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Glucocorticoid receptor deletion from the dorsal raphe nucleus of mice reduces dysphoria-like behavior and impairs hypothalamic-pituitary-adrenocortical axis feedback inhibition

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Abstract

Glucocorticoids can cause depression and anxiety. Mechanisms for glucocorticoid effects on mood are largely undefined. The dorsal raphe nucleus (DRN) produces the majority of serotonin in the brain, and expresses glucocorticoid receptors (GR). Since we previously showed that antidepressants used to treat depression and anxiety decrease DRN GR expression, we hypothesized that deleting DRN GR would have anxiolytic- and antidepressant-like effects. We also hypothesized that DRN GR deletion would disinhibit activity of the **hypothalamic pituitary adrenal (HPA) axis**. Adeno-associated virus pseudotype AAV2/9 expressing either Cre recombinase (DRN^{GR}KO mice) or GFP (DRN-GFP mice) was injected into the DRN of floxed GR mice to test these hypotheses. Three weeks after injection, mice underwent 10d of social defeat or control handling and tested for anxiety-like behavior (open field test, elevated plus maze), depression-like behavior (sucrose preference, forced swim test (FST), tail suspension (TST)), social interaction, and circadian and stress-induced HPA activity. DRN GR deletion decreased anxiety-like behavior in control but not in defeated mice. DRN GR deletion decreased FST and tended to decrease TST despair-like behavior in both control and defeated mice, but did not affect sucrose preference. Exploration of social (a novel mouse) as well as neutral targets (an empty box) was increased in DRN^{GR}KO mice, suggesting that DRN GR deletion also promotes active coping. DRN GR deletion increased stress-regulated HPA activity without strongly altering circadian HPA activity. We have shown a novel role for DRN GR to mediate anxiety- and despair-like behavior and to regulate HPA negative feedback during acute stress.

Keywords

depression; anxiety; stress; corticosterone; serotonin

INTRODUCTION

Activity of the hypothalamic-pituitary-adrenocortical (**HPA**) axis, which regulates the secretion of adrenocortical glucocorticoids, has received interest as a biomarker in

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depression (Heuser *et al.*, 1994). HPA hyperactivity occurs in 50–80% of depressed patients (Heuser *et al.*, 1994) and has also been reported, although less consistently, in anxiety disorders (Catapano *et al.*, 1990; Martin *et al.*, 2009). In both of these disorders, HPA hyperactivity after treatment correlates with increased risk of relapse (Cookson *et al.*, 1985; Zobel *et al.*, 2001; Abelson *et al.*, 2007; Künzel *et al.*, 2009), suggesting that HPA activity might be a useful marker of treatment response.

However, there is evidence that glucocorticoids not only reflect, but may also contribute to mood and anxiety disorders. Glucocorticoids themselves have been shown to cause dysphoria (depression and anxiety) symptoms (Brown *et al.*, 1999; Loosen, 2002). Depression occurs in over 50% of patients with Cushing's syndrome (Kelly, 1996; Dimopoulou *et al.*, 2013) and up to 20% of patients that receive exogenous glucocorticoids for immunosuppressive therapy (Sirois, 2003; Kenna *et al.*, 2011). Anxiety also occurs in these populations, albeit at a lower rate (Kenna *et al.*, 2011). Chronic glucocorticoid administration also promotes depression-like and anxiety-like behaviors in animals (Gregus *et al.*, 2005; Ardayfio & Kim, 2006; Sterner & Kalynchuk, 2010). While many studies have focused on the role of forebrain glucocorticoid receptors (**GR**) in dysphoria-like behavior (Wei *et al.*, 2004; Boyle *et al.*, 2005; Boyle *et al.*, 2006; McKlveen *et al.*, 2013; Vincent *et al.*, 2013), less is known about GR expressing brainstem regions mediating depression-like and anxiety-like effects of glucocorticoids.

The dorsal raphe nucleus (**DRN**) is a brainstem region that expresses GR (Harfstrand *et al.*, 1986) and provides the majority of serotonergic projections to forebrain areas implicated in mood and anxiety (Mamounas *et al.*, 1991; Lowry, 2002; Abrams *et al.*, 2004; Fu *et al.*, 2010; Waselus M, 2011). Dysregulated levels of serotonin have been postulated to contribute to the pathology of mood and anxiety disorders (Nutt, 2002; Delgado, 2006; Kent & Shapiro, 2009; Insel *et al.*, 2010). In addition, the most common treatments for both depression and anxiety disorders include serotonin selective reuptake inhibitors, which block the reuptake of serotonin and increase serotonin levels (Vaswani *et al.*, 2003). We have previously shown that antidepressants that block serotonin reuptake decrease DRN GR gene expression (Heydendael & Jacobson, 2009; 2010). While it remains unknown if decreasing DRN GR has antidepressant-like effects, there is evidence that GR impairment in serotonin synthesizing cells reduces dysphoria-like behavior induced either by repeated social defeat stress or by chronic glucocorticoid administration (Espallergues *et al.*, 2012). However, since there are 9 serotonergic nuclei (Franklin & Paxinos, 2007), the location of GR involved in these dysphoria-like behaviors remains unknown.

The finding of antidepressant-like effects from impairing GR in serotonin cells (Espallergues *et al.*, 2012), in combination with our data showing antidepressant-induced decreases in DRN GR (Heydendael & Jacobson, 2009; 2010) led us to hypothesize that deleting GR specifically in the DRN would result in antidepressant-like and anxiolytic-like effects. We tested this hypothesis in floxed GR mice by analyzing depression-like and anxiety-like behaviors in control-handled and socially-defeated mice. Since GR in many brain regions have also been shown to mediate glucocorticoid feedback inhibition of HPA activity (Keller-Wood & Dallman, 1984; Diorio *et al.*, 1993; Jacobson, 2005), we also tested

if DRN GR deletion affects glucocorticoid secretion during the circadian rhythm or after an acute stress.

EXPERIMENTAL PROCEDURES

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of Albany Medical College and followed the standards of the NIH *Guide for the Care and Use of Animals* (Institute of Laboratory Animal Resources, 2010). Mice were housed on a 12:12 light: dark cycle (lights on at 7:00 a.m.) with ad libitum access to rodent chow. Floxed GR male mice (B6.129S6-*Nr3c1*^{tm2.1Ljm/J}) were bred from floxed GR male and female mice on a pure C57BL/6 background (founders generously donated by Dr. Louis Muglia, University of Cincinnati, Cincinnati, OH, USA).

