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Loss of *Gabrd* in CRH neurons blunts the corticosterone response to stress and diminishes stress-related behaviors

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Summary

The hypothalamic-pituitary-adrenal (HPA) axis is under tight regulation by strong GABAergic inhibition onto corticotropin-releasing hormone (CRH) neurons in the paraventricular nucleus (PVN) of the hypothalamus. CRH neurons receive two forms of GABAergic inhibition, phasic and tonic, but the specific roles of these two types of signaling have not yet been studied in this cell type. Our lab recently demonstrated a role for the GABA_A δ subunit in the tonic GABAergic regulation of CRH neurons. Using a floxed *Gabrd* mouse model established in our laboratory, we generated mice in which the GABA_A δ subunit is selectively removed from CRH neurons (*Gabrd/Crh* mice), resulting in a loss of tonic GABAergic inhibition in these neurons.

Interestingly, the loss of this tonic GABAergic constraint did not significantly alter basal levels of corticosterone (CORT). However, the loss of the GABA_A δ subunit in CRH neurons blunted the CORT response to stress, likely due to the loss of the disinhibitory effect of GABA following acute stress. This blunting of HPA axis reactivity was associated with a decrease in depression-like and anxiety-like behaviors. Exogenous CORT was sufficient to increase anxiety-like and depression-like behaviors in *Gabrd/Crh* mice. Together, these results show the importance of the GABA_A δ subunit in the regulation of CRH neurons, and thus the HPA axis, and demonstrate that dysregulation of CRH neurons alters stress-related behaviors.

Keywords

GABA; tonic inhibition; CRH neurons; paraventricular nucleus; hypothalamus; HPA axis; stress; corticosterone; depression; anxiety

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1. Introduction

During stress, signals from different neural pathways converge on corticotropin-releasing hormone (CRH) neurons in the paraventricular nucleus (PVN) of the hypothalamus. Although these neuroendocrine cells are usually under robust GABAergic inhibition (Decavel and van den Pol, 1990; Herman et al., 2004), stress activates the CRH neurons, triggering the hypothalamic-pituitary-adrenal (HPA) axis to release stress hormones, including cortisol in humans and corticosterone (CORT) in mice. Healthy responses to stress require appropriate release of CRH neurons from their inhibition and subsequent restoration of inhibition, while dysfunction of the HPA axis has been linked with mood and sleep disorders as well as cardiovascular disease, metabolic disease, and infertility (Chrousos, 2009). However, the mechanisms that regulate GABAergic control of CRH neurons remain unclear.

GABA_A receptors (GABA_ARs) are composed of different subunits arranged in pentameric assemblies, with specific subunit compositions determining their regional distribution, kinetics, and pharmacology (Olsen and Sieghart, 2008). GABA_ARs with unique subunit assemblies mediate two distinct forms of inhibition, tonic and phasic (Farrant and Nusser, 2005). Phasic GABAergic inhibition refers to the transient currents that result from GABA binding to synaptic GABA_ARs (IPSCs); whereas, tonic GABAergic inhibition is a sustained conductance mediated by extrasynaptic GABA_ARs, including those containing a GABA_AR δ subunit, responding to ambient levels of GABA in the extracellular space. The role of tonic GABAergic inhibition in CRH neurons and the impact on HPA axis reactivity has not been fully explored.

We, as well as others, recently demonstrated that the GABA_AR δ subunit plays a role in regulating the activity of CRH neurons (Sarkar et al., 2011; Gunn et al., 2013). To further investigate the function of the GABA_AR δ subunit in the regulation of CRH neurons, we generated a mouse model with a loss of the GABA_AR δ subunit specifically in CRH neurons (*Gabrd/Crh* mice). Here we utilize this mouse model to directly examine the role of the GABA_AR δ subunit-mediated inhibition on CRH neurons. We show that the tonic inhibition mediated by the GABA_AR δ subunit in CRH neurons plays a critical role in regulating the ability of the HPA axis to respond to stress. Furthermore, we demonstrate that dysregulation of CRH neurons plays a role in depression- and anxiety-like behaviors.

2. Methods and Methods

2.1. Animals

Adult (8-12 week old) male mice were housed at the Tufts University School of Medicine, Division of Laboratory Animal Medicine, in clear plastic cages (5 mice/cage) in a temperature- and humidity-controlled environment with a 12 h light/dark cycle (lights on at 0700h) and *ad libitum* access to food and water. Animals were handled according to protocols approved by the Tufts University Institutional Animal Care and Use Committee.

CRH cell-specific GABA_AR δ subunit knockout mice (*Gabrd/Crh* mice) were generated by crossing floxed *Gabrd* mice (Lee and Maguire, 2013) with CRH-Cre mice obtained from the Mutant Mouse Regional Research Center. Both *Gabrd/Crh* mice and Cre^{-/-} littermates are maintained on a 129Sv/SvJ genetic background. We have previously confirmed the specificity of Cre recombinase expression in CRH neurons in this CRH-Cre line (Sarkar et al., 2011). For all experiments, *Gabrd/Crh* mice were compared to Cre^{-/-} littermate controls. All experiments, unless otherwise stated, were performed as close to 1200h as possible to maintain consistency in HPA axis activity across experiments and experimental groups.

Restraint stress for 30 min was used as an acute stressor. Mice were placed into a 50 ml conical tube with nose holes for 30 min. After 30 min, the mice were removed from the restraint tubes and immediately used for experimentation.

2.2. Western blot

Western blot analysis was performed as previously described (Maguire et al., 2005; Maguire and Mody, 2007; Maguire and Mody, 2008; Maguire et al., 2009; Sarkar et al., 2011; Lee and Maguire, 2013). Animals were deeply anesthetized with isoflurane and euthanized by rapid decapitation. The PVN and hippocampus were rapidly removed, placed on ice in homogenization buffer (containing 10 mM NaPO₄, 100 mM NaCl, 10 mM sodium pyrophosphate, 25 mM NaF, 5 mM EDTA, 5 mM EGTA, 2% Triton X-100, 0.5% deoxycholate, and 1 mM sodium vanadate; pH 7.4) in the presence of protease inhibitors (cOmplete Mini, Roche, and fresh phenylmethylsulfonyl fluoride), and briefly sonicated. The lysate was incubated on ice for 30 min, centrifuged at 14,000 rpm for 5 min, and the supernatant was collected. Protein concentrations were determined using the DC Protein Assay (Bio-Rad). Total protein (25 µg) was loaded onto a 10% SDS-polyacrylamide gel, subjected to gel electrophoresis, transferred to an Immobilon-P membrane (Millipore), blocked in 10% nonfat milk, and probed with an antibody specific for the GABA_A δ subunit (1:500, PhosphoSolutions 868-GDN). Blots were incubated with peroxidase-labeled anti-rabbit IgG (1:2000, GE Healthcare) and immunoreactive proteins were visualized using enhanced chemiluminescence (GE Healthcare). Optical density (OD) measurements were determined using NIH ImageJ software. All experimental groups were run in parallel in cohorts of equal number.

