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Serotonergic systems in the balance: CRHR1 and CRHR2 differentially control stress-induced serotonin synthesis

Nina C. Donner[1,2,*], **Philip H. Siebler**[1], **Danté T. Johnson**[1], **Marcos D. Villarreal**[1], **Sofia Mani**[1], **Allison J. Matti**[1], and **Christopher A. Lowry**[1]

Nina C. Donner: nina_donner@psych.mpg.de

[1]Department of Integrative Physiology and Center for Neuroscience, University of Colorado Boulder, 1725 Pleasant Street, 114 Clare Small, UCB 354, 80309 Boulder, CO, USA

[2]Department of Stress Neurobiology and Neurogenetics, Max Planck Institute of Psychiatry, Kraepelinstr. 2-10, 80804 Munich, Germany

Abstract

Anxiety and affective disorders are often associated with hypercortisolism and dysfunctional serotonergic systems, including increased expression of *TPH2*, the gene encoding the rate-limiting enzyme of neuronal serotonin synthesis. We previously reported that chronic glucocorticoid exposure is anxiogenic and increases rat *Tph2* mRNA expression, but it was still unclear if this also translates to increased TPH2 protein levels and *in vivo* activity of the enzyme. Here, we found that adult male rats treated with corticosterone (CORT, 100 μg/ml) via the drinking water for 21 days indeed show increased TPH2 protein expression in the dorsal and ventral part of the dorsal raphe nucleus (DRD, DRV) during the light phase, abolishing the enzyme's diurnal rhythm. In a second study, we systemically blocked the conversion of 5-hydroxytryptophan (5-HTP) to serotonin immediately before rats treated with CORT or vehicle were either exposed to 30 min acoustic startle stress or home cage control conditions. This allowed us to measure 5-HTP accumulation as a direct readout of basal versus stress-induced *in vivo* TPH2 activity. As expected, basal TPH2 activity was elevated in the DRD, DRV and MnR of CORT-treated rats. In response to stress, a multitude of serotonergic systems reacted with increased TPH2 activity, but the stress-, anxiety-, and learned helplessness-related dorsal and caudal DR (DRD/DRC) displayed

[*]Corresponding author at: Nina C. Donner, Department of Stress Neurobiology and Neurogenetics, Max Planck Institute of Psychiatry, Kraepelinstr. 2-10, 80804 Munich, Germany. Tel.: +49 (0)89 30622 554.

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Conflict of Interest

The authors have no commercial interest and report no conflict of interest with regard to the submitted manuscript and the experiments conducted.

Contributors

Nina Donner and Christopher Lowry designed the study. Nina Donner performed all three experiments, and prepared the manuscript. Christopher Lowry edited the manuscript. Philip Siebler assisted with tissue collection and with the adrenal functionality assay in *Experiment 2*. Dante Johnson, Marcos Villarreal and Allison Matti assisted with brain sectioning, western blots and HPLC sample preparation in *Experiments 1* and *2*. Sofia Mani assisted with animal care in all three experiments, and with HPLC sample preparation in *Experiment 3*. All authors have approved the final version of the submitted manuscript.

stress-induced increases in TPH2 activity only after chronic CORT-treatment. To address the mechanisms underlying this region-specific CORT-dependent sensitization, we stereotaxically implanted CORT-treated rats with cannulae targeting the DR, and pharmacologically blocked either corticotropin-releasing hormone receptor type 1 (CRHR1) or type 2 (CRHR2) 10 min prior to acoustic startle stress. CRHR2 blockade prevented stress-induced increases of TPH2 activity within the DRD/DRC, while blockade of CRHR1 potentiated stress-induced TPH2 activity in the entire DR. Stress-induced TPH2 activity in the DRD/DRC furthermore predicted TPH2 activity in the amygdala and in the caudal pontine reticular nucleus (PnC), while serotonin synthesis in the PnC was strongly correlated with the maximum startle response. Our data demonstrate that chronically elevated glucocorticoids sensitize stress- and anxiety-related serotonergic systems, and for the first time reveal competing roles of CRHR1 and CRHR2 on stress-induced *in vivo* serotonin synthesis.

Keywords

serotonin; tryptophan hydroxylase; corticotropin receptor; stress; glucocorticoids; dorsal raphe nucleus

1. INTRODUCTION

Both hypothalamic-pituitary-adrenal (HPA) axis function and serotonergic function are commonly dysregulated in stress-related psychiatric disorders. Elevated basal glucocorticoid levels or dysfunctional negative feedback control of the HPA axis are often symptomatic of major depressive disorder (MDD) (Gillespie and Nemeroff, 2005), bipolar disorder (Scott et al., 2013), and anxiety disorders (Graeff, 2007). Also, chronic medication with synthetic glucocorticoids increases the risk of developing mania, MDD, and psychosis (Fietta and Delsante, 2009). Most strikingly, up to 81% of patients with Cushing's syndrome, defined by chronic elevation of endogenous cortisol levels, suffer from comorbid depression (Feelders et al., 2012), compared to a 17% lifetime MDD prevalence in the general population.

At the same time, brain serotonergic function is dysregulated in many stress-related psychiatric diseases, and serves as a key target for treatment. Importantly, *post mortem* studies of tryptophan hydroxylase 2 (TPH2), the rate-limiting enzyme for brain serotonin (5-hydroxytryptamine, 5-HT) synthesis, have identified elevated *TPH2* mRNA (Bach-Mizrachi et al., 2006) and increased TPH2 protein expression (Underwood et al., 2010) as potential biomarkers of depression, bipolar disorder and suicide.

Thus, revealing how chronic glucocorticoid exposure interacts with the expression and function of *TPH2* is of clinical relevance. Previously, we found that chronic glucocorticoid exposure is anxiogenic and depressogenic, and elevates rat *Tph2* mRNA during the light phase of the diurnal light cycle (Donner et al., 2012). Since the functional consequences of increased *Tph2* mRNA expression are still unclear, we here measured the diurnal expression of TPH2 protein and basal versus acoustic startle (AS) stress-induced *in vivo* TPH2 activity.

We chose AS stress as the stressor following chronic glucocorticoid treatment because the neuroanatomical circuitry of noise stress, including the AS reflex, is better understood than that of other, often multimodal stressors (Davis et al., 1982). A noise signal is transduced into physiological and behavioral responses in two categorically different ways. First, noise stress elicits a short-latency startle reflex, transduced from the cochlear nucleus via the nucleus of the lateral lemniscus to magnocellular neurons of the caudal pontine reticular nucleus (PnC), from where giant PnC neurons send projections to motor neuron cell bodies in the spinal cord that control the behavioral response to AS, namely the muscle twitching of the startle reflex. Second, separate projections to subcortical nuclei, such as the central amygdaloid nucleus (CE), activate more complex neuronal networks, including brain serotonergic systems (Evans et al., 2009) that themselves innervate the PnC (Hobson et al., 1986).

In the 1990s, the laboratory of Dr. Margaret Boadle-Biber contributed a series of experiments revealing that brief glucocorticoid exposure is permissive for increased *ex vivo* TPH activity for up to 24 h following acute or repeated AS stress, without altering basal TPH activity (Singh et al., 1990). However, little is known about the interaction of chronic glucocorticoid treatment and acute stress on the enzyme's *in vivo* activity. Also, previous studies often failed to consider the functional topography of midbrain serotonergic systems, and since the identification of peripheral TPH1 and brain-specific TPH2 immunohistochemistry can now differentiate between the two isoforms (Walther et al., 2003).

To test the hypotheses that 1) chronic glucocorticoid exposure increases TPH2 protein expression and sensitizes the enzymatic activity of TPH2 in anxiety-related serotonergic systems, and that 2) stress-induced TPH2 activity depends on local corticotropin-releasing hormone receptor type 1 (CRHR1) or 2 (CRHR2) activation within the dorsal raphe nucleus (DR), male rats were treated with 100 μg/ml corticosterone (CORT) via the drinking water for 21 days to replicate previously reported behavioral findings, and to measure light- and dark-phase expression of TPH2 protein. A second study assessed basal and AS stress-induced *in vivo* TPH2 activity in five functionally distinct serotonergic regions of the DR and in the median raphe nucleus (MnR) and within 15 relevant mesolimbocortical fore- and hindbrain regions. In a third study, rats were cannulated to perform intra-DR microinjections of selective CRHR1 or CRHR2 antagonists, addressing the mechanism controlling stress-induced 5-HT synthesis following CORT-induced sensitization.

2. MATERIALS & METHODS

2.1. Animals

Adrenal-intact, adult male Sprague Dawley rats (Harlan Labs, Indianapolis, IN, USA; 200–225 g at arrival day) were kept with *ad libitum* access to food and water/treatment on a reversed 12:12 h light/dark cycle (lights on at 1800 h). In *Experiments 1* and *2*, rats were pair-housed according to the same treatment and killing time point, and either treated with vehicle (n =16) or 100 μg/ml CORT (n = 16) for 21 days. On day 16, their anxiety-like behavior was assessed on the elevated plus-maze (EPM) for 5 min. On day 17, all rats were swim-trained for 15 min, and on day 18 tested for 5 min in the forced swim test (FST).