Stereotaxic Surgery—All mice were 2–3 months old at the time of surgery. We first established a model of DRN GR deletion using recombinant adeno-associated virus (AAV) pseudotype 2/9 (the AAV2 genome packaged in the AAV9 capsid (Sun *et al.*, 2010)). Mice were injected with recombinant AAV2/9 vectors expressing Cre recombinase (AAV2/9-Cre) and a fluorescent marker or only the fluorescent marker GFP (AAV2/9-GFP) as a control. Viral vectors were obtained from the University of Massachusetts Medical School (Worcester, MA, USA). Virus stocks were diluted on the day of surgery to 1.45×10^{13} genomic copies/ml with sterile 1X Dulbecco's phosphate buffered saline from Cellgro (Manassas, VA, USA).

Mice were anesthetized with 125 mg/kg ketamine, 12.5mg/kg xylazine and mounted in a Kopf stereotaxic apparatus with model 922 tapered mouse ear bars. Following local anesthesia of the scalp with bupivacaine, the skull was exposed, and bregma and lambda were visualized with a dissecting microscope (model PZMIII-AAC) from World Precision Instruments (Sarasota, FL, USA). Coordinates were located from bregma or lambda with a Stoelting digitizer (Wood Dale, IL, USA) attached to the micromanipulator of the stereotaxic apparatus. Burr holes were drilled in the skull with a flexible shaft Dremel tool equipped with a 0.75 mm carbide bit (Stoelting). 500 nl of virus was injected at 10 nl/sec through 33 ga. stainless steel beveled tubing (Vita Needle, Needham, MA, USA) attached by PE 20 tubing (Fisher, Pittsburgh, PA, USA) to a 2 μ l Hamilton Syringe (Fisher). Adeno-associated virus was delivered by two midline injections, with coordinates optimized from Thevenot *et al.* (Thévenot *et al.*, 2003) at A/P –4.7 mm, M/L 0.0 mm, D/V –3.5 mm from bregma and A/P –4.4mm, M/L 0.0 mm, D/V –3.5 mm from bregma. The injector was left in place 7–9 min before being withdrawn at 0.1 mm/10 sec. After the scalp incision was sutured, analgesia was provided by 1 mg/kg indomethacin ip (Alexis Biochemicals, San Diego, CA, USA) in 10ml/kg of sterile-filtered 0.1M sodium carbonate, followed by 0.001M indomethacin available as a separate drinking fluid for 3 d after surgery. Mice were individually housed after surgery for the rest of the experiment.

Social Defeat—To examine the role of DRN GR on behavior and HPA regulation under control and chronic stress conditions, mice underwent daily control handling or social defeat beginning 3 weeks after virus injection surgery. In the socially-defeated group, mice were

placed in the home cage of an aggressive CD1 retired male breeder for 1 minute, during which the experimental mouse was rapidly attacked and exhibited submissive behaviors representative of defeat (fleeing, freezing, or supine posture). Only CD1 mice that attacked in less than 30 sec were used as aggressors. Defeated mice were then placed back in their home cage with a handful of bedding from the CD1 aggressor's cage to provide a scent memory of the attack (Dr. Herb Covington III, personal communication). Since initial results indicated that partitioned housing of socially defeated mice with their aggressors (Berton *et al.*, 2006) did not affect social interaction behavior, socially defeated mice were placed back in their respective home cage after social defeat.

Non-aggressive CD1 mice never attacked or had an attack latency greater than 3 min. Non-defeated control mice were placed for 1 min in the vacant home cage of a non-aggressive CD1 male that had never been used for defeat (Martinez *et al.*, 1998) and had a handful of bedding from this cage placed in their home cage. Non-defeated control mice therefore experienced handling, novel environments, and novel scents without the physical or olfactory exposure to aggression. Socially-defeated and control mice were rotated through different aggressors or cages, respectively, on a daily basis for 21 d; behavioral and hormonal tests were conducted during the last 6 d of defeat or control handling, after the main behavioral effects of social defeat have been shown to occur (Krishnan *et al.*, 2007; Cao *et al.*, 2010).

Behavioral tests

All tests were conducted within 3h of lights-on, with the exception of the social interaction test, which was performed within 7h of lights-on. Behavioral tests were videotaped and as indicated, scored, using Ethovision XT 8.1 software (Noldus Information Technology, Leesburg, VA, USA). Tests were conducted on separate days in the order of least (sucrose preference, open field test, elevated plus maze, social interaction) to most stressful (forced swim, tail suspension). Some tests were not performed in all mice. For example, the elevated plus maze was added after it appeared that time in the center of an open field was too low to give accurate an estimate of anxiety-like behavior. The tail suspension test was substituted for the forced swim test in some studies to give another measure of despair-like immobility. However, when the same tests were performed across studies, tests were performed in the same relative order, and these data were pooled to increase the number of mice with complete DRN GR deletion available for analysis.

Sucrose Preference Test—Mice were given a choice between two, 50 ml bottles containing either 1% sucrose or water. To control for side preference, the position of the bottles was switched daily. The tubes were weighed every day to determine intake. Sucrose preference data are presented as % sucrose intake = sucrose consumed (g) / [water consumed (g) + sucrose consumed (g)].

Open Field Test—Mice were placed into a corner of a 42cm × 35cm × 13.5cm basin and videotaped for 5 min. Total distance traveled, and total duration in the center 32 cm × 25 cm of the basin was scored automatically with Ethovision 8.1 software.

Elevated Plus Maze—The dimensions of the elevated plus maze were 65 cm × 65 cm × 40 cm. Each arm was 30 cm long, 5 cm wide. The open arm had a 0.5 cm edge and the closed arm wall height was 15 cm. Mice were placed on the central platform, facing an open arm. Ethovision software was used to videotape and analyze the mouse behavior in a 5 min test. An arm entry was scored when all four paws of the mouse entered an arm, and a partial open arm entry was scored when two paws entered the open arm. The time spent in the open arms, center and closed arms was recorded, along with the total distance traveled. An observer blind to genotype and social defeat conditions manually scored the full open and closed arm entries, partial open arm entries, and head dips on the open arm.