2.3. Immunohistochemistry

Immunohistochemistry was performed as previously described (Maguire et al., 2009; Sarkar et al., 2011; Lee and Maguire, 2013). Mice were deeply anesthetized with isoflurane and euthanized by rapid decapitation. Brains were rapidly removed, fixed by immersion fixation in 4% paraformaldehyde overnight at 4°C, cryoprotected in 10–30% sucrose, and frozen at -80°C until use. Coronal sections (40 µm) were prepared using a Leica cryostat. For GABA_A δ subunit staining, sections were treated with 3% H₂O₂/MeOH for 30 min, blocked with 10% normal goat serum for 1 h, and probed with polyclonal antibodies specific for the GABA_A δ subunit (1:500, Millipore AB9752) and HRP-labeled anti-rabbit IgG (ABC Elite, Vector Laboratories). DAB reactivity was visualized by light microscopy and OD measurements were determined in the region of interest in an equal number of serial sections using NIH ImageJ software (PVN: n = 80–100 hemisections, 4–5 mice per experimental group; dentate gyrus: n = 30–40 hemisections, 4–5 mice per experimental group; amygdala: n = 30–40 hemisections, 4–5 mice per experimental group; BnST: n = 20–25 hemisections, 4–5 mice per experimental group). For the PVN, the region of interest was determined by fluorogold labeling as previously described (Larsen et al., 2003; Sarkar et al., 2011). Briefly, mice were administered 200 µl of 10% fluorogold solution intraperitoneally 3–5 days prior to tissue processing. The experimental groups were processed in parallel to ensure equivalent treatment. For the c-fos staining, sections were blocked with 10% normal goat serum for 1 h, and probed with a primary polyclonal anti-c-fos antibody (1:10,000, Sigma) and a secondary anti-rabbit Alexa 488 antibody (Invitrogen). Sections were mounted with a mounting medium containing DAPI (Vector Laboratories). Fluorescence was visualized using a Nikon A1R confocal and cell counts were performed in the region of interest in an equal number of serial sections using NIH ImageJ software. The total area of c-fos immunolabeling was measured and divided by the average area of an individual c-fos-labeled neuron to quantify the number of c-fos-positive neurons in the region of interest (n = 3 mice per experimental group).

2.4. Electrophysiology

Electrophysiological recordings were performed as previously described (Sarkar et al., 2011; Lee and Maguire, 2013). Adult mice were anesthetized with isoflurane and decapitated. The brain was rapidly removed and placed immediately in ice-cold, oxygenated normal artificial cerebral spinal fluid (nACSF) (containing 126 mM NaCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, and 10 mM dextrose; 300–310 mOsm) with 3 mM kynurenic acid. Coronal sections (350 μ m) were prepared using a Leica VT1000S vibratome and stored in oxygenated nACSF at 33°C for at least 1 h before recording. Slices were placed into a recording chamber maintained at 33°C (in-line heater, Warner Instruments) and perfused at a high flow rate ($\sim$ 6 ml/min) throughout the experiment. Adequate O₂ tension and physiological pH (7.3–7.4) were maintained by continually bubbling the media with 95% O₂ / 5% CO₂. Visual identification of CRH neurons (Sarkar et al., 2011) as well as morphological and electrophysiological methods were used to identify CRH neurons in the PVN (Luther et al., 2002).

For voltage clamp recordings, intracellular recording solution contained 140 mM CsCl, 1 mM MgCl₂, 10 mM HEPES, 4 mM NaCl, 0.1 mM EGTA, 2 mM Mg-ATP, and 0.3 mM Na-GTP (pH 7.25; 280–290 mOsm). Electrodes with DC resistance of 5–8 M Ω were used for recording spontaneous IPSCs at V_H = -70 mV in the presence of 3 mM kynurenic acid. The frequency and peak amplitude of spontaneous inhibitory postsynaptic currents (sIPSCs) were measured over a 5 min period. Tonic GABAergic currents were measured as previously described (Stell et al., 2003; Maguire et al., 2005; Maguire and Mody, 2007; Maguire et al., 2009; Sarkar et al., 2011; Lee and Maguire, 2013). The mean holding current was measured during 10 ms epochs collected every 100 ms throughout the experiment. A Gaussian was fit to these points to determine the mean holding current in nACSF and in the presence of a concentration of SR95531 demonstrated to block both phasic and tonic GABAergic inhibition ($\sim$ 200 μ M) (Stell and Mody, 2002). The difference in the holding current in the presence or absence of SR95531 is determined as a measure of tonic GABAergic inhibition. Series resistance and whole-cell capacitance were continually monitored and compensated throughout the course of the experiment. Recordings were eliminated from data analysis if series resistance increased by > 20%.

Cell-attached recordings were performed in the voltage-clamp configuration with I_{amp} = 0 pA using electrodes with DC resistance of 5–8 M Ω and an intracellular solution containing 130 mM K-gluconate, 10 mM KCl, 4 mM NaCl, 10 mM HEPES, 0.1 mM EGTA, 2 mM Mg-ATP, and 0.3 mM Na-GTP (pH 7.25; 280–290 mOsm). The spontaneous firing rate of CRH neurons was measured over a 5 min period in *Gabrd/Crh* mice and *Cre*^{-/-} littermates subjected to 30 min of acute restraint stress as well as in minimally-handled controls.

Current clamp recordings were carried out in the $I = 0$ configuration using electrodes with DC resistance of 5–8 M Ω and an intracellular solution containing 130 mM K-gluconate, 10 mM KCl, 4 mM NaCl, 10 mM HEPES, 0.1 mM EGTA, 2 mM Mg-ATP, and 0.3 mM Na-GTP (pH 7.25; 280–290 mOsm). The spontaneous firing rate of CRH neurons was measured over a 5 min period in *Gabrd/Crh* mice and *Cre*^{-/-} littermates subjected to 30 min of acute restraint stress as well as in minimally-handled controls.

2.5. CORT measurements

CORT levels were quantified as previously described (Sarkar et al., 2011). Plasma was isolated from the trunk blood by high-speed centrifugation and CORT levels were measured by enzyme immunoassay according to manufacturer's specifications (Enzo Life Sciences). Duplicate 5 μ l plasma samples were assayed and absorbance measurements at 415 nm were compared with a standard curve. Samples from different experimental groups were run in

parallel. Samples were collected at 1200h for basal and stress-induced CORT measurements. For diurnal CORT measurements, blood samples were collected every two hours from 0600h to 2200h (ZT 23 through the light period to ZT 15), which encompassed 3 time points during the dark period and 6 time points during the light period, including the rise in CORT levels leading up to the dark period and the fall in CORT during the dark phase.

