Impaired Glucocorticoid Production and Response to Stress in *Arntl*-Deficient Male Mice

Alexei Leliavski, Anton Shostak, Jana Husse, and Henrik Oster

Chronophysiology Group (A.L., H.O.), Medical Department I, University of Lübeck, Lübeck, Germany; and Circadian Rhythms Group (A.L., A.S., J.H., H.O.), Max Planck Institute for Biophysical Chemistry, Göttingen, Germany

The basic helix-loop-helix transcription factor Aryl Hydrocarbon Receptor Nuclear Translocator-Like (ARNTL, also known as BMAL1 or MOP3) is a core component of the circadian timing system in mammals, which orchestrates 24-hour rhythms of physiology and behavior. Genetic ablation of *Arntl* in mice leads to behavioral and physiological arrhythmicity, including loss of circadian baseline regulation of glucocorticoids (GCs). GCs are important downstream regulators of circadian tissue clocks and have essential functions in the physiological adaptation to stress. The role of the clock machinery in the regulation of stress-induced GC release, however, is not well understood. Here we show that already under unstressed conditions *Arntl*-deficient mice suffer from hypocortisolism with impaired adrenal responsiveness to ACTH and down-regulated transcription of genes involved in cholesterol transport in adrenocortical cells. Under stress they show diminished GC and behavioral responses and develop behavioral resistance to acute and subchronic stressors, as shown using forced swim, tail suspension, and sucrose preference tests. These data suggest that the clock gene *Arntl* regulates circadian and acute secretion of GCs by the adrenal gland. *Arntl* disruption, probably via its effect on adrenal clock function, modulates stress axis activity and, thus, may promote resistance to both acute and repeated stress. **(*Endocrinology* 155: 133–142, 2014)**

Glucocorticoid (GC) hormones play an essential role in the orchestration of physiology and behavior in response to stress (1–3), while at the same time GCs have been implicated in the entrainment of circadian rhythms (4, 5). Excessive GC production is associated with a variety of pathologies including metabolic deregulation and mood disorders such as depression and anxiety (2, 3). GCs, mainly cortisol in humans and corticosterone (CORT) in rodents, are predominantly produced by the adrenal glands in a pulsatile fashion with an underlying circadian rhythm (6). Adrenal GC secretion reflects the activation of the hypothalamic-pituitary-adrenal (HPA) axis. Hypophyseal ACTH binds to melanocortin-2 receptors (MC2Rs) in adrenocortical cells where it stimulates transport of cholesterol into mitochondria where CORT biosynthesis takes place. Daily GC peak levels are observed in the beginning of the activity phase, ie, in the early morning in humans and in the evening in nocturnal animals. Different mechanisms are involved in the circadian regulation of GC rhythms (reviewed in Refs. 6 and 7) including molecular circadian clocks located in neurons of the hypothalamic suprachiasmatic nuclei (8) and in adrenocortical cells (9–11).

At the molecular level, these clocks are composed of transcriptional-translational feedback loops (12, 13), in which the transcription factors Circadian Locomotor Output Cycles Kaput (CLOCK) and Aryl Hydrocarbon Receptor Nuclear Translocator-Like (ARNTL) activate *Per* and *Cry* genes, the products of which feed back on their own transcription by inhibiting CLOCK/ARNTL. In addition, the CLOCK/ARNTL complex induces rhythmic transcription of a plethora of other genes, translating the activity of the molecular oscillator to rhythmic physiology. ARNTL plays a key role in the circadian clockwork, because, in mice, its deficiency leads to abrogation of endogenous behavioral and molecular rhythms (14, 15). GCs have been implicated in synchronizing circadian clocks in peripheral tissues and in the central nervous sys-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Received June 5, 2013. Accepted October 23, 2013.
First Published Online November 4, 2013

Abbreviations: ARNTL, Aryl Hydrocarbon Receptor Nuclear Translocator-Like; CLOCK, Circadian Locomotor Cycles Output Kaput; CORT, corticosterone; CT, circadian time; DD, constant dark; FST, forced swim test; GC, glucocorticoid; HPA axis, hypothalamic–pituitary-adrenal axis; LD, light-dark; SPT, sucrose preference test; TST, tail suspension test; ZT, Zeitgeber time.

tem (CNS) (16, 17). Activated GC receptors induce transcription of *Per* genes (18) or directly interact with clock proteins (19, 20).

Stress-induced HPA axis activation and subsequent GC release mediate behavioral and physiological adaptation to various threatening conditions. Elevated blood GC levels stimulate the mobilization of energy substrates from liver and adipose stores, enhance memory formation, and promote effective coping with stress (1, 21). On the other hand, chronic stress/HPA axis activation may lead to various behavioral pathologies in mice and humans such as impaired memory and cognition, vulnerability to depression and anxiety, and abnormal reward seeking (2, 22, 23).

In the present study we focus on how *Arntl* regulates GC release from the adrenal gland. We find impaired CORT production and ACTH responses in *Arntl*-deficient mice together with blunted behavioral effects of acute and subchronic stress, suggesting a critical role of the circadian clock gene *Arntl* in the physiological adaptation to stress.

Materials and Methods

Animals and housing

All animal experiments were ethically approved and licensed by the local state authorities and executed according to the regulations of the German Animal Welfare Act (TierSchG). Male wild-type and *Arntl*$^{-/-}$ mice (3–4 months old) on a C57BL6J background were individually housed under 12-hour light, 12-hour dark conditions (LD; 300–400 lux) with ad libitum access to food and water. For adrenal slice culture experiments and analysis of gene expression in constant darkness conditions (DD), single-housed animals were LD-entrained for at least 1 week and then released into DD. Tissues were collected 36 and 48 hours after "lights off", which roughly corresponds to circadian times (CT) 0 and CT12, ie, the beginning and end of the animal's rest phase, in wild-type animals. For hormonal measurements, blood samples were also collected at CT6 and CT18 (42 and 54 hours after lights off).

Behavioral tests

Forced swim test (FST)

The test was performed at the end of the light phase (between Zeitgeber time (ZT) 10 and ZT12, ie, 10–12 hours after "lights on") as described in Ref. 24, with minor modifications. Briefly, animals were placed for 6 minutes into a standard 3-L glass beaker filled with tap water (25 ± 2°C) from which they could not escape. Every session was video recorded, and the duration of immobility over every minute of the 6-minute test was estimated using the CowLog open source software (http://cowlog.org) (25).

Repeated restraint stress

Mice were exposed to confinement stress once daily for 2 hours between ZT10 and ZT12 for 7 consecutive days (26) by keeping them in small transparent plastic restrainers (95 × 30 × 32 mm). Sucrose preference tests (SPTs; see below) were conducted 1 day before the restraint period (baseline sucrose preference, see below) and after 7 restraint sessions. Immobility behavior during a tail suspension test (TST; see below) was assessed twice, 1 day before the beginning of the first and 1 day after the last restraint session.