Social Interaction Test—Mice were placed in a 42 cm × 35 cm × 13.5 cm basin across from a 12 cm × 8 cm × 13.5 cm wire mesh test box in one corner of the basin. Each mouse was videotaped for two consecutive 2.5 min sessions. During the first session the mouse was exposed to the empty wire mesh test box (12 cm × 8 cm × 13.5 cm). During the second session, the mouse was exposed an unfamiliar, non-aggressive CD1 male mouse enclosed in a wire mesh box as a social target. The test mouse was considered in the “interaction zone” if the mouse entered within 10 cm of the box, both with and without the mouse in the test box. The amount of time the test mouse spent in the interaction zone was scored automatically with Ethovision software. An observer blind to genotype and social defeat conditions manually scored the number of times the nose of the experimental mouse touched the test box or social target as an additional measure of target investigation and interaction.

Forced Swim Test—We used a 2-day forced swim test because it has been shown to be sensitive to glucocorticoids (Veldhuis *et al.*, 1985; Mitchell & Meaney, 1991) and yields similar results as a single forced swim test with regard to depression-like behavior (Cryan *et al.*, 2002). Mice were placed in a 1000 ml beaker (10 cm wide × 19.5 cm high) filled with 25±1°C water (Vincent *et al.*, 2013). Mice were subjected to a 15-minute pre-swim, and immobility was scored in a 5-min test swim 24 h later. Immobility was manually scored from Ethovision videotapes by a trained observer blind to treatment groups as the time the mouse spent motionless or made only brief, non-rhythmical movement of the paws or tail to keep its head above water.

Tail Suspension Test—As previously described (Vincent *et al.*, 2013), mice were taped by their tail to a metal rod and allowed to hang upside down for 8 minutes. Immobility behavior was manually scored from Ethovision videotapes as the time mice were completely motionless or swinging from the momentum of previous struggling without moving any part of their body.

Plasma hormone assay

Blood was obtained by submandibular puncture with 5 mm Goldenrod animal lancets (Medipoint Inc., Mineola, NY, USA) and collected through heparinized capillary tubes into microfuge tubes within 45 sec of moving the mouse's cage. A single sample (0.05–0.1 ml) was collected for determination of either stress, circadian nadir, or circadian peak hormone levels. Stress-induced plasma corticosterone levels were determined in a blood sample collected immediately after the 8 min tail suspension test. Circadian nadir and peak plasma

corticosterone levels were determined in blood samples collected within 1 hr of lights-on and 1 h of lights-off, respectively, 3 d after behavioral tests were complete but while daily defeats or control handling continued. Plasma was separated by centrifugation and assayed for corticosterone, using previously described modifications (Kier *et al.*, 2005) of a commercial radioimmunoassay kit (MP Biomedical, Solon, OH, USA).

Immunocytochemical analysis of GR expression

All brains were collected after perfusion fixation, stored in 4% paraformaldehyde overnight at 4°C, and then cryoprotected in 30% sucrose at 4°C as previously described (Vincent *et al.*, 2013). Within two weeks of cryoprotection, brains were sectioned coronally at 25 µm on a cryostat (Leica, Buffalo Grove, IL, USA), and distributed sequentially over the wells of a 6-well tissue culture plate (Corning Incorporated Life Sciences, Lowell, MA, USA) containing cryoprotectant solution (50% 0.05M Tris-buffered saline, pH 7.5, 30% ethylene glycol, 20% glycerol) and stored at –20°C.

As previously described (Vincent *et al.*, 2013), GR expression was analyzed with Tyramide Single Amplification (TSA; Perkin Elmer, Boston, Massachusetts, USA) in all for mice injected with AAV2/9-Cre and a representative set of DRN-GFP mice injected with AAV2/9-GFP for comparison. On day 1, free floating sections were incubated in 1% hydrogen peroxide, blocked with filtered TNB (0.1M TRISHCl, 0.15 NaCl, 0.5% blocking reagent, pH 7; Perkin Elmer) before processing for GR immunocytochemistry and incubated overnight at 4°C in rabbit anti-GR (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). On day 2, sections were incubated in horseradish peroxidase (HRP)-goat anti-rabbit (Perkin Elmer Boston, Massachusetts, USA) in TNB (1:1000), and incubated for 10 minutes at room temperature in TSA-Cy5 antibody in amplification diluent (1:50; Perkin Elmer). Sections were mounted on slides and coverslipped with N-propyl-gallate (Sigma Aldrich, St. Louis, MO, USA).

Data Analysis

Immunocytochemistry—All images were captured at 50X magnification on an Olympus BX50 fluorescence microscope (Center Valley, PA, USA). Based on the anatomical designations described in Abrams *et al.* (Abrams *et al.*, 2004), a total of 12 rostrocaudal sections representative of every 100 µm from –4.12 to –5.32 mm bregma were examined for GR immunofluorescence. Complete GR deletion was defined as the lack of GR immunofluorescence in the bilateral dorsolateral wings, and in the dorsomedial, ventromedial, and interfascicular regions of the DRN in all 12 rostrocaudal sections.

Statistics—Statistical analysis was performed with Statview 5.0 software (SAS Institute, Cary, NC, USA). 2-way factorial ANOVAs were performed to identify main effects of DRN GR deletion and social defeat. *Post-hoc* comparisons of data were performed by unpaired t-test with Bonferroni correction for multiple comparisons. These post-hoc comparisons gave similar results as non-parametric Mann-Whitney tests (not shown), indicating that potential violations of normal distribution or homogeneity of variance by the smaller *n* values of mice with complete DRN GR deletion did not confound our data analysis. All results are expressed as a mean ± SEM. Significance was set at $P < 0.05$.

RESULTS

Effect of partial DRN GR deletion on dysphoria-like behavior and HPA activity

Using the rostral, middle and caudal DRN designations of Abrams *et al.* (Abrams *et al.*, 2004), we found that GR immunoreactivity (Figure 1, left) extended from the caudal area of the rostral DRN (from bregma -4.36mm) to the dorsal part of the caudal DRN (from bregma -4.96mm). GR was not strongly expressed in the anterior portions of the rostral DRN (-4.16mm to 4.24mm) or posterior portions of the caudal DRN (-5.02 to -5.34mm). Complete GR deletion was defined as lack of GR expression over the entire the rostrocaudal extent of the DRN (Figure 1, right). However, many of the AAV2/9-Cre injections only resulted in partial GR deletion, with GR expression remaining in one or both lateral wings and/ or the intrafascicular region of the DRN. Although partial DRN GR deletion tended to increase circadian peak (evening) and stress-induced plasma corticosterone, partial DRN GR deletion did not alter any behavioral measures or AM plasma corticosterone (Table 1). Therefore, we focused subsequent analyses only on comparisons between mice with complete DRN GR deletion (**DRN^{GRKO}**) and mice injected with the AAV2/9-GFP control vector (**DRN-GFP**).

