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Chronic non-invasive corticosterone administration abolishes the diurnal pattern of *tph2* expression

Nina C. Donner *, Christian D. Montoya, Jodi L. Lukkes, Christopher A. Lowry

Department of Integrative Physiology and Center for Neuroscience, University of Colorado Boulder, Boulder, CO 80309-0354, USA

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Glucose

Summary Both hypothalamic—pituitary—adrenal (HPA) axis activity and serotonergic systems are commonly dysregulated in stress-related psychiatric disorders. We describe here a non-invasive rat model for hypercortisolism, as observed in major depression, and its effects on physiology, behavior, and the expression of *tph2*, the gene encoding tryptophan hydroxylase 2, the rate-limiting enzyme for brain serotonin (5-hydroxytryptamine; 5-HT) synthesis. We delivered corticosterone (40 μg/ml, 100 μg/ml or 400 μg/ml) or vehicle to adrenal-intact adult, male rats via the drinking water for 3 weeks. On days 15, 16, 17 and 18, respectively, the rats' emotionality was assessed in the open-field (OF), social interaction (SI), elevated plus-maze (EPM), and forced swim tests (FST). On day 21, half of the rats in each group were killed 2 h into the dark phase of a 12/12 h reversed light/dark cycle; the other half were killed 2 h into the light phase. We then measured indices of HPA axis activity, plasma glucose and interleukin-6 (IL-6) availability, and neuronal *tph2* expression at each time point. Chronic corticosterone intake was sufficient to cause increased anxiety- and depressive-like behavior in a dose-dependent manner. It also disrupted the diurnal pattern of plasma adrenocorticotropin (ACTH), corticosterone, and glucose concentrations, caused adrenal atrophy, and prevented regular weight gain. No diurnal or treatment-dependent changes were found for plasma concentrations of IL-6. Remarkably, all doses of corticosterone treatment abolished the diurnal variation of *tph2* mRNA expression in the brainstem dorsal raphe nucleus (DR) by elevating the gene's expression during the animals' inactive (light) phase. Our data demonstrate that chronic elevation of corticosterone creates a vulnerability to a depression-like syndrome that is associated with increased *tph2* expression, similar to that observed in depressed patients.

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); ACTH, adrenocorticotropin; ANOVA, analysis of variance; Cort, corticosterone; CRF, corticotropin-releasing factor; DR, dorsal raphe nucleus; 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, dorsal raphe nucleus, ventrolateral part; EPM, elevated plus-maze; FST, forced swim test; HPA, hypothalamic—pituitary—adrenal; MDD, major depressive disorder; OF, open-field; PVC, polyvinyl chloride; SEM, standard error of the mean; SI, social interaction; SCN, suprachiasmatic nucleus; TPH, tryptophan hydroxylase protein; *tph2*, neuronal tryptophan hydroxylase 2 gene; TPH2, neuronal tryptophan hydroxylase 2 protein; VLPAG, ventrolateral part of the periaqueductal gray.

* Corresponding author at: Department of Integrative Physiology and Center for Neuroscience, University of Colorado Boulder, Boulder, CO 80309-0354, USA. Tel.: +1 303 492 8154; fax: +1 303 492 0811.
E-mail address: nina.donner@colorado.edu (N.C. Donner).

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1. Introduction

Dysregulation of hypothalamic–pituitary–adrenal (HPA) axis functionality (Daban et al., 2005; Graeff, 2007; Pariante and Lightman, 2008; Walker et al., 2008) and brain serotonergic systems (Mann et al., 1989; Owens and Nemeroff, 1994; Naughton et al., 2000; Arango et al., 2002; Zill et al., 2004; De Luca et al., 2005; You et al., 2005) is a common feature of many stress-related psychiatric disorders, including major depressive disorder (MDD), bipolar disorder, and anxiety disorders. Meanwhile, various animal models of depression have been validated, in part, because of their impairments in emotionality together with HPA axis- and serotonergic dysfunctions (Neumann et al., 2011). It is unclear, however, if HPA axis abnormalities such as hypercortisolemia are a cause or an effect of the observed serotonergic irregularities and behavioral symptoms (Lowry, 2002; Porter et al., 2007; Lanfumey et al., 2008; Heydendael and Jacobson, 2009). The fact that patients with Cushing's syndrome, a disease characterized by chronic elevation of endogenous cortisol, have an increased prevalence of depression suggests that hypercortisolism itself may increase the vulnerability to development of psychiatric disease (Kelly et al., 1980; Sonino et al., 1998; Michaud et al., 2009; Pereira et al., 2010).

Several lines of evidence report dysregulated HPA axis activity in MDD, a disease that affects about 20% of the US population and is the leading cause for disability between the ages of 15 and 44 (Kessler et al., 2005). Depressed patients often display hypercortisolism under basal conditions (Plotsky et al., 1998; Barden, 2004; Gillespie and Nemeroff, 2005), a flattened diurnal variation of circulating cortisol (Weber et al., 2000b; Putnam et al., 2008; Van den Bergh and Van Calster, 2009), and an abnormal ultradian pulsatility profile of cortisol (Deuschle et al., 1997; Wichers et al., 2008b). A classic diagnostic characteristic of melancholic depression is impaired negative feedback inhibition of the HPA axis in form of non-suppression of adrenocorticotropin (ACTH) and cortisol release after the combined dexamethasone/corticotropin-releasing factor suppression test (Holsboer et al., 1982; Heuser et al., 1994). As a possible compensatory response to the hypercortisolism, the ratio of glucocorticoid to mineralocorticoid receptor expression appears to be lower in depressed patients (Juruena et al., 2004). Meanwhile, promising treatment effects on both HPA axis activity and mood are observed in a new class of antidepressants that are designed to reduce the amount of circulating glucocorticoids (O'Dwyer et al., 1995; Laakmann et al., 2004; Carvalho and Pariante, 2008).

With respect to the serotonergic system, *tph2*, the gene encoding the rate-limiting enzyme for brain serotonin (5-hydroxytryptamine; 5-HT) synthesis (Walther and Bader, 2003; Walther et al., 2003; Patel et al., 2004; Zhang et al., 2004), may be a reliable biomarker for MDD: levels of *tph2* mRNA and protein expression consistently have been found to be elevated in depressed suicide victims, both in the brainstem dorsal raphe nucleus (DR) (Underwood et al., 1999; Boldrini et al., 2005; Bach-Mizrachi et al., 2006, 2008; Bonkale et al., 2006; Underwood et al., 2010), the main source of 5-HT in the brain, and in DR projection regions (Perroud et al., 2010). Increased *tph2* expression also concurs with the recent finding that depressed patients have increased brain 5-HT turnover that returns to basal levels following successful antidepressant treatment (Barton et al., 2008).

Importantly, *tph2* expression appears to depend largely on the circadian nature of circulating glucocorticoids. In adrenal-intact rats, *tph2* mRNA expression (Malek et al., 2005) and protein (Malek et al., 2004) are elevated before and during the first hours of the active phase (dark phase in rodents), which correlates with peak plasma concentrations of corticosterone, the rat equivalent of human cortisol. Adrenalectomy abolishes the daily pattern of *tph2* expression (Singh et al., 1990b; Malek et al., 2007), while corticosterone restores the diurnal *tph2* mRNA rhythm when replaced via the drinking water for 12 h during the active phase. In contrast, a constant, clamped substitution of corticosterone through subcutaneous pellets fails to restore the diurnal pattern of *tph2* expression (Malek et al., 2007).

Based on these findings, we hypothesized that chronic elevation of glucocorticoids is sufficient to upregulate *tph2* gene expression and cause a depressive-like phenotype. To test this hypothesis, we delivered three different doses of corticosterone for 3 weeks to adrenal-intact, male rats via the drinking water, and assessed their emotionality, endocrine function, and *tph2* mRNA expression in the brainstem DR.

2. Materials and methods

2.1. Animals

Sixty-four adrenal-intact, adult male Sprague Dawley rats (Harlan, Indianapolis, IN, USA; 200–225 g) were kept with *ad libitum* access to vehicle or corticosterone solution, and standard rat chow (Teklad 22/5 Rodent diet(W) 8640, Harlan) on a reversed 12:12 h light/dark cycle (lights on at 1900 h). Twenty-two age-matched conspecifics were used as partner rats in the SI test.

2.2. Treatment

Rats were pair-housed according to treatment group, and treated for 21 days with corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione, Cat. No. C2505, Sigma–Aldrich, St. Louis, MO, USA) or vehicle (tap water containing 0.45% 2-hydroxypropyl-β-cyclodextrin, Cat. No. 332607, Sigma–Aldrich; $n = 16$). All corticosterone-containing drinking bottles were wrapped in aluminum foil to prevent light-induced degradation of the hormone, and clean bottles with freshly prepared vehicle or corticosterone solutions were supplied every 4 days. Similar to doses used in previously reported rodent models (Conrad et al., 2007; Malek et al., 2007; Gourley et al., 2008b; David et al., 2009; Gourley and Taylor, 2009; Lee et al., 2010), rats in the experimental groups were either treated with 40 μg/ml corticosterone (Cort-40, approximately 7 mg/kg/day (David et al., 2009), $n = 16$), 100 μg/ml corticosterone (Cort-100, approximately 30 mg/kg/day (Gourley et al., 2008b), $n = 16$), or 400 μg/ml corticosterone (Cort-400, approximately 70–120 mg/kg/day (Conrad et al., 2007), $n = 16$). We chose this non-invasive drinking-water-mediated paradigm to retain the overall integrity of the diurnal corticosterone rhythmicity present

in normal animals (Windle et al., 1998; Droste et al., 2008; Karatsoreos et al., 2010).