Please refer to supplemental materials for behavioral testing details. In *Experiment 3*, rats were single-housed following surgery. Between arrival and experimental procedures, and between surgery and treatment start, rats were allowed to recover for at least four days. CORT-water bottles were replaced with vehicle bottles 90 min prior to sacrifice in all experiments to reduce variability based on differences in individual water consumption. For timeline illustrations of *Experiments 1*, *2* and *3*, see Fig. 1.

2.2. Experimental design

Previously we discovered that chronic CORT, compared to vehicle, elevates *Tph2* mRNA expression 2 h into the rats' light phase (Donner et al., 2012). Meanwhile, peak TPH2 protein expression follows approximately 8 h after the diurnal peak in *Tph2* mRNA (Malek et al., 2004). To compare TPH2 protein expression and TPH2 activity after chronic CORT treatment with our published findings on *Tph2* mRNA expression, we therefore euthanized rats of all experiments +10 h into the light phase (at 0400 h), and a second group +10 h into the dark phase in *Experiment 1* (at 1600 h).

Experiment 1: Diurnal *TPH2* protein expression after chronic CORT (Fig. 1, panel A, N = 32)—On day 21, half of the rats per treatment group were killed via rapid decapitation at 0400 h during the light phase, the other half at 1600 h during the dark phase. Brains were fresh frozen and stored at −80 °C until further processing for western blot analysis of TPH2 protein expression in the DR and MnR.

Experiment 2: TPH2 activity after chronic CORT (Fig. 1, panel B, N = 32)—Following the behavioral testing days, rats continued to be handled for 30 s per day on days 19 and 20 to habituate them to subsequent injection procedures. On day 21, all rats received an intraperitoneal (i.p.) injection of 100 mg/kg of the aromatic L-amino acid decarboxylase (AADC) inhibitor NSD-1015 at 0330 h to block AADC-dependent conversion of 5-hydroxytryptophan (5-HTP) to 5-HT, before one half of each treatment group was subjected to AS stress for 30 min (60 dB background, 100 millisecond-long 120 dB startle stimuli at 3.0 kHz, random intervals) while the other half remained in their home cage. Immediately after the termination of AS at 0400 h, all rats were sacrificed via rapid decapitation to collect brain, thymus, adrenal glands and trunk blood. Brains were fresh frozen and stored at −80 °C until microdissection of fore- and hindbrain regions to analyze 5-HTP accumulation as a readout of TPH2 activity. See supplemental information for details regarding the AS stress exposure and the processing of tissue and blood.

Experiment 3: Mechanism of AS-induced TPH2 activity increase after chronic CORT (Fig. 1, panel C, N = 60)—Sixty rats received stereotaxic implants with a unilateral guide cannula targeting the dorsocaudal DR (DRD/DRC), using non-traumatic ear bars (45° angle, Cat. No. 51612, Stoelting, Wood Dale, IL, USA), and were treated with 100 μg/ml CORT via the drinking water for 21 days. A vehicle control group was omitted because the objective was to elucidate the mechanism underlying the AS-induced increase in TPH2 activity in the DRD/DRC, for which the presence of chronic CORT was necessary and permissive, as demonstrated in *Experiment 2*. Rats were handled daily for 30 s each during the last 5 days of treatment to habituate them to subsequent injection procedures. On

day 21, rats received either an intracranial microinjection of 1) vehicle (Veh, n = 20), 2) the selective CRHR1 receptor antagonist antalarmin (Ant, n = 20) or 3) the selective CRHR2 receptor antagonist antisauvagine-30 (A30, n = 20) at 0320 h, and an intraperitoneal (i.p.) injection of 100 mg/kg NSD-1015 at 0330 h. Half of each treatment group was then subjected to 30 min of AS, while the other half remained in their home cages. Immediately after the termination of AS at 0400 h, all rats were euthanized via rapid decapitation. Brains were extracted, fresh-frozen and stored at −80 °C until analysis of 5-HTP accumulation as an index of TPH2 activity.

2.3. Drugs and treatment

The crystalline hormone corticosterone (CORT, 11-β,21-dihydroxy-4-pregnene-3,20-dione; minimum ≥ 92%; Cat. No. C2505, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in tap water containing 0.45% 2-hydroxypropyl-β-cyclodextrin (Cat. No. 332607, Sigma-Aldrich). To prevent light-induced degradation of CORT, spray-painted, black drinking bottles were used. Immediately before AS, 100 mg/kg of the AADC-inhibitor NSD-1015 (3-hydroxybenzylhydrazine dihydrochloride, Cat. No. 54880, Sigma-Aldrich) was injected i.p. in 0.9% sterile saline to block AADC-dependent conversion of 5-HTP to 5-HT. In *Experiment 3*, rats received microinjections of vehicle (0.5 μL 2% EtOH/2% Cremophor EL in artificial cerebrospinal fluid, 50 ng of the selective CRHR1 receptor antagonist antalarmin (Cat. No. A8727, Sigma-Aldrich) in 0.5 μL vehicle, or 2 μg of the selective CRHR2 antagonist antisauvagine-30 (Cat. No. A4727, Sigma-Aldrich) in 0.5 μL vehicle 10 min prior to the onset of AS. Please refer to the supplemental information regarding the justification of dosage for each compound.

2.4. Stereotaxic surgery and verification of cannula placement

Under isoflurane anesthesia and four days before the start of chronic CORT treatment, a permanent, unilateral guide cannula (Cat. No. C315GA/SPC, 26 ga, Plastics One Products, Roanoke, VA, USA) was stereotaxically implanted into the right hemisphere at the following coordinates with reference to bregma. AP: −8.30 mm, ML: +4.00 mm, DV: −6.65 mm, angle: 32.0° from the midline, incisor bar: −3.3 mm (Paxinos and Watson, 1998). The angle of the guide cannula prevented penetration of the mesencephalic aqueduct by the cannula track. Placement of each microinjection cannula tip (extending 1 mm past the guide cannula tip) was verified microscopically post mortem in 25 μm DR-sections, mounted on glass slides (Cat. No. 16004-382; VWR, West Chester, PA, USA), using cresyl violet staining (see supplemental materials section). In 78.33% of the cases (47 of N = 60) stereotaxic surgery was considered successful, meaning a) that the microinjection cannula tip was located either within or adjacent to the dorsocaudal DR (DRD/DRC) region, b) that neither the guide nor the injection cannula track penetrated the aqueduct, and c) that the injection site was located within a 0.5 mm radius from the DRD/DRC center. Although we cannot exclude compound diffusion into the mesencephalic aqueduct (Aq), the small injection volume ensured concentrated, local diffusion prior to potential leakage into the ventricular system (Edeline et al., 2002).

2.5. Brain sectioning

For *Experiments 1* and *2*, 300 μm coronal brain sections were collected from the entire brain. For *Experiment 3*, sections throughout the entire DR region (−7.50 mm to −9.00 mm bregma) were collected by alternating one 100 μm section with two 25 μm sections, while regular 300 μm sections were collected from the remaining brain. This sectioning pattern allowed for both measurement of TPH2 activity and verification of the injection site. Sections were kept at −80 °C until they were microdissected at −10 °C, using a cold plate, blunt dissection needles ranging from 410 μm to 1 mm in inner diameter (Fine Science Tools, Foster City, CA, USA), and a stereotaxic rat brain atlas for reference (Paxinos and Watson, 1998). Brain regions of interest were grouped into a) brainstem serotonergic systems, namely the median raphe nucleus (MnR), the dorsal part (DRD) of the DR, the ventral DR (DRV), the ventrolateral DR/ventrolateral periaqueductal gray (DRVL/VLPAG), the interfascicular part of the DR (DRI), and the caudal DR (DRC); and b) 15 different mesolimbocortical stress-, anxiety-, or learned helplessness-related regions innervated by midbrain serotonergic neurons. Please see supplemental material and supplemental Fig. S1 for details.

2.6. Western blot for TPH2 protein

Microdissected brain tissue samples were collected into 60 μl HEPES (Cat. No. 90909C, Sigma Aldrich) buffer containing 0.25% Protease-Inhibitor Cocktail Set III (Cat. No. 539134, Millipore, Billerica, MA, USA), and homogenized. Total protein content was determined in 5 μl of each sample, using a commercially available, colorimetric protein assay (Micro BCA Protein Assay Kit, Cat. No. 1856210, Thermo Scientific, Rockford, IL, USA), while approximately 30 μg protein per sample were analyzed in the western blot assay for TPH2 (56 kDa), using a 1:200 diluted antibody against TPH2 (rabbit anti-rat TPH2, Cat. No. ABN60, Millipore) and a horseradish peroxidase-conjugated secondary antibody (1:500, Cat. No. AP182P, Millipore), both in 5% dry milk TBST buffer. β-actin (42 kDa) served as the loading control, and blotted bands were visualized using chemoluminescence. See supplemental information for details.