Effect of DRN GR deletion on anxiety-like behavior

Although whole brain GR deletion has been reported to decrease anxiety-like behavior, forebrain GR deletion was found to have inconsistent effects on anxiety-like behavior (Tronche *et al.*, 1999; Boyle *et al.*, 2006). Since the presence of brainstem GR was the most obvious difference between these two studies, we surmised that deletion of GR from brainstem structures such as the DRN might decrease anxiety-like behavior. We tested the effects of DRN GR deletion under baseline conditions in control-handled mice and after the chronic stress of repeated social defeat, a paradigm that is ethologically relevant to the social stresses that can precipitate or exacerbate human depression and anxiety disorders (Berton *et al.*, 2006). In the open field test (Table 2), in which time spent in the less sheltered center of the field is interpreted as reduced anxiety-like behavior (Holmes, 2003), there were marginal main effects of DRN GR deletion to increase the time **DRN^{GRKO}** mice spent in the center of the open field ($F_{1,13}=3.755$; $P=0.0747$) and to decrease latency to enter the center ($F_{1,13}=3.967$; $P=0.0678$). There was no significant effect of social defeat or DRN GR deletion $\times$ social defeat interaction on open field center time or entry latency. Since the open field test can also be used as a measure of overall activity (Holmes, 2003), we measured the total distance traveled in the open field test. There were no significant effects of DRN GR deletion, social defeat, or DRN GR deletion $\times$ defeat interaction on open field locomotion (Table 2).

We used the elevated plus maze as an additional test of anxiety-like behavior to avoid the possibility that low center time in the open field may have created a floor effect that prevented detection of the effects of GR deletion or social defeat. In the elevated plus maze, time spent in the open arms is interpreted as a measure of reduced anxiety-like behavior (Holmes, 2003). There was a significant main effect of DRN GR deletion ($F_{1,48}=7.655$; $P=0.0080$) and social defeat ($F_{1,48}=5.29$; $P=0.0257$) on the percent of time mice spent in the open arms, with a marginally significant interaction of DRN GR deletion $\times$ social defeat

($F_{1,48}=3.278$; $P=0.0765$; Figure 2). *Post-hoc* analysis showed that GR DRN deletion significantly increased the amount of time that non-defeated DRNGRKO mice spent in the open arms compared to non-defeated DFN-GFP mice. However, there was no difference in open arm time between DRNGRKO and DRN-GFP mice in the social defeat group (Figure 2). Although there was a significant main effect of social defeat on percent of time mice spent in the open arms (see above), this effect was not significant at the *post-hoc* level. There was also a significant main effect of DRN GR deletion on total full (4 limb) entries into the open arms ($F_{1,48}=5.455$; $P=0.0237$), which was marginally significant in non-defeated mice ($P=0.0633$; Table 3). There was no significant effect of social defeat or DRN GR deletion $\times$ social defeat interaction on full open arm entries in the elevated plus maze. In addition, there was a significant main effect of DRN GR deletion ($F_{1,48}=4.265$; $P=0.0443$) to increase and of social defeat to decrease ($F_{1,48}=6.534$; $P=0.0138$) exploratory head dips in the open arms (Table 3). The number of head dips was marginally higher in non-defeated DRNGRKO vs. DRN-GFP mice ($P=0.0542$), but effects of social defeat on exploratory head dips did not approach significance at the *post-hoc* level. There were also no significant effects of DRN GR deletion, social defeat, or DRN GR deletion $\times$ social defeat interaction on closed arm entries, partial (2 limb) open arm entries, closed arm time, center time or total distance traveled in the elevated plus maze (Table 3).

Effect of DRN GR deletion on depression-related behaviors

We have previously shown that DRN GR expression is decreased by antidepressants that block serotonin reuptake (Heydendael & Jacobson, 2009; 2010), and Espallergues *et al.* have shown antidepressant-like effects from impairing GR function in serotonergic neurons (Espallergues *et al.*, 2012). Therefore, we hypothesized that DRN GR deletion would have antidepressant-like effects to decrease depression-like behavior. To test this hypothesis, we analyzed sucrose preference and despair-like immobility in DRN-GFP and DRNGRKO mice after social defeat or control handling. These tests respectively model the anhedonia (loss of pleasure) and apathy observed in depressed patients (American Psychiatric Association, 1994; Lucki, 1997; Willner *et al.*, 1998). We also analyzed the effects of DRN GR deletion on social and non-social investigative activity, which has been used as a measure of both depression- (Krishnan *et al.*, 2007) and anxiety-like behavior (File, 1980).

After social defeat, there was a significant main effect of social defeat to decrease sucrose intake ($F_{1,46}=16.166$; $P<0.0002$), but there was no significant effect of DRN GR deletion or DRN GR deletion $\times$ social defeat interaction (Figure 3). *Post-hoc* analysis revealed that socially-defeated mice exhibited significantly lower sucrose preference than their non-defeated controls, but neither baseline sucrose preference nor defeat-induced decreases in sucrose preference was altered by DRN GR deletion (Figure 3).

To determine if DRN GR deletion affects depression-like immobility behavior, we tested DRN-GFP and DRNGRKO mice in a forced swim or a tail suspension test after social defeat or control handling. In both of these tests, greater immobility is a rodent correlate of despair- or depression-like behavior (Porsolt *et al.*, 1977; Cryan *et al.*, 2005). There was a significant main effect of DRN GR deletion ($F_{1,42}=16.105$; $P=0.0002$) to decrease forced swim test immobility. *Post-hoc* analysis showed that immobility was significantly lower in

DRNGRKO vs. DRN-GFP mice in both the control and defeated groups (Figure 4, top). There was no significant effect of social defeat or DRN GR deletion $\times$ social defeat interaction on forced swim test immobility. A similar trend was observed in the tail suspension test (Figure 4, bottom), in which there was a marginal main effect of DRN GR deletion to reduce tail suspension test immobility behavior ($F_{1,77}=3.293$; $P=0.0735$), with no significant effects of social defeat or DRN GR deletion $\times$ social defeat interaction on tail suspension test immobility.