TST

TSTs were conducted according to a protocol described elsewhere (24). Between ZT10 and ZT12, mice were suspended for 6 minutes by the tail on a horizontal bar at a height of 20–25 cm. Every session was video recorded in the absence of the experimenter. The duration of immobility (ie, passive hanging without movements) over the course of the 6-minute test was measured with assistance of the CowLog software.

SPT

To estimate baseline sucrose preference (27), mice were provided a choice between 2 bottles filled with 1% sucrose solution and tap water. To avoid positional preference, bottle positions were changed twice a day, in the middle of activity and rest phases, respectively. The bottles were weighed once a day (at the end of light phase) for 3 consecutive days, and sucrose and water intake were averaged. Sucrose preference after restraint stress was evaluated over a 24-hour period. To calculate the percentage of sucrose preference, the amount of consumed sucrose solution was divided by the amount of total liquid intake and multiplied by 100.

Quantitative RT-PCR

Quantitative analysis of mRNA levels was performed as described elsewhere (28). Total RNA was extracted from whole adrenal tissues using TRIzol Reagent (Life Technologies) according to the manufacturer's instructions. cDNA was synthesized using High Capacity cDNA Reverse Transcription kit (Life Technologies). Quantitative real-time PCR was performed on a C1000 Thermal Cycler and CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories) with GoTaq qPCR Master Mix (Promega Corp.), and relative expression was assessed by comparison with *Eef1a1* using the ΔΔCT method (9). Primer sequences are listed in Supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Adrenal responsiveness to ACTH ex vivo

Adrenal slice culture and adrenal ACTH stimulation ex vivo were performed as described previously (10). Briefly, 200-μm slices were precultured for 20 minutes on Millicell-CM membranes (Millipore Corp.) in DMEM (PAA Laboratories) supplemented with 0.1% dimethyl sulfoxide, 50 μM 2-mercaptoethanol, 2% fetal bovine serum, and 0.12 mg/mL penicillin/streptomycin at 37°C and 5% CO_2. Slices were stimulated with 20 nM ACTH and medium was collected immediately (0 minutes), 30 minutes, 90 minutes, and 210 minutes later. To study dose response to ACTH, adrenal slices were stimulated with 0,

2, and 20 nM ACTH, and medium was collected 90 minutes later. Samples were stored at −80°C until further processing.

Dexamethasone suppression test

Mice received ip injection of dexamethasone solution (100 μg per kg of body weight in 0.9% saline) at ZT8, and trunk blood was collected 6 hours later for corticosterone (CORT) analysis, as described below. A control group was injected with 200 μL of 0.9% saline solution. The selected dosage was previously shown to be efficient in suppressing CORT production down to about 20% compared with saline-injected controls (29).

Sample preparation and hormone measurements

Animals were removed from their cages and immediately culled by cervical dislocation. Trunk blood was collected in Microvette 300 EDTA-coated tubes (Sarstedt), centrifuged at 2200 × *g* for 20 minutes at 4°C, and plasma was frozen at −80°C until use. The time passed between opening the cage and finishing blood collection was usually around 1 minute, but never more than 2 minutes. Fecal samples were collected at 4-hour intervals and stored at −80°C until extraction. Fecal corticoid extraction was done according to a previously published protocol (30). CORT/corticoid concentrations were measured using a commercially available RIA kit from MP Biomedicals (catalog no. 07–120103). Plasma samples were diluted at 1:200, fecal extracts at 1:5, and medium samples at 1:10, respectively. ACTH plasma concentrations were analyzed using the IMMULITE 1000 Immunoassay System (Siemens) at 1:2 to 1:4 dilutions.

Histologic analysis

Isolated adrenal glands were removed from surrounding fat, weighed, fixed in 4% paraformaldehyde, and embedded in paraffin. Adrenal sections (8-μm) were stained with hematoxylin-eosin. To evaluate adrenal cortex-to-medulla ratio, cortical and medullar areas were measured from every specimen in at least 3 different sections close to the middle of the adrenal gland. Lipid staining was performed on frozen adrenals obtained from untreated or repeatedly stressed mice (3 sequential 10-minute forced swim sessions with 30-minute rest intervals). Cryosections (10-μm) were rinsed in 60% isopropanol and stained with Oil Red O solution (Sigma-Aldrich) for 10 minutes. Image analysis was performed with Image J software (National Institutes of Health, Bethesda, Maryland).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad). All data are represented as means ± SEM. Whenever applicable, normality of data distribution was confirmed using the D'Agostino-Pearson omnibus test. For smaller cohort sizes outlier tests were performed, revealing no indications of non-normal distribution. Two-group comparisons were done using unpaired *t* tests. For multiple comparisons one- or two-way ANOVA with Bonferroni post hoc test was used as indicated in the figure legends. Time course analyses (Figures 1, A, C, and E, 3C, and 5; and Supplemental Figure 1) were performed using repeated-measures two-way ANOVA. *P* values below 0.05 were considered significant.

