Chronic mild stress-induced alterations of clock gene expression in rat prefrontal cortex: modulatory effects of prolonged lurasidone treatment

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Running title: Lurasidone modulate clock gene expression in the CMS model

Abstract

Disruptions of biological rhythms are known to be associated with depressive disorders, suggesting that abnormalities in the molecular clock may contribute to the development of these disorders. These mechanisms have been extensively characterized in the suprachiasmatic nucleus, but little is know about the role exerted by individual clock genes in brain structures that are important for depressive disorders. Using the chronic mild stress model we found a significant reduction of BMAL1 and CLOCK protein levels in the nuclear compartment of the prefrontal cortex of CMS rats, which was paralleled by a down-regulation of the expression of several target genes, including *Per*s and *Cry*s but also *Reverbβ* and *Pparα*.

Interestingly, chronic treatment with the multi receptor modulator lurasidone (3 mg/kg for 5 weeks) was able to normalize the molecular changes induced by CMS exposure in prefrontal cortex, but it was also able to regulate some of these genes within the hippocampus.

We believe that changes in clock genes expression after CMS exposure may contribute to the disturbances associated with depressive disorders and that the ability of chronic lurasidone to normalize such alterations may be relevant for its therapeutic properties in ameliorating functions that are deteriorated in patients with major depression and other stress-related disorders.

Introduction

Depressive disorders are characterized by complex and heterogeneous symptoms, including alterations of biological rhythms that can manifest, for example, with changes in sleep-wake cycle (1). At the bases of these modifications there is a disruption of the circadian clock since it has been demonstrated that the intensity of major depressive symptoms in human is correlated with the misalignment of circadian rhythms (2). Furthermore mutations in circadian genes are found in depressed subjects and may contribute to specific symptoms (3). Moreover, considering the importance of cell birth and proliferation in the hippocampus in mood and in the antidepressant activity, the alterations in neurogenesis observed following chronic circadian disruption (4) confirm that this process may contribute to the development or exacerbation of depressive disorders.

While the pacemaker cells controlling most of the circadian rhythms are located in the suprachiasmatic nucleus (SCN), it has been recently demonstrated that patients with major depressive disorder show profound alterations of hundreds of genes that have a rhythmic transcriptional activity in regions outside the SCN (5).

At molecular level, the circadian clock involves periodic changes in gene expression achieved by transcription-translation feedback loops whereby the protein product of transcribed genes auto-regulate their own transcription. In mammals, the circadian clock is composed of an auto-regulatory transcriptional network with an interlocked feedback loop. Specifically, the core transcriptional circuit comprises the transcription factors BMAL1 and CLOCK that heterodimerize and activate the transcription of *Period* (*Per*) and *Cryptochrome* (*Cry*) genes. PER/CRY proteins then repress their own transcription by inhibiting the activity of CLOCK:BMAL1 until they are degraded to allow a new cycle of transcription to begin (6). In addition, the interlocking feedback loop regulated rhythmic expression of *Bmal1* through opposing action of the ROR and REV-ERB families that activate and repress *Bmal1* transcription respectively and whose expression is controlled by the core loop (6).

Since depression can be the consequence of altered and often maladaptive response to stress, models in which animals are exposed to different paradigms of stress during adult life are supposed to disrupt normal homeostasis, leading to pathologic alterations. Among the wide array of experimental paradigms employed in rodents to investigate the mechanisms that may contribute to stress-related disease susceptibility, the chronic mild stress (CMS) model is a well-validated paradigm to induce depressive-like behavior, which is also associated with significant alterations of circadian rhythms (7).

On these bases, in the present study we have used the CMS paradigm to establish possible changes in the expression of the clock genes in brain regions different from the SCN, such as prefrontal cortex and hippocampus, which play a key role in core symptoms of major depressive disorders. Furthermore, we have investigated the ability of the multi-receptor modulator lurasidone to normalize stress-induced changes of clock genes expression. Even if lurasidone was initially introduced as an antipsychotic drug, recent evidence indicate that it also has antidepressant (8, 9) and procognitive activity both in rats and primates (10, 11).

Moreover, we have recently shown that prolonged treatment with lurasidone normalizes the behavioral as well as the molecular changes at synaptic levels due the exposure to CMS (12).