Social interaction has been used as a measure of both the social withdrawal of depression (Krishnan *et al.*, 2007) and the conflict avoidance of anxiety (File, 1980), with lower interaction interpreted as greater depression-like withdrawal or anxiety-like avoidance. There were significant main effects of GR DRN deletion ($F_{1,126}=11.812$; $P=0.0008$) and social defeat ($F_{1,126}=14.788$; $P=0.0002$), but no significant effect of DRN GR deletion $\times$ social defeat interaction on time spent investigating a novel, confined mouse. Non-defeated DRNGRKO mice exhibited significantly greater mouse interaction time than did non-defeated DRN-GFP mice (Figure 5, left). Socially defeating mice significantly decreased the amount of time mice spent interacting with the target mouse, but mouse interaction time of defeated DRNGRKO mice was still significantly higher than that of defeated DRN-GFP mice (Figure 5, left). However, there was also a significant effect of DRN GR deletion on interaction with an empty box ($F_{1,126}=34.278$; $P=0.0001$), with both non-defeated and socially-defeated DRNGRKO mice spending significantly more time investigating the box compared to their DRN-GFP counterparts (Figure 5, right). There were no significant effects of social defeat or an interaction of DRN GR deletion $\times$ social defeat on box interaction time. The number of nose touches made by the test mouse with the caged mouse or the empty box were also analyzed as an additional measure of active investigative behavior, and gave results similar to analysis of interaction zone time (data not shown).

Effect of GR DRN deletion on HPA activity

GR in the brain are also important for mediating glucocorticoid negative feedback during the peak of the circadian rhythm or in response to stress (Jacobson, 2005; Ulrich-Lai & Herman, 2009). Most animal studies have focused on the role of GR in forebrain regions, including the hippocampus, prefrontal cortex and amygdala (Herman *et al.*, 1996), with less focus on brainstem GR (an exception being (Scheuer *et al.*, 2004)). Therefore, we tested if deleting GR in the DRN would affect glucocorticoid secretion during the circadian rhythm or after an acute stress.

There was a significant main effect of social defeat on circadian nadir (AM) plasma corticosterone levels ($F_{1,51}=10.361$; $P=0.0022$), without any significant effects of DRN GR deletion or DRN GR deletion $\times$ social defeat interaction (Figure 6, left). *Post-hoc* analysis showed that defeated DRN-GFP mice had significantly higher AM corticosterone levels compared to their non-defeated DRN-GFP controls. Although AM corticosterone levels also appeared to be higher in defeated vs. non-defeated DRNGRKO mice, this effect was not significant at the *post-hoc* level (Figure 6, left).

There were significant main effects of DRN GR deletion ($F_{1,51}=6.888$; $P=0.0114$) and social defeat ($F_{1,51}=0.981$; $P=0.0029$) on circadian peak (PM) plasma corticosterone levels. There

was no significant effect of DRN GR deletion $\times$ social defeat interaction on PM plasma corticosterone (Figure 6, right). *Post-hoc* analysis showed that defeated DRN-GFP mice also had significantly higher PM corticosterone levels compared to their non-defeated DRN-GFP controls (Figure 6, right). There were no significant *post-hoc* differences in PM corticosterone between non-defeated DRN-GFP and DRNGRKO mice, between defeated DRN-GFP and DRNGRKO mice, or between non-defeated and defeated DRNGRKO mice (Fig. 6, right).

We also analyzed plasma corticosterone immediately after an 8 min tail suspension test to determine if DRN GR deletion affected acute stress-induced HPA activity. There were significant main effects of DRN GR deletion ($F_{1,67}=9.387$; $P=0.0031$) and social defeat ($F_{1,67}=9.387$; $P=0.0015$) but no GR deletion $\times$ social defeat interaction on plasma corticosterone after tail suspension. Deleting DRN GR caused plasma corticosterone levels to be significantly higher after tail suspension in non-defeated DRNGRKO vs. DRN-GFP mice (Figure 7). In addition, social defeat significantly increased post-tail suspension plasma corticosterone levels in DRN-GFP compared to those in non-defeated DRN-GFP mice (Figure 7). Although plasma corticosterone appeared to be higher after tail suspension in socially-defeated DRNGRKO vs. non-defeated DRNGRKO mice, these groups did not differ significantly at the *post-hoc* level (Figure 7).

DISCUSSION

We have shown that deleting DRN GR has anxiolytic- and antidepressant-like effects on behavior and impairs HPA negative feedback during stress. DRN GR deletion reduced anxiety-like behavior in the elevated plus maze in control-handled mice and increased exploratory behavior in both control and socially-defeated mice. DRN GR deletion did not affect all depression-like behaviors, but in tests showing significant effects of DRN GR deletion, antidepressant-like effects of DRN GR deletion were present in chronically defeated as well as non-defeated mice and were not due to generalized changes in locomotor activity. Although the interaction-enhancing effects of DRN GR deletion in the social interaction test were more exploratory than specifically social in nature, lower exploratory activity has been linked with passive coping behavior that is relevant to depression as well as anxiety (Steimer T *et al.*, 1997; Koolhaas JM *et al.*, 1999; Cryan *et al.*, 2002; Dimopoulou *et al.*, 2013). Thus, the increased exploratory behavior in DRNGRKO mice, reflecting active coping behavior, is consistent with the other anxiolytic- and antidepressant-like effects observed after DRN GR deletion. In addition, DRN GR deletion increased corticosterone responses to stress without strongly altering circadian HPA activity. In sum, our results support a novel role for DRN GR in mediating dysphoria-like behavior and stress-related HPA feedback inhibition.

DRN GR deletion reduces anxiety-like behaviors

DRN GR deletion had anxiolytic-like effects to increase open arm time of non-defeated mice in the elevated plus maze and to increase exploratory behavior in the social interaction test in both non-defeated and defeated mice. Consistent with the ability of DRN GR deletion to increase open arm time in the elevated plus maze, DRN GR deletion increased

exploratory head-dipping behavior in the open arms, a well-characterized test of anxiety-like behavior in rodents (Holmes, 2003). The lack of effect of DRN GR deletion on elevated plus maze behavior in socially-defeated mice suggests that chronic stress may override the effects of DRN GR deletion on some anxiety-like behaviors.