2.7. Assessment of TPH2 activity using high pressure liquid chromatography with electrochemical detection (HPLC-ED)

Following i.p. injection of the AADC-inhibitor NSD-1015, 5-HTP accumulation was analyzed as an index of TPH2 activity. Brain microdissections were collected into acetate buffer (pH 5.0; 992.7 mL HPLC-grade H_2O, 3.0 g sodium acetate, 4.3 ml glacial acetic acid) and snap-frozen on dry ice. For the assay, they were thawed and centrifuged, reconstituted with 0.2 M NaOH, and assayed for total protein content using a commercially available kit (Micro BCA Protein Assay Kit, Cat. No. 1856210, Thermo Scientific). Then, 50 μl of the supernatant were placed in an ESA 542 autosampler (ESA Analytical Ltd., Huntington, UK) to be analyzed for 5-HTP content using HPLC-ED (sample temperature 4 °C, column oven temperature 29 °C, flow rate 1.50 ml/min). TPH2 activity was expressed as a ratio of total 5-HTP content over the total amount of protein per sample. For details refer to supplemental information.

2.8. Statistics

Prior to statistical analysis using SPSS (version 20 for Windows, SPSS Inc., Chicago, IL, USA), outliers were identified and removed from the data set using the Grubbs test. Behavioral parameters (time or number of events in the EPM and FST), thymus and adrenal gland weight, and the difference in rectal temperature from before and after the FST, as well as the number of fecal boli left in the swim tank, were compared using Student's *t*-tests. Maximum acoustic startle response (ASR) was compared using Student's *t*-test. TPH2 protein expression in *Experiment 1* was analyzed with a linear mixed model analysis (LMM), using *treatment* and *light/dark* as between-subjects factors, and *region* as the within-subjects factor. If a main effect or interaction was detected in any of the LMMs, groups were further analyzed using a two-way ANOVA for each brain region, followed by Fisher's Protected LSD *post hoc* tests where appropriate. LMMs with *treatment* (or *antagonist*) and *stress* as between-subjects factors, and *region* as the within-subjects factor were performed to analyze the 5-HTP data sets of *Experiments 2* and *3*. Significant correlations of 5-HTP content in serotonergic regions and 5-HTP content in target regions, and of 5-HTP content in the PnC and maximum ASR were identified and graphed using SigmaPlot (version 11, Systat Software, Inc., Chicago, IL, USA). Significance was accepted at $p < 0.05$, and results are shown as the mean + the standard error of the mean (SEM).

3. RESULTS

For effects of chronic CORT treatment on body weight gain, weekly water and food consumption, the diurnal rhythm of water and food consumption, thymus weight, adrenal weight, basal and stress-induced plasma CORT concentrations, and on *ex vivo* adrenal functionality under basal conditions or following AS stress please refer to supplemental results online (see Fig. S2, Fig. S3 and Table S1).

3.1. Chronic CORT increases light phase TPH2 protein expression in anxiety-related serotonergic systems

Experiment 1 revealed that chronic CORT treatment abolishes the diurnal variation of TPH2 protein expression in the DRD and DRV, but not in the MnR (*treatment*, $F(1, 30) = 6.48$, $p < 0.05$; *light/dark*, $F(1, 30) = 8.01$, $p < 0.01$; *treatment x light/dark*, $F(1, 28) = 3.20$, $p = 0.34$). This was due to increased light phase TPH2 protein expression in the DRD and DRV of CORT-treated rats (Fig. 2, panels A and B). In contrast, a decrease in dark phase TPH2 protein expression in the MnR in chronically CORT-treated rats, compared to vehicle controls, approached statistical significance ($p = 0.063$) (Fig. 2, panels A and B). Other midbrain serotonergic regions (DRC, DRI, DRVL/VLPAG) neither displayed a significant diurnal variance of TPH2 protein nor a treatment effect.

3.2. Anxiety- and depressive-like behavior after chronic CORT treatment

On the EPM, CORT-treated rats of *Experiments1* and *2* displayed increased anxiety-like behavior as they spent less time exploring the open arms ($p = 0.003$, Fig. 2, panel C, left side; $p = 0.007$, Fig. 2, panel D, left side) and entered the open arms less often ($p = 0.037$, Fig. 2, panel C, right side; $p = 0.009$, Fig. 2, panel D, right side) compared to vehicle-treated rats. Conversely, CORT-treated rats displayed more time exploring the closed arms

compared to vehicle-treated rats ($p = 0.042$, Fig. 2, panel D, left side) and reared more often at the walls of the closed arms ($p = 0.048$, Fig. 2, panel C, right side; $p = 0.005$, Fig. 2, panel D, right side).

In the FST, CORT-treated rats of *Experiments 1* and *2* spent more time immobile ($p = 0.008$, Fig. 2, panel E; $p = 0.042$, Fig. 2 panel F). In *Experiment 1*, this was due to decreased climbing behavior ($p = 0.048$, Fig. 2, panel E). In *Experiment 2*, vehicle-treated rats displayed a more active escape-seeking behavior by diving more often to the bottom of the tank, while CORT-treatment prevented diving ($p = 0.040$, data not shown). No treatment effect of CORT was detected on the decrease in body temperature while swimming (delta rectal temperature, Fig. 2, panels E and F) or on the number of fecal boli left in the swim tank after each individual FST session.

3.3. Chronic CORT alters basal and stress-induced TPH2 activity in anxiety-related serotonergic systems

Two major effects of chronic CORT-treatment on TPH2 activity were detected within brainstem serotonergic systems in *Experiment 2* (*treatment*, $F(1, 30) = 8.37$, $p < 0.01$; *stress*, $F(1, 30) = 9.14$, $p < 0.01$). First, in the DRD, DRV and MnR chronic CORT-treatment elevated the basal activity of TPH2 (Fig. 3 panels A and B). In the DRV this elevation of basal TPH2 activity after chronic CORT resulted in lost responsiveness to AS stress. Both the DRV and the MnR of vehicle-treated controls responded with significantly enhanced TPH2 activity following AS stress. Second, chronic CORT-treatment sensitized specific subdivisions of the DR to AS stress. The DRD and DRC of vehicle-treated rats did not respond with changes in TPH2 activity following AS stress, but the DRD and DRC of CORT-treated rats responded with stress-induced increases in TPH2 activity (Fig. 3, panel A, gray background), indicating that chronic CORT exposure is necessary or permissive for an increase in TPH2 activity in these anxiety-relevant regions, following a novel stressor. A significant interaction of *stress* and *treatment* was indeed responsible for the AS-induced increase in TPH2 activity in the DRD ($F(1, 28) = 3.42$, $p < 0.05$), but not in the DRC. In contrast, the MnR of both vehicle- and CORT-treated rats displayed increased TPH2 activity in response to AS stress (Fig. 3, panel B). Neither the DRVL/VLPAG nor the DRI or the average of all subdivisions (entire DR) displayed altered TPH2 activity in response to chronic CORT-treatment or AS stress.

Many mesolimbocortical target areas of midbrain serotonergic systems, including the ventral orbital cortex (VO), the prelimbic cortex (PrL), the bed nucleus of the stria terminalis (BNST), the basolateral amygdala (BL), the central amygdala (CE), the entorhinal cortex (Ent), and the dorsolateral and lateral periaqueductal gray (DLPAG/LPAG) responded with increased TPH2 activity to AS stress in vehicle-treated rats (*stress*, $F(1, 30) = 13.56$, $p < 0.01$). Please see Fig. S4 in the online supplemental material for details. Depending on the region, chronic CORT treatment had varying effects on AS-induced TPH2 activity (*treatment*, $F(1, 30) = 2.36$, $p = 0.09$; *treatment x region x stress*, $F(1, 28) = 3.55$, $p = 0.01$). In the BL and Ent the AS-induced TPH2 increase was blunted by chronic CORT treatment (Fig. S4, supplemental material), but in the lateral orbital cortex (LO), the infralimbic cortex (IL), the CA1 region of the dorsal hippocampus (dCA1), and in the caudal pontine reticular

nucleus (PnC) chronic CORT-treatment was permissive for an AS-induced increase of TPH2 activity (Fig. 3, panels D, E, F and G). The Ent was the only non-serotonergic brain region where chronic CORT-induced a basal increase of TPH2 activity (Fig. S4, supplemental material).

3.4. Local CRHR2 blockade prevents, while CRHR1 blockade potentiates stress-induced TPH2 activity

In *Experiment 3* all rats received chronic CORT via the drinking water. By locally blocking CRHR1 or CRHR2 within the DR of chronically CORT-treated rats, we discovered both an antagonist effect and an interaction between antagonist and AS stress (*antagonist*, $F(2, 42) = 11.77$, $p < 0.001$; *antagonist x stress*, $F(1, 39) = 14.97$, $p < 0.001$). As in *Experiment 2*, AS stress increased TPH2 activity in the DRD, DRC and MnR of rats that were microinjected with vehicle (Fig. 4, panels A and B). In the anxiety-related DRD and DRC this AS-induced increase of TPH2 activity was prevented by intra-DR microinjection of A30, the CRHR2 antagonist. A30 had no effect on AS-induced TPH2 activity in the MnR, which is located further from the injection site relative to the DR. Antalarmin, the CRHR1 antagonist, on the contrary, interacted with AS stress to increase or further potentiate TPH2 activity in the entire DR, including the DRD, DRC, DRV and DRVL/VLPAG (Fig. 4, panel A). Antalarmin had no effect on AS-induced TPH2 activity in the MnR, which, as mentioned above, is positioned further from the injection site. No significant antagonist or stress effect was detected in the DRI, and neither antagonist altered TPH2 activity under home cage control conditions. For results within the selected 15 mesolimbocortical target regions innervated by midbrain serotonergic systems please refer to Figure S5 in the supplemental materials section online.