2.3. Behavioral testing

The behavioral tests were intentionally arranged in the order of the least stressful (OF) to the most stressful (FST) event to prevent effects of one test on the next day's behavior. Detailed testing procedures are described below. All behavioral tests occurred between 0900 and 1600 h during the rats' active phase within the dark cycle, and were recorded using low light conditions (red light, range: 3.5—27.5 lux, see detailed descriptions below for lux measurements in each behavioral apparatus), and a low light-sensitive web cam (Microsoft® LifeCam VX-6000, Microsoft, Seattle, WA, USA) that was mounted above the respective behavioral apparatus. Each behavioral apparatus was cleaned evenly with a damp cloth and a small amount of Windex® before individual animals were tested. Again, each experiment was run in randomized blocks on each of two consecutive days such that four rats of each treatment group were studied at each time point on each day. All behaviors were scored while blinded to the treatment groups.

2.4. Open-field (OF) test

The rats' explorative and anxiety-related behaviors were tested in the OF test, as described previously (Hale et al., 2008), on day 15. Our OF consisted of a 90 cm × 90 cm polyvinyl chloride (PVC) box with 40-cm high walls and an open top, and the total duration was reduced to 10 instead of 15 min. The light intensity varied from 16.7 ± 0.7 lux in the center of the OF to an average of 10.9 ± 1.1 lux in the corners of the OF. As an optical aid for scoring, the OF was divided into 6 × 6 equal-sized squares with tape. To analyze the rats' exploration of the OF quantitatively, an outer zone was defined as the sum of all squares adjacent to the walls, an inner zone as the sum of all squares adjacent to the outer squares, and a center zone as the sum of the four inner-most squares using the spatial analysis software 'EthoVision XT' (version 6.0; Noldus Information Technologies, Wageningen, The Netherlands). In the beginning of the test, each rat was placed into the same corner of the outer zone facing towards the center. The time spent in each zone, as well as the time spent immobile, the total distance moved, the number of rears at the walls, and the latency to enter the inner and center zones were the parameters we chose to score and report. Increased time spent in the inner or center zone is associated with increased anxiety states (Walsh and Cummins, 1976). As in our previous studies (Spiga et al., 2006), the open-field test also served as a familiarization of the test rat with the same environment that was used the following day for the social interaction test.

2.5. Social interaction (SI)

The rats' social behavior was assessed in the SI test (File, 1980; Spiga et al., 2006) for 10 min on day 16. During the SI test, each test rat was exposed to an age-matched unfamiliar male conspecific, the so-called partner rat, in the same PVC box and lighting conditions used the day before for the OF test. The test rat was placed in one corner, and the partner rat was placed in the opposing corner of the OF. During the test the following behaviors were classified as active SI only when initiated by the test rat: sniffing, following, grooming, wrestling with, and crawling over or under the partner. The number of rears at the walls of the OF were analyzed as a general explorative parameter. Social interaction time and the number of rears (of the test rat) were evaluated using 'The Observer' software (version 5.0, Noldus Information Technologies). No aggressive behavior was observed. An increase in the percent time spent interacting is indicative of an anxiolytic treatment effect, a decrease signifies an anxiogenic effect (File, 1980).

2.6. Elevated plus-maze (EPM)

On day 17, all rats were tested once more for their explorative versus anxiety-related behavior (Pellow et al., 1985), this time in a slightly more contrasting behavioral paradigm, namely the EPM. Each rat was exposed to the EPM for 5 min. The EPM consisted of an elevated plus-shaped PVC platform with two closed arms (10 cm × 50 cm arm area with 40-cm tall walls on both sides and at the end; average light intensity: 3.5 ± 0.2 lux) and two open arms (no walls; average light intensity: 26.0 ± 0.8 lux), connected in the center by a neutral, wall-free zone (10 cm × 10 cm; light intensity: 17.5 ± 0.9 lux). All materials for the EPM were provided by Alpha Plastic & Design (Fort Collins, CO, USA). The contrast between the protected closed arms and the exposed open arms challenges the animal with a conflict situation between its exploratory drive and its innate fear of open and exposed areas (Pellow et al., 1985). Each rat was placed in the neutral zone with its head facing into the same closed arm. The following parameters for anxiety-related behavior were scored using 'The Observer' software (version 5.0; Noldus Information Technologies): the amount of time spent in the neutral center area, the time spent in the open versus the closed arms of the EPM, the number of entries (partial entry, meaning past shoulders) and full entries (past hind legs) into the closed and open arms, the latency to first enter an open arm, the number of rears at the walls of the closed arms, and the time spent grooming.

2.7. Forced swim test (FST)

To assess the rats' active versus passive stress coping behavior, all rats were exposed to the FST for 5 min on day 18 of treatment, after being trained for the novel task the day before, 3 h after testing in the EPM, in a non-recorded pretest session for 15 min. In many behavioral and pharmacological labs, the FST is routinely used to analyze despair-like behavior (reactive versus proactive stress coping behavior) and effects of antidepressants in rodents (Porsolt et al., 2001). Two rats at a time were tested in the FST in separate round PVC buckets (30.5 cm diameter) that were filled with 25 °C tap water at 35 cm depth. From a camera above, the rats' behavior was recorded, and later analyzed with 'The Observer' software (version 5.0, Noldus Information Technologies). The time spent climbing (proactive coping behavior involving high-pace front leg paddling that breaks the water surface, strong hind leg strokes), the time spent swimming (proactive

coping behavior involving slow-pace front and hind leg movements, no breaking of the water surface), the time spent immobile (reactive, despair-like behavior involving minimal hind leg movements required only to stay afloat and keep the nose above water, and stiff body posture) and the number of dives (interpreted as proactive, explorative escape-seeking behavior) were scored (Cryan et al., 2005b).

2.8. Measuring body weight and food and water intake

The rats' body weight was measured on day 0 (treatment start), and on days 7, 14 and 21. The total amount of food and drinking water consumed per cage each week was calculated based on the weight of the remaining food or drinking bottles, before the drinking bottle weight was converted into volume (density of 1 g/1 ml).

2.9. Experimental procedure

To investigate diurnal variations in hormone concentrations and gene expression, half of the rats in each treatment group were killed between 0830 h and 0930 h (approximately 2 h into the active, dark phase), the other half between 2030 and 2130 h (approximately 2 h into the inactive, light phase) via rapid decapitation on day 21 of treatment. Corticosterone drinking bottles were replaced with vehicle 90 min before sacrifice to reduce individual variability in plasma hormone concentrations. Brains were removed immediately, fresh-frozen on dry ice, and stored at −80 °C until sectioning. Trunk blood was collected into sterile Falcon tubes containing 200 μl of 2% ethylene-diamine-tetraacetic acid (EDTA; Cat. No. EDS, Sigma−Aldrich) and 5% heparin (Cat. No. H6279, Sigma−Aldrich) in 0.05 M phosphate buffered saline. Blood samples were kept on ice until subsequent centrifugation in a microfuge (Beckman Coulter, Fullerton, CA, USA) for 15 min (10,000 rpm, 4 °C). On ice, plasma samples from each rat were aliquoted and stored at −80 °C. The pituitary and adrenal glands were dissected from surrounding tissue, their wet weight measured, and post-fixed in 0.1 M sodium phosphate buffer with 4% paraformaldehyde fixative (paraformaldehyde Cat. No. 158127, Sigma−Aldrich).

2.10. Plasma hormone, glucose and cytokine assays

Plasma ACTH concentrations were determined using an enzyme-linked immunoassay kit (Cat. No. EK-001-21, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA). The immunoassay has reportedly a sensitivity of 70 pg/ml, a 5−10% intra-assay coefficient of variation, and an inter-assay coefficient of variation of less than 15%. The assay was conducted in duplicates, and plasma samples were used in original, undiluted form without prior extraction.

Corticosterone concentrations within the plasma samples were analyzed with an enzyme-linked immunoassay kit from Assay Designs (Cat. No. 900-097, Ann Arbor, MI, USA). According to the manufacturer, this assay has a sensitivity of 0.027 ng/ml, an intra-assay coefficient of variation of 6−8%, and an inter-assay coefficient of variation of 8−13%. Prior to being analyzed in duplicates in the assay, all corticosterone plasma samples were diluted 1:40 in assay buffer containing 2.5% steroid displacement reagent (provided by the kit).

Plasma glucose concentrations were analyzed using an enzymatic assay kit from Cayman Chemical Company (Cat. No. 10009582, Ann Arbor, MI, USA). This assay has a range of 0−250 mg/dl, an intra-assay coefficient of variation of 5−8%, and an inter-assay coefficient of variation of 2−11%. Duplicates of glucose assay plasma samples were used in original, undiluted form.

Plasma concentrations of the pro inflammatory cytokine interleukin 6 (IL-6) were determined in original, undiluted duplicate samples with an enzyme-linked immunoassay from Invitrogen (Cat. No. KRD0061C, Carlsbad, CO, USA). This IL-6 assay has a range of 5.0−1500 pg/ml, an intra-assay coefficient of variation of 3−6%, and an inter-assay coefficient of variation of 5−9%.