2.6. CORT treatment

Mice with intact adrenal glands were either sham-implanted or implanted with a 21-day slow release, 10 mg corticosterone pellet (Innovative Research of America). Mice were anesthetized with a ketamine/xylazine cocktail until unresponsive to a foot pinch. A small 1 cm incision was made on the back of the neck and a small slow-release pellet (or nothing for sham) was placed underneath the skin. The incision was then closed with sutures. The animals were allowed to recover and were exposed to corticosterone for 14 days until experimentation.

2.7. Behavioral tests

All behavioral experiments were conducted and scored with the experimenter blind to genotype. Mice were tested in a random order to control for unknown confounding factors. Four tests were performed over the course of four days. The following sequence was chosen to minimize distress to the animals.

Open field—The open field test was performed as previously described (Sarkar et al., 2011). Mice were tested for 10 min in a 40 cm × 40 cm open field photobeam frame with 16 × 16 equally spaced photocells (Hamilton-Kinder). Mice were placed individually into the center of the open field. The number of entries, the total distance traveled, and the amount of time spent in the center of the open field as well as the total number of beam breaks were measured using MotorMonitor software (Hamilton-Kinder).

Light/dark box—Mice were tested for 10 min in a 22 cm × 43 cm photobeam frame with 4 × 8 equally spaced photocells (Hamilton-Kinder), where one half is an open, illuminated compartment and the other half is an enclosed, dark compartment. Mice were placed individually into the dark box at the beginning of the trial. The number of entries into the light box and the total distance traveled in the light compartment were measured using MotorMonitor software (Hamilton-Kinder).

Tail suspension—Mice were suspended by the tip of the tail from a bar at a height of 36 cm above a table. Each trial was videotaped and subsequently scored for the latency to the first bout of immobility and the cumulative time spent immobile during the 6 min test.

Forced swim—The forced swim test was performed as previously described (Maguire and Mody, 2008). Mice were placed individually into cylinders (21 cm diameter) containing 15 cm of water at room temperature (22–25°C). Each trial was videotaped and subsequently scored for the latency to immobility and the cumulative time spent immobile during the 6 min test.

2.8. Statistical analysis

Statistical significance was determined using a Student's t-test to compare two experimental groups or a one-way ANOVA with Tukey's correction for multiple comparisons to compare more than two experimental groups. Statistical tests were performed using Prism software (GraphPad). The statistical test used is noted in the text and the figure legends for each experiment.

3. Results

3.1. Decreased GABA_AR δ subunit expression in PVN of *Gabrd/Crh* mice

Gabrd/Crh mice exhibited no gross anatomical deficits and had comparable body weights at 3 months of age (25.8 ± 0.9 g) to *Cre*^{-/-} littermate controls (25.6 ± 1.2 g) (data not shown; $n = 12$ mice per experimental group). We examined GABA_AR δ subunit expression in brain regions with populations of CRH neurons, including the BnST, amygdala, and PVN, using immunohistochemistry. Expression in these regions was compared to the dentate gyrus, which has a high level of GABA_AR δ subunit expression but does not contain a population of CRH neurons. Given that CRH neurons are only a subpopulation of neurons in the BnST and amygdala, we were unable to observe a significant difference in GABA_AR δ subunit expression in *Gabrd/Crh* mice (amygdala: 73.1 ± 9.6 OD units; BnST: 23.8 ± 9.7 OD units) compared to controls (amygdala: 99.6 ± 5.7 OD units; BnST: 14.7 ± 8.0 OD units) (Fig. 1; amygdala: $n = 30$ –40 hemisections, 4–5 mice per experimental group; BnST: $n = 20$ –25 hemisections, 4–5 mice per experimental group; Student's t-test; $p > 0.05$). Similarly, we were unable to detect a difference in GABA_AR δ subunit expression in the dentate gyrus of *Gabrd/Crh* mice (125.7 ± 6.5 OD units) compared to *Cre*^{-/-} littermates (115.9 ± 6.8 OD units), which is a region that does not contain CRH neurons (Fig. 1; dentate gyrus: $n = 30$ –40 hemisections, 4–5 mice per experimental group; Student's t-test; $p > 0.05$). Although the PVN is also a heterogeneous nucleus, we were able to detect a decrease in expression of the GABA_AR δ subunit in the PVN of *Gabrd/Crh* mice (27.5 ± 2.5 OD units) compared to *Cre*^{-/-} controls (62.4 ± 9.6 OD units) (Fig. 1; PVN: $n = 80$ –100 hemisections, 4–5 mice per experimental group; Student's t-test; $* p < 0.05$). The loss of the GABA_AR δ subunit in the PVN was confirmed by Western blot analysis. Expression of the GABA_AR δ subunit was decreased in the total protein isolated from the PVN of *Gabrd/Crh* mice (21.3 ± 1.8 OD units/25 μ g protein) compared to *Cre*^{-/-} littermates (38.1 ± 3.8 OD units/25 μ g protein) (Fig. 2; Student's t-test; $* p < 0.05$). The partial reduction in GABA_AR δ subunit expression is anticipated since the PVN is a heterogeneous nucleus. In contrast, there was no difference in the expression of the GABA_AR δ subunit in the hippocampus of *Gabrd/Crh* mice (31.2 ± 2.8 OD units/25 μ g protein) compared to *Cre*^{-/-} littermates (32.6 ± 3.5 OD units/25 μ g protein) (Fig. 2; $n = 7$ mice per experimental group; Student's t-test; $p > 0.05$). These data are consistent with the removal of the *Gabrd* gene specifically from CRH neurons.

3.2. Loss of tonic inhibition in CRH neurons of *Gabrd/Crh* mice

To functionally assess the loss of the GABA_AR δ subunit in CRH neurons, we performed whole-cell patch-clamp recording on CRH neurons in the PVN to measure phasic and tonic GABAergic inhibition. *Gabrd/Crh* mice did not exhibit any changes in phasic GABAergic inhibition. No significant differences were observed in the frequency (*Gabrd/Crh* mice: 6.8 ± 1.9 Hz; *Cre*^{-/-}: 5.9 ± 1.0 Hz) or peak amplitude (*Gabrd/Crh* mice: 61.1 ± 7.4 pA; *Cre*^{-/-}: 58.2 ± 5.5 pA) of spontaneous IPSCs in CRH neurons (Fig. 3a,b; $n = 16$ –20 cells, 4 mice per experimental group; Student's t-test; $p > 0.05$). However, a significant reduction in the tonic GABAergic current was measured in slices from *Gabrd/Crh* mice (0.7 ± 0.2 pA) compared with *Cre*^{-/-} littermates (13.8 ± 3.4 pA) (Fig. 3c,d; $n = 15$ –20 cells, 4–5 mice per experimental group; Student's t-test; $* p < 0.05$), consistent with the loss of GABA_AR δ subunit expression from CRH neurons. These data confirm a role for the GABA_AR δ subunit in regulating CRH neurons through tonic GABAergic conductance as previously reported (Sarkar et al., 2011).