3.5. Correlation of TPH2 activity in the DR with amygdalar regions and the PnC

To identify simultaneous serotonergic activity in anxiety-related networks under control conditions and during AS stress, we averaged the 5-HTP content in the DRD and DRC (DRD/DRC), and performed correlation analyses with 5-HTP content in all of the 15 selected projection regions, including the amygdala (BL and CE), of both home cage control rats and rats exposed to AS stress, irrespective of drug treatment.

In *Experiment 2*, increased TPH2 activity in the DRD/DRC under home cage control conditions was significantly correlated with increased TPH2 activity in the BL ($p = 0.019$, Fig. 3, panel H). In *Experiment 3*, elevated TPH2 activity in the DRD/DRC under home cage conditions also strongly predicted elevated TPH2 activity the BL ($p < 0.001$, Fig. 4, panel E) as well as in the CE ($p = 0.002$, Fig. 4, panel F). During AS stress in *Experiment 2*, TPH2 activity in the DRD/DRC was strongly correlated with TPH2 activity in the CE ($p = 0.001$, Fig. 3, panel I). These correlations of synchronous 5-HT synthesis in the dorsocaudal DR and the amygdalar complex are in concert with studies revealing that both the BL and the CE are innervated by the dorsocaudal DR (Commons et al., 2003; Hale et al., 2008a), and suggest that in our model serotonergic projections from the DRD/DRC to the BL may be more active under control/resting conditions, whereas AS stress may cause serotonergic projections from the DRD/DRC to the CE to become more active.

In *Experiment 2*, stress-induced TPH2 activity in the DRD (by itself) was correlated with TPH2 activity in the PnC ($p = 0.029$, Fig. 3, panel J), suggesting that AS stress causes PnC-projecting serotonergic neurons in the DRD to become more active and contribute to the PnC-mediated control of the acoustic startle reflex.

3.6. Chronic CORT and CRHR1 blockade potentiate the acoustic startle response

Chronic exposure to CORT in the drinking water during *Experiment 2* increased the maximum acoustic startle response (ASR) compared to vehicle controls (Fig. 5, panel A; $p < 0.001$). Furthermore, a strong correlation of 5-HTP in the PnC and the maximum ASR was found when the values of both vehicle- and CORT-treated rats were plotted (Fig. 5, panel B; $r = 0.868$, $r^2 = 0.753$, $p < 0.001$, $N = 16$). In *Experiment 3*, intra-DR microinjection of the CRHR1 antagonist antalarmin potentiated the maximum ASR compared to rats that received intra-DR microinjections of vehicle (Fig. 5, panel C; $F = 4.16$, $p < 0.05$). Again, we found a strong correlation of 5-HTP in the PnC and the maximum ASR when the values of all groups were plotted (Fig. 5, panel D; $r = 0.862$, $r^2 = 0.743$, $p < 0.001$, $N = 22$). Although interpretation of the startle results may be confounded by the systemic NSD-1015 injection of all rats immediately prior to AS stress, a to date unpublished study by Lowry et al. found that high concentrations of intracerebroventricular CRH, which are likely to saturate CRHR1 and then activate CRHR2, leads to a pronounced increase of 5-hydroxyindoleacetic acid (5-HIAA), the main metabolite of 5-HT, in the PnC (see Fig. S6 in supplemental materials, Lowry et al., unpublished observation).

4. DISCUSSION

We show that stress-induced increases in TPH2 activity are enhanced following chronic glucocorticoid administration, that this effect is region-specific, and that it depends on the balance of local CRHR1 and CRHR2 actions. Chronic CORT administration caused anxiety- and depressive-like behavior and increased basal TPH2 protein expression and also the enzyme's activity in anxiety-related serotonergic systems. Most notably, chronic CORT was necessary for stress-induced increases in TPH2 activity in the DRD/DRC, a region crucially involved in learned helplessness. We also revealed that stress-induced increases in TPH2 activity in the DRD/DRC of CORT-primed rats depend on local CRHR2 activation. In contrast, blockade of CRHR1 potentiated AS-induced TPH2 activity.

Chronic CORT administration increased anxiety-like behavior in the EPM, enhanced startle, and caused increased immobility in the FST. These results reproduce our previous findings (Donner et al., 2012) and resemble comparable rodent glucocorticoid paradigms (Murray et al., 2008). Lee et al. (2010) (Lee et al., 2010) delivered the same CORT dose (100 ug/ml) via the drinking water to adrenal-intact, adult mice for 28 days, resulting in physiological stress levels of CORT in the plasma at the end of treatment (on average 250 ng/ml versus 100 ng/ml in vehicle-treated controls). Elevated anxiety in our model may be due to glucocorticoid-induced increases in *Crh* expression in the BNST, which projects heavily to the DRD/DRC region (Peyron et al., 1998). Both chronic stress and chronic glucocorticoid exposure elevate *Crh* mRNA expression in the BNST (Makino et al., 1994), and overexpression of *Crh* in the BNST alters CRHR2 binding specifically in the DRD/DRC (Sink et al., 2012). The BNST mediates anxiety-like behavioral responses, but is also

required for learned helplessness as BNST lesions prevent exaggerated fear conditioning and escape deficits observed 24 h following inescapable stress (Hammack et al., 2004). Learned helplessness in turn is thought to be dependent on functional desensitization of 5-HT_{1A} receptors within the DRD/DRC (Rozeske et al., 2011), resulting in a sensitization of the anxiety-related DRD/DRC serotonergic neurons.

Consistent with these behavioral effects, chronic CORT increased basal (non-stress) light phase TPH2 protein expression and TPH2 activity in anxiety-related subdivisions of the DR, most notably in the DRD. Previously, we found that the same CORT dose also increases light phase *Tph2* mRNA expression, most significantly in the DRD/DRC (Donner et al., 2012). *Tph2* displays a natural diurnal rhythm with higher expression during the dark phase than during the light phase (Malek et al., 2004; Malek et al., 2007). Interestingly, expression of *Tph2* mRNA and protein during the dark phase, when the water/CORT intake of nocturnally active rats is much higher than during the light phase, when rats mostly rest, remained largely unaffected by chronic CORT treatment. TPH2 is stabilized and its output increased by phosphorylation of serine 19 (Murphy et al., 2008), so this phosphorylation could reach a ceiling effect during the dark phase.

In the present experiments, basal TPH2 activity in the DRD/DRC was furthermore positively correlated with basal TPH2 activity in the BL, which is innervated by serotonergic neurons in the DRD/DRC and is a nodal structure in control of anxiety states and anxiety-related behavior (2008). Within the DR, the DRD/DRC gives rise to the highest number of BL-projecting neurons, and the vast majority of neurons projecting from the DRD to the BL is serotonergic, suggesting that tonic serotonergic input from the DRD/DRC influences the BL (Asan et al., 2013). Likewise, the CRHR2-selective neuropeptide urocortin 2, when microinjected into the DR, increases 5-HT release in the BL (Amat et al., 2004), where 5-HT actions on excitatory 5-HT_{2C} receptors are required to produce the anxiety-like behavioral effects of learned helplessness (Christianson et al., 2010).

Basal TPH2 activity in the DRD/DRC following chronic CORT was also correlated with TPH2 activity in the CE. Tracing studies have revealed direct projections from the DRD/DRC to both the BL and CE (Halberstadt and Balaban, 2006; Hale et al., 2008). Basal light phase *Tph2* mRNA (Donner et al., 2012) and TPH2 activity (present study) were also elevated in the DRV following chronic CORT treatment, which may influence sensorimotor function and complex cognitive tasks (Hale and Lowry, 2011).

Chronic CORT was permissive for AS-induced increases in TPH2 activity, specifically in the DRD/DRC. This result is consistent with findings by Singh et al. who reported that repeated systemic dexamethasone injections can restore noise-stress induced increases in *ex vivo* TPH activity in the brain tissue of adrenalectomized rats, whereas adrenalectomy, treatment with a glucocorticoid receptor antagonist, and amygdalar lesions all block *ex vivo* TPH activity (1990; 1994). Activation of c-Fos expression in DRD/DRC serotonergic neurons is observed following exposure to noise stressors (Evans et al., 2009), anxiogenic stimuli such as exposure to an open-field (Hale et al., 2008), intracerebral injections of CRH-related peptides including the CRHR2-selective agonist urocortin 2 (Evans et al., 2009), systemic treatment with various anxiogenic drugs (Abrams et al., 2005), inescapable

shock (Rozeske et al., 2011), and social defeat (Paul et al., 2011). Collateral projections from the DRD/DRC innervate functionally related targets that are in control of anxiety-like behavior and stress responses (Hale and Lowry, 2011). In a transgenerational rat model of anxiety due to intimate partner violence, serotonergic neurons in the DRD/DRC of both the aggressor-exposed F0 mothers and the unexposed F1 daughters displayed a four-fold increase of c-Fos activation when confronted with an unfamiliar male (Cordero et al., 2012), suggesting that the excitability of DRD/DRC serotonergic neurons is subject to epigenetic imprinting. Serotonergic neurons in the DRD/DRC possess a distinct neurochemical profile. In contrast to other DR subdivisions, the DRD/DRC contains serotonergic neurons that co-express CRH, and these CRH/5-HT neurons are heavily innervated by neurokinin 1 (NK1) receptor-immunoreactive glutamatergic fibers (Commons et al., 2003). Local application of substance P, a natural NK1 receptor agonist, activates 5-HT neurons in the DRD/DRC, but suppresses activation of other DR subdivisions (Valentino and Commons, 2005). The DRD/DRC also densely expresses vesicular glutamate transporter 3 (VGLUT3), and genetic VGLUT3 deletion results in increased anxiety and reduced 5-HT_{1A}-mediated autoinhibition of serotonergic neurotransmission in limbic target sites (Amilhon et al., 2010). Delivery of CRH into the DR causes immediate 5-HT release in the CE and subsequent 5-HT release in the medial prefrontal cortex, the latter of which is blocked by intra-DR CRHR2 antagonism or by chemical inactivation of the MnR (Forster et al., 2008). In concert with these findings, stress-induced increases of TPH2 activity in the DRD/DRC predicted TPH2 activity in the CE in our studies.