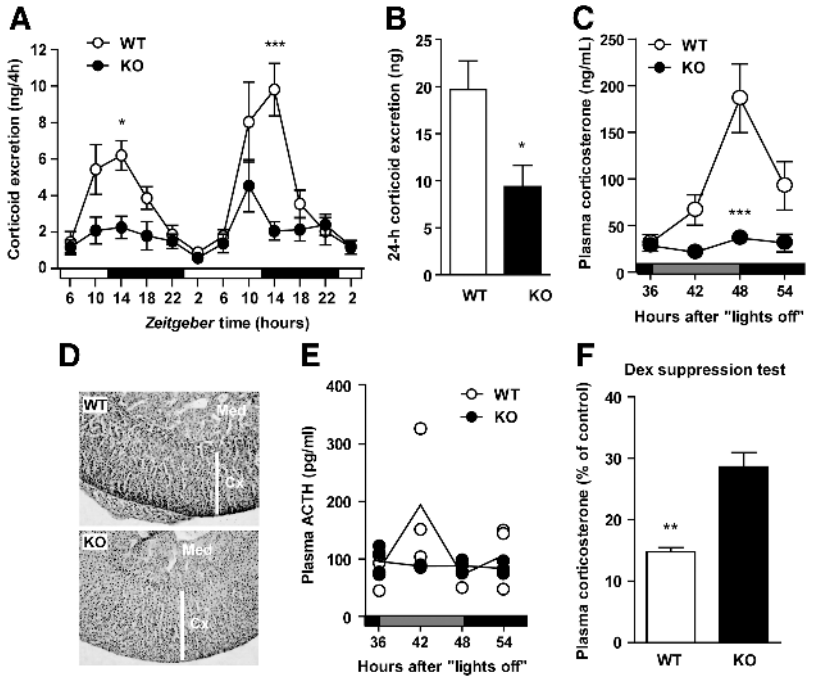

Figure 1. Hypocortisolism in *Arntl*$^{-/-}$ mice A and B, Profile (48 hour) of corticoid excretion (A) and total amount of excreted corticoids per day (B) in fecal samples from wild-type (WT) and *Arntl*$^{-/-}$ (knockout [KO]) mice kept in LD (n = 5–6). C and D, Plasma CORT (C) and plasma ACTH (E) levels in WT and KO mice on the second day in DD (n = 3–5). D, Hematoxylin and eosin staining of WT and KO adrenals (Cx, adrenal cortex; Med, medulla); magnification, 20×. F, Dexamethasone (Dex) suppression test. Suppressive effect of dexamethasone on CORT production is normalized to CORT levels of saline-injected control mice (n = 4). *, $P < .05$; **, $P < .01$; ***, $P < .001$ (two-way ANOVA with Bonferroni post hoc test [panels A, C, and E]; Student's *t* test [B and F]).

Results

Hypocortisolism in Arntl$^{-/-}$ mice

To test whether *Arntl* deficiency affects daily dynamics of GC production, we measured corticoid excretion in feces of *Arntl*$^{-/-}$ and congenic wild-type mice at 4-hour intervals over the course of 2 days (30). Fecal corticoid excretion profiles have been shown to faithfully mimic blood CORT levels, with a delay of 4–6 hours, while allowing for repeated noninvasive sampling from individual animals and reducing variability caused by ultradian CORT oscillations (30, 31). As expected, corticoid excretion in *Arntl*-deficient mice showed strongly dampened diurnal rhythmicity (Figure 1A). Moreover, overall corticoid excretion in mutants was reduced by about 50% in comparison with age-matched wild-type controls (Figure 1B). Similarly, nonrhythmic and

overall low CORT levels were observed in plasma and fecal samples of *Arntl*$^{-/-}$ mice kept in DD (Figure 1C and Supplemental Figure 1).

Histologic examination of *Arntl*-deficient adrenals did not reveal any gross defects in adrenal morphology (Figure 1D and Supplemental Figure 2B). Relative adrenal weight was even slightly increased in *Arntl*$^{-/-}$ animals compared with wild-type controls (Supplemental Figure 2A). Hypocortisolism could result from deregulation of the HPA axis upstream of the adrenal gland, eg, by diminished ACTH synthesis or release from the pituitary (32). To address this point, we measured plasma ACTH levels in wild-type and *Arntl*-deficient mice at 4 different times on the second day in DD. We found no significant differences in ACTH concentrations between wild-type and *Arntl*$^{-/-}$ mice (Figure 1E) at any of the time points examined, although individual variation at 42 hours in wild-type mice was quite high, suggesting that reduced CORT production may not simply represent the result of reduced ACTH signal. The combination of largely unchanged ACTH levels with a hypocortisolic state in *Arntl*$^{-/-}$ mice may indicate blunted sensitivity of the HPA axis to negative CORT feedback. Indeed, we found that dexamethasone was less effective in inhibiting CORT production in knockout mice (Figure 1F).

ACTH hyposensitivity in Arntl$^{-/-}$ adrenal slice culture

These findings let us to hypothesize that hypocortisolism in *Arntl*$^{-/-}$ mice may result, at least in part, from reduced sensitivity of the adrenal cortex to ACTH stimulation. To test this, we cultured adrenal tissue slices from wild-type and *Arntl*$^{-/-}$ mice culled at 36 and 48 hours after lights off and stimulated them with 20 nM ACTH to measure CORT responses ex vivo. In wild-type explants CORT production was rapidly induced upon ACTH stimulation at both time points, with higher responsiveness at 48 hours correlating with high in vivo CORT levels at this time point (10) (Figure 2A). In accordance with our hypothesis, the ability of *Arntl*$^{-/-}$ adrenals to respond to ACTH stimulation did not differ between the 2 time points and was dramatically reduced compared with wild types (Figure 2B). A dose response determined at 48 hours confirmed the reduced CORT response of *Arntl*$^{-/-}$ adrenal slices to ACTH concentrations at various concentrations (Figure 2C).

Arntl$^{-/-}$ mice show decreased CORT and behavioral responses to acute stress

The altered CORT-to-ACTH ratio under undisturbed conditions in vivo, together with the blunted ACTH sensitivity of adrenal slices, suggested that *Arntl* deficiency

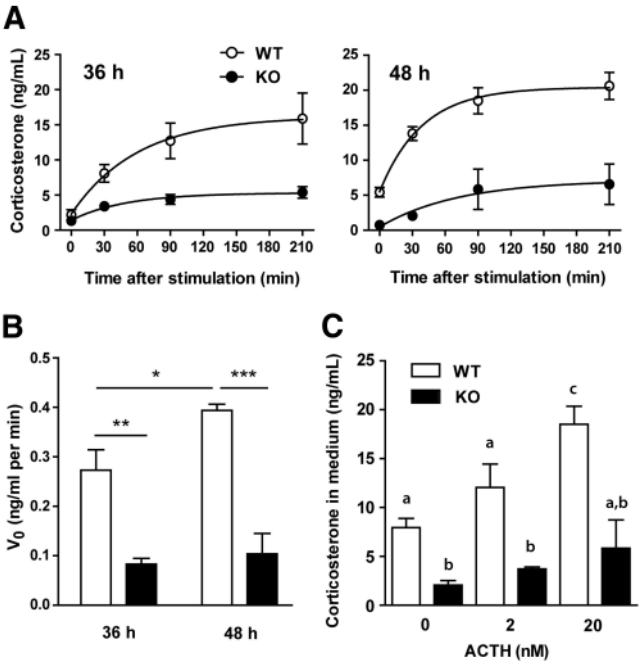

Figure 2. Reduced responsiveness of *Arntl*$^{-/-}$ adrenal explants to ACTH stimulation A, Dynamics of CORT release into the medium after stimulation of wild-type (WT) and knockout (KO) adrenal slices with 20 nM ACTH (n = 3–6) in adrenal slice explants prepared at 36 hours (left panel) or 48 hours (right panel) after lights off. B, Initial rate of CORT release from ACTH-treated WT and KO adrenal explants. Two-way ANOVA revealed significant effect of genotype on the initial rate ($P < .0001$). C, Dose response to ACTH stimulation in WT and KO adrenal slices. Identical letters indicate the absence of significant differences between columns. V0, Release rate at t = 0; *, $P < .05$; **,$P < .01$; ***, $P < .001$ (Bonferroni post hoc test).