3.3. Blunted HPA axis reactivity to stress in *Gabrd/Crh* mice

To determine the impact of the loss of the GABA_AR δ subunit on the activity of CRH neurons, we measured the spontaneous firing rate of CRH neurons in slices from *Gabrd/Crh* mice and *Cre*^{-/-} littermates subjected to 30 min of restraint stress or from minimally-handled

controls. There was no significant difference in the basal firing rate of CRH neurons recorded in the cell-attached configuration between non-stressed *Gabrd/Crh* mice (5.3 ± 1.0 Hz) and *Cre*^{-/-} littermates (4.6 ± 0.5 Hz) (Fig. 3e,f; one-way ANOVA with Tukey's test; $p > 0.05$). Interestingly, following acute restraint stress, the firing rate of CRH neurons in the PVN recorded in the cell-attached mode was decreased in *Gabrd/Crh* mice (7.3 ± 0.8 Hz) compared to *Cre*^{-/-} littermates (11.0 ± 1.6 Hz) (Fig. 3e,f; $n = 12$ – 18 cells, 3 – 4 mice per experimental group; one-way ANOVA with Tukey's test; $F(3, 53) = 8.621$; * $p < 0.05$). Similarly, we observed a significant decrease in the firing rate of CRH neurons in the PVN recorded in the whole-cell current-clamp $I = 0$ configuration following acute restraint stress in *Gabrd/Crh* mice (6.4 ± 1.1 Hz) compared to *Cre*^{-/-} littermates (10.1 ± 1.3 Hz), while non-stressed basal firing rates were not significantly different (*Cre*^{-/-}: 4.1 ± 0.6 Hz; *Gabrd/Crh*: 5.4 ± 0.9 Hz) (data not shown; $n = 18$ – 27 cells, 5 – 6 mice per experimental group; one-way ANOVA with Tukey's test; $F(3, 80) = 6.304$; * $p < 0.05$). However, there was no difference in the resting membrane potential (RMP) between *Gabrd/Crh* mice (control: -51.3 ± 1.9 mV; stress: -50.6 ± 1.4 mV) and *Cre*^{-/-} littermates (control: -50.1 ± 1.0 mV; stress: -53.8 ± 1.8 mV). These data demonstrate that the stress-induced activation of CRH neurons is blunted in *Gabrd/Crh* mice.

To investigate the impact of the loss of tonic GABAergic inhibition of CRH neurons on HPA axis activity, we examined c-fos activation in the PVN, BnST, and amygdala, regions with subpopulations of CRH neurons, following acute restraint stress. We observed a significant decrease in the average number of c-fos positive neurons per section in the PVN 30 min following acute restraint stress in *Gabrd/Crh* mice (97.1 ± 22.7) compared to *Cre*^{-/-} littermates (172.2 ± 24.5), which was increased compared to non-stress control mice (*Cre*^{-/-}: 12.4 ± 1.6 ; *Gabrd/Crh*: 17.0 ± 2.4) (Fig. 4a,b; $n = 3$ mice per experimental group; Student's t-test; * $p < 0.05$). However, we did not observe a significant difference in c-fos labeling following acute restraint stress in the amygdala or BnST of *Gabrd/Crh* mice (BnST: 36.1 ± 3.0 ; amygdala: 58.4 ± 2.8) compared to *Cre*^{-/-} littermates (BnST: 40.6 ± 3.3 ; amygdala: 66.6 ± 8.7) (data not shown; $n = 3$ mice per experimental group; Student's t-test; * $p < 0.05$). Consistent with decreased activation of CRH neurons in the PVN following stress in *Gabrd/Crh* mice, stress-induced CORT levels are decreased in *Gabrd/Crh* mice (248.7 ± 16.3 ng/ml) compared to *Cre*^{-/-} littermates (327.3 ± 16.0 ng/ml) and non-stressed controls (*Cre*^{-/-}: 63.1 ± 18.2 ng/ml; *Gabrd/Crh*: 69.9 ± 15.6 ng/ml) (Fig. 4c; 11 – 16 mice per experimental group; one-way ANOVA with Tukey's test; * $p < 0.05$ compared to non-stressed controls; # $p < 0.05$ compared to stressed *Cre*^{-/-} littermates). Our lab previously demonstrated that excitatory actions of GABA play a role in activating the HPA axis in response to acute stress (Sarkar et al., 2011). Consistent with those findings, these data demonstrate that removing the GABA_AR δ subunit from CRH neurons results in a suppression of HPA axis responsiveness to stress. These data demonstrate that the loss of the GABA_AR δ subunit in CRH neurons blunts the corticosterone response to stress.

However, loss of the GABA_AR δ subunit in CRH neurons does not appear to alter diurnal CORT secretion. Both *Gabrd/Crh* mice and *Cre*^{-/-} littermates exhibited the same pattern of CORT levels throughout the day, including an increase in CORT levels just prior to the dark period and a fall in CORT levels during the dark phase. Furthermore, we did not observe any significant differences in diurnal CORT levels at any time point in *Gabrd/Crh* mice (0600h: 2.7 ± 1.3 ng/ml; 0800h: 36.3 ± 18.2 ng/ml; 1000h: 16.7 ± 2.1 ng/ml; 1200h: 35.9 ± 12.2 ng/ml; 1400h: 50.4 ± 16.0 ng/ml; 1600h: 65.3 ± 28.0 ng/ml; 1800h: 100.6 ± 37.6 ng/ml; 2000h: 180.6 ± 52.6 ng/ml; 2200h: 3.3 ± 1.8 ng/ml) compared to *Cre*^{-/-} littermates (0600h: 7.9 ± 4.8 ng/ml; 0800h: 13.2 ± 2.6 ng/ml; 1000h: 30.5 ± 14.5 ng/ml; 1200h: 33.8 ± 8.5 ng/ml; 1400h: 60.4 ± 26.8 ng/ml; 1600h: 58.9 ± 32.8 ng/ml; 1800h: 158.8 ± 46.7 ng/ml; 2000h: 162.9 ± 43.9 ng/ml; 2200h: 5.3 ± 2.0 ng/ml) (Fig. 4d). In addition, there was no change in adrenal weight (*Cre*^{-/-}: 3.5 ± 0.4 g; *Gabrd/Crh*: 4.1 ± 0.3 g) or the adrenal

weight:body weight ratio ($Cre^{-/-}$: 0.12 ± 0.01 ; $Gabrd/Crh$: 0.15 ± 0.01) observed between experimental groups (Fig. 4e,f) ($n = 10$ – 12 mice per experimental group; Student's t-test; $p > 0.05$). Together, these data suggest that a loss of the GABA_AR δ subunit in CRH neurons blunts stress-induced CORT release.