2.11. *In situ* hybridization histochemistry and analysis of *tph2* mRNA expression

Brains were cryosectioned at 12 μm in series of six between bregma −7.568 mm and −8.648 mm (Paxinos and Watson, 1998), thaw-mounted onto VistaVision HistoBond® microscope slides (VWR Scientific, West Chester, PA, USA), and used in an *in situ* hybridization histochemistry assay with a cRNA probe complementary to bases 761−1343 of rat *tph2* mRNA, similar to a previously described protocol (Donner and Handa, 2009). Briefly, brain slices were post-fixed, washed in 2× standard saline citrate (SSC) buffer, and hybridized with 1×10^6 cpm of [35-S]-UTP-labeled cRNA (per slide) in a humidified chamber at 55 °C. The next day, hybridization buffer was washed off with 2× SSC, and tissue was treated with RNase A to degrade non-hybridized cRNA. Slides were then desalted twice in 0.1× SSC buffer, dehydrated in an ascending alcohol series, air-dried, and apposed to a Kodak BioMax autoradiography film (PerkinElmer, Waltham, MA, USA) for 7 days.

Digital autoradiography images were captured and analyzed with ImageJ (NIH, Bethesda, MD, USA) to measure background-corrected "mean gray value" using matrices in the shape of each subdivision of the brainstem DR. Based on Gardner et al. (2009), Abrams et al. (2004), and Paxinos and Watson (1998), a total of 16 rostro-caudal levels containing five functional subdivisions of the DR (dorsal raphe nucleus, caudal part, DRC; dorsal raphe nucleus, dorsal part, DRD; dorsal raphe nucleus, interfascicular part, DRI; dorsal raphe nucleus, ventral part, DRV; dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray region, DRVL/VLPAG) were analyzed.

2.12. Morphological analysis of adrenal glands

Post-fixed adrenal glands were cryosectioned at 30 μm, mounted on VistaVision HistoBond® microscope slides (VWR), and stained with 0.5% cresyl violet acetate (Paxinos and Watson, 1998) (Cat. No. 1791, Sigma−Aldrich). Based on digital photomicrographs, the width of the three adrenocortical layers (Vinson, 2003), zona glomerulosa, zona fasciculata and zona reticularis (at 100× total magnification), and the diameter of the adrenal medulla (at 40× total magnification)

were measured using ImageJ (Ichimura et al., 1995). At 100× total magnification, an investigator blinded to the treatment groups also counted the number of cells within an area of 50 μm × 50 μm in each of the three adrenocortical subdivisions, using a microscope eyepiece with an integrated 10 mm × 10 mm reticule (Type 020-518.500, Leica Microsystems, Wetzlar, Germany).

2.13. Statistics

All data were analyzed using SPSS (version 17, SPSS Inc., Chicago, IL, USA). Due to outlier identification via the Grubb's test (Grubbs, 1969), we excluded 6.2% of the data points for 'immobility', 7.8% of the data points for the 'latency until first inner entry', and 3.1% each of the data points for 'latency until first center zone entry', and 'time in center zone' from the OF data set. Similarly, we eliminated 4.7% from the plasma corticosterone data set and 1.6% of the values from the plasma glucose data set. Body weight gain, food and water consumption were compared using a multifactor analysis of variance approach with *treatment* as the between-subjects factor and *time* (weeks 1, 2 and 3) as the within-subject factor. Behavioral parameters and pituitary and adrenal data were analyzed using a one-way ANOVA with the between-subjects factor *treatment*. *Tph2* expression in the entire DR, and plasma hormone, glucose and IL-6 concentrations were analyzed using two-way ANOVA with *treatment* and *killing time point* as between-subjects factors. *Tph2* expression within the different subdivisions of the DR was compared using an ANOVA for repeated measurements (within-subject factor *subdivision*) with *treatment* and *killing time point* as between-subjects factors. Where appropriate, a Fisher's Protected LSD *post hoc* analysis was performed. Significance was accepted at $p < 0.05$, and values are shown as the mean ± the standard error of the mean (SEM). Graphs for our figures and correlations were either designed in Excel 2007 (Microsoft), SigmaPlot (version 11.0, Systat Software Inc., Evanston, IL, USA), or SPSS (version 17, SPSS Inc., Chicago, IL, USA).

Detailed methods are described in supplemental material online.

3. Results

3.1. Weight gain

Over the course of the 3-week treatment, vehicle control rats gained an average of 85.8 g in body weight, while Cort-40 and Cort-100 rats gained significantly less weight (Fig. 1A). Cort-400 animals continuously lost weight during the 3 week period (*treatment*, $F_{(3,\ 60)} = 416.87$, $p < 0.001$; *time*, $F_{(2,\ 61)} = 425.04$, $p < 0.001$; *interaction*, $F_{(6,\ 52)} = 19.51$, $p < 0.001$). Corticosterone treatment also resulted in altered physical appearance such as a visible reduction in abdominal diameter (Fig. 1B).

3.2. Body-weight-corrected food and water intake

When corrected for body weight, corticosterone treatment increased both food consumption (*treatment*, $F_{(3,\ 28)} = 2.41$, $p = 0.09$; *time*, $F_{(2,\ 29)} = 988.12$, $p < 0.001$; *interaction*, $F_{(6,\ 20)} = 2.97$, $p < 0.05$) and water intake (*treatment*, $F_{(3,\ 28)} = 20.35$, $p < 0.001$; *time*, $F_{(2,\ 29)} = 1723.11$, $p < 0.001$; *interaction*, $F_{(6,\ 20)} = 7.76$, $p < 0.001$) dose-dependently during the 3 weeks of treatment. In other words, when body weight was taken into account, Cort-400 rats consumed more food than vehicle controls after 2 weeks of treatment, and significantly more food than all other treatment groups after 3 weeks of treatment (Fig. 1C). At the same time, Cort-100 and Cort-400 groups drank more water after 2 and 3 weeks than all other (on average heavier) treatment groups (Fig. 1D).

3.3. Absolute food and water intake

After 2 and 3 weeks of treatment, absolute food intake (Fig. S1 A, supplemental material), i.e., not corrected for body weight, was reduced in both the Cort-100 and the Cort-400 group, compared to vehicle controls and Cort-40 rats (*treatment*, $F_{(3,\ 28)} = 3.33$, $p < 0.05$; *time*, $F_{(2,\ 29)} = 1214.57$, $p < 0.001$, *interaction*, $F_{(6,\ 20)} = 5.44$, $p < 0.001$). Similarly, in absolute terms the Cort-400 group drank less water than all other treatment groups (*treatment*, $F_{(3,\ 28)} = 17.24$, $p < 0.001$; *time*, $F_{(2,\ 29)} = 2547.48$, $p < 0.001$; *interaction*, $F_{(6,\ 20)} = 3.63$, $p < 0.01$; Fig. S1 B, supplemental material).

3.4. Open-field (OF)

Cort-400 rats spent significantly less time in the center zone of the OF (Fig. 2A) than vehicle controls ($F_{(3,\ 41)} = 3.46$, $p < 0.05$), and also remained immobile longer than the Cort-40 and Cort-100 groups ($F_{(3,\ 56)} = 3.27$, $p < 0.05$). Cort-400 rats also took more time before entering the inner ($F_{(3,\ 55)} = 3.16$, $p < 0.05$) and center zones of the open-field for the first time ($F_{(3,\ 41)} = 3.07$, $p < 0.05$). The observed treatment differences are indicative of a dose-dependent anxiogenic effect of corticosterone.

3.5. Elevated plus-maze (EPM)

The EPM revealed a strong anxiogenic effect of chronic corticosterone treatment, as reflected in an incremental dose–response within most behavioral parameters (Fig. 2B). Compared to the vehicle group, Cort-100 and Cort-400 rats, but not Cort-40 rats, spent more time in the protected closed arms ($F_{(3,\ 60)} = 4.35$, $p < 0.01$), and less time in the unprotected open arms ($F_{(3,\ 60)} = 5.97$, $p < 0.01$). All doses reduced the number of full open arm entries ($F_{(3,\ 60)} = 6.32$, $p < 0.01$) and increased rears at the walls of the closed arms ($F_{(3,\ 60)} = 9.39$, $p < 0.001$) relative to the vehicle group. Rats of the Cort-400 group also entered the open arms of the EPM less often than the vehicle and Cort-40 groups ($F_{(3,\ 60)} = 2.97$, $p < 0.05$). No treatment-dependent differences were detected for the time spent in the neutral central area, grooming, or the number of entries into the closed arms (a measure of locomotor activity).