may also lead to altered CORT responses after acute stress. To test this, wild-type and *Arntl*$^{-/-}$ mice were subjected to an FST as an acute stressor (33), and plasma CORT levels were measured in different cohorts before and after the FST. Because differences in adrenal sensitivity to ACTH between wild-type and mutants were highest at 48 hours in DD (Figure 2), all further experiments were performed at this time point. As expected, in wild-type mice FST caused a more than 3-fold elevation of CORT levels compared with baseline conditions (Figure 3A). In contrast, no significant up-regulation of CORT levels, but preserved ACTH responses, were observed in stressed *Arntl*$^{-/-}$ mice (Figure 3, A and B). In line with this, wild-type mice showed increasing amounts of immobility over the first 6 minutes of the FST, which is usually interpreted as despair-like behavior (Figure 3C). In contrast, and analogous to their absent CORT response, *Arntl*$^{-/-}$ mice stayed invariably active during the whole course of the test (Figure 3C). Of note, body position in water during immobility bouts was unaltered in *Arntl*-deficient mice (Supplemental Figure 3), and the duration of immobility bouts in a repeated FST was comparable to those in wild-type counterparts (data not shown). This suggests that the ob-

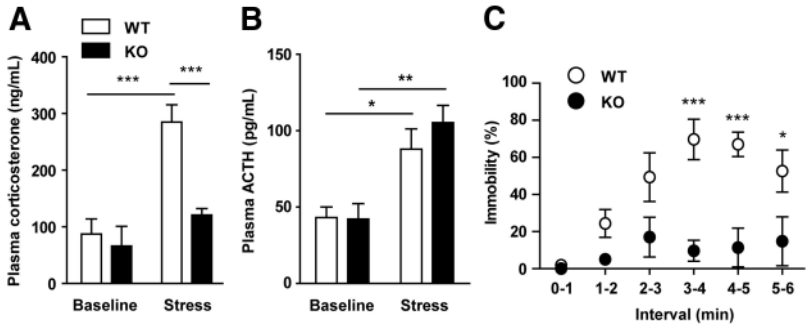

Figure 3. Reduced hormonal and behavioral responses to acute stress in *Arntl*$^{-/-}$ mice A, Plasma CORT and ACTH concentrations in wild-type (WT) and knockout (KO) animals before and after acute stress (forced swimming) at 48 hours after lights off (n = 4–10). In panel A, two-way ANOVA revealed significant effects of genotype and treatment and interaction between both factors ($P = 0.008$; $P = 0.0007$; and $P = 0.034$, respectively). In panel B, a significant effect of treatment was found ($P = 0.0003$). C, Time course of immobility behavior (in percent) during 6 minutes of forced swimming in WT and KO mice (n = 4–5). *, $P < .05$; ***, $P < .001$ (Bonferroni post hoc test).

served vigorous swimming was not merely a behavioral adaptation of *Arntl*-deficient mice to a compromised ability of staying afloat due to unrelated physical and metabolic abnormalities (34, 35). Reduced body temperature (Supplemental Figure 3C) and impaired muscle strength, as reported in *Arntl* mutants (34), would rather promote the time spent immobile in the FST (36), but the opposite phenotype was observed in *Arntl*$^{-/-}$ mice. Together, we conclude that *Arntl*-deficient mice show HPA axis insensitivity correlating with behavioral resistance in response to acute stress evoked by forced swimming.

Altered adrenal expression of transcripts involved in cholesterol transport and ACTH signaling

To identify a potential mechanism underlying the observed changes in the regulation of CORT production in *Arntl*$^{-/-}$ mice, we measured the mRNA levels of genes involved in adrenocortical physiology (summarized in Supplemental Figure 4) using quantitative RT-PCR. We hypothesized that potential ARNTL target genes, being under control of this essential transcription factor of the circadian clock, are likely to be expressed in a circadian manner (eg, *Mc2r*, *Mrap*, *Prkce*, *Sp1*, *Nr5a1*, *Nr0b1, Star*, *Ldlr*, *Stard4*, *Por*), as has been reported in previous studies (9, 10, 37). In addition, we included genes that encode key steroidogenic enzymes (*Cyp11a1*, *Cyp11b1, Hsd3b1*) and proteins involved in transport of cholesterol as the main substrate of CORT biosynthesis (*Scarb1*, *Nr1h3*). We found that the mRNA levels of most genes associated with adrenal development and steroidogenesis (*Cyp11a1*, *Cyp11b1, Nr5a1*, *Nr0b1, Nr1h3*) remained largely unaltered in *Arntl*$^{-/-}$ adrenal glands (Figure 4A). In contrast, several key genes involved in cholesterol trafficking (*Star*, *Ldlr*, *Stard4*) were down-regulated by 50% or more in *Arntl*-deficient adrenals (Figure 4C), while, at the same time, the expression of *Mc2r*, which encodes the ACTH receptor, appeared elevated in *Arntl*$^{-/-}$ compared with wild-type adrenals (Figure 4B).

Translocation of cholesterol to the mitochondrion is the rate-limiting step of steroidogenesis and, therefore, down-regulation of the cholesterol transport machinery may explain the blunted CORT responsiveness to ACTH stimulation or stress observed in *Arntl*$^{-/-}$ adrenals. To test this more directly, we analyzed adrenal lipid content in untreated and stressed mice using Oil Red O staining. In wild-type mice cholesterol esters that make up the vast majority of lipids stored in adrenocortical lipid droplets became depleted dramatically after repeated swimming stress. In the mutants baseline levels were already reduced compared with wild types, but importantly, little effect was observed after stress (Figure 4D), which would be in line with an incapacity to transport cholesterol into mitochondria for conversion into CORT.

To test whether altered levels of gene expression in *Arntl*$^{-/-}$ adrenals may reflect a general metabolic deficiency, we analyzed levels of the regulated transcripts in different metabolic tissues. Whereas *Por* was similarly down-regulated in liver, adipose tissue, and muscle, differential effects on *Ldlr*, *Star* and *Stard4* expression after deletion of *Arntl* were found in different tissues (Supplemental Figure 5). These data do not support the hypothesis that systemic metabolic changes in *Arntl*-deficient mice are responsible for the reduced expression of cholesterol transport genes.

In summary, our results suggest that deregulation of gene expression, particularly genes involved in cholesterol transport, in *Arntl*-deficient adrenal glands may contribute to compromised adrenal responsiveness to ACTH and, hence, reduced CORT response to stress.

