+0 8+1 8+2 8+6 8+8
E.
Steroidogenic area (mm²)
F.
Normalized stim. CORT / steroidogenic area

Figure 3. Stimulated CORT secretion is disproportionately low relative to adrenocortical cell mass for at least 2 weeks after withdrawal of long-term DEX. A) IF without TrueBlack for tyrosine hydroxylase (TH, red) DAPI (blue) and 488-streptavidin (green; columns 1 and 2) counterstains. Adrenal macrophages (AMs) were detected by autofluorescence (cyan; columns 3 and 4). Mice were either untreated (DN) or treated for 8 weeks DEX followed by withdrawal for 0-8 weeks (8+0, 8+1, 8+2, 8+6, and 8+8). Shown as 100 µM-thick, whole adrenal slices (columns 1 and 3, scale bar=500 microns) with magnification of the region in the white box (columns 2 and 4, scale bar=100 microns). Columns 1 and 2 imaged at wavelengths 405 nm, 488 nm, and 647 nm; columns 3 and 4 imaged at wavelengths 405 nm, 440 nm, and 647 nm. Morphometric analysis of B) pan-cortical area, which includes both streptavidin-positive and autofluorescent regions ($p=0.18$ by one-way ANOVA), C) Autofluorescent AM area ($p=0.18$), D) steroidogenic cell type area, calculated as pan-cortical area minus AMs ($p<0.01$), and E) medullary (TH- positive) area ($p=0.17$ by one-way ANOVA). F) Ratio of steroidogenic cell type area to cosyntropin-stimulated CORT secretion, normalized to DN controls. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. DN (B-F)

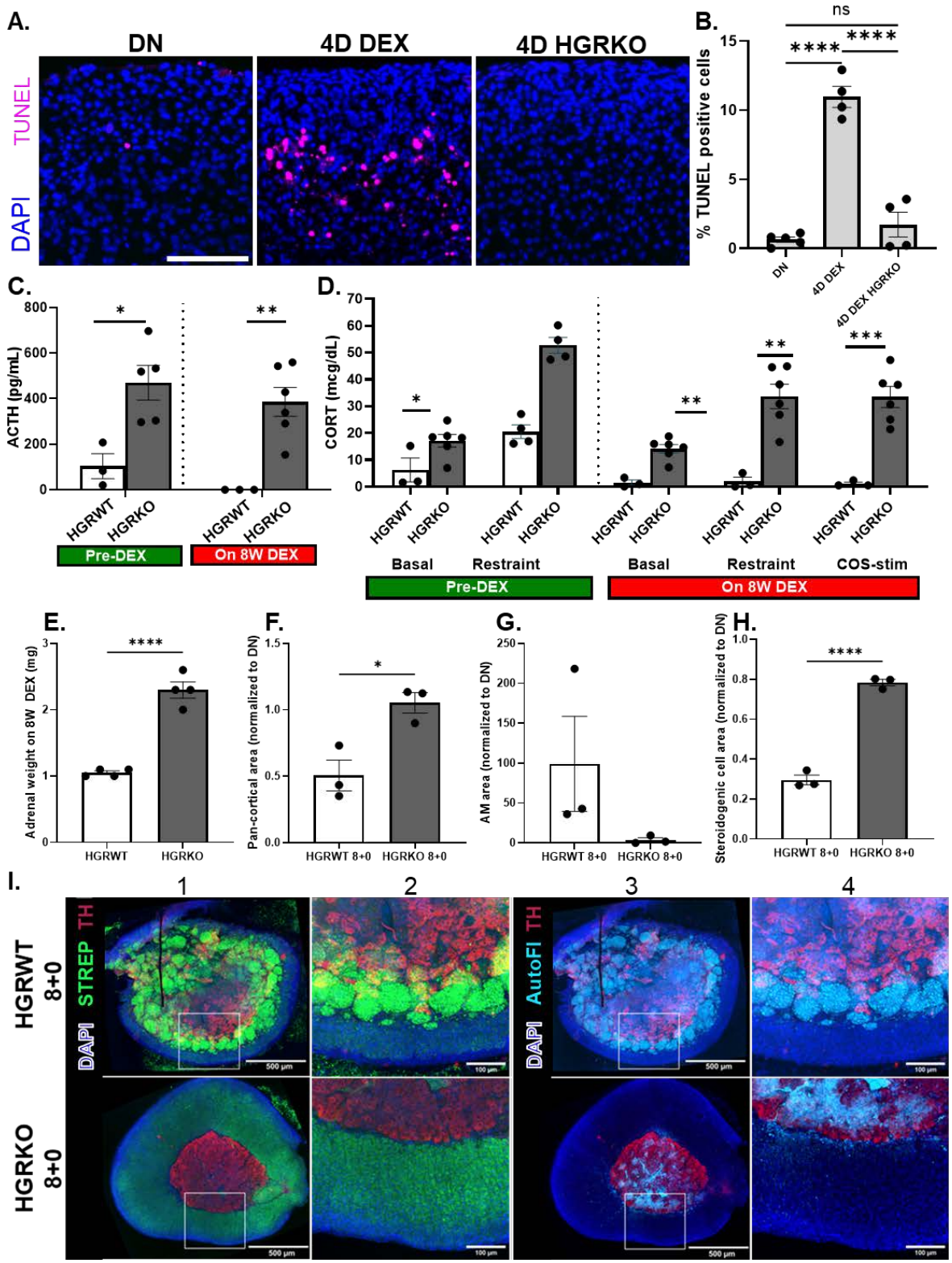

Figure 4. Selective deletion of the hypothalamic glucocorticoid receptor (GR) confers protection against DEX-induced adrenal suppression. A) Short-term (4 days DEX) induces

apoptosis in DEX-treated wild-type (4D DEX) but not Sim1^{Cre}:GR$^{fl/fl}$ (HGRKO) mice, as shown by TUNEL staining (magenta) with B) quantification. $p<0.0001$ by one-way ANOVA. **** denotes $p<0.0001$ for select pairwise comparisons. HGRKO mice and Cre-negative, functionally wild-type littermate controls (HGRWT) mice were then treated with DEX for 8 weeks. Hormonal function was assessed pre-treatment on long-term DEX. C) Basal (unstressed circadian peak) ACTH levels in HGRWT vs. HGRKO animals prior to DEX (left of dashed line) and on 8 weeks of DEX (right of dashed line). $p=0.19$ for DEX status and $p<0.0001$ for genotype by two-way ANOVA. H) CORT secretion in the basal state, following 20 minutes of physical restraint (Restraint), and following cosyntropin stimulation (COS-stim.) in HGRWT vs HGRKO animals, either prior to DEX treatment (left of dashed line) vs. on 8 weeks of DEX (right of dashed line). E) Adrenal weights and morphometric analyses for F) pan-cortical area, G) AM area, and H) steroidogenic cell type area in HGRWT and HGRKO adrenals on long-term DEX (8+0), all computed as per Fig. 3 and normalized to DN adrenals from Fig. 3. I) Representative thick, floating section IF without TrueBlack for tyrosine hydroxylase (TH, red), with DAPI (blue), and 488-streptavidin (green); methods and visualization as per Fig. 3. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ by t-test (HGRWT vs. HGRKO (C-H).