3.6. Social interaction (SI)

Cort-400 rats interacted less with the conspecific male than vehicle controls ($F_{(3,\ 60)} = 3.10$, $p < 0.05$; Fig. 3), indicating increased anxiety-like behavior. No differences in SI were observed between the Cort-40, Cort-100 and the vehicle

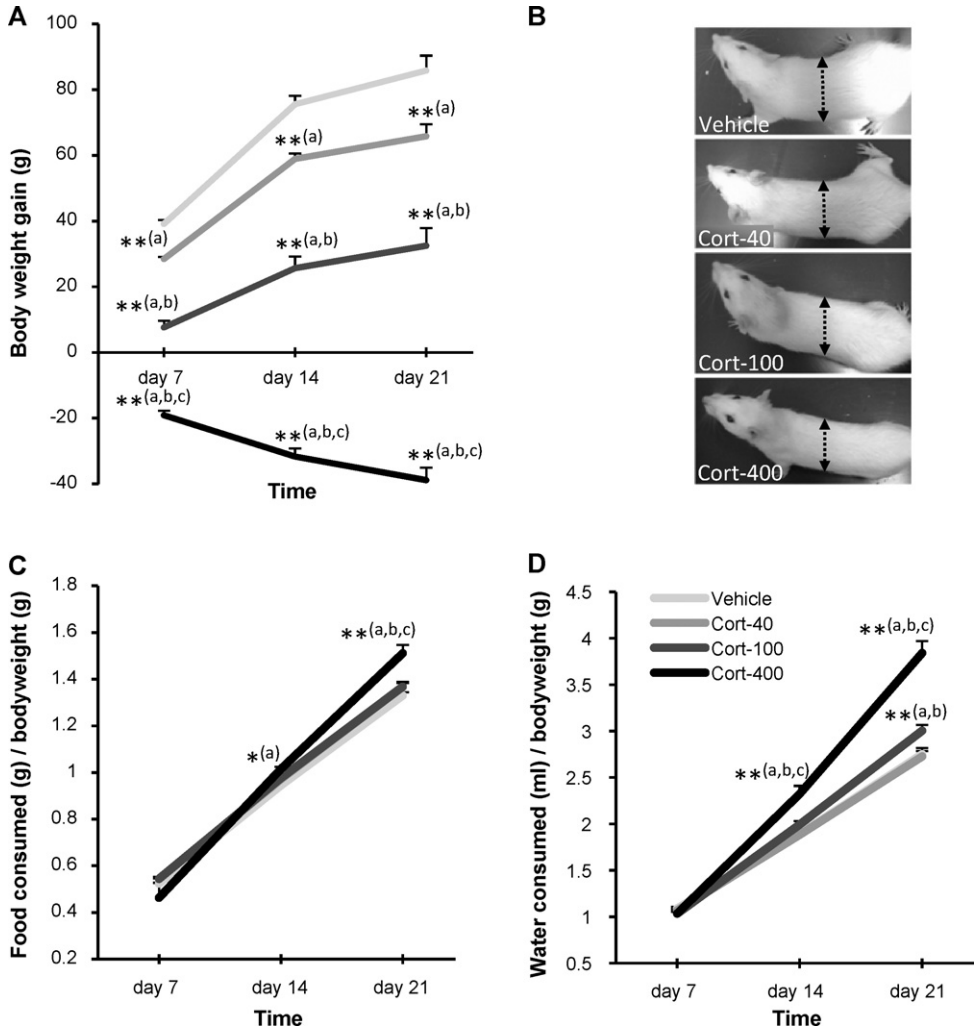

Figure 1 Body weight gain (A), physical appearance (B), food intake (C) and water consumption (D) of adrenal-intact male rats during treatment with either vehicle (0.45% hydroxypropyl-beta-cyclodextrin) or corticosterone (40, 100 or 400 μg/ml) via the drinking water for 21 days. All data represent cumulative values, meaning the difference compared to day 0 of the experiment. The arrows in (B) indicate the abdominal diameter, based on photographs of representative rats from each treatment group. All data are displayed as means + SEM. *$p < 0.05$, **$p < 0.01$ versus (a) vehicle, (b) Cort-40, and (c) Cort-100, after *post hoc* analysis with Fisher's Protected LSD test (vehicle, $n = 16$; Cort-40, $n = 16$; Cort-100, $n = 16$, Cort-400, $n = 16$).

group, and no treatment effects on rearing behavior were observed.

3.7. Forced swim test (FST)

Compared to vehicle controls, Cort-100 and Cort-400 animals displayed less climbing behavior ($F_{(3,\ 60)} = 4.24$, $p < 0.01$), and more immobility ($F_{(3,\ 60)} = 3.97$, $p < 0.05$; Fig. 4) in the FST. Corticosterone treatment also tended to increase the number of dives, although this comparison only approached significance ($F_{(3,\ 60)} = 2.62$, $p = 0.059$; Fig. 4). Scores for swimming behavior were similar amongst all treatment groups.

3.8. Plasma ACTH, corticosterone, glucose and IL-6 concentrations

In vehicle controls, plasma ACTH was significantly higher during the active, dark phase, compared to rats killed during their inactive, light phase (Fig. 5A). In comparison, Cort-40 rats did not show the described diurnal pattern. Cort-100 and Cort-400 groups also lacked a diurnal variance, and their absolute plasma ACTH concentrations were decreased in a dose-dependent manner compared to vehicle controls (*treatment*, $F_{(3,\ 60)} = 46.16$, $p < 0.001$; *killing time point*, $F_{(1,\ 62)} = 3.80$, $p = 0.05$; *interaction*, $F_{(3,\ 56)} = 3.99$, $p < 0.05$).

Similarly, plasma corticosterone concentrations of vehicle and Cort-40 rats were significantly elevated in dark-phase compared to light-phase animals (Fig. 5B). This diurnal variation was absent in Cort-100 and Cort-400 animals (*treatment*, $F_{(3,\ 57)} = 8.98$, $p < 0.001$; *killing time point*, $F_{(1,\ 59)} = 16.21$, $p < 0.001$ *interaction*, $F_{(3,\ 53)} = 9.46$, $p < 0.001$). Cort-400 treatment resulted in light-phase plasma corticosterone concentrations that were elevated compared to all other groups at that time point, most likely reflecting excess exogenous corticosterone from the drinking water.

In vehicle and Cort-40 rats, plasma glucose concentrations also varied diurnally (Fig. 5C), with elevated plasma glucose

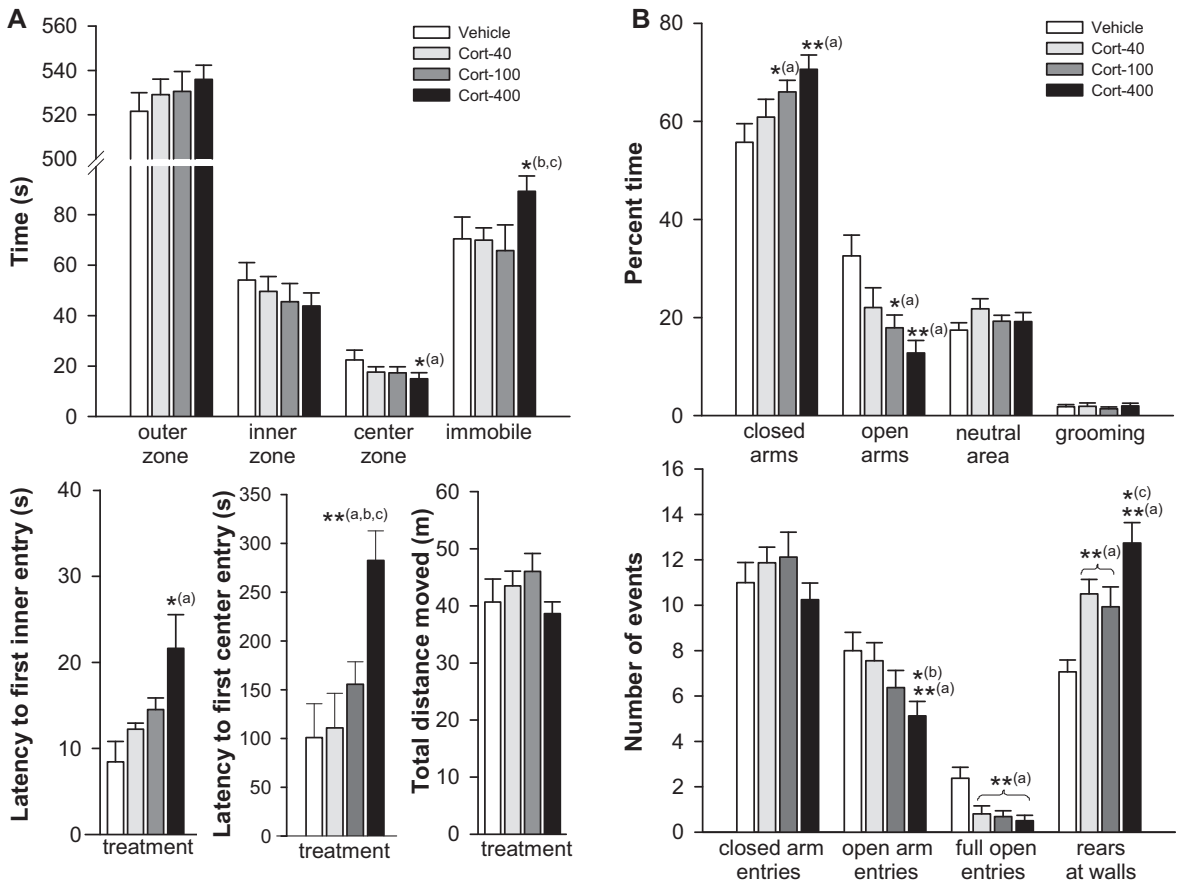

Figure 2 Anxiety-related behavioral parameters of all rats were assessed in the open-field (panel A) on day 15, and on the elevated plus-maze (panel B) on day 17 of treatment with either vehicle (0.45% hydroxypropyl-beta-cyclodextrin) or corticosterone (40, 100 or 400 μg/ml). Both tests were performed during the rats' active phase. Panel (A) displays the amount of time the rats spent in the outer, inner and center zones of the open-field, as well as the total duration of immobility during the test. Panel (A) also shows the latency to first enter the inner and center zones, and the total distance moved. Panel (B) depicts the percent time the rats spent either in the closed arms, the open arms, or in the neutral middle-square of the elevated plus-maze. The percent time spent grooming, the number of closed, open, and full open arm entries, and the number of rears at the walls are also indicated in panel (B). Data are displayed as means + SEM. *$p < 0.05$, **$p < 0.01$ versus (a) vehicle, (b) Cort-40, and (c) Cort-100, after *post hoc* analysis with Fisher's Protected LSD test (vehicle, $n = 16$; Cort-40, $n = 16$; Cort-100, $n = 16$, Cort-400, $n = 16$).

during the dark phase. Again, this diurnal pattern was abolished in the Cort-100 and Cort-400 groups (*treatment*, $F_{(3,\ 59)} = 34.97$, $p < 0.001$; *killing time point*, $F_{(1,\ 61)} = 6.96$, $p < 0.05$; *interaction*, $F_{(3,\ 55)} = 2.71$, $p = 0.05$).