Experiment 3 revealed that chronic CORT-mediated increases of DRD/DRC TPH2 activity following AS stress depend on local CRHR2 actions. *In situ* hybridization histochemistry, immunohistochemistry, western blotting, and quantitative RT-PCR confirm that CRHR2 is expressed within the DR of rodents and primates, including DRD/DRC serotonergic neurons (Day et al., 2004; Sanchez et al., 2010; Lukkes et al., 2011), and intracerebroventricular infusion of urocortin 2, a natural CRHR2 agonist, activates c-Fos expression in the DRD/DRC (Hale et al., 2010). Studies demonstrating that chronic stress or glucocorticoid exposure elevate *Crh* mRNA expression in the BNST (Makino et al., 1994), and that experimental overexpression of CRH in the lateral BNST increases contextual fear while altering CRHR2 binding specifically in the caudal DRD/DRC (Sink et al., 2012), corroborate the notion that a dysfunctional, CRHR2-mediated signaling pathway within the BNST-DRD/DRC circuitry promotes chronic anxiety states. In comparison, infusion of CRH into the rostral DR fails to produce learned helplessness, but CRH or urocortin 2 infusion into the caudal DR mimics, and CRHR2 antagonism blocks learned helplessness (Hammack et al., 2003b). Interestingly, low doses of intra-DR CRH prevent learned helplessness-induced escape deficits (Hammack et al., 2003a), indicating that at low concentrations CRH exerts opposite effects, probably by acting on CRHR1, which binds CRH with a higher affinity than CRHR2 (Hillhouse and Grammatopoulos, 2006). Similarly, Lukkes et al. (2008) found that intra-DR antagonism of CRHR1 abolishes intra-DR CRH-induced decreases in nucleus accumbens 5-HT release, while CRHR2 antagonism blocks intra-DR CRH-induced increases in accumbal 5-HT release. Also, stress exposure, such as FST swimming, changes the distribution and availability of CRH receptors by trafficking more CRHR2 from the cytosol to the cell membrane of serotonergic DR neurons, while

CRHR1 internalizes into the cytosol, potentiating CRHR2-mediated actions under conditions of repeated or chronic stress (Waselus et al., 2009).

According to the proposed opposing actions of CRHR1 and CRHR2 in control of anxiety-related serotonergic systems, local blockade of CRHR1 in the DR increased acoustic startle-induced TPH2 activity in the DRD/DRC and in the DRV. Comparably small amounts of CRHR1 are expressed within the DR and its inhibitory surround (Day et al., 2004), and more so on non-serotonergic, for example GABAergic neurons (Kirby et al., 2008). *Ex vivo*, CRH has been shown to inhibit 5-HT-neuronal activation through CRHR1-induced GABA release onto 5-HT neurons, and *in vivo* both swim stress and CRH act on CRHR1 within the DR to decrease 5-HT-neuronal activation and 5-HT release in relevant forebrain targets (Valentino et al., 2010).

After integrating our results into the published literature, we developed a hypothetical model of how stress-induced CRHR1- versus CRHR2-activation on separate neuron types within the DRD/DRC region may keep stress- and anxiety-related serotonergic systems in the balance, and how an increased CRF drive from the BNST, for example caused by chronically elevated glucocorticoid levels, may shift this balance towards proportionally more CRHR2 activation in the DRD/DRC, leading to more BL-dependent conflict anxiety (see EPM results) and to a more pronounced PnC-dependent startle response (see Fig. 6 for details). The PnC is a hindbrain region that integrates acoustic stimuli, directly commands the startle response (Simons-Weidenmaier et al., 2006), and may also elicit muscle twitching during REM sleep (Homma et al., 2002). Anxiety is known to enhance the startle response, 5-HT itself enhances the startle reflex (Davis, 1980), and tracing studies confirmed that neurons from the serotonergic DR innervate the pontine reticular formation (Hobson et al., 1986). Within the PnC, immunohistochemistry identified excitatory 5-HT_{2C} receptors on giant PnC neurons, suggesting that serotonin serves to increase the excitability of giant PnC neurons (Weber et al., 2008).

In summary, our data strongly support the hypothesis that glucocorticoids are permissive for CRHR2-dependent, stress-induced 5-HT synthesis in anxiety-related serotonergic systems, and that CRHR1 serves to oppose or restrain these effects. This is the first study demonstrating direct interplay of CRHR1 and CRHR2 within the DR in balancing stress-induced *in vivo* 5-HT synthesis, and suggests a role for these interactions in the pathophysiology of anxiety and affective disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. Chronic corticosterone abolished the diurnal variance of TPH2 protein expression.
2. Chronic corticosterone sensitized *in vivo* TPH2 activity towards acoustic startle.
3. Local CRHR1 blockade potentiated stress-induced TPH2 activity increases in the DR.
4. Local CRHR2 blockade prevented stress-induced TPH2 activity increases in the DR.
5. TPH2 activity in the caudal pontine reticular nucleus predicted startle response.

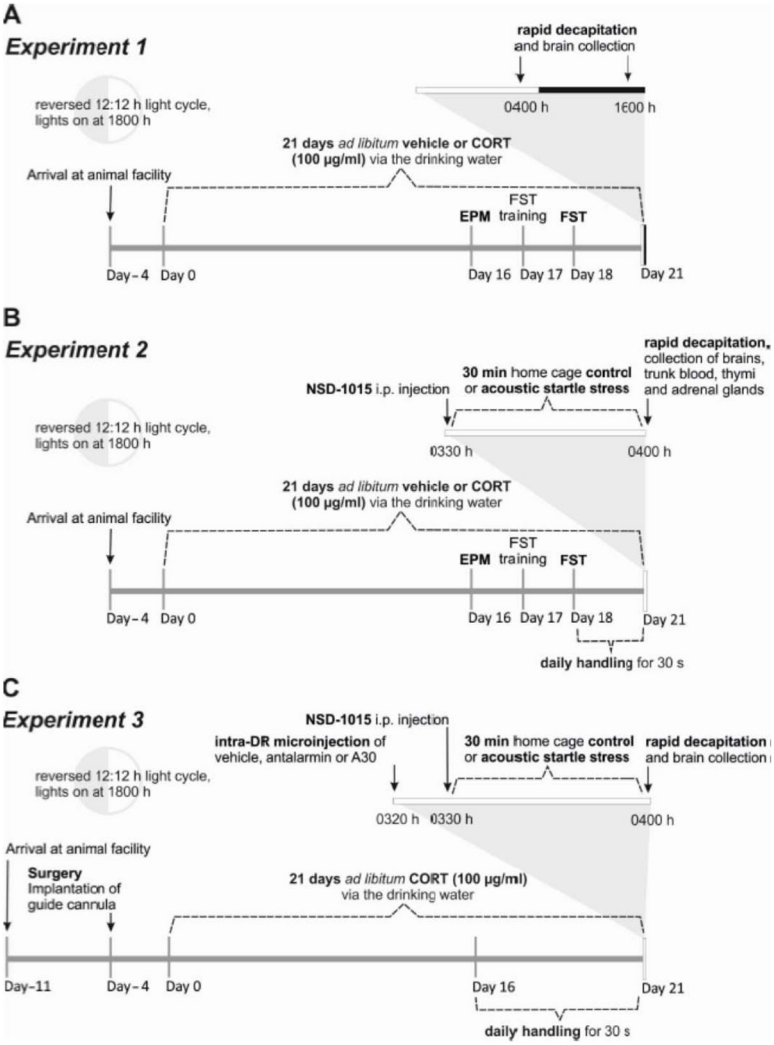

Figure 1. Experimental Design

Timelines of *Experiments* (A) *1*, (B) *2*, and (C) *3*. (A) Adult, male rats (N = 32) were treated with vehicle (n = 16) or corticosterone (CORT, 100 µg/ml, n = 16) via the drinking water for 21 days to quantify TPH2 protein expression during the light and dark phases of the light cycle. (B) Adult, male rats (N = 32) were treated with vehicle (n = 16) or CORT (n = 16) via the drinking water for 21 days to assess the rats' emotionality on the elevated plus-maze (EPM) and in the forced swim test (FST), to measure basal and acoustic startle stress (AS)-induced TPH2 activity, and to record treatment effects on plasma CORT concentrations, adrenal weight, and thymus weight. (C) Adult, male rats (N = 60) received stereotaxic implants of a unilateral guide cannula targeting the dorsocaudal parts of the dorsal raphe nucleus (DRD/DRC). After all rats were chronically treated with CORT for 21 days, they received either an intra-DR microinjection of vehicle (n = 20), antalarmin (n = 20) or antisauvagine-30 (A30, n = 20) 10 min prior to 30 min of AS stress or home cage control conditions (3 × 2 design). TPH2 activity was measured in the form of *in vivo* 5-hydroxytryptophan (5-HTP) accumulation after i.p. injection of 100 mg/kg of the aromatic L-amino acid decarboxylase inhibitor NSD-1015 immediately prior to AS stress.