3.4. Decreased depression-like behavior in *Gabrd/Crh* mice

Dysregulation of the HPA axis has been associated with mood problems, including depression and anxiety disorders. To determine whether there is a direct relationship between dysregulation of the HPA axis and depression, we assessed depression-like behavior in *Gabrd/Crh* mice and $Cre^{-/-}$ littermates using both the tail suspension and forced swim tests. In the tail suspension test, *Gabrd/Crh* mice exhibited an increased latency to the first bout of immobility (147.2 ± 29.3 s) and spent less cumulative time immobile (89.5 ± 17.2 s) compared to $Cre^{-/-}$ littermates (latency: 64.0 ± 5.1 s; total time: 159.2 ± 10.4 s) (Fig. 5a). These data suggest that *Gabrd/Crh* mice exhibit decreased depression-like behavior, consistent with blunted HPA axis activity (Fig. 4). To test whether the decreased depression-like behavior in *Gabrd/Crh* mice is due to the suppressed CORT response to stress, we assessed depression-like behavior in *Gabrd/Crh* mice and $Cre^{-/-}$ littermates treated with exogenous CORT through a slow-release pellet for 14 days. Implantation with corticosterone pellets significantly increased corticosterone levels (253.6 ± 50.2 ng/ml) compared to sham-implanted mice (57.9 ± 13.7 ng/ml) (data not shown). This continuous infusion of CORT decreased the latency to the first bout of immobility and increased the total time spent immobile in both *Gabrd/Crh* mice (latency: 69.6 ± 5.6 s; total time: 143.7 ± 11.3 s) and $Cre^{-/-}$ littermates (latency: 43.0 ± 3.0 s; total time: 198.2 ± 5.3 s) (Fig. 5a). Interestingly, CORT treatment in *Gabrd/Crh* mice resulted in levels of depression-like behavior in the tail suspension test (latency: 69.6 ± 5.6 s; total time: 143.7 ± 11.3 s) similar to untreated $Cre^{-/-}$ littermates (latency: 64.0 ± 5.1 s; total time: 159.2 ± 10.4 s) (Fig. 5a; $n = 10$ – 18 mice per experimental group; one-way ANOVA with Tukey's test; latency: $F(3, 50) = 8.712$; total time: $F(3, 50) = 11.25$; * denotes $p < 0.05$ compared to $Cre^{-/-}$ mice; # denotes $p < 0.05$ compared to *Gabrd/Crh* mice).

Likewise, in the forced swim test, *Gabrd/Crh* mice exhibited an increased latency to the first bout of immobility (166.0 ± 24.8 s) and spent less total time immobile throughout the trial (74.5 ± 14.6 s) compared to $Cre^{-/-}$ littermates (latency: 97.9 ± 17.3 s; total time: 165.9 ± 22.1 s) (Fig. 5b). CORT replacement decreased the latency to immobility and increased the total time spent immobile in both *Gabrd/Crh* mice (latency: 104.6 ± 14.4 s; total time: 128.7 ± 16.4 s) and $Cre^{-/-}$ littermates (latency: 48.9 ± 5.1 s; total time: 218.3 ± 8.2) (Fig. 5b). Interestingly, CORT treatment in *Gabrd/Crh* mice resulted in levels of depression-like behavior in the forced swim test (latency: 104.6 ± 14.4 s; total time: 128.7 ± 16.4 s) similar to untreated $Cre^{-/-}$ littermates (latency: 97.9 ± 17.3 s; total time: 165.9 ± 22.1 s) (Fig. 5b; $n = 10$ – 18 mice per experimental group; one-way ANOVA with Tukey's test; latency: $F(3, 47) = 6.083$; total time: $F(3, 47) = 9.467$; * denotes $p < 0.05$ compared to $Cre^{-/-}$ mice; # denotes $p < 0.05$ compared to *Gabrd/Crh* mice). These data demonstrate that dysregulation of CRH neurons alters stress-related behaviors, causing a decrease in depression-like activity in *Gabrd/Crh* mice that can be increased with exogenous CORT administration.

3.5. Decreased anxiety-like behavior in *Gabrd/Crh* mice

To determine if suppression of the HPA axis alters anxiety-like behavior, we assessed anxiety-like behaviors in *Gabrd/Crh* mice and $Cre^{-/-}$ littermates. In the light/dark box test, *Gabrd/Crh* mice left the enclosed, dark compartment to enter the illuminated compartment more frequently (35.0 ± 2.8 entries) than $Cre^{-/-}$ littermates (25.9 ± 2.0 entries), and traveled an increased distance in the light compartment (1719.8 ± 87.5 cm) compared to $Cre^{-/-}$ littermates (1254.4 ± 90.2 cm) (Fig. 6a), with no significant change in total locomotor

behavior (*Gabrd/Crh*: 1296.9 ± 77.1 beam breaks; *Cre^{-/-}* littermates: 1085.8 ± 85.8 beam breaks) (data not shown). These data suggest that *Gabrd/Crh* mice exhibit decreased anxiety-like behavior. Supplementation with CORT enhanced the anxiety-like behaviors in both *Gabrd/Crh* mice and *Cre^{-/-}* littermates, reducing the number of entries (*Cre^{-/-}*: 10.6 ± 1.3 entries; *Gabrd/Crh*: 25.3 ± 2.4 entries) and the distance traveled in the light compartment (*Cre^{-/-}*: 566.1 ± 34.1 cm; *Gabrd/Crh*: 1073.5 ± 158.2 cm) (Fig. 6a). CORT treatment results in anxiety-like levels in *Gabrd/Crh* mice (entries: 25.3 ± 2.4 entries; distance: 1073.5 ± 158.2 cm) similar to untreated *Cre^{-/-}* littermates (entries: 25.9 ± 2.0 ; distance: 1254.4 ± 90.2 cm) (Fig. 6a; $n = 10$ – 18 mice per experimental group; one-way ANOVA with Tukey's test; entries: $F(3, 62) = 13.43$; distance: $F(3, 62) = 13.00$; beam breaks: $F(3, 62) = 2.571$; * denotes $p < 0.05$ compared to *Cre^{-/-}* mice; # denotes $p < 0.05$ compared to *Gabrd/Crh* mice).