No treatment- or time-dependent differences were detected for plasma concentrations of IL-6 (*treatment*, $F_{(3,\ 60)} = 0.49$, $p = 0.687$; *killing time point*, $F_{(1,\ 62)} = 0.60$, $p = 0.444$; *interaction*, $F_{(3,\ 56)} = 0.97$, $p = 0.414$; Table S1, supplemental material).

3.9. Adrenal atrophy

Table 1 summarizes the dose-dependent effects of corticosterone to induce adrenal atrophy. Three weeks of corticosterone treatment significantly reduced adrenal gland weight ($F_{(3,\ 60)} = 13.25$, $p < 0.001$), diameter ($F_{(3,\ 60)} = 30.62$, $p < 0.001$), and adrenal cortex width ($F_{(3,\ 60)} = 48.25$, $p < 0.001$). Within the adrenal cortex, the strongest treatment effects on zone width ($F_{(3,\ 60)} = 51.95$, $p < 0.001$) and cell density (increased: $F_{(3,\ 60)} = 48.62$, $p < 0.001$) were detected within the zona fasciculata (Fig. 6), where most glucocorticoid-producing cells are located. The observed adrenal atrophy is most likely due to negative feedback regulation of HPA axis tissues by means of the exogenous corticosterone (Plaschke et al., 2006). For a detailed description of the atrophy results see supplemental material.

3.10. *tph2* mRNA expression in the DR

Corticosterone treatment significantly altered *tph2* expression in the entire DR, meaning when all subregions of the DR (Fig. 7) were averaged for each individual (*treatment*, $F_{(3,\ 60)} = 3.05$, $p < 0.05$; *killing time point*, $F_{(1,\ 62)} = 1.37$, $p = 0.242$; *interaction*, $F_{(3,\ 56)} = 2.71$, $p < 0.01$; Fig. 8). Specifically, the circadian difference (27%) in *tph2* expression as observed between light and dark phase vehicle-animals ($p = 0.002$) was no longer present in any of the corticosterone-treated groups. Within the light phase, Cort-40 ($p < 0.05$), Cort-100 ($p < 0.01$), and Cort-400 ($p = 0.01$) rats all expressed significantly more *tph2* mRNA than vehicle

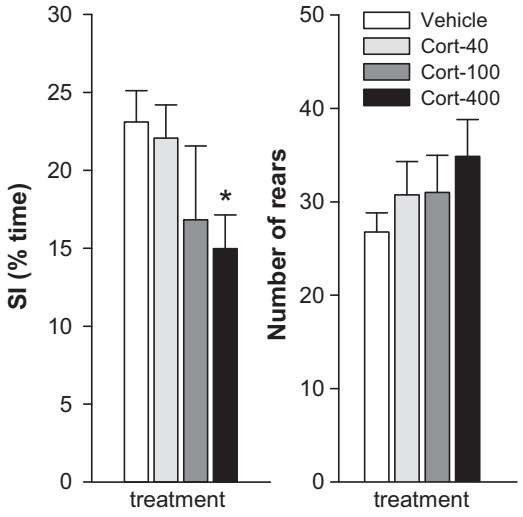

Figure 3 Social interaction scores on day 16 of treatment in rats treated with either vehicle (0.45% hydroxypropyl-beta-cyclodextrin) or corticosterone in the drinking water (40, 100 or 400 μg/ml). Shown is the percentage of time each group spent actively engaging in social interaction with the partner rat, and the number of rears at the walls of the open-field. Data are displayed as means + SEM. $^{*}p < 0.05$ versus vehicle, after *post hoc* analysis with Fisher's Protected LSD test (vehicle, $n = 16$; Cort-40, $n = 16$; Cort-100, $n = 16$, Cort-400, $n = 16$).

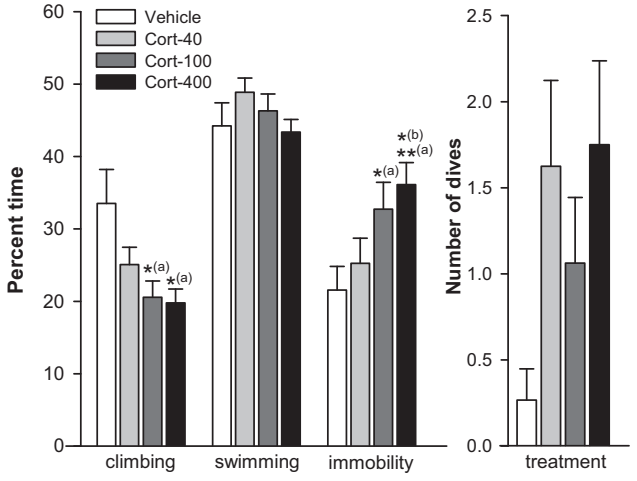

Figure 4 The rats' stress coping behavior was assessed in the forced swim test on day 18 of treatment with vehicle (0.45% hydroxypropyl-beta-cyclodextrin) or corticosterone in the drinking water (40, 100 or 400 μg/ml). Shown is the percentage of time the rats spent either actively climbing or swimming, or immobile (passive coping behavior). The number of dives (proactive escape seeking behavior) for each treatment group is displayed on the right side. Data are displayed as means + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ versus (a) vehicle or (b) Cort-40, after *post hoc* analysis with Fisher's Protected LSD test (vehicle, $n = 16$; Cort-40, $n = 16$; Cort-100, $n = 16$, Cort-400, $n = 16$).

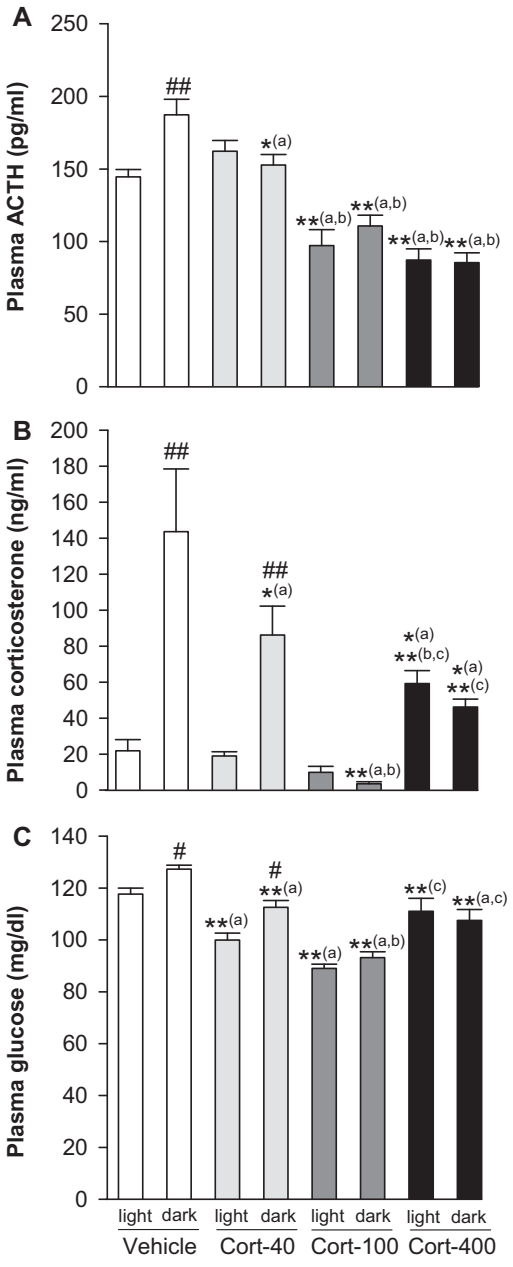

Figure 5 Concentrations of adrenocorticotropin (ACTH; A), corticosterone (B), and glucose (C) in plasma sampled from adrenal-intact male rats during the light and dark phases after treatment with either vehicle (0.45% hydroxypropyl-beta-cyclodextrin) or corticosterone in the drinking water (40, 100 or 400 μg/ml) for 21 days. Half of each treatment group was killed during their inactive phase (2100 h, 2 h after lights on), the other half during their active phase (0900 h, 2 h after lights off). All data are displayed as means + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ versus the corresponding light or dark phase value of (a) vehicle, (b) Cort-40, or (c) Cort-100. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus the corresponding light phase value within the same treatment group, after *post hoc* analysis with Fisher's Protected LSD test (vehicle-light, $n = 8$; vehicle-dark, $n = 8$; Cort-40-light, $n = 8$; Cort-40-dark, $n = 8$; Cort-100-light, $n = 8$, Cort-100-dark, $n = 8$; Cort-400-light, $n = 8$; Cort-400-dark, $n = 8$).