We analyzed the transcriptional levels of genes involved in the core (*Bmal1*, *Clock*, *Per1*, *Per2*, *Cry1* and *Cry2*) and in the

interlocking feedback loop (*Rev-Erbα* and *Rev-Erbβ*) in the prefrontal cortex as well as in dorsal and ventral hippocampus. Moreover, we investigated the mRNA levels of *Pparα*, *Nampt* and *Bhlhe40* whose expression is regulated by the heterodimer CLOCK:BMAL1. Finally, we analyzed the protein levels of BMAL1, CLOCK, CRY1 and PER1 in the prefrontal cortex in order to obtain a detailed picture of the clock machinery under stress conditions as well as in response to pharmacological treatment.

Materials and Methods

General reagents were purchased from Sigma-Aldrich (Milan, Italy) whereas molecular biology reagents were obtained from Life Technologies (Monza, Italy), PerkinElmer (Milan, Italy), Roche (Monza, Italy), Eurofins MWG-Operon (Ebersberg, Germany) and Bio-Rad Laboratories S.r.l. (Segrate, Italy). Lurasidone was kindly provided by Sumitomo Dainippon Pharma Co. Ltd (Japan).

Animals

Adult male Wistar rats (Charles River, Germany) were brought into the laboratory one month before the beginning of the experiment. The animals were single-housed with food and water freely available, and were maintained on 12-h light/dark cycle with a constant temperature (22 2°C) and humidity (50 ± 5%) conditions. All procedures used in this study are conformed to the rules and principles of the 2010/63/EU Directive, were approved by the Local Bioethical Committee at the Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland, and adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Stress procedure and pharmacological treatment

Animals were divided into two groups: No Stress (control animals) and Stress. Animals were subjected to the stress procedure for 7 weeks. Each week of the stress regime consisted of two periods of food or water deprivation, two periods of 45 degree cage tilt, two periods of intermittent illumination (lights on and off every 2h), two periods of soiled cage (250 mL water in sawdust bedding), one period of paired housing, two periods of low intensity stroboscopic illumination (150 flashes/min), and three periods of no stress. All stressors were 10-14 h of duration and were applied individually and continuously, day and night. Control animals were housed in separate rooms and had no contact with the stressed animals.

Based on the results of the final sucrose test carried out the following first 2 weeks of stress (12), both control and stress-reactive groups were further divided into matched subgroups (n=10) and for the subsequent five weeks they received oral administration (by gavage) of vehicle (hydrossi-ethil-cellulose, HEC 1%) or lurasidone (3 mg/kg daily). The stress was continued throughout the entire period of drugs administration. After five weeks, the treatments were terminated and all the animals were killed by decapitation 24h after (between 11:00 and 14:00) the last drug administration. The hippocampus (dorsal and ventral) and the prefrontal cortex were rapidly dissected. The dorsal hippocampus corresponds to the plates 25-33 according to the atlas of Paxinos and Watson (Paxinos and Watson, 1996), whereas the ventral hippocampus corresponds to the plates 34-43. The prefrontal cortex (defined as Cg1, Cg3, and IL subregions corresponding to the plates 6-10 according to the atlas of Paxinos and Watson) was dissected from 2-mm-thick slices, whereas the hippocampus was dissected from the whole brain. The brain specimens were frozen on dry ice and stored at -80ºC for further analysis.

Sucrose intake test

After a period of adaptation to laboratory and housing conditions, the animals were trained to consume a palatable sucrose solution (1%). Training consisted of nine 1 hour-baseline tests in which the sucrose solution was presented to the rats in their home cage after 14 hours of food and water deprivation. Sucrose intake was measured as differences in bottle weight before and after each test. Subsequently, sucrose consumption was monitored, under similar conditions, at weekly intervals throughout the whole experiment.

RNA preparation and gene expression analyses

For gene expression analyses, total RNA was isolated from the different brain regions by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories S.r.l.; Segrate, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis. The samples were then processed for real-time polymerase chain reaction (PCR) as previously reported (13) to assess mRNA levels of: *Bmal1*, *Clock*, *Cry1*, *Cry2*, *Per1*, *Per2*, *Rev-Erbα*, *Rev-Erbβ*, *Pparα*, *Nampt* and *Bhlhe40*.

Briefly, an aliquot of each sample was treated with DNase to avoid DNA contamination and subsequently analyzed by TaqMan qRT–PCR instrument (CFX384 real-time system, Bio-Rad Laboratories S.r.l.) using the iScript one-step RT–PCR kit for probes (Bio-Rad Laboratories S.r.l.). Samples were run in 384-well format in triplicates as multiplexed reactions with a normalizing internal control (36B4). Thermal cycling was initiated with incubation at 50°C for 10 min (RNA retrotranscription), and then at 95°C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 s to enable the melting process and then for 30 s at 60°C for the annealing and extension reactions. A comparative cycle threshold (Ct) method was used to calculate the relative target gene expression. Primer sequences used (Table 1) were purchased from Eurofins MWG-Operon.