Likewise, in the open field test, *Gabrd/Crh* mice made an increased number of entries into the center (31.9 ± 3.8 entries), spent more time in the center (78.1 ± 9.0 s), and traveled an increased distance in the center of the open field (695.2 ± 97.5 cm) compared to *Cre^{-/-}* littermates (entries: 20.4 ± 2.9 ; time in center: 50.1 ± 5.4 s; distance in center: 405.7 ± 60.1 cm) (Fig. 6b). The increased time spent in the center of the open field cannot be attributed to hyperactivity since the overall locomotor activity was not significantly different between the two experimental groups (*Gabrd/Crh* mice: 2144.8 ± 72.8 beam breaks; *Cre^{-/-}*: 1930.5 ± 88.8 beam breaks) (Fig. 6b). Treatment with exogenous CORT increased the anxiety-like behavior in *Gabrd/Crh* mice (entries: 17.9 ± 3.4 entries; total time in center: 42.4 ± 9.3 s; distance in center: 433.9 ± 81.5 cm) to levels similar to *Cre^{-/-}* littermates (entries: 20.4 ± 2.9 ; time in center: 50.1 ± 5.4 s; distance in center: 405.7 ± 60.1 cm) with no difference in locomotor activity (*Gabrd/Crh* + CORT: 1825.5 ± 100.3 beam breaks; *Cre^{-/-}* littermates: 1930.5 ± 88.8 beam breaks) (Fig. 6b; $n = 13$ – 18 mice per experimental group; one-way ANOVA with Tukey's test; entries: $F(3, 55) = 3.817$; total time: $F(3, 55) = 6.137$; distance: $F(3, 55) = 3.230$; beam breaks: $F(3, 55) = 2.471$; * denotes $p < 0.05$ compared to *Cre^{-/-}* mice; # denotes $p < 0.05$ compared to *Gabrd/Crh* mice). These data demonstrate that the blunted responsiveness of the HPA axis in *Gabrd/Crh* mice is associated with decreased anxiety-like behaviors.

4. Discussion

Here we characterize a novel mouse model generated in our lab, *Gabrd/Crh* mice, which serve as a useful tool for investigating the role the GABA_AR δ subunit in CRH neurons on HPA axis regulation. The development of the *Gabrd/Crh* mouse is an advance over the global *Gabrd^{-/-}* knockout strain, in which the GABA_AR δ subunit is also absent from other cell types throughout the hippocampus, thalamus, and cortex, regions that may be involved in stress-related behaviors and indirectly regulate HPA axis function. With the specificity of this conditional knockout mouse, the removal of the GABA_AR δ subunit is limited to CRH neurons, thus avoiding potentially confounding factors from effects on other cell types or across brain regions. Although this paper focuses on the role of the GABA_AR δ subunit in CRH neurons in the PVN, we cannot rule out effects on other populations of CRH neurons, such as in the BnST and amygdala, which may indirectly affect HPA axis responsiveness and stress-related behaviors. For example, previous studies have demonstrated that loss of GABAergic control of forebrain neurons resulted in HPA axis hyperexcitability and anxiety-like behaviors in mice (Earnheart et al., 2007). This study merely demonstrates that the loss of the GABA_AR δ subunit in CRH neurons decreases anxiety- and depression-like behaviors, which may be HPA axis-independent despite the observed blunted CORT response in *Gabrd/Crh* mice. However, the evidence that CORT administration increases anxiety- and depression-like behaviors in both *Gabrd/Crh* mice and *Cre^{-/-}* littermates suggests a permissive role of elevated glucocorticoids in the manifestation of stress-related

behaviors similar to previous findings (Kalinichev et al., 2002; Kalynchuk et al., 2004; Gregus et al., 2005; Johnson et al., 2006; Mitra and Sapolsky, 2008; Marks et al., 2009; Rasmussen et al., 2011). This current study did not directly assess the role of GABAergic control of extrahypothalamic CRH neurons in altered HPA axis responsiveness and stress-related behaviors; however, no change in GABA_AR δ subunit expression was observed in the BnST or amygdala, which may suggest that the GABA_AR δ subunit is not expressed in these neurons or is not expressed at a high enough density to result in a quantifiable difference. Further studies are required to fully appreciate the role of GABA_AR δ subunit-mediated inhibition of CRH neurons in extrahypothalamic populations. Here we focused on CRH neurons in the PVN, which have previously been shown to be regulated by GABA_AR δ subunit-containing receptors (Sarkar et al., 2011).

A tonic conductance mediated by the GABA_AR δ subunit has been identified in cells that release CRH (Hewitt et al., 2009; Sarkar et al., 2011), and loss of the GABA_AR δ subunit dramatically alters the activity and modulation of these neurons by neurosteroids (Sarkar et al., 2011; Gunn et al., 2013). Interestingly, it has been shown that the expression of the GABA_AR δ subunit is downregulated in the PVN following chronic stress (Verkuyt et al., 2004), implicating these receptors in stress reactivity. Since multiple cell types express the GABA_AR δ subunit, our *Gabrd/Crh* mice are useful in directly testing the role of the GABA_AR δ subunit specifically in CRH neurons on HPA axis function. Our data demonstrate that removing the GABA_AR δ subunit from CRH neurons eliminates the tonic inhibition of these neurons without affecting the phasic component (Fig. 3). Although transgenic mouse models may be subject to different developmental trajectories, the GABA_AR δ subunit is absent during embryonic and early postnatal development, is only weakly expressed during late postnatal development, and reaches its highest level of expression in the adult (Laurie et al., 1992), which decreases the likelihood for developmental alterations due to the lack of the GABA_AR δ subunit. As such, we did not observe any gross abnormalities in development, including adult body weight or cytoarchitecture of the brain, in *Gabrd/Crh* mice. The loss of GABA_AR δ subunit expression could also potentially result in compensatory alterations in the expression of other GABA_AR subunits; however, we did not observe any compensation for the tonic component nor changes in the phasic component of GABAergic inhibition in CRH neurons from *Gabrd/Crh* mice (Fig. 3). Therefore, our data do not support developmental defects which could account for the phenotypic changes in *Gabrd/Crh* mice compared to *Cre*^{-/-} littermates.