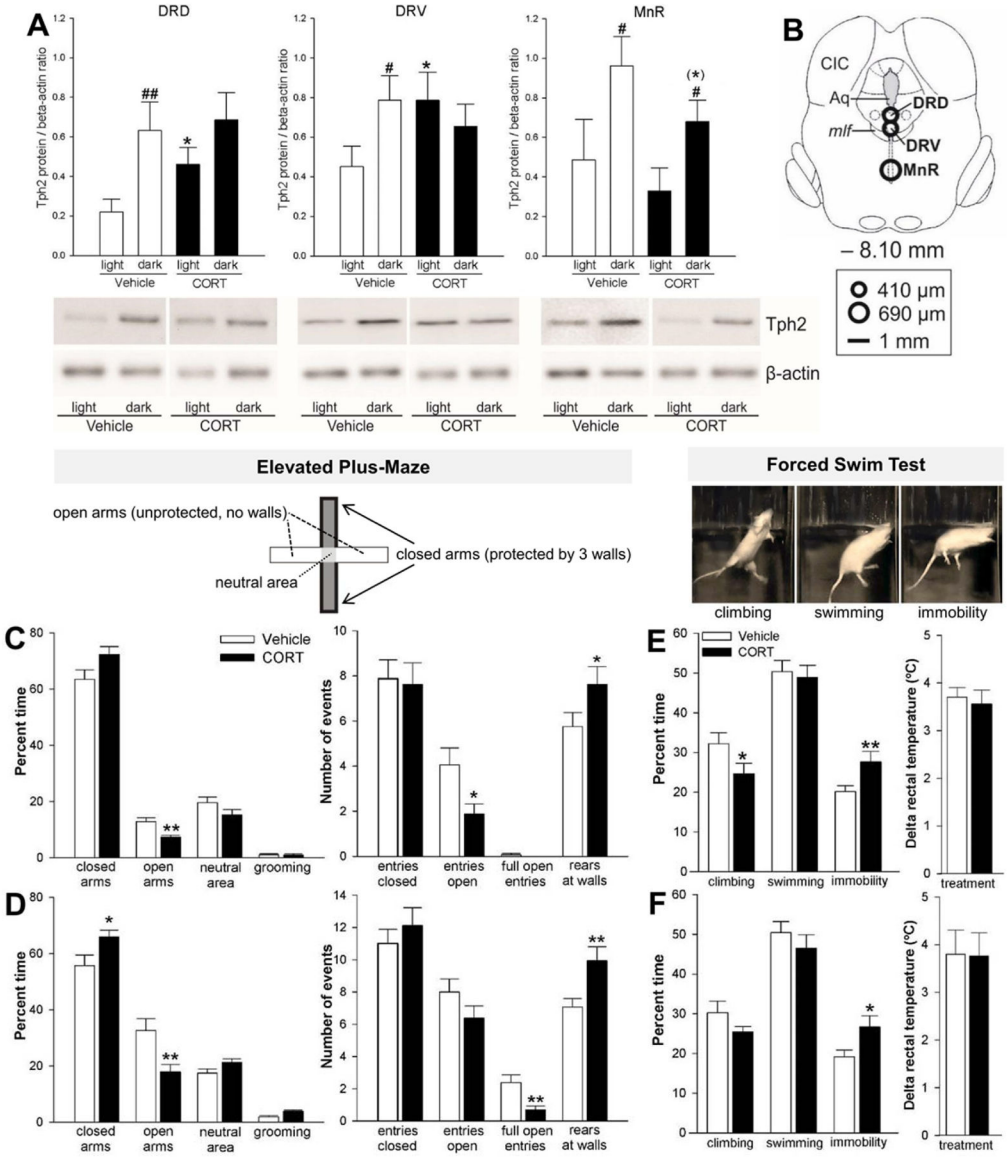

Figure 2. Effects of chronic CORT on behavior and TPH2 protein expression

(A) Light phase and dark phase TPH2 protein expression in the dorsal (DRD) and ventral part (DRV) of the dorsal raphe nucleus, and in the median raphe nucleus (MnR) following treatment with chronic corticosterone (CORT) or vehicle. Half of each treatment group was sacrificed +10 h into the (active) dark phase at 1600 h, the other half +10 h into the (inactive) light phase at 0400 h. The photomicrographs depict representative, color-inverted images of enhanced chemoluminescence detection of TPH2 protein content in bands from region-specific western blots in comparison to β-actin protein, the loading control. #$p <$ 0.05, ##$p < 0.01$ light/dark effect within the same treatment group; *$p < 0.05$ CORT-treatment effect versus vehicle-light group, $^{(*)}p = 0.063$ versus vehicle-dark group, after *post hoc* analysis with Fisher's Protected LSD test (Vehicle-light, n = 8; Vehicle-dark, n = 8; CORT-light, n = 8; CORT-dark, n = 8). (B) Diameter and location of microdissection samples at a representative neuroanatomical section adapted from Paxinos and Watson

(1998) at bregma level −8.10 mm. (C) Anxiety-related behavioral parameters of rats from *Experiment 1* and (D) *Experiment 2*, as assessed on the elevated plus-maze (EPM) during the rats' active (dark) phase on day 16 of treatment with either vehicle or CORT. Panels on the left side depict the percent time the rats spent either in the closed arms, the open arms, the neutral area, or grooming. The number of entries into either arm type, and the number of rears at the walls are indicated in the panels on the right side. Active (climbing and swimming) versus passive (floating) stress coping behavior was assessed on day 18 of treatment in the forced swim test (FST), again in both (E) *Experiment 1* and (F) *Experiment 2*. On the right side are the percentages of time the rats spent climbing, swimming, or immobile. The panels on the right side display the difference in rectal temperature (delta temperature, °C) from before and after the FST. Further abbreviations: Aq, cerebral aqueduct; CIC, central nucleus of the inferior colliculus; *mlf*, medial longitudinal fasciculus. Data are displayed as means + SEM. *$p < 0.05$, **$p < 0.01$, versus Vehicle (Vehicle, n = 16; CORT, n = 16).