DRN GR deletion also increased both social and non-social exploratory behavior in the social interaction test. Social interaction has been used as an anxiolytic-sensitive measure of anxiety-like behavior in rodents (File, 1980), while increases and decreases in non-social exploratory behavior have respectively been used as measures of active and passive coping that correlate with lower or higher anxiety-like behavior (Koolhaas JM *et al.*, 1999; Cryan *et al.*, 2002). Our results are consistent with the finding that brain GR promote anxiety-like behavior (Tronche *et al.*, 1999) and suggest that the DRN is a site of GR expression contributing to anxiety-like behavior.

Our findings are also consistent with reports that glucocorticoids can induce anxiety in humans and animals (Korte, 2001; Brown, 2009; Sterner & Kalynchuk, 2010; Kenna *et al.*, 2011). Although we did not observe significant effects of social defeat to increase anxiety-like behavior at the *post-hoc* level, we did find a significant main effect of social defeat on anxiety-like behavior in the elevated plus maze that corroborates the effects of social defeat reported by other laboratories (Tronche *et al.*, 1999; Wei *et al.*, 2004; Boyle *et al.*, 2006; Krishnan *et al.*, 2007; Kinsey *et al.*, 2007; Kolber *et al.*, 2008; Blugeot *et al.*, 2011). Our finding that DRN GR deletion reduces anxiety-like behavior differs from that of Espallergues *et al.*, who did not observe differences in baseline anxiety-like behavior after impairing GR in serotonergic neurons (Espallergues *et al.*, 2012). These differences could be due to more selective and localized manipulation of GR function in our model. Since Espallergues *et al.* deleted histone deacetylase 6 to modify GR activity (Espallergues *et al.*, 2012), it is also possible that there are other effects of histone deacetylase 6 deletion unrelated to GR that prevented baseline anxiolytic-like effects from being observed, or that interfering with GR function in all serotonergic nuclei obscures the effects of DRN GR deletion on anxiety-like behavior. Lastly, since Espallergues *et al.* used Pet1 to drive Cre expression (Espallergues *et al.*, 2012), GR function in serotonergic synthesizing cells that do not depend on Pet1 expression, which include those projecting to emotionally-relevant regions such as the extended amygdala (Kiyasova V *et al.*, 2011; Kiyasova & Gaspar, 2011), may not have been affected. Although our targeting strategy was not selective for particular cell types within the DRN, our data demonstrate that GR in the DRN can contribute to anxiety-like behavior.

DRN GR deletion increases active coping behaviors

DRN GR deletion had antidepressant-like effects on several but not all measures of depression-like behavior. We observed significant effects of DRN GR deletion to reduce despair-like immobility in the forced swim test and a tendency to reduce immobility in the tail suspension test regardless of prior stress, but no effect on sucrose preference. Effects of DRN GR deletion on despair-like immobility were more evident in the forced swim test than in the tail suspension test, possibly because the forced swim test involved a prior swim and the tail suspension test was an isolated test, or possibly because these tests rely on different

neural circuits (Jacobson & Cryan, 2007). While we did not find increases in despair-like immobility after social defeat, this result is in keeping with many findings in social defeat paradigms (Krishnan *et al.*, 2007; Kinsey *et al.*, 2007; Blugeot *et al.*, 2011). Our findings that social defeat reduced sucrose preference and specifically social interaction indicate that our social defeat paradigm had emotionally-relevant behavioral effects comparable to those in the literature (Krishnan *et al.*, 2007).

Although effects of DRN GR deletion to enhance interaction in the social interaction test were more related to general exploration rather than specifically social investigation, exploratory behavior has been correlated with depression as well as anxiety. Reductions in exploratory behavior in humans are associated with depression-related characteristics of negative outlook and lower social engagement (Dimopoulou *et al.*, 2013). In rodents, reductions in exploratory behavior, reflecting passive coping, also correlate with greater despair-like behavior (Koolhaas JM *et al.*, 1999; Cryan *et al.*, 2002). The increase in exploratory behavior observed after DRN GR deletion suggests that DRN GR facilitate dysphoria-like behavior. This finding is consistent with reports that patients with Cushing's syndrome, who have elevated glucocorticoid levels as a result of a tumor, exhibit decreased exploratory behavior (Dimopoulou *et al.*, 2013). The increase in exploration in DRNGRKO mice was not due to simple hyperactivity because there was no change in locomotor activity in the open field test or the elevated plus maze. In sum, our immobility and exploration data implicate DRN GR in promoting passive coping behaviors that are relevant to depression and anxiety under both baseline and chronic stress conditions.

DRN GR deletion enhances HPA responses to acute stress without strongly affecting circadian HPA activity

DRN GR deletion did not have robust effects on basal circadian HPA activity. Social defeat caused a significant increase in circadian nadir plasma corticosterone levels that was consistent with those observed in other social defeat (Keeney *et al.*, 2006; Barik *et al.*, 2013) and chronic stress models (Herman *et al.*, 1995; Blugeot *et al.*, 2011). Chronic defeat also increased circadian peak glucocorticoid levels; increases in circadian HPA activity induced by defeat resembled the increases in HPA activity often observed in depressed patients (Heuser *et al.*, 1994; Rush *et al.*, 1996; Linkowski, 2003), but were not significantly affected by DRN GR deletion at the *post-hoc* level. It is possible that we might have detected significant differences of DRN GR deletion with additional sampling points **or by increasing the number of DRNGRKO mice.**

Effects of DRN GR deletion to increase HPA activity were much more pronounced during acute stress. Complete DRN GR deletion significantly increased plasma corticosterone responses to the acute stress of tail suspension in mice subjected to control handling. Social defeat also increased plasma corticosterone responses to the acute stress of tail suspension in DRN-GFP mice, consistent with the augmented HPA responses reported in other models of social defeat (Keeney *et al.*, 2006; Barik *et al.*, 2013) and with the ability of a repeated stress to augment the HPA response to a novel (heterotypic) stress (Bhatnagar & Dallman, 1998). In socially-defeated mice, complete DRN GR deletion appeared to increase tail suspension corticosterone levels, but this was not significant at the *post-hoc* level. As with circadian

peak HPA activity, significant effects of complete DRN GR deletion on stress-induced HPA activity in defeated mice might have been detected by sampling at more time points **or increasing the number of DRNGRKO mice.**

Because only a single, post-stress sample was used to assess HPA reactivity to stress, the enhancement of HPA responses to tail suspension by DRN GR deletion could have been attributed either to overall increases in baseline HPA activity or to interference with glucocorticoid feedback inhibition during stress. However, DRN GR deletion did not increase HPA activity at the circadian nadir, when tail suspension was conducted. Therefore, our data indicate that DRN GR deletion impairs glucocorticoid feedback inhibition of HPA activity during acute stress. This finding establishes a novel role for DRN GR in HPA feedback regulation.