Using this novel mouse model, we were able to investigate the role of the GABA_AR δ subunit in HPA axis regulation. Interestingly, the GABA_AR δ subunit does not appear to play a role in the regulation of basal or diurnal corticosterone secretion. Rather, the loss of the GABA_AR δ subunit in CRH neurons removes a disinhibitory influence of GABA following acute stress (Hewitt et al., 2009; Sarkar et al., 2011) (for review see (Wamsteeker and Bains, 2010)), resulting in a blunted CORT response. It may seem counterintuitive that removing tonic GABAergic inhibition from CRH neurons would result in a blunted, rather than exacerbated, CORT response to stress. However, there is growing evidence that there is a collapse of the chloride gradient in CRH neurons following stress (Hewitt et al., 2009; Sarkar et al., 2011) (for review see (Wamsteeker and Bains, 2010)). The chloride gradient necessary for the inhibitory actions of GABA is maintained by KCC2, the main K⁺/Cl⁻ cotransporter in the adult brain (Rivera et al., 1999; Payne et al., 2003; Rivera et al., 2005). We have previously demonstrated that acute stress causes a decrease in levels of total, phosphorylated, and cell surface levels of KCC2, resulting in excitatory actions of GABA (Sarkar et al., 2011). This downregulation of KCC2 has been proposed to be a mechanism to rapidly overcome the GABAergic constraint on CRH neurons to mount the physiological response to stress (Sarkar et al., 2011), and our findings here lend further support for this theory (for review see (Wamsteeker and Bains, 2010)). The deletion of the GABA_AR δ

subunit removes a disinhibitory influence of GABA in *Gabrd/Crh* mice, resulting in the diminished response to stress observed in these mice.

The study presented here conflicts in some ways with the current dogma concerning the role of GABA in the regulation of the HPA axis. Pharmacological studies have demonstrated that microinfusion of GABA antagonists, such as bicuculline, into the PVN enhances the CORT response to stress (Cole and Sawchenko, 2002; Cullinan et al., 2008), c-fos activation of neurons in the PVN, and increased CRH gene transcription (Cole and Sawchenko, 2002). Direct microinfusion of a low dose of the agonist muscimol, which binds preferentially to GABA_AR δ subunit-containing receptors (Chandra et al., 2010; Mihalek et al., 1999), into the PVN blunts the CORT response to acute restraint stress (Cullinan et al., 2008), in contrast to our findings that the CORT response is blunted by the removal of GABA_AR δ subunit-containing receptors. Although there is considerable evidence for the inhibitory affects of GABA on HPA axis regulation, there are also numerous studies suggesting that GABA may also play a stimulatory role. Previous studies from our lab (Sarkar et al., 2011) and others (Hewitt et al., 2009) demonstrate excitatory actions of GABA immediately following an acute stress. Consistent with these findings, microinfusion studies have concluded that GABA is moderately inhibitory at the level of the pituitary but stimulatory at the level of the hypothalamus (Borycz et al., 1992). These conflicting observations may be due to the timing in which the role of GABA on HPA axis function was assessed. Recent studies demonstrate differences in the magnitude of GABAergic inhibition immediately following acute restraint stress compared to later time points (Wamsteeker Cusulin et al., 2013). Interestingly, these findings demonstrate that immediately following acute stress, there is a potentiation of excitatory GABAergic signaling; whereas, an hour following the stressful episode, there is a reduction in the magnitude of GABAergic inhibition (Wamsteeker Cusulin et al., 2013). Taken together, these studies suggest that GABA may exert an immediate disinhibitory effect following stress, playing a permissive role in mounting the physiological response to stress (for review see (Wamsteeker and Bains, 2010)), and subsequently exert an inhibitory influence to restore CORT levels to baseline. In fact, the GABAergic regulation of the HPA axis is highly plastic (for review see (Wamsteeker and Bains, 2010; Levy and Tasker, 2012)), and the full impact of this regulation has yet to be fully understood. Recent studies suggest that CRH neurons in the PVN and in extrahypothalamic regions may be a heterogeneous population, including some which are GABAergic (Dabrowska et al., 2013). These studies highlight the complexity of GABAergic control of the HPA axis and question our current understanding of GABAergic regulation of the HPA axis.

Associated with this blunted reactivity to stress, *Gabrd/Crh* mice exhibited decreased depression- and anxiety-like behavior. Replacement of CORT increased depression- and anxiety-like behaviors to Cre^{-/-} littermate levels, also demonstrating that glucocorticoid sensitivity is preserved in these mice. Taken together, the behavioral phenotypes of *Gabrd/Crh* mice show that the removal of the GABA_AR δ subunit from CRH neurons has consequences on the ability of the HPA axis to respond appropriately to stressful situations, highlighting the significance of GABAergic signaling, and specifically that of tonic GABAergic conductance in the regulation of the HPA axis. The behavioral profile of the *Gabrd/Crh* mouse suggests that a suppressed HPA axis response may be adaptive. This is consistent with previous studies suggesting that elevated levels of corticosterone (both induced by environmental and genetic manipulations as well as exogenous administration) have been previously implicated in anxiety- and depression-like behaviors (Kalinichev et al., 2002; Kalynchuk et al., 2004; Gregus et al., 2005; Johnson et al., 2006; Mitra and Sapolsky, 2008; Marks et al., 2009; Rasmussen et al., 2011). If blocking CORT release is sufficient to decrease the impact of stressful situations, these results may have therapeutic implications for the treatment of stress-related disorders. Stress is a risk factor for cognitive and

emotional dysfunction such as depression, anxiety disorders, and memory impairments, as well as sleep disorders, drug abuse, and seizures (Lupien et al., 2009; Koe et al., 2009; Kenny, 2011). Chronic stress has also been shown to have effects outside of the central nervous system, such as cardiovascular disease, abnormalities in growth and metabolism, reproductive impairments, immune dysfunction, and other somatic manifestations (Chrousos, 2009). Thus, identifying new targets to diminish the impact of stress would be highly beneficial.

Since the GABA_A δ subunit confers neurosteroid sensitivity, perhaps these compounds may provide therapeutic promise in the treatment of stress-related disorders (for review see (Gunn et al., 2011)). Indeed, the activity of CRH neurons has been shown to be regulated by neurosteroids (Sarkar et al., 2011; Gunn et al., 2013). Previous studies have implicated neurosteroids in the modulation of CORT output after acute stress (Guo et al., 1995; Smith et al., 2006; Sarkar et al., 2011), which points to the therapeutic potential of neuroactive steroids in modulating HPA axis activity. However, the desired effects of steroid treatment are complicated by the opposing actions of GABAergic activity during basal conditions and during stress. Without the GABA_A δ subunit, *Gabrd/Crh* mice may lack the ability to respond appropriately to neurosteroid enhancement of CRH neuron activity. These mice can inform testing of novel treatments with neuroactive steroids by providing data for off-target effects that are not specific to the HPA axis. The regulation of CRH neurons by the GABA_A δ subunit (Sarkar et al., 2011; Gunn et al., 2013), whether through tonic inhibition or neurosteroid action, represents a mechanism that is distinct from the classical action of glucocorticoid signaling through gene transcription and highlights the synaptic regulation of HPA axis function (Tasker and Herman, 2011). Further investigation into the synaptic mechanisms regulating HPA axis function will drive the development of novel treatments for stress-related disorders.