Table 1 Dose-dependent effect of corticosterone on pituitary and adrenal glands.[a]

	Vehicle	Cort-40	Cort-100	Cort-400
Weight of pituitary (mg)	9.6 ± 0.4	10.1 ± 0.3	10.3 ± 0.7	9.9 ± 2.0
Weight of both adrenal glands (mg)	50.8 ± 2.1	38.4 ± 3.8$^{**(a)}$	32.0 ± 3.4$^{**(a)}$	25.0 ± 2.4$^{**(a,b)}$
Diameter of entire adrenal gland (μm)	3661 ± 113	3365 ± 112$^{*(a)}$	3022 ± 74$^{*(b),**(a)}$	2382 ± 94$^{**(a,b,c)}$
Adrenal medulla diameter (μm)	1293 ± 90	1187 ± 71	1383 ± 79	1134 ± 78
Adrenal cortex width (μm)	1184 ± 34	1089 ± 52	820 ± 28$^{**(a,b)}$	624 ± 28$^{**(a,b,c)}$
Zona glomerulosa				
Width (μm)	102 ± 5	95 ± 5	89 ± 11	56 ± 4$^{**(a,b,c)}$
Cell density (cells/(50 μm)^2)	33.8 ± 3.0	38.2 ± 0.9	40.1 ± 1.4$^{*(a)}$	46.8 ± 1.1$^{*(c),**(a,b)}$
Zona fasciculata				
Width (μm)	548 ± 18	409 ± 36$^{*(a)}$	171 ± 9$^{**(a,b)}$	135 ± 5$^{**(a,b)}$
Cell density (cells/(50 μm)^2)	17.3 ± 0.7	22 ± 1.3$^{**(a)}$	28.1 ± 1.6$^{**(a,b)}$	36.2 ± 0.8$^{**(a,b,c)}$
Zona reticularis				
Width (μm)	534 ± 17	585 ± 38	560 ± 26	433 ± 24$^{*(a),**(b,c)}$
Cell density (cells/(50 μm)^2)	33.1 ± 1.7	34.6 ± 1.3	40.3 ± 0.7$^{**(a,b)}$	43.6 ± 0.6$^{**(a,b)}$

[a] Listed are the weights of the pituitary and both adrenal glands, as well as the average adrenal gland size, diameter of the adrenal gland and adrenal medulla, widths of the subregions of the adrenal cortex, and cell densities in each adrenocortical subregion of adult male rats that were either treated with vehicle (0.45% hydroxypropyl-beta-cyclodextrin) or corticosterone (40, 100 or 400 μg/ml) via the drinking water for 21 days. Data are displayed as means ± SEM.

* $p < 0.05$ versus (a) vehicle, (b) Cort-40, and (c) Cort-100, after *post hoc* analysis with Fisher's Protected LSD test (vehicle, $n = 16$; Cort-40, $n = 16$; Cort-100, $n = 16$, Cort-400, $n = 16$).

** $p < 0.01$ versus (a) vehicle, (b) Cort-40, and (c) Cort-100, after *post hoc* analysis with Fisher's Protected LSD test (vehicle, $n = 16$; Cort-40, $n = 16$; Cort-100, $n = 16$, Cort-400, $n = 16$).

controls (Fig. 8). No treatment-dependent differences in *tph2* expression were detected in rats that were killed during their active, dark phase.

With the exception of the DRI, this treatment effect of corticosterone was maintained throughout all DR subdivisions when analyzed separately, indicating a global, not circuit- or subregion-specific effect of corticosterone on basal *tph2* expression. For subregional results of *tph2* expression in the DRD, DRV, DRVL/VLPAG, DRC and DRI please consult the supplemental information online (Fig. S2A–G), which also contains correlation analyses of behavioral parameters with subregional and overall *tph2* mRNA expression in the DR (Fig. S3A–G).

4. Discussion

Administration of corticosterone in the drinking water for three consecutive weeks dose-dependently altered body weight gain, induced despair- and anxiety-like behavior, flattened the diurnal HPA axis rhythm, and elevated *tph2* expression in the DR of male rats.

4.1. Depressive-like behavior in the FST

Our results demonstrate that chronic glucocorticoid delivery to adrenal-intact rats is sufficient to induce despair-like

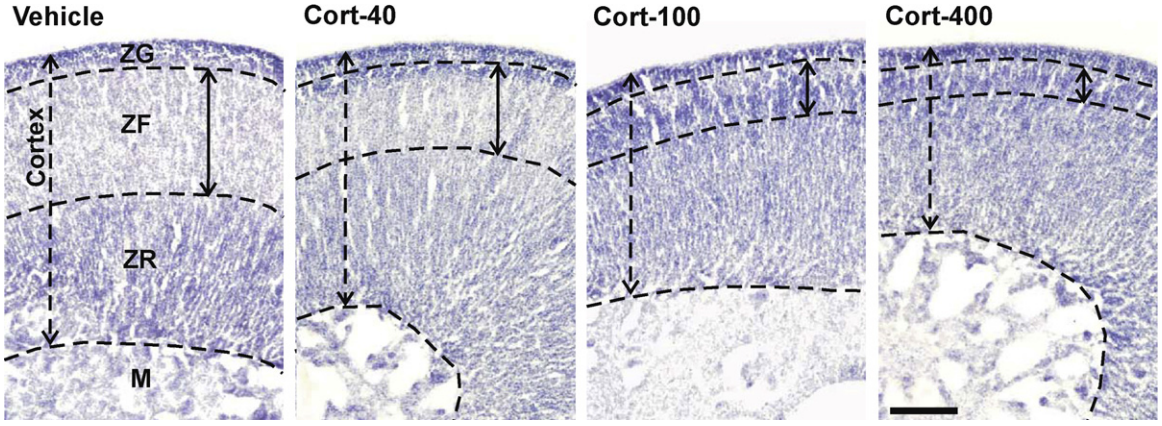

Figure 6 Photomicrographs of adrenal gland sections that were stained with cresyl violet to visualize the diameter of the adrenal cortex (dashed double arrow) and its subdivisions. Male rats were either treated with vehicle (0.45% hydroxypropyl-beta-cyclodextrin) or corticosterone in the drinking water (40, 100 or 400 μg/ml) for 21 days. The borders between the three cortical subdivisions in each treatment group are indicated by dashed lines. The continuous double arrow indicates the diameter of the zona fasciculata, the principal region of glucocorticoid production. Abbreviations: ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis; M, adrenal medulla. Scale bar: 100 μm.

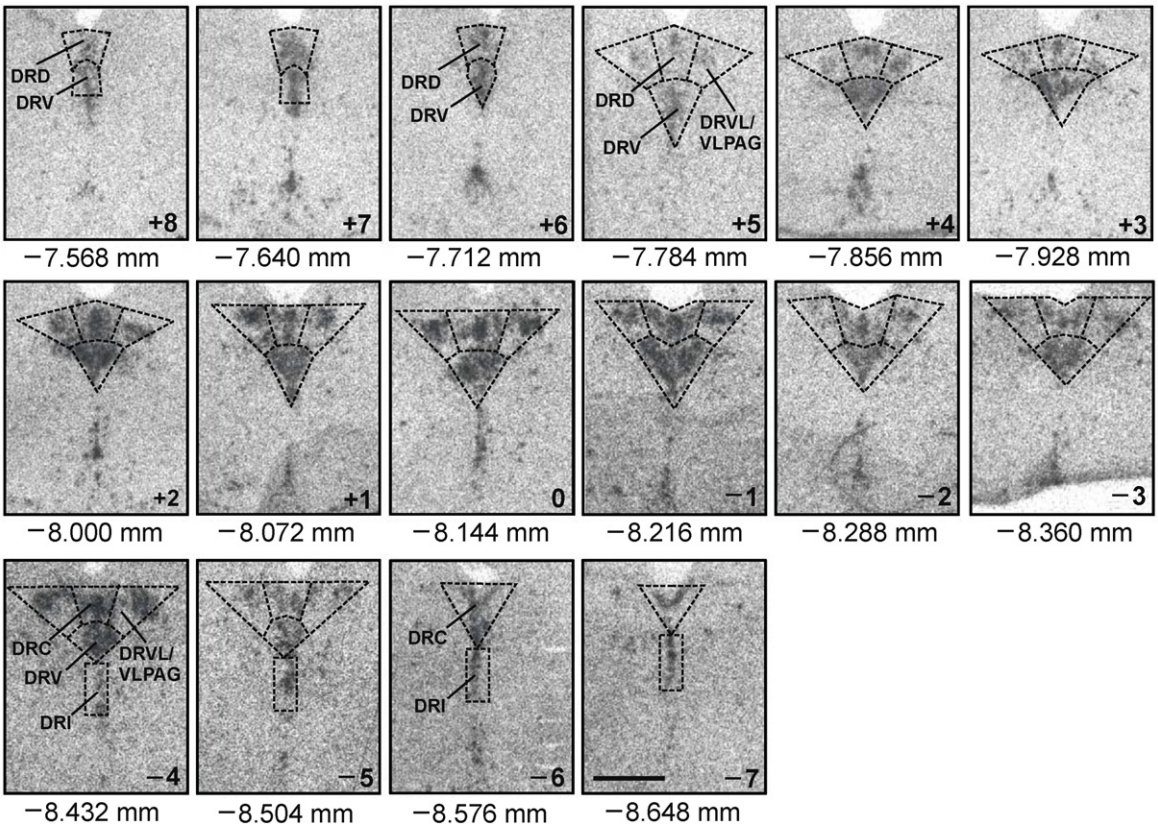

Figure 7 Neuroanatomical atlas of rat *tph2* mRNA expression used for analysis of the dorsal raphe nucleus. Displayed (from top left to bottom right) are the sixteen coronal levels that were used to measure *tph2* mRNA expression in all subregions of the dorsal raphe nucleus from rostral (−7.568 mm bregma, designated level +8) to caudal (−8.648 mm bregma, designated level −7). Photographs are autoradiographic images revealing the localization of bound 35-S-labeled cRNA probe complementary to *tph2* mRNA. Abbreviations are applicable to each following level until indicated differently. Abbreviations: 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, dorsal raphe nucleus, ventrolateral part; VLPAG, ventrolateral periaqueductal gray. Scale bar: 1 mm.