Arntl$^{-/-}$ mice are resistant to behavioral changes induced by repeated restraint stress

It is well accepted that chronic/prolonged stress induces dramatic changes in animal and human behavior, including increased susceptibility to depression, anxiety, and drug addiction (1, 2, 23). We hypothesized that, complementary to reduced acute stress responses, behavioral changes in response to a subchronic stressor might also be altered in *Arntl*-deficient mice. To address this point we used a 1-week repeated restraint stress paradigm with

SPTs and TSTs tests as behavioral outputs (38–40). Under baseline conditions sucrose preference as well as absolute intake of 1% sucrose solution were reduced in *Arntl*$^{-/-}$ mice compared with age-matched wild-type controls (Figure 5, A and B). After 1 week of daily restraint wild-type mice significantly increased sucrose intake and sucrose preference, whereas in *Arntl*$^{-/-}$ mice, no change in sucrose intake behavior was observed (Figure 5, A and B). Moreover whereas wild-type mice showed increased immobility in the TST repeated after 1 week of constraint stress, *Arntl*-deficient animals were significantly less immobile at the end of the stress period (Figure 5C). Taken together, we conclude that the circadian clock gene *Arntl* regulates behavioral responses to acute and chronic stressors, potentially via modulation of adrenal CORT secretion.

Discussion

In the current study we demonstrated that *Arntl* is necessary for normal CORT production and responses to acute and repeated stress. *Arntl*-deficient mice show hypocortisolism without changes in ACTH secretion. *Arntl*$^{-/-}$ adrenals are less sensitive to ACTH stimulation ex vivo correlating with reduced expression of cholesterol transport genes *Star*, *Stard4*, and *Ldlr*. Together these changes may lead to blunted GC and behavioral responses to stress.

We observed that *Arntl*$^{-/-}$ mice show low levels of plasma CORT and blunted circadian corticoid rhythms under LD and DD conditions (Figure 1 and Supplemental Figure 1), confirming previous findings that *Arntl* is indispensable for maintenance of physiological circadian rhythms. Of note, Rudic et al (41) did not observe a loss of daily GC variation in *Arntl*$^{-/-}$ mice in DD, but plasma CORT was measured only at 2 time points (CT4 and CT16), whereas the normal peak and trough of GC secretion (CT0 and CT12) were not assessed. Hypocortisolism has also been reported in mice carrying a mutation in the gene encoding for the ARNTL partner CLOCK (42), whereas, to the contrary, a lack of *Cry* genes results in up-regulated CORT levels (20, 28). This is consistent with the view that the components of the positive limb of the circadian clockwork, ARNTL and CLOCK, promote GC production, whereas members of the negative branch have opposite effects. Similarly, constant dis-inhibition of ARNTL/CLOCK activity in *Cry1/2* double-mutant mice promotes overproduction of another adrenal corticoid, aldosterone (43). Blood CORT levels are low in *Per2* single- and *Per2/Cry1* double-mutant mice (10, 44), possibly reflecting the positive impact of PER proteins on *Arntl* transcription (45).

Hypocortisolism, a main feature of adrenal insufficiency, can be caused by a variety of primary and secondary factors, including impaired HPA axis activity and steroidogenesis or GC metabolism, but also defects in adrenal development (46). Deletion of *Arntl* did not cause any significant alterations in ACTH levels. Together with reduced ACTH sensitivity (Figure 2), this suggests that blunted CORT secretion in *Arntl*$^{-/-}$ mice may be rooted in the adrenal itself, ie, representing a case of primary hypocortisolism. However, low GC levels were not mirrored by a dis-inhibition of ACTH release from the pituitary, which is in accordance with impaired sensitivity of the HPA axis to inhibitory CORT feedback (Figure 1, E and F). This could have developmental reasons or may simply reflect an additional effect of *Arntl* deficiency on GC feedback target regions, ie, the hypothalamus or the pituitary. In line with this, it was found that *Arntl* is required for induction of *Per2* expression by GCs (19).

In addition to regulating circadian clock function, *Arntl* plays an important role in tissue development, eg, in skeletal muscle and adipose tissues (34, 47). However, our findings, together with published observations (10, 11), suggest that hypocortisolism in *Arntl*$^{-/-}$ mice seems to represent a functional defect, rather than a result of aberrant adrenal development. Indeed, analysis of *Arntl*$^{-/-}$ adrenal weight, morphology, and cortex-to-medulla ratio did not indicate gross developmental abnormalities. Additionally, the expression of the key transcription factors SF1 (encoded by *Nr5a1*) and DEX1 (*Nr0b1*) regulating adrenal gland development was not altered in *Arntl*-deficient adrenals (Figure 4).

Our data and previous studies suggest that circadian clock gene deficiency promotes adrenal ACTH resistance (10, 48). The clock machinery regulates cellular physiology via transcriptional programs (12). This lead us to hypothesize that hypocortisolism in *Arntl*$^{-/-}$ mice may be the consequence of altered expression of clock target genes involved in regulating the steroidogenesis (9–11, 37). Indeed, the down-regulation of cholesterol transport (*Ldlr*, *Star*, *Stard4*) and steroidogenic (*Por*) genes in *Arntl*-deficient adrenals, together with a lack of cholesterol depletion upon stress (Figure 4), may provide an explanation for the observed ACTH resistance and blunted GC synthesis. In line with our findings, both *Por* and *Star* have previously been shown to be directly clock-controlled genes (11, 49). Therefore, a similar reduction of the transcript levels in muscle and adipose tissue of *Arntl*$^{-/-}$ mice (Supplemental Figure 5) is not unexpected. To the contrary, the ACTH receptor gene *Mc2r* was up-regulated, an effect that is likely to be attributable to reduced GC feedback but might also be related to an indirect control of its expression by the circadian clock.