Protein extraction and western blot analysis.

Western blot analysis was used to investigate BMAL1, CLOCK, CRY1, PER1, GR and CREB in the nuclear fraction. Tissues were manually homogenized using a glass-glass potter in a pH 7.4 cold buffer containing 0.32 M sucrose, 0.1 mM EGTA, 0.1 mM PMSF, 1 mM HEPES solution in presence of a complete set of protease (Roche) and phosphatase (Sigma-Aldrich) inhibitors. The total homogenate was centrifuged at 2500 rpm for 10 min at 4°C to obtain the pellet corresponding to the nuclear fraction (13) which was re-suspended in a buffer (20 mM HEPES, 0.1 mM dithiothreitol (DTT), 0.1 mM EGTA) with protease and phosphatase inhibitors. Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad Laboratories), using bovine serum albumin as calibration standard. Equal amounts of protein were run under reducing conditions on 10% SDS-polyacrylamide gels and then electrophoretically transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The blots were blocked with 10% nonfat dry milk and then incubated over night at 4°C with the primary antibodies: BMAL1: 1:5000 (Cell Signaling), CLOCK: 1:5000 (Abcam),

CRY1: 1:5000 (Thermo Scientific), PER1: 1:5000 (GeneTex), GR 1:500 (Thermo Scientific), and CREB 1:1000 (Cell Signaling). Membranes were then incubated for 1 h at room temperature with the opportune secondary antibody: BMAL1: 1:2500 (anti-rabbit), CLOCK: 1:2500 (anti-rabbit), CRY1: 1:2500 (anti-rabbit), PER1: 1:2500 (anti-rabbit), GR 1:2000 (anti-rabbit) and CREB 1:2000 (anti-rabbit).

Immunocomplexes were visualized by chemiluminescence using the Western Lightning Plus ECL (PerkinElmer) and the Chemidoc MP imaging system (Bio-Rad Laboratories). Results were normalized using β-actin as an internal standard because its expression is not regulated by the experimental paradigm used.

Statistical analyses

All the analyses were carried out on individual animals (independent determinations) and analyzed with a two-way ANOVA, with stress (no stress vs. stress) and treatment (vehicle vs. lurasidone) as independent factors. When appropriate, further differences were analyzed by Fisher's Protected Least Significant Difference (PLSD). Sucrose intake after two weeks of CMS was analyzed with Student's t test. Significance for all tests was assumed for $P<0.05$. For graphic clarity, data are presented as means percent $\pm$ standard error (SEM) of control group (No Stress/Veh).

Results

Behavioral characterization of animals exposed to CMS and chronically treated with lurasidone

Sucrose intake was used to establish the behavioral outcome of CMS exposure. As summarized in Table 2, animals were exposed to 2 weeks of CMS develop anhedonia, as indicated by the significant reduction of sucrose intake (-4.5g; p< 0.001 vs Control; Student's t test). The pathological phenotype was still present after 7 weeks of CMS (-5g; p< 0.001 vs Control/Vehicle; two-way ANOVA with Fisher PLSD) and was corrected by a concomitant treatment with lurasidone (+6.1g; p< 0.001 vs Stress/Vehicle; two-way ANOVA with Fisher PLSD).

Analysis of clock gene expression in the rat prefrontal cortex

Firstly, we investigated the expression of master clock genes in CMS rats, as compared to controls, treated with vehicle or lurasidone. Specifically we investigated two up-stream regulators, *Bmal1* and *Clock*, as well as their downstream targets, namely *Cry1*, *Cry2* and *Per1*, *Per2*.

In prefrontal cortex, as shown in Figure 1A, there was no main effect of stress ($F_{1,39}$<0.001, *P*>0.05) or treatment ($F_{1,39}$=0.139, *P*>0.05) on the expression of *Bmal1*, although we found a significant stress *x* treatment interaction ($F_{1,39}$=9.843, *P*<0.01). Indeed, chronic lurasidone increased *Bmal1* mRNA levels in control rats (+52%, *P*<0.05 *vs* No Stress/Veh, Fischer's PLSD). Conversely, the enhanced expression of *Bmal1* found in CMS rats (+47%, *P*<0.05 *vs* No Stress/Veh, Fischer's PLSD) was normalized by drug exposure (-28%, *P*=0.062 *vs* Stress/Veh, Fischer's PLSD).