behavior. This is consistent with recent studies reporting comparable behavioral effects after drinking-water-mediated corticosterone administration (Gourley et al., 2008a,b; Gourley and Taylor, 2009; Lee et al., 2010). Here, Cort-100 and Cort-400 rats responded with decreased climbing behavior and increased immobility in the FST, but showed no change in swimming behavior. Our results are inconsistent with a study in which adrenal-intact mice treated with corticosterone (35 μg/ml via the drinking water) for 7 weeks showed no differences in mobility in the FST (David et al., 2009). The conflicting findings in the FST may be due to species differences, the time course of treatment, or methodology. For example, the murine FST only scores the final 4 min of the 5 min test session, while the majority of climbing behavior (60—95% in this study) tends to occur during the first minute of the test. On the other hand, our findings are consistent with those of Brotto et al. (2001) who also found that chronic corticosterone (20 mg/kg, s.c., daily during the active, dark phase for 10 days) decreases climbing behavior, without affecting swimming behavior, in adrenal-intact male rats. An increase in swimming behavior is thought to be associated with serotonergic activity, while altered climbing is correlated with noradrenergic or dopaminergic intervention (Detke et al., 1995; Cryan et al., 2005a; Perona et al., 2008). Indeed, chronic corticosterone treatment decreases hippocampal tyrosine hydroxylase mRNA and protein expression (Zhao et al., 2008). Consequently the corticosterone-induced decrease in climbing behavior observed in the FST in our study may be due to decreases in tyrosine hydroxylase mRNA and protein expression. This does not, however, preclude the possibility that elimination of the diurnal rhythm of *tph2* mRNA contributes to specific symptoms in hypercortisolism-induced depressive disorders (apart from those that might be reflected by reduced climbing behavior in the FST), such as increases in anxiety (Cloninger, 1990; Brady and Kendall, 1992; Kessler et al., 2005; Godart et al., 2006) or disruption of circadian rhythms (Soria and Urretavizcaya, 2009; Westrich and Sprouse, 2010), manifesting, for example, in sleep problems (Pandi-Perumal et al., 2009). More mechanistic studies are necessary to reveal whether or not the serotonergic system plays a crucial role in mediating the behavioral consequences of chronic glucocorticoid administration.

4.2. Anxiety-like behavior in the OF, EPM, and SI test

Concordant with previous studies (Ardayfio and Kim, 2006; Murray et al., 2008; Lee et al., 2010), chronic corticosterone treatment induced an anxiety-like state. In the SI and OF

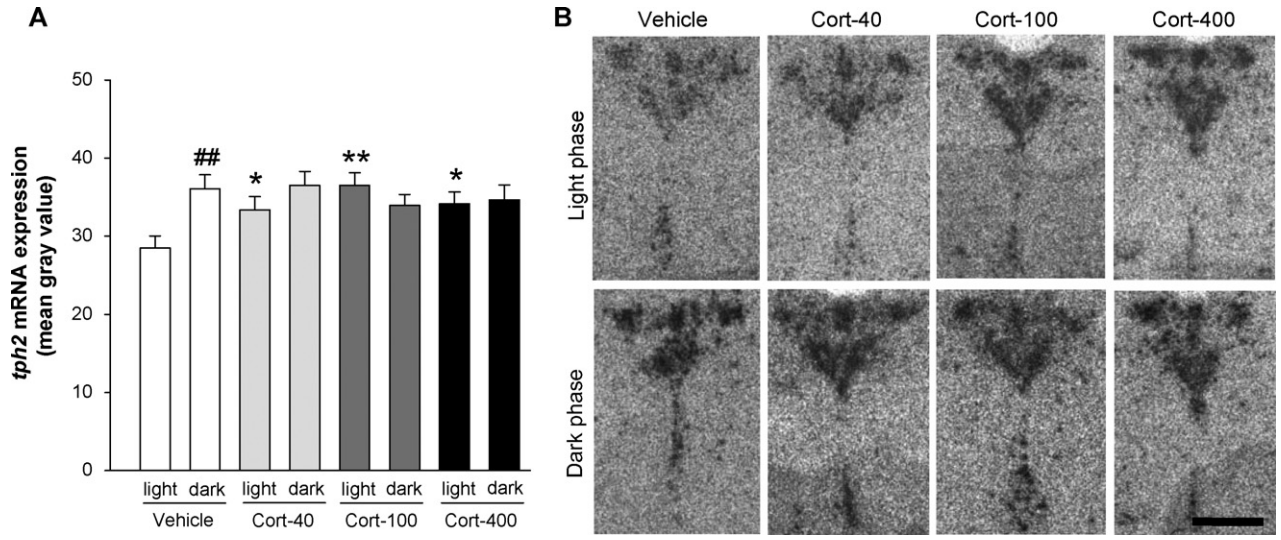

Figure 8 Average expression of *tph2* mRNA (A; mean gray value) in the entire brainstem dorsal raphe nucleus of male rats that were either treated with vehicle (0.45% hydroxypropyl-beta-cyclodextrin) or corticosterone (40, 100 or 400 μg/ml) for 21 days. Half of each treatment group was killed 2 h into the animals' inactive, light phase, the other half 2 h into the rats' active, dark phase. Also shown are representative photomicrographs of light- and dark-phase *tph2* mRNA expression in each treatment group at about−8.144 mm bregma (B). $^{*}p < 0.05$, $^{**}p < 0.01$ versus the corresponding light phase value of Vehicle. $^{\#\#}p < 0.01$ versus vehicle-light phase, after *post hoc* analysis with Fisher's Protected LSD test (vehicle-light, $n = 8$; vehicle-dark, $n = 8$; Cort-40-light, $n = 8$; Cort-40-dark, $n = 8$; Cort-100-light, $n = 8$, Cort-100-dark, $n = 8$; Cort-400-light, $n = 8$; Cort-400-dark, $n = 8$). Scale bar: 1 mm.

tests, increased anxiety-like behavior was only evident in Cort-400 rats. On the EPM, however, both Cort-100 and Cort-400 produced a strong anxiogenic effect (e.g. percent time in the open arms), and the number of full open arm entries was reduced following all chronic doses of corticosterone. The EPM is thought to include both conflict anxiety and panic- or escape-like behaviors (Pellow et al., 1985; McNaughton and Zangrossi Junior, 2008), and the greater sensitivity of this test, compared to SI or OF, may reflect effects of corticosterone on specific anxiety-related neuronal circuits (Lowry et al., 2005, 2008b; Hale et al., 2008). In our study, we did not observe altered locomotor activity in the OF test, and the increased number of rears of all corticosterone treatment groups in the EPM test may simply be confounded by the fact that these rats also spent more time in the closed arms, where most of the explorative rearing behavior naturally occurs. Corticosterone's effect on locomotion, however, remains controversial. In some studies (Veldhuis et al., 1982; Cador et al., 1993; Sandi and Guaza, 1994; Sandi et al., 1996a,b) acute or chronic corticosterone treatment has been reported to increase rat locomotor activity, while in others, chronic corticosterone administration had no effect on locomotion (Ehlers et al., 1992; Kalynchuk et al., 2004; Marks et al., 2009).

4.3. Elevated *tph2* mRNA during the light phase and abolished diurnal variation of *tph2* mRNA

In our model, chronic administration of corticosterone elevated *tph2* expression in the DR. In contrast, previous studies have found that *tph2* expression is resistant to stress-related stimuli. For example, *tph2* expression is unaltered following exposure of rats to social defeat (Gardner et al., 2009), chronic restraint stress (Abumaria et al., 2008), or chronic social stress (Abumaria et al., 2006). However, chronic unpredictable stress in adult male mice results in an increased corticosterone stress response and elevates *tph2* mRNA expression in the DR (McEuen et al., 2008), suggesting that *tph2* expression is only altered if habituation to a chronic stressor is prevented. In addition, studies in our laboratory have shown that adverse early life experience (e.g. maternal separation) creates a vulnerability to stress-induced increases in *tph2* mRNA later in life, suggesting that interactions between development and environment are crucial in determining *tph2* mRNA expression (Gardner et al., 2009; Hale et al., 2011).

Previous studies by Malek and colleagues revealed that the diurnal pattern of *tph2* expression depends on the diurnal rhythm of corticosterone (Malek et al., 2007). Here, we demonstrate that chronic corticosterone elevation abolishes the diurnal rhythm of the gene's expression by enhancing *tph2* expression during the inactive phase, while no differences were detected during the active phase. In contrast, Clark et al. have identified a decrease in both *tph2* expression in the DR and 5-HT synthesis in the frontal cortex after mice were exposed to 4 days of low dose dexamethasone (0.1–3.0 mg/kg, s.c. injection once per day), an effect that was blocked by co-administration of the glucocorticoid receptor antagonist mifepristone (Clark et al., 2005, 2008). Consistent with our findings, however, another study using the same species and delivery paradigm demonstrated that dexamethasone treatment of adrenalectomized rats via the drinking water increases TPH protein in the DR (Azmitia et al., 1993). While rats normally consume most of their water during drinking bouts throughout the active, dark phase (Johnson and Johnson, 1990), it is possible that chronic corticosterone administration in our study disrupted the circadian rhythm of drinking behavior, particularly as disruption of *tph2* mRNA rhythmicity may lead to disruption of TPH protein rhythmicity in the suprachiasmatic nucleus (SCN) (Barassin et al., 2002), the predominant circadian pacemaker

of the brain. Serotonergic function in the SCN plays an important role in the control of circadian rhythms (for review see Lowry (2002)), including perhaps the circadian pattern of drinking behavior (Edgar et al., 1997; Boer et al., 1998).