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Figure 1. GABA_A R δ subunit expression in populations of CRH neurons in *Gabrd/Crh* mice
 (a) Representative immunostaining of GABA_A R δ subunit expression in the BnST, amygdala, hippocampus, and PVN of *Gabrd/Crh* mice and *Cre*^{-/-} littermates. The region of interest used for optical density measurements are highlighted (white dotted line). (b) The average optical density measurements demonstrate a reduction in GABA_A R δ subunit expression in the PVN, but not in the BnST, amygdala, or dentate gyrus of *Gabrd/Crh* mice. n = 20–100 sections, 4–5 mice per experimental group. * *p* < 0.05 compared to *Cre*^{-/-} littermates using a Student's *t*-test.

Figure 2. Decreased GABA_AR δ subunit expression in PVN of *Gabrd/Crh* mice

(a) Representative western blots of GABA_AR δ subunit expression in total protein isolated from the PVN and hippocampus in *Gabrd/Crh* mice and *Cre*^{-/-} littermates. (b) The average optical density (OD) measurements from western blots demonstrate reduced GABA_AR δ subunit expression in the PVN of *Gabrd/Crh* mice compared to *Cre*^{-/-} littermates, while expression in the hippocampus is not significantly different. *n* = 7 mice per experimental group. * *p* < 0.05 compared to *Cre*^{-/-} littermates using a Student's *t*-test.

Figure 3. Disinhibition of CRH neurons due to a loss of tonic GABAergic inhibition in *Gabrd/Crh* mice

(a) Representative whole-cell recordings of spontaneous IPSCs from CRH neurons from *Gabrd/Crh* mice and *Cre^{-/-}* littermates. (b) The average frequency and peak amplitude of spontaneous IPSCs were not significantly different in *Gabrd/Crh* mice and *Cre^{-/-}* littermates. $n = 16\text{--}20$ cells, 4 mice per experimental group. (c) Representative traces of the tonic inhibition in CRH neurons from *Cre^{-/-}* littermate controls and *Gabrd/Crh* mice, held at -70 mV. Each dot represents the average holding current every 100 ms before and after the addition of the GABA_AR antagonist SR95531 (black line) to reveal the tonic current. (d) The average tonic GABAergic inhibition was significantly reduced in *Gabrd/Crh* mice

compared with controls, consistent with the loss of GABA_AR δ subunit expression from CRH neurons. $n = 15\text{--}20$ cells, 4–5 mice per experimental group. * $p < 0.05$ compared to Cre^{-/-} littermates using a Student's t-test. (e) Representative cell-attached recordings from control (non-stressed) *Gabrd/Crh* mice and Cre^{-/-} littermates or mice subjected to a single episode of 30 min restraint stress. (f) The average firing rates of CRH neurons recorded in the cell-attached mode were not different between *Gabrd/Crh* mice and Cre^{-/-} littermates that were naïve to stress. Following a 30 min restraint stress, the average firing rate of CRH neurons is decreased in *Gabrd/Crh* mice compared to Cre^{-/-} littermates. $n = 12\text{--}18$ cells, 3–4 mice per experimental group. * $p < 0.05$ compared to non-stressed Cre^{-/-} littermate controls; # $p < 0.05$ compared to stressed Cre^{-/-} littermates using a one-way ANOVA with Tukey's test for multiple comparisons.

Figure 4. Blunted HPA axis reactivity to stress in *Gabrd/Crh* mice

(a) Representative c-fos expression in the PVN 30 min following acute restraint stress in *Gabrd/Crh* mice and *Cre^{-/-}* littermates. (b) The average number of c-fos positive neurons in the PVN following acute restraint stress is decreased in *Gabrd/Crh* mice compared to *Cre^{-/-}* littermates. $n = 3$ mice per experimental group. * $p < 0.05$ compared to *Cre^{-/-}* littermates using a Student's t-test. (c) Basal CORT levels in *Gabrd/Crh* mice and *Cre^{-/-}* littermates naïve to stress are not significantly different. In contrast, following a 30 min restraint stress, CORT levels are increased in *Cre^{-/-}* littermates compared to naïve mice. CORT levels were blunted in *Gabrd/Crh* mice compared to *Cre^{-/-}* littermates, showing a suppression of HPA

axis responsiveness to stress. $n = 11-16$ mice per experimental group. * $p < 0.05$ compared to basal levels; # $p < 0.05$ compared to stressed $Cre^{-/-}$ littermates using a one-way ANOVA with Tukey's test for multiple comparisons. (d) Diurnal CORT levels are not significantly different between *Gabrd/Crh* mice and $Cre^{-/-}$ littermates. There is no significant difference in CORT levels at any time point between *Gabrd/Crh* mice and $Cre^{-/-}$ littermates. Similarly, there is no difference in the average adrenal weight (e) or adrenal:body weight ratio (f) between *Gabrd/Crh* mice and $Cre^{-/-}$ littermates using a Student's t-test. $n = 10-12$ mice per experimental group.

Figure 5. Decreased depression-like behavior in *Gabrd/Crh* mice

(a) The average latency to immobility is increased and the total time spent immobile in the tail suspension test is decreased in *Gabrd/Crh* mice compared to *Cre*^{-/-} littermates, suggesting decreased depression-like behavior. CORT treatment decreased the latency to immobility and increased the total time spent immobile in both *Gabrd/Crh* mice and *Cre*^{-/-} littermates. (b) Similarly, in the forced swim test, *Gabrd/Crh* mice demonstrated an increase in the average latency to immobility and less time spent immobile compared to *Cre*^{-/-} littermates. Exogenous administration of CORT decreased the latency to the first bout of immobility and increased the total time spent immobile in both *Gabrd/Crh* mice and *Cre*^{-/-} littermates. *n* = 10–18 mice per experimental group. * denotes *p* < 0.05 compared to *Cre*^{-/-} mice and # denotes *p* < 0.05 compared to *Gabrd/Crh* mice using a one-way ANOVA with Tukey's test for multiple comparisons.

Figure 6. Decreased anxiety-like behavior in *Gabrd/Crh* mice

(a) In the light/dark box test, *Gabrd/Crh* mice demonstrated more entries into and greater distance traveled in the light compartment compared to *Cre*^{-/-} littermates. CORT supplementation decreased the number of entries into and decreased the distance traveled in the light compartment in both *Gabrd/Crh* mice and *Cre*^{-/-} littermates. *n* = 10–18 mice per experimental groups. (b) In the open field test, *Gabrd/Crh* mice spent more time and traveled an increased distance in the center of the open field compared to *Cre*^{-/-} littermates. CORT treatment in *Gabrd/Crh* mice decreased the amount of time spent and distance traveled in the open field. General locomotor activity was not different across groups. *n* =

13–18 mice per experimental groups. * denotes $p < 0.05$ compared to Cre^{-/-} mice and # denotes $p < 0.05$ compared to *Gabrd/Crh* mice using a one-way ANOVA with Tukey's test for multiple comparisons.