When investigating *Clock* mRNA levels (Fig. 1B), we found a main effect of treatment ($F_{1,39}$=14.945, *P*<0.001), but not of stress ($F_{1,39}$=1.357, *P*>0.05) and no significant stress *x* treatment interaction ($F_{1,39}$=0.010, *P*>0.05). Indeed, lurasidone administration increased *Clock* expression in sham (+38%, *P*<0.01 *vs* No Stress/Veh, Fischer's PLSD) as well as in CMS rats (+40%, *P*<0.05 *vs* Stress/Veh, Fischer's PLSD). Similarly, *Cry1* expression (Fig. 1C) was modulated by lurasidone treatment ($F_{1,39}$=11.605, *P*<0.01) but not by CMS exposure ($F_{1,39}$=2.953, *P*>0.05), with no stress *x* treatment interaction ($F_{1,39}$=0.254, *P*>0.05). Indeed, lurasidone significantly up-regulated *Cry1* mRNA levels, independently from the chronic stress (+21%, *P*<0.05 *vs* No Stress/Veh and +33%, *P*=0.01 *vs* Stress/Veh, Fischer's PLSD). Conversely, *Cry2* expression was modulated by CMS exposure ($F_{1,39}$= 5.483, *P*<0.05) as well as by drug intervention ($F_{1,39}$=12.665, *P*=0.001), with no stress *x* drug interaction ($F_{1,39}$=1.170, *P*>0.05) (Fig. 1D). Interestingly, *Cry2* mRNA levels were significantly reduced in CMS rats (-27%, *P*<0.05 *vs* No Stress/Veh, Fischer's PLSD), an effect that was normalized by chronic lurasidone administration (+51%, *P*<0.01 *vs* Stress/Veh, Fischer's PLSD).

Per1 mRNA levels (Fig. 1E) were also significantly affected by chronic lurasidone treatment ($F_{1,39}$=13.616, *P*=0.001), which produced a marked elevation of its expression only in CMS exposed animals (+132%, P=0.001 *vs* Stress/Veh animals, Fischer's PLSD). Lastly, we found a significant effect of CMS ($F_{1,39}$=31.662, *P*<0.001) and treatment ($F_{1,39}$=21.377, *P*<0.001) on *Period 2* (*Per2*)

expression (Fig. 1F). CMS significantly reduced its mRNA levels (-47%, *P*<0.001 *vs* No Stress/Veh, Fischer's PLSD), whereas long-term lurasidone administration was able to normalize these changes (+77%, *P*<0.001 *vs* Stress/Veh, Fischer's PLSD). Moreover, drug treatment was able to produce a small but significant elevation of *Per2* mRNA levels also in sham animals (+18%, *P*=0.05 *vs* No Stress/Veh, Fischer's PLSD). **Interestingly, comparing the expression of these gene in animals exposed to stress and the results of the sucrose consumption test, we found a significant positive correlation between the gene expression of Per1 (r=0.5722, *P*<0.05) (Fig. 2B), Per2 (r=0.5694 *P*<0.05) (Fig. 2C) and the sucrose preference (Fig. 2), while a negative correlation was observed for Bma1 (r=-0.4907 *P*<0.05) (Fig. 2A).**

Analysis of Rev-Erbα and Rev-Erbβ mRNA expression in prefrontal cortex

Next, in order to investigate the interlocking feedback loop that regulated the core clock cycle, we analyzed the mRNA levels of *Rev-Erbα* and *Rev-Erbβ*, whose expression is regulated by the heterodimer CLOCK:BMAL1 and that in turn control *Bmal1* transcription. We found that CMS produced a slight, but significant, decrease of *Rev-Erbβ* mRNA levels (-15%, *P*<0.05 *vs* No Stress/Veh, Fischer's PLSD), an effect that was not present when the animals had received chronic lurasidone (+15%, *P*<0.05 *vs* Stress/Veh, Fischer's PLSD) (Fig.3B). No significant changes were found for *Rev-Erbα* expression (Fig.3A).

Analysis of CLOCK:BMAL1 regulated-genes in prefrontal cortex

In order to obtain further information on potential changes of genes downstream from CLOCK:BMAL1 in prefrontal cortex, we investigated the mRNA levels of genes that contain the E-box sequence and whose expression can be regulated by the heterodimer, including *Pparα* (Fig. 4A), *Nampt* (Fig. 4B) and *Bhlhe40* (Fig. 4C).

We found a significant effect of stress