4.4. Correlation of *tph2* expression and anxiety- and depressive-like behavior

The fact that all doses of corticosterone treatment sufficiently abolished the normal diurnal variation in *tph2* mRNA emphasizes the potential of *tph2* mRNA diurnal rhythmicity as a sensitive biomarker for the development of depression. Yet, higher corticosterone doses were generally required to alter behavioral parameters, suggesting that together with altered serotonergic function, other glucocorticoid-sensitive brain circuitries are necessary to facilitate the behavioral phenotype of anxiety and depressive disorders.

In our study, increased *tph2* mRNA expression was associated with increased anxiety-related behavior and increased immobility in the FST. Our findings are seemingly inconsistent with previous reports that found increased *tph2* expression in the DR to be associated with antidepressant-like or anxiolytic effects in rodent models. For example, previous studies have found correlations between increased *tph2* mRNA expression and antidepressant-like or anxiolytic behavior following chronic treatment with antidepressants, such as imipramine or fluoxetine (Shishkina et al., 2007; Heydendael and Jacobson, 2009, 2010), or with sex steroids (Hiroi et al., 2006; Donner and Handa, 2009). In contrast, our data are consistent with the finding that chronic treatment with selective serotonin reuptake inhibitors like citalopram and/or fluoxetine result in decreased *tph2* mRNA (Shishkina et al., 2011) and TPH protein in the DR (MacGillivray et al., 2010), and that chronic citalopram decreases serotonin synthesis in DR target regions (Honig et al., 2009). In addition, our findings are consistent with studies of post-mortem tissues from depressed suicide patients, which have consistently found elevated *tph2* mRNA and protein expression in the DR (Underwood et al., 1999; Boldrini et al., 2005; Bach-Mizrachi et al., 2006, 2008; Bonkale et al., 2006; Underwood et al., 2010).

4.5. Proposed mechanism of corticosterone action to regulate *tph2* expression

Based on the global increase in *tph2* expression throughout all major subdivisions of the DR, we hypothesize that corticosterone acts directly on glucocorticoid or mineralocorticoid receptors within the DR to regulate *tph2* gene expression rather than indirectly, for example, on CRF neurons in the central nucleus of the amygdala. Also, direct, chronic infusion of CRF into the DR is insufficient to significantly alter *tph2* expression (Clark et al., 2007). A potential role for CRF in the central nucleus of the amygdala in glucocorticoid-induced increases in *tph2* expression would be supported by findings that glucocorticoids given chronically to adrenal-intact rats increase CRF mRNA expression in the central nucleus of the amygdala (Makino et al., 1994). CRF neurons within the central nucleus of the amygdala project to the DR (Gray and Magnuson, 1992; Gray, 1993), and activate serotonergic systems in a glucocorticoid-dependent manner (Singh et al., 1990a, 1992; Forster et al., 2008). However, a stress challenge may be required to increase *tph2* expression via this pathway, and would be expected to result in site-specific increases in *tph2* expression, due to the fact that the central nucleus of the amygdala has topographically organized projections to the DR, mainly to the ventrolateral and dorsal parts (DRVL/VLPAG and DRD) (for review see Hale and Lowry, 2011). Indeed, in previous studies involving models of chronic anxiety states and vulnerability to a depression-like syndrome, we have found site specific increases in *tph2* expression in the DRVL/VLPAG and DRD regions after stress challenge (Gardner et al., 2009; and unpublished observations).

4.6. Importance of *tph2*-neuronal projections and rhythmicity for hypothalamic regulation of circadian function

Barassin et al. (2002) reported that in addition to the diurnal variation in *tph2* mRNA expression as observed in the control animals in our study, TPH protein displays a significant light/dark variation in the median raphe nucleus (MnR), the SCN, and specifically the lateral wings (DRVL/VLPAG) of the DR, but not in other DR subdivisions nor in the entire DR combined. Interestingly, both midbrain raphe regions that were shown to have diurnal variation in TPH expression (MnR and DRVL/VLPAG) are known to project to the SCN (Lowry et al., 2008a). Thus, although *tph2* mRNA expression varies in a diurnal manner throughout the entire DR, diurnal variations in TPH protein seem most pronounced in neural systems regulating circadian function, such as the SCN. This has important implications for patients suffering from affective disorders as many of them suffer from dysfunctions of circadian rhythms, including HPA axis rhythmicity (Deuschle et al., 1997; Gillespie and Nemeroff, 2005; Graeff, 2007; Wichers et al., 2008a), sleep-wake cycles (Pandi-Perumal et al., 2009; Westrich and Sprouse, 2010), and body temperature (Rausch et al., 2003; Bunney and Potkin, 2008).

4.7. Abolished diurnal variation of plasma ACTH

While not all depressed patients display HPA axis abnormalities, adverse early life experiences are most predictive of elevated HPA axis activity and development of affective disorders in adulthood (Sanchez et al., 2001; Heim and Nemeroff, 2002; Heim et al., 2008). Consistent with findings in depressed patients that display hypercortisolism (Deuschle et al., 1997; Weber et al., 2000b; Wichers et al., 2008b; Van den Bergh and Van Calster, 2009), chronic glucocorticoid elevation in this study also flattened the diurnal HPA axis rhythm. In our study, normal circadian variation of plasma ACTH was completely abolished by all doses of corticosterone. More specifically, ACTH concentrations were reduced during the active phase in Cort-40 rats, and further decreased during both the inactive phase and the active phase in Cort-100 and Cort-400 animals. The basal elevation of glucocorticoids and flattening of the HPA axis rhythm in human depressed patients is thought to be due to increased cortisol release frequency during the nadir of HPA axis activity (Deuschle et al., 1997), and impaired negative feedback of HPA axis activity (Heuser et al., 1994), possibly due to reduced sensitivity of glucocorticoid receptors either peripherally at the pituitary or centrally at the level of the

hypothalamus and limbic structures (Holsboer, 2000; Pariante and Miller, 2001; Pariante, 2009). In our study, corticosterone treatment resulted in a dose-dependent overall decrease in plasma ACTH, potentially indicative of intact negative feedback mechanisms within the HPA axis of our experimental rats, even though circadian HPA axis rhythmicity was lost.

4.8. Metabolic effects of corticosterone

In male rats, reduced body weight gain is commonly used to assess the efficacy of repeated or chronic glucocorticoid treatment (Conrad et al., 2007; Gourley and Taylor, 2009). Low doses of repeated glucocorticoid treatment are known to exert anabolic actions while chronic medium to high doses enhance catabolic processes including lipolysis and muscle protein breakdown (Tomas et al., 1979; Devenport et al., 1989; Santana et al., 1995). Meanwhile, chronic medium to high doses of glucocorticoids act, for example, on hypothalamic neuropeptide Y to increase food intake (Tempel and Leibowitz, 1994). Accordingly, Cort-100 and Cort-400 rats in our study gained much less weight or continuously lost weight, even though they ate and drank more, when corrected for body weight, than vehicle controls after 2 and 3 weeks of treatment. Chronic corticosterone administration also flattened the diurnal rhythm of plasma glucose dose-dependently, and resulted in decreased plasma glucose availability, an effect that seems counterintuitive given the anti-insulin actions of glucocorticoids (Warne et al., 2009). However, complex species-, dose-, and diet-dependent metabolic changes take place during chronic glucocorticoid exposure. For example, opposite effects of chronic corticosterone administration (25 or 100 μg/ml in the drinking water for 4 weeks) on weight gain have been reported in mice (Karatsoreos et al., 2010), and depressed, hypercortisolic human patients display elevated fasting plasma glucose (Roberge et al., 2007; Cassels, 2009), suffer from glucose and insulin resistance (Weber et al., 2000a), and have a higher prevalence for developing obesity, type 2 diabetes, or metabolic syndrome (Tsigos and Chrousos, 2002; Roberge et al., 2007; Anagnostis et al., 2009; Kyrou and Tsigos, 2009; Cagampang et al., 2011).

5. Conclusion

In conclusion, our data suggest that chronic exposure to increased concentrations of glucocorticoids, as observed in various affective disorders (Plotsky et al., 1998; Barden, 2004; Gillespie and Nemeroff, 2005; Graeff, 2007; Gunduz-Bruce et al., 2007), Cushing's syndrome (Sonino et al., 1998; Michaud et al., 2009; Pereira et al., 2010), or after prolonged stress exposure (Leonard, 2005; Herman et al., 2008; McEuen et al., 2008; Kyrou and Tsigos, 2009), is sufficient to elevate *tph2* expression, induce a depression-like syndrome, flatten the diurnal HPA axis rhythm, and alter both glucose metabolism and weight gain. Our results indicate that dysregulation of circulating glucocorticoid concentrations and rhythmicity, independent from exposure to external stressors, may disrupt the diurnal pattern of serotonin synthesis, and thus contribute to the dysfunction of circadian rhythms symptomatic of many psychiatric disorders, specifically affective disorders. In addition, our data provide further rationale for continued exploration of the use of HPA-axis-targeting medication for patients suffering from affective disorders (O'Dwyer et al., 1995; Belanoff et al., 2002; Pariante et al., 2003; Young et al., 2004).

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Conflict of interest

The authors report no biomedical financial interests or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.psyneuen.2011.08.008.

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