Although DRN GR deletion increased stress-induced and possibly circadian HPA activity, it is unlikely that the behavioral effects of DRN GR deletion resulted indirectly from the effects of elevated glucocorticoids acting outside of the DRN. Widespread GR activation in brain, resulting either from chronic systemic glucocorticoid exposure (Gregus *et al.*, 2005; Ardayfio & Kim, 2006; Sterner & Kalynchuk, 2010) or GR overexpression (Wei *et al.*, 2004), is associated with increased anxiety- and depression-like behaviors, which did not occur with DRN GR deletion. Furthermore, although we saw similar increases in HPA activity after partial DRN GR deletion, partial DRN deletion did not affect dysphoria-like behaviors. The effects of partial GR deletion suggest not only that HPA activity is more sensitive to DRN GR loss than are anxiety- or depression-like behaviors but also that altered HPA activity was probably not responsible for changes in these behaviors after complete DRN GR deletion.

Topographical characterization of the DRN has revealed several subregions with functional distinctions. Whereas serotonergic neurons in the rostral DRN appear to be involved in motor activity (Waselus M, 2011), serotonin neurons of the middle and caudal DRN are implicated in physiological and behavioral responses to emotional stimuli by their neuroanatomical connections and activity (Lowry, 2002). Mapping studies suggest roles for the dorsal region of the middle DRN in facilitating anxiety-like responses and for the lateral wings and caudal (interfascicular) region of the DRN in promoting anxiolytic- and antidepressant-like responses (Hale MW, 2012). The middle and caudal DRN also provides input, albeit largely indirect, to the paraventricular hypothalamus, which contains the adrenocorticotropin-releasing factors governing HPA activity (Lowry, 2002). We found GR expression in the mouse DRN to be concentrated in the caudal area of the rostral region (from bregma -4.36) to the dorsal part of the caudal DRN (from bregma -4.96), positioning it well to regulate dysphoria-like behavior and HPA activity. Of note, however, dorsomedial DRN GR deletion was common to all partial DRN GR deletions, and yet did not affect anxiety-like behavior. In contrast, partial DRN GR deletion, which consistently included dorsomedial DRN GR deletion, appeared to increase HPA activity. Since mapping of GR function within the DRN was not our original goal, there were not enough mice with sufficiently localized GR deletion for detailed analysis of this or other subregions on other behavioral or endocrine endpoints. Nevertheless, these limited data suggest the possibility

that GR in the dorsomedial DRN may be more important for regulating HPA activity than anxiety behavior.

By virally transducing Cre to delete DRN GR in adult mice, we have minimized possible compensatory or developmental effects potentially associated with transgenic strategies for Cre-mediated gene deletion. However, our approach also resulted in GR deletion in all cell types within the DRN. It will be of interest in future studies to determine if GR expression in specific neurons or glia within individual DRN subregions is involved in dysphoria-like behavior and HPA activity.

CONCLUSIONS

Our data indicate that DRN GR contribute both to dysphoria-like behavior and to feedback regulation of acute stress-induced HPA activity. In the absence of stress, our data indicate that DRN GR promote passive coping behavior, including reduced exploration, increased despair-like immobility and increased anxiety-like behavior. We have also found a novel contribution of DRN GR to glucocorticoid inhibition of HPA responses during acute stress. This finding adds the DRN, with other GR-expressing regions of the brainstem (Scheuer *et al.*, 2004), to the growing list of brain sites mediating negative feedback regulation of the HPA axis. A better understanding of glucocorticoid effects on DRN signaling could ultimately aid in treating patients prone to affective disorders after stress, as well as patients susceptible to psychiatric side effects from immunosuppressive glucocorticoid therapy.

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ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
DRN	dorsal raphe nucleus
DRN-GFP	dorsal raphe nucleus green fluorescent protein
DRNGRKO	dorsal raphe nucleus glucocorticoid receptor knock out
GR	glucocorticoid receptors
HPA	hypothalamic pituitary adrenal
TSA	tyramide single amplification

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Figure 1.

Representative images of GR immunofluorescence in the dorsal raphe nucleus of mice injected with AAV2/9-GFP (**DRN-GFP**) or mice injected with AAV2/9-Cre with complete GR deletion (**DRNGRKO mice**). Images are shown for two levels of each of the rostral, middle and caudal DRN regions as defined by Abrams *et al.*, with respective distances (mm) from bregma indicated at the left of the figure (Abrams *et al.*, 2004). The boundaries of the DRN, indicated by the dashed lines, were determined using the Franklin and Paxinos mouse brain atlas (Franklin and Paxinos, 2007). Dotted lines outline the aqueduct.

Figure 2.
Time spent in the open arms of an elevated plus maze by DRN-GFP mice (white bars) and DRNGRKO (black bars) after control handling (Control) or repeated daily social defeat. Group *N*s are shown within the data bars. *, $P < 0.05$ vs. DRN-GFP mice in the same defeat group.

Figure 3. Sucrose preference in DRN-GFP and DRNGRKO mice, calculated as the percentage of total fluid intake represented by consumption of 1% sucrose. Groups are shown as in Figure 2. #, $P < 0.05$ vs. control, non-defeated mice in the same virus injection group.

Figure 4. Depression-like immobility in (top) a 5 min forced swim test (FST) and (bottom) an 8 min tail suspension test (TST) in DRN-GFP and DRNGRKO mice. Groups are shown as in Figure 2. *, $P < 0.05$ compared to DRN-GFP mice in the same defeat group.