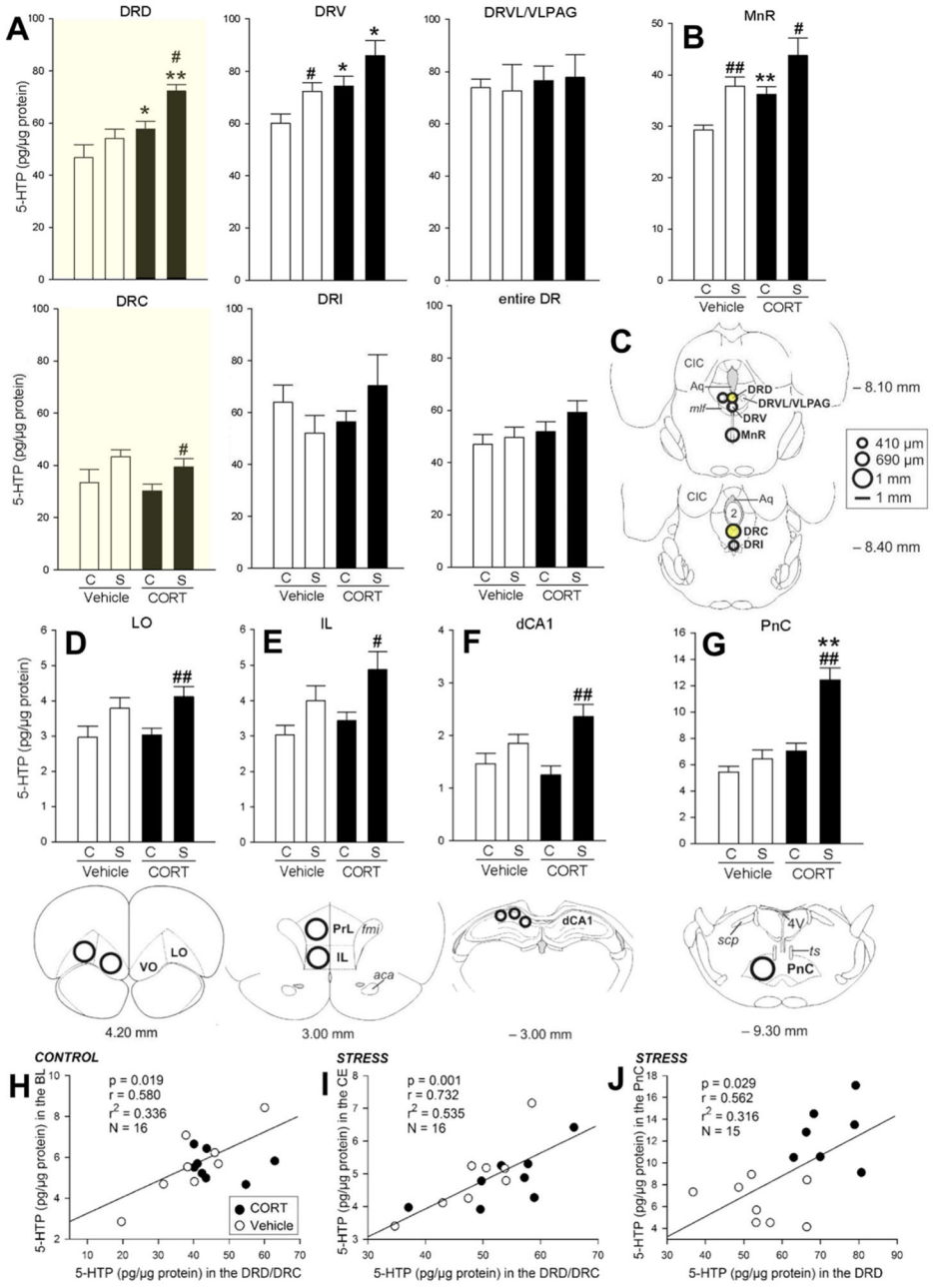

Figure 3. Effects of chronic CORT and acute stress on TPH2 activity

The bar graphs display the region-specific amount of 5-hydroxytryptophan (5-HTP) in pg/µg total protein, as a measure of the enzymatic activity of TPH2, following a 2 × 2 design of treatment (Vehicle or corticosterone (CORT), via the drinking water for 21 days) and home cage control conditions ('C') versus acoustic startle stress ('S') on day 21. Displayed are TPH2 activity in (A) each functional subdivision of the dorsal raphe nucleus (DR), including the entire DR, (B) the median raphe nucleus (MnR), (D) the lateral orbital cortex (LO), (E) the infralimbic cortex (IL), (F) the CA1 region of the dorsal hippocampus (dCA1), and (G) the caudal pontine reticular nucleus (PnC). Panels H, I and J display significant correlations

of TPH2 activity in the DRD/DRC or the DRD by itself with TPH2 activity in the basolateral amygdala (BL), the central amygdala (CE) and the PnC. Panel C maps the neuroanatomical location and diameter of microdissections within the serotonergic DR and MnR. Further abbreviations: 2, second cerebellar lobule; 4V, fourth ventricle; aca, anterior commissure, anterior part; Aq, cerebral aqueduct; CIC, central nucleus of the inferior colliculus; DRC, dorsal raphe nucleus, caudal part; DRD dorsal raphe nucleus, dorsal part; DRI, dorsal raphe nucleus, interfascicular part; DRV, dorsal raphe nucleus, ventral part; DRVL/VLPAG, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; *fmi*, forceps minor of the corpus callosum; *mlf*, medial longitudinal fasciculus; PrL, prelimbic cortex; *ts*, tectospinal tract; VO, ventral orbital cortex. Data are displayed as means + SEM. #$p < 0.05$, ##$p < 0.01$ stress effect within the same treatment group; *$p < 0.05$, **$p < 0.01$ treatment effect versus respective vehicle group, after *post hoc* analysis with Fisher's Protected LSD test (Vehicle-Control, n = 8; Vehicle-Stress, n = 8; CORT-Control, n = 8; CORT-Stress, n = 8).

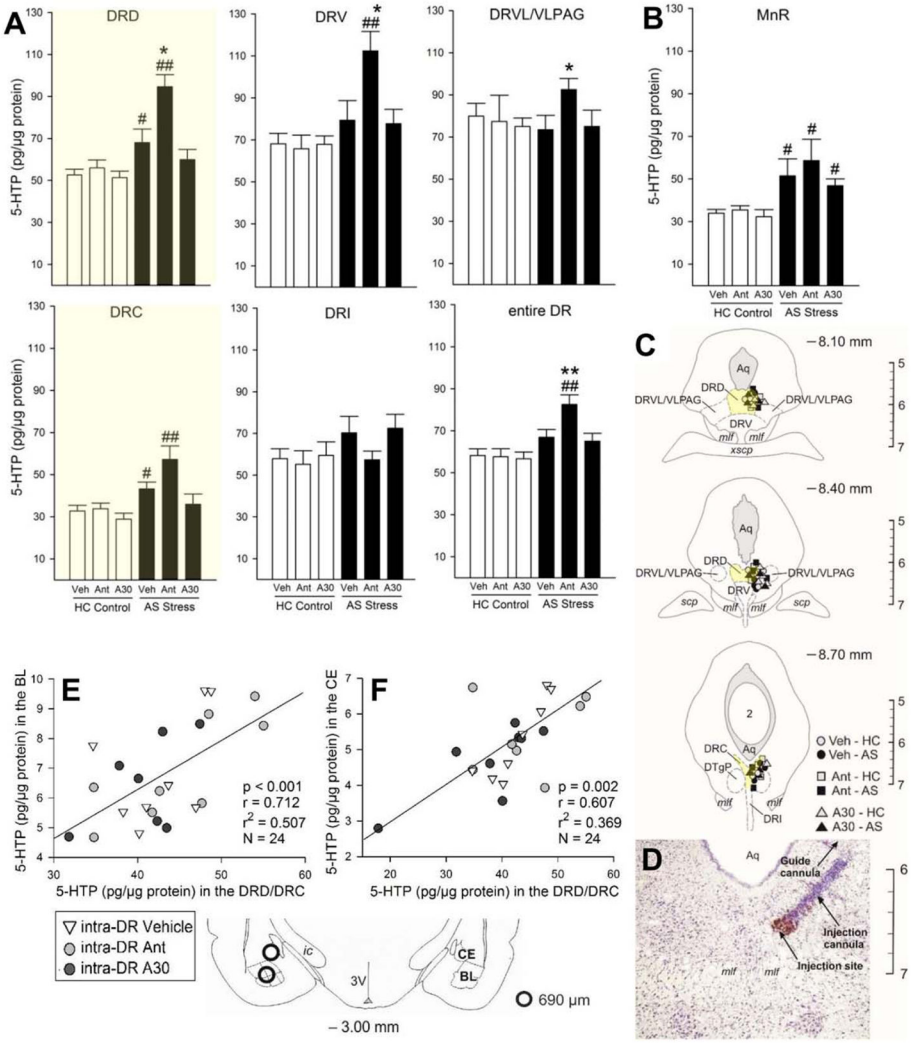

Figure 4. Effects of intra-DR microinjections of CRHR1 and CRHR2 antagonists on stress-induced TPH2 activity

Graphs illustrating the amount of 5-hydroxytryptophan (5-HTP) measured in pg/µg total protein in (A) each functional subdivision of the dorsal raphe nucleus (DR) and in (B) the median raphe nucleus (MnR), following a 2 × 3 design of stress (home cage, HC control; versus acoustic startle, AS stress) and intra-DR antagonist microinjection (vehicle, Veh; antalarmin, Ant; and antisauvagine-30, A30). All rats in *Experiment 3* were exposed to chronic corticosterone (CORT) via the drinking water for 21 days. Data are displayed as means + SEM. #$p < 0.05$, ##$p < 0.01$ stress effect versus the HC control group of the same antagonist microinjection; *$p < 0.05$, **$p < 0.01$ antagonist effect versus the AS stress-group that received microinjection of vehicle, after *post hoc* analysis with Fisher's Protected LSD test (Veh-HC, n = 8; Veh-AS, n = 7; Ant-HC, n = 8; Ant-AS, n = 7; A30-HC, n = 8; A30-AS, n = 9). Panel C summarizes the verification and mapping of cannulae placements. Unilateral stereotaxic placements of guide and injection cannulae were verified via histochemistry and comparison with a rat brain atlas (Paxinos and Watson, 1998). Depicted are those injection sites between −8.10 mm and −8.70 mm from bregma that were

considered within target. (D) Representative photomicrograph of guide and injection cannula tracks and of an injection site itself at −8.40 mm from bregma in a 25 μm-thick brain section stained histochemically with cresyl violet. The vertical scales in panels C and D indicate the dorsoventral coordinates in millimeters. Panels E and F display significant correlations of TPH2 activity in the dorsocaudal DR (DRD/DRC) and TPH2 activity in the basolateral (BL) and central amygdala (CE) under home cage control conditions, and representative BL and CE microdissection sites and diameters at bregma level -3.00 mm. Further abbreviations: 2, second cerebellar lobule; Aq, cerebral aqueduct; DRD, dorsal raphe nucleus, dorsal part; DRV, dorsal raphe nucleus, ventral part; DRVL/VLPAG, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; DRC, dorsal raphe nucleus, caudal part; DRI, dorsal raphe nucleus, interfascicular part; DTgP, dorsal tegmental nucleus, pericentral part; *mlf*, medial longitudinal fasciculus; *scp*, superior cerebellar peduncle; *xscp*, decussation of the superior cerebellar peduncle (Veh-HC, n = 8; Veh-AS, n = 7; Ant-HC, n = 8; Ant-AS, n = 7; A30-HC, n = 8; A30-AS, n = 9).