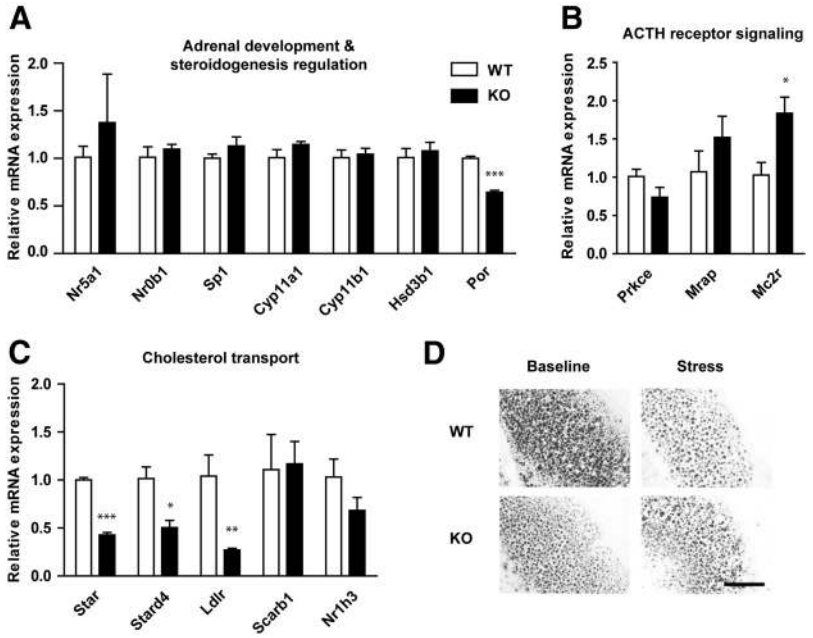

Figure 4. *Arntl*-deficient mice show altered steroidogenesis-associated gene expression in the adrenal gland A–C, Comparison of mRNA levels of genes involved in adrenal function and steroid biosynthesis in adrenals from wild-type (WT) and knockout (KO) mice at 48 hours after lights off (n = 3–4). D, Oil Red O staining of adrenal sections from untreated and stressed WT and KO mice. Scale bar, 200 μm. *, $P < .05$; **, $P < .01$; ***, $P < .001$ (Student's *t* test [panels A–C]).

Impaired ACTH sensitivity may not only cause hypocortisolism but may also lead to compromised GC and behavioral responses to stress. We used the forced swimming paradigm as an acute, predominantly physical stressor (33). Consistent with reduced adrenal responsiveness to ACTH stimulation ex vivo, *Arntl*-deficient mice had dramatically blunted CORT, but not ACTH, responses to acute stress compared with wild-type controls (Figure 3). Both duration and type of stressor are critical for programming the intensity of evoked GC responses (1, 2). In line with this, a longer and more intense stressor (immobilization) is still able to induce GC responses in *Arntl*$^{-/-}$ mice (50) or in mice with a compromised adrenal clock (11).

GCs exert a plethora of effects on animal behavior via binding to corticoid receptors in the brain (1). Clinical and experimental data support a key role of excessive GC production in the pathogenesis of depression (2). In contrast, chronically reduced GC levels may lead to opposite changes, such as mania-like behavior. For instance, ablation of CORT production in rodents by adrenalectomy or metyrapone treatment, as well as blockade of central GC effects by glucocorticoid receptor deletion in the brain, led to reduced immobility in the FST (51–53). The same test revealed a drastic reduction in immobility in *Arntl*$^{-/-}$ mice (Figure 3), indicating resistance to acute stress effects, despite the fact that *Arntl* deficiency causes development of progressive arthropathy and impairment of locomotor activity (35). Similarly, a tendency toward reduced immobility was seen in *Arntl*$^{-/-}$ mice during the TST, which became significant during repeated testing (Figure 5). Conversely, *Arntl*-deficient animals were found to be anhedonic, reflected by a reduced baseline sucrose preference. This could be, in part, an effect of altered olfaction or taste in *Arntl*$^{-/-}$ mice (54). It further implies that the mood phenotype of *Arntl*$^{-/-}$ mice may vary depending on whether activation of the stress axis is involved. In other words, *Arntl* deficiency may not be protective for the development of depression but may confer resistance against the mood effects of stress. Detrimental effects of repeated stress are based on recruitment of neural pathways that are distinct from those involved in acute stress responses (reviewed in Ref. 3). We applied repeated restraint stress to reveal whether *Arntl*-deficient mice are also less sensitive to prolonged stress effects (55). Extensive chronic stress leads to signs of anhedonia in rodents such as reduced sucrose preference (39). To the contrary, a

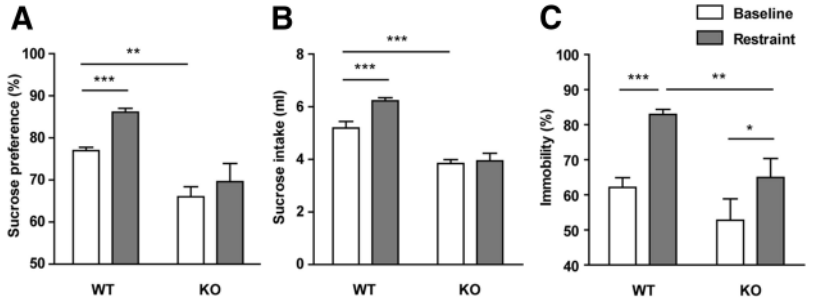

Figure 5. *Arntl*$^{-/-}$ mice are resistant to behavioral changes induced by repeated restraint stress A and B, Sucrose preference (A) and sucrose consumption (B) in wild-type (WT) and knockout (KO) mice before and after 7 days of chronic restraint stress (n = 6–9). Effects of genotype and stress were significant for sucrose preference ($P < 0.0001$ and $P = 0.003$, respectively). Effects of genotype and stress and interaction between 2 factors were significant for sucrose consumption ($P < 0.0001$; $P = 0.002$; and $P = 0.006$, respectively). C, TST immobility behavior (in percent) before and after repeated restraint measured in the same cohorts of mice as in panels A and B. Effects of genotype and stress were significant ($P = .012$ and $P < .0001$, respectively). **,$P <$ .01; ***, $P < .001$ (repeated measures two-way ANOVA with Bonferroni posttest).

shorter, subchronic stress (up to 3 weeks) is usually associated with increased reward-seeking behavior (39, 56), which is interpreted as a compensation for stress-associated deficits in reward signaling (23). In line with this, wild-type mice responded to repeated restraint by a gradual increase in sucrose consumption and sucrose preference (Figure 5). In contrast, repeated restraint did not affect sucrose intake in *Arntl*-deficient mice, which is consistent with the view that GCs regulate the activation of mesolimbic reward circuits and dopamine release in the nucleus accumbens (57). Reduced immobility in the FST has previously been observed in *Clock*$^{\Delta 19}$ and *Per2*^{Brdm1} mutant mice (58, 59). In both cases, the phenotype has been contributed to by local clock gene effects in the brain. However, both strains also show decreased daily CORT production (20, 42), which together with our data suggests that peripheral clock regulation may also play a role in this context (see also Ref. 60). At the same time, and in line with our dexamethasone suppression data, it suggests that the observed behavioral resistance of *Arntl*$^{-/-}$ mice to stress may also be influenced by deregulated glucocorticoid receptor signaling in the brain (19, 20). Tissue-specific genetic targeting of the clock gene machinery will help to better clarify the contribution of different sites of action of *Arntl* in this context.

In conclusion, our data on adrenal CORT regulation in *Arntl*$^{-/-}$ mice provide a complementary perspective on the regulation of stress responses and mood. It has been documented that disruption of the normal light-dark cycle in humans and rodents can lead to excessive HPA axis activation and symptoms of depression (Ref. 27 and reviewed in Ref. 60). In contrast, we and others observed that a genetic disruption of the molecular clock in mice can also confer hormonal and behavioral resistance to stress. This effect may be mediated, at least in part, by regulation of adrenocortical clocks, thus potentially providing a new and easily accessible target for the treatment of stress-associated disorders.