Figure 5. Social interaction and exploratory behavior in DRN-GFP and DRNGRKO mice. Left and right panels respectively show the time spent interacting with a mouse enclosed in a wire mesh box or with an empty wire mesh box. Groups are shown as in Figure 2. *, $P < 0.05$ compared to DRN-GFP mice in the same defeat group; #, $P < 0.05$ vs. control, non-defeated mice in the same virus injection group.

Figure 6. Circadian nadir (AM) and peak (PM) plasma corticosterone in DRN-GFP and DRNGRKO mice. Groups are shown as in Figure 2. #, $P < 0.05$ vs. control, non-defeated mice in the same virus injection group.

Figure 7.

Plasma corticosterone levels immediately after the acute stress of an 8 min tail suspension test (TST) in DRN-GFP and DRNGRKO mice. Groups are shown as in Figure 2. Group sizes differ for corticosterone and TST immobility (Figure 4, bottom) because we were unable to collect plasma from some mice. *, $P < 0.05$ compared to DRN-GFP mice in the same defeat group; #, $P < 0.05$ vs. control, non-defeated mice in the same virus injection group.

Table 1

Effects of partial DRN GR deletion on time spent in the open arms of elevated plus maze (EPM), time investigating a mouse or an empty box in the social interaction test (SIT), immobility time during a 5 min forced swim test (FST), and circadian nadir (AM), peak (PM) or tail suspension stress-induced plasma corticosterone (cort) levels. Partial DRN GR deletion was defined as GR expression remaining in one or both lateral wings or the intrafascicular region of the DRN. Data from DRN-GFP mice are repeated for comparison. Behavioral and hormonal measurements were performed as described in Methods.

	Control		Social Defeat	
	DRN-GFP mice	Partial GR deletion	DRN-GFP mice	Partial GR deletion
EPM Open Arm Time (sec)	13.71 ± 2.58 <i>N</i> = 21	12.03 ± 4.98 <i>N</i> = 18	10.98 ± 3.18 <i>N</i> = 17	10.74 ± 2.67 <i>N</i> = 15
SIT MOUSE Interaction Time (sec)	35.60 ± 4.71 <i>N</i> = 57	25.49 ± 3.22 <i>N</i> = 56	16.26 ± 3.18 <i>N</i> = 46	15.30 ± 3.40 <i>N</i> = 43
SIT Box Interaction Time (sec)	11.86 ± 2.43 <i>N</i> = 57	14.39 ± 3.66 <i>N</i> = 56	12.83 ± 3.61 <i>N</i> = 46	16.37 ± 4.81 <i>N</i> = 43
FST Immobility (sec)	203.86 ± 17.36 <i>N</i> = 18	181.53 ± 14.84 <i>N</i> = 21	199.91 ± 14.62 <i>N</i> = 17	155.90 ± 16.87 <i>N</i> = 17
AM plasma cort (µg/dl)	1.20 ± 0.20 <i>N</i> = 28	2.48 ± 0.46 <i>N</i> = 11	4.25 ± 0.76 <i>N</i> = 19	2.89 ± 1.00 <i>N</i> = 9
PM plasma cort (µg/dl)	14.49 ± 0.89 <i>N</i> = 28	18.89 ± 1.13 <i>N</i> = 21	18.24 ± 1.34 <i>N</i> = 19	20.60 ± 2.21 <i>N</i> = 15
8' TST plasma cort (µg/dl)	17.45 ± 0.81 <i>N</i> = 34	21.81 ± 1.36 <i>N</i> = 11	23.36 ± 1.01 <i>N</i> = 24	31.52 ± 1.63 <i>N</i> = 10

Table 2

Measures of anxiety-like behavior in the open field test (OFT) in DRN-GFP and DRNGRKO mice after control handling or repeated daily social defeat.

	Control		Social Defeat	
	DRN-GFP mice	DRNGRKO mice	DRN-GFP mice	DRNGRKO mice
OFT Center time (sec)	5.21 ± 2.03 N = 6	9.86 ± 0.83 N = 3	4.78 ± 1.29 N = 5	7.90 ± 2.62 N = 3
OFT Latency to center (sec)	125.20 ± 49.01 N = 6	41.34 ± 1.82 N = 3	146.27 ± 47.48 N = 5	36.95 ± 14.16 N = 3
OFT Distance (cm)	1441.44 ± 145.81 N = 6	1886.64 ± 242.72 N = 3	1284.17 ± 229.84 N = 5	1480.73 ± 224.67 N = 3

Table 3

Additional measures of elevated plus maze (EPM) behavior in DRN-GFP mice and DRNGRKO mice after control handling or repeated social defeat. Open arm entries, exploratory head dips in the open arms, full (4 limb) closed arm entries, partial (2 limb) open arm entries, time spent in the closed arms and center zone, and total distance traveled were measured as described in Methods.

	Control		Social Defeat	
	DRN-GFP mice	DRNGRKO mice	DRN-GFP mice	DRNGRKO mice
Open Arm Entries	1.00 ± 0.17 N = 21	2.27 ± 0.92 N = 7	1.18 ± 0.32 N = 17	1.86 ± 0.51 N = 7
Open Arm Head Dips	2.57 ± 0.66 N = 21	7.14 ± 2.84 N = 7	1.77 ± 0.66 N = 17	1.75 ± 0.70 N = 7
Closed Arm Entries	7.67 ± 0.61 N = 21	6.00 ± 1.02 N = 7	6.17 ± 1.04 N = 17	8.00 ± 1.11 N = 7
Partial Open Arm Entries	3.76 ± 0.50 N = 21	2.57 ± 1.00 N = 7	3.88 ± 0.92 N = 17	4.28 ± 1.04 N = 7
EPM Closed Arm Time (sec)	258.93 ± 4.57 N = 21	244.89 ± 16.48 N = 7	260.48 ± 8.95 N = 17	255.98 ± 9.13 N = 7
EPM Center Time (sec)	26.27 ± 2.61 N = 21	16.03 ± 4.06 N = 7	27.59 ± 7.82 N = 17	26.76 ± 4.83 N = 7
EPM Distance Traveled (cm)	1224.28 ± 127.92 N = 21	1030.69 ± 174.76 N = 7	1369.15 ± 185.64 N = 17	1084.62 ± 196.39 N = 7