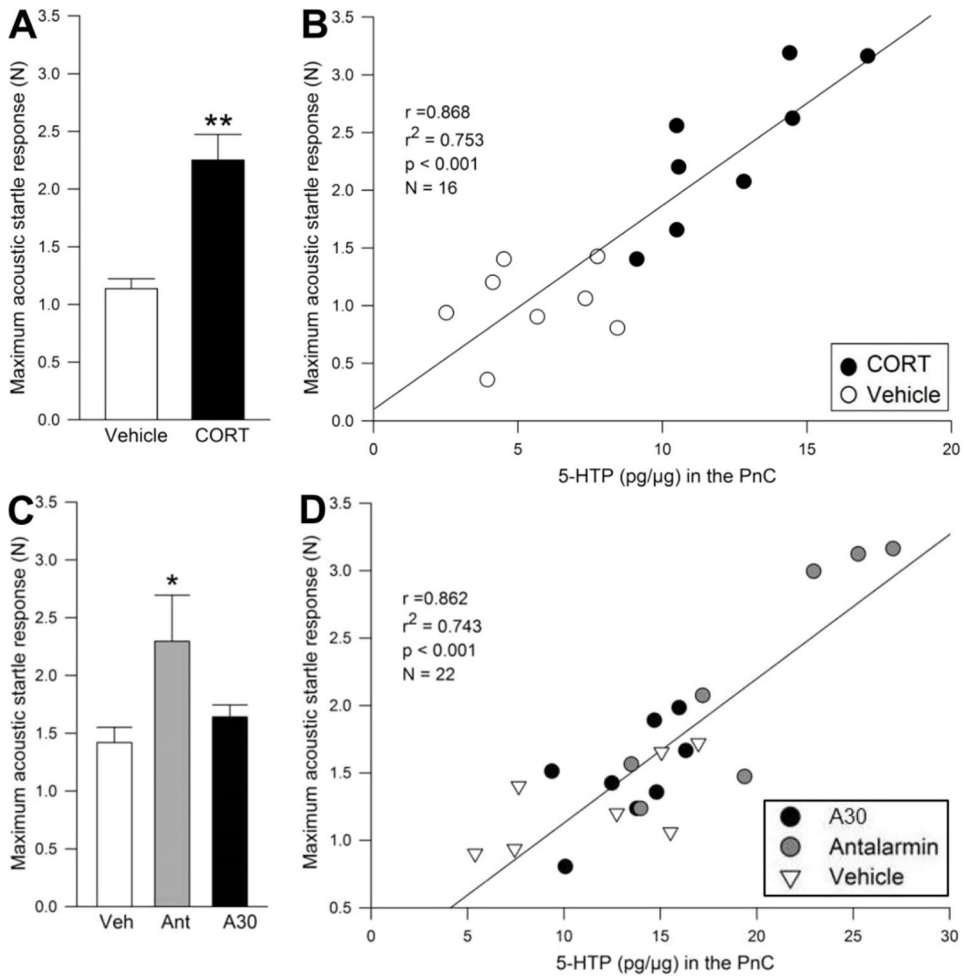

Figure 5. Effects of chronic CORT and CRHR1 antagonist on the startle response

Acoustic startle (AS) responses as measured during the 30 min exposure to AS stress on day 21 in (A) *Experiment 2* and (C) *Experiment 3*, and correlation of 5-hydroxytryptophan (5-HTP in pg/µg total protein) measured in the caudal pontine reticular nucleus (PnC), and the maximum startle response in *Experiments 2* and *3* (panels B and D, respectively). The maximum acoustic startle response is displayed in Newton, N. Further abbreviations: A30, antisauvagine-30; Ant, antalarmin, CORT, corticosterone. Data are displayed as means + SEM. *$p < 0.05$ versus Veh (Veh, n = 7; Ant, n = 7; A30, n = 9); **$p < 0.01$ versus Vehicle (Vehicle, n = 8; CORT, n = 8).

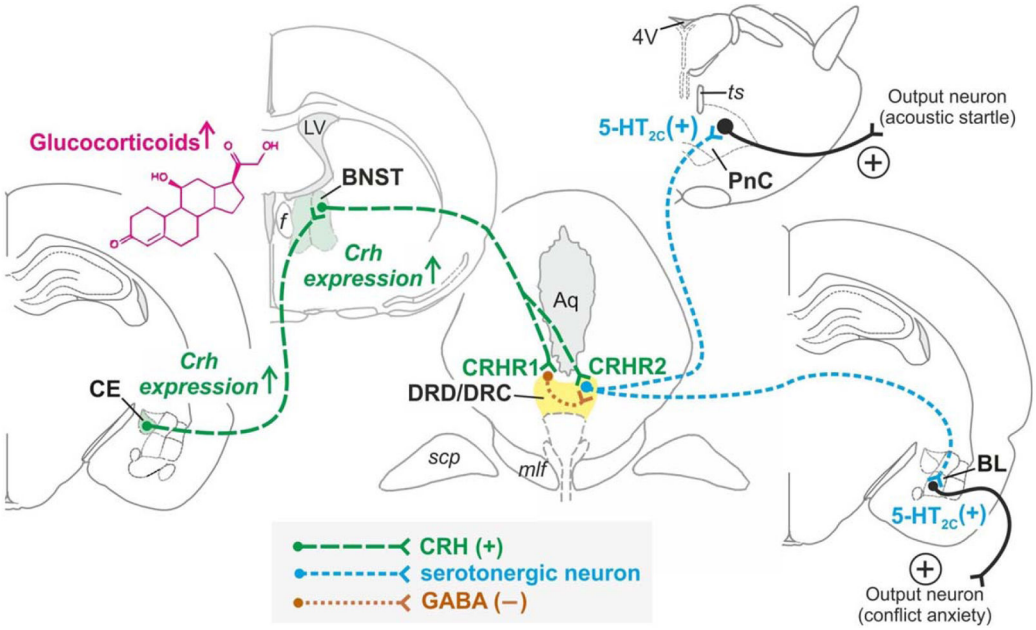

Figure 6. Mechanistic model

Hypothetical model of corticotropin-releasing hormone (CRH)-mediated serotonergic control executed from the dorsocaudal subdivisions of the dorsal raphe nucleus (DRD/DRC) towards the anxiety-related basolateral amygdala (BL) and the acoustic startle-controlling caudal pontine reticular nucleus (PnC). Chronic glucocorticoid exposure increases CRH expression in the central amygdala (CE) and in the bed nucleus of the stria terminalis (BNST) (Makino et al., 1994; Shepard et al., 2000), while sustained input from CE-derived CRH neurons drives CRH expression in the BNST, particularly in the (dorso)lateral part ($BNST_L$) (Shepard et al., 2006; Walker et al., 2009). $BNST_L$-derived CRH is then released into the DRD/DRC (Sink et al., 2012), acting onto CRH receptor type 2 (CRHR2) to increase, and on CRH receptor type 1 (CRHR1) to restrain, TPH2 activity and thus serotonin synthesis in DRD/DRC serotonergic neurons during an acute stressor. Both CRHR1 and CRHR2 are G protein-coupled receptors that mainly activate Gs. Most CRHR1 is not expressed in serotonergic neurons, but is located in γ-aminobutyric acid (GABA)ergic interneurons (Valentino et al., 2010), likely acting to increase GABAergic inhibition of TPH2 activity. In contrast, CRHR2 has been detected within the soma of serotonergic neurons (Waselus et al., 2009; Lukkes et al., 2011). The DRD/DRC innervates, amongst other forebrain target regions, the BL. In the BL, activation of the excitatory serotonin receptor $5\text{-}HT_{2C}$ has anxiogenic effects and mediates the exaggerated anxiety in a model of learned helplessness (Christianson et al., 2010). Within the PnC, immunohistochemistry has identified excitatory $5\text{-}HT_{2C}$ receptors on giant PnC neurons (Weber et al., 2008). Electrophysiological studies furthermore suggest that serotonin increases the excitability of giant PnC neurons via two-pore K^+ channels, namely the TWIK-related acid-sensitive K^+ channel type 3 (Weber et al., 2008), indicating how serotonergic projections from the DR to the PnC (Hobson et al., 1986) may potentiate the AS response. During an acute stressor, enhanced translocation of CRHR2 from the cytoplasm to the cell membrane (Waselus et al., 2009) may further potentiate stress-induced serotonin synthesis within DRD/DRC

serotonergic neurons projecting to the BL and PnC, activating conflict anxiety-controlling output neurons and enhancing the startle response. Within the DRD/DRC, chronic corticosterone (CORT) exposure increases basal TPH2 expression, and facilitates stress-induced serotonin synthesis by increasing the sensitivity of local, CRHR2-expressing serotonergic neurons in response to an acute stressor, resulting in passive stress-coping behavior, increased conflict anxiety, and learned helplessness (Christianson et al., 2010). Further abbreviations: 4V, fourth ventricle; Aq, cerebral aqueduct; *f*, fornix; LV, lateral ventricle; *mlf*, medial longitudinal fasciculus; *scp*, superior cerebellar peduncle; *ts*, tectospinal tract.