Acknowledgments

We thank Dr. Johanna Barclay (University of Queensland, Queensland, Australia) for critical reading of the manuscript, Drs. Olaf Jöhren and Volker Ott and Ines Stölting (Institutes of Neuroendocrinology and Experimental and Clinical Pharmacology and Toxicology, University of Lübeck, Lübeck, Germany) for generous technical support, and all members of the Circadian Rhythms Group for helpful discussions.

Address all correspondence and requests for reprints to: Professor Dr. Henrik Oster, Medical Department I, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany, E-mail: henrik.oster@uksh.de.

This work was supported by the German Research Foundation (DFG) (to H.O.), the Max Planck Society, and a Gerhard Hunsmann Fellowship (to A.L.). H.O. is a Lichtenberg fellow of the Volkswagen Foundation.

Present addresses for A.S.: Circadian Rhythms and Molecular Clocks Group, Biochemistry Center, Heidelberg University, Heidelberg, Germany.

Present address for J.H.: Program in Neurosciences and Mental Health, The Hospital for Sick Children, Toronto, Canada.

Disclosure Summary: The authors have nothing to disclose.

References

1. **de Kloet ER, Joëls M, Holsboer F.** Stress and the brain: from adaptation to disease. *Nat Rev Neurosci*. 2005;6(6):463–475.
2. **Holsboer F.** The corticosteroid receptor hypothesis of depression. *Neuropsychopharmacology*. 2000;23(5):477–501.
3. **Ulrich-Lai YM, Herman JP.** Neural regulation of endocrine and autonomic stress responses. *Nat Rev Neurosci*. 2009;10(6):397–409.
4. **Stratmann M, Schibler U.** Properties, entrainment, and physiological functions of mammalian peripheral oscillators. *J Biol Rhythms*. 2006;21(6):494–506.
5. **Barclay JL, Tsang AH, Oster H.** Interaction of central and peripheral clocks in physiological regulation. *Prog Brain Res*. 2012;199:163–181.
6. **Dickmeis T.** Glucocorticoids and the circadian clock. *J Endocrinol*. 2009;200(1):3–22.
7. **Kalsbeek A, van der Spek R, Lei J, Endert E, Buijs RM, Fliers E.** Circadian rhythms in the hypothalamo-pituitary-adrenal (HPA) axis. *Mol Cell Endocrinol*. 2012;349(1):20–29.
8. **Moore RY, Eichler VB.** Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Res*. 1972;42(1):201–206.
9. **Oster H, Damerow S, Hut RA, Eichele G.** Transcriptional profiling in the adrenal gland reveals circadian regulation of hormone biosynthesis genes and nucleosome assembly genes. *J Biol Rhythms*. 2006;21(5):350–361.
10. **Oster H, Damerow S, Kiessling S, et al.** The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. *Cell Metab*. 2006;4(2):163–173.
11. **Son GH, Chung S, Choe HK, et al.** Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. *Proc Natl Acad Sci USA*. 2008; 105(52):20970–20975.
12. **Buhr ED, Takahashi JS.** Molecular components of the Mammalian circadian clock. *Handb Exp Pharmacol*. 2013;217:3–27.
13. **Oster H.** The genetic basis of circadian behavior. *Genes Brain Behav*. 2006;5 Suppl 2:73–79.
14. **Bunger MK, Wilsbacher LD, Moran SM, et al.** Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell*. 2000;103(7):1009–1017.
15. **Ko CH, Yamada YR, Welsh DK, et al.** Emergence of noise-induced oscillations in the central circadian pacemaker. *PLoS Biol*. 2010; 8(10):e1000513.
16. **Balsalobre A, Brown SA, Marcacci L, et al.** Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science*. 2000; 289(5488):2344–2347.

17. **Segall LA, Perrin JS, Walker CD, Stewart J, Amir S.** Glucocorticoid rhythms control the rhythm of expression of the clock protein, Period2, in oval nucleus of the bed nucleus of the stria terminalis and central nucleus of the amygdala in rats. *Neuroscience*. 2006;140(3):753–757.
18. **So AY, Bernal TU, Pillsbury ML, Yamamoto KR, Feldman BJ.** Glucocorticoid regulation of the circadian clock modulates glucose homeostasis. *Proc Natl Acad Sci USA*. 2009;106(41):17582–17587.
19. **Cheon S, Park N, Cho S, Kim K.** Glucocorticoid-mediated Period2 induction delays the phase of circadian rhythm. *Nucleic Acids Res*. 2013;41(12):6161–6174.
20. **Lamia KA, Papp SJ, Yu RT, et al.** Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. *Nature*. 2011;480(7378):552–556.
21. **Peckett AJ, Wright DC, Riddell MC.** The effects of glucocorticoids on adipose tissue lipid metabolism. *Metabolism*. 2011;60(11):1500–1510.
22. **McEwen BS, Sapolsky RM.** Stress and cognitive function. *Curr Opin Neurobiol*. 1995;5(2):205–216.
23. **Parylak SL, Koob GF, Zorrilla EP.** The dark side of food addiction. *Physiol Behav*. 2011;104(1):149–156.
24. **Castagne V, Moser P, Roux S, Porsolt RD.** Rodent models of depression: forced swim and tail suspension behavioral despair tests in rats and mice. *Curr Protoc Pharmacol*. 2001;Chapter 5:Unit 5 8.
25. **Hänninen L, Pastell M.** CowLog: open-source software for coding behaviors from digital video. *Behav Res Methods*. 2009;41(2):472–476.
26. **Swiergiel AH, Leskov IL, Dunn AJ.** Effects of chronic and acute stressors and CRF on depression-like behavior in mice. *Behav Brain Res*. 2008;186(1):32–40.
27. **LeGates TA, Altimus CM, Wang H, et al.** Aberrant light directly impairs mood and learning through melanopsin-expressing neurons. *Nature*. 2012;491(7425):594–598.
28. **Barclay JL, Shostak A, Leliavski A, et al.** High fat diet-induced hyperinsulinemia and tissue-specific insulin resistance in Cry deficient mice. *Am J Physiol Endocrinol Metab*. 2013;304(10):E1053–E1063.
29. **Bartolomucci A, Pederzani T, Sacerdote P, Panerai AE, Parmigiani S, Palanza P.** Behavioral and physiological characterization of male mice under chronic psychosocial stress. *Psychoneuroendocrinology*. 2004;29(7):899–910.
30. **Abraham D, Dallmann R, Steinlechner S, Albrecht U, Eichele G, Oster H.** Restoration of circadian rhythmicity in circadian clock-deficient mice in constant light. *J Biol Rhythms*. 2006;21(3):169–176.
31. **Cavigelli SA, Monfort SL, Whitney TK, Mechref YS, Novotny M, McClintock MK.** Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms. *J Endocrinol*. 2005;184(1):153–163.
32. **Karpac J, Ostwald D, Bui S, Hunnewell P, Shankar M, Hochgeschwender U.** Development, maintenance, and function of the adrenal gland in early postnatal proopiomelanocortin-null mutant mice. *Endocrinology*. 2005;146(6):2555–2562.
33. **Ishida A, Mutoh T, Ueyama T, et al.** Light activates the adrenal gland: timing of gene expression and glucocorticoid release. *Cell Metab*. 2005;2(5):297–307.
34. **Andrews JL, Zhang X, McCarthy JJ, et al.** CLOCK and BMAL1 regulate MyoD and are necessary for maintenance of skeletal muscle phenotype and function. *Proc Natl Acad Sci USA*. 2010;107(44):19090–19095.
35. **Bunger MK, Walisser JA, Sullivan R, et al.** Progressive arthropathy in mice with a targeted disruption of the Mop3/Bmal-1 locus. *Genesis*. 2005;41(3):122–132.
36. **Arai I, Tsuyuki Y, Shiomoto H, Satoh M, Otomo S.** Decreased body temperature dependent appearance of behavioral despair in the forced swimming test in mice. *Pharmacol Res*. 2000;42(2):171–176.
37. **Park SY, Walker JJ, Johnson NW, Zhao Z, Lightman SL, Spiga F.** Constant light disrupts the circadian rhythm of steroidogenic proteins in the rat adrenal gland. *Mol Cell Endocrinol*. 2013;371(1–2):114–123.
38. **Chiba S, Numakawa T, Ninomiya M, Richards MC, Wakabayashi C, Kunugi H.** Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex. *Prog Neuropsychopharmacol Biol Psychiatry*. 2012;39(1):112–119.
39. **Strekalova T, Couch Y, Kholod N, et al.** Update in the methodology of the chronic stress paradigm: internal control matters. *Behav Brain Funct*. 2011;7:9.
40. **Yi LT, Li J, Li HC, et al.** Antidepressant-like behavioral, neurochemical and neuroendocrine effects of naringenin in the mouse repeated tail suspension test. *Prog Neuropsychopharmacol Biol Psychiatry*. 2012;39(1):175–181.
41. **Rudic RD, McNamara P, Curtis AM, et al.** BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS Biol*. 2004;2(11):e377.
42. **Turek FW, Joshu C, Kohsaka A, et al.** Obesity and metabolic syndrome in circadian Clock mutant mice. *Science*. 2005;308(5724):1043–1045.
43. **Doi M, Takahashi Y, Komatsu R, et al.** Salt-sensitive hypertension in circadian clock-deficient Cry-null mice involves dysregulated adrenal Hsd3b6. *Nat Med*. 2010;16(1):67–74.
44. **Yang S, Liu A, Weidenhammer A, et al.** The role of mPer2 clock gene in glucocorticoid and feeding rhythms. *Endocrinology*. 2009;150(5):2153–2160.
45. **Shearman LP, Sriram S, Weaver DR, et al.** Interacting molecular loops in the mammalian circadian clock. *Science*. 2000;288(5468):1013–1019.
46. **Bornstein SR.** Predisposing factors for adrenal insufficiency. *N Engl J Med*. 2009;360(22):2328–2339.
47. **Shimba S, Ishii N, Ohta Y, et al.** Brain and muscle Arnt-like protein-1 (BMAL1), a component of the molecular clock, regulates adipogenesis. *Proc Natl Acad Sci USA*. 2005;102(34):12071–12076.
48. **Torres-Farfan C, Abarzua-Catalan L, Valenzuela FJ, et al.** Cryptochrome 2 expression level is critical for adrenocorticotropin stimulation of cortisol production in the capuchin monkey adrenal. *Endocrinology*. 2009;150(6):2717–2722.
49. **Cho H, Zhao X, Hatori M, et al.** Regulation of circadian behaviour and metabolism by REV-ERB-α and REV-ERB-β. *Nature*. 2012;485(7396):123–127.
50. **Curtis AM, Cheng Y, Kapoor S, Reilly D, Price TS, Fitzgerald GA.** Circadian variation of blood pressure and the vascular response to asynchronous stress. *Proc Natl Acad Sci USA*. 2007;104(9):3450–3455.
51. **Rogóz Z, Skuza G, Leskiewicz M, Budziszewska B.** Effects of coadministration of fluoxetine or tianeptine with metyrapone on immobility time and plasma corticosterone concentration in rats subjected to the forced swim test. *Pharmacol Rep*. 2008;60(6):880–888.
52. **Mitchell JB, Meaney MJ.** Effects of corticosterone on response consolidation and retrieval in the forced swim test. *Behav Neurosci*. 1991;105(6):798–803.
53. **Tronche F, Kellendonk C, Kretz O, et al.** Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet*. 1999;23(1):99–103.
54. **Granados-Fuentes D, Tseng A, Herzog ED.** A circadian clock in the olfactory bulb controls olfactory responsivity. *J Neurosci*. 2006;26(47):12219–12225.
55. **Stewart LQ, Roper JA, Young WS, 3rd, O'Carroll AM, Lolait SJ.**

Pituitary-adrenal response to acute and repeated mild restraint, forced swim and change in environment stress in arginine vasopressin receptor 1b knockout mice. *J Neuroendocrinol*. 2008;20(5):597–605.

56. **Dubreucq S, Matias I, Cardinal P, et al.** Genetic dissection of the role of cannabinoid type-1 receptors in the emotional consequences of repeated social stress in mice. *Neuropsychopharmacology*. 2012;37(8):1885–1900.
57. **Piazza PV, Barrot M, Rougé-Pont F, et al.** Suppression of glucocorticoid secretion and antipsychotic drugs have similar effects on the mesolimbic dopaminergic transmission. *Proc Natl Acad Sci USA*. 1996;93(26):15445–15450.
58. **Roybal K, Theobold D, Graham A, et al.** Mania-like behavior induced by disruption of CLOCK. *Proc Natl Acad Sci USA*. 2007;104(15):6406–6411.
59. **Hampp G, Ripperger JA, Houben T, et al.** Regulation of monoamine oxidase A by circadian-clock components implies clock influence on mood. *Curr Biol*. 2008;18(9):678–683.
60. **Albrecht U.** Circadian clocks and mood-related behaviors. *Handb Exp Pharmacol*. 2013;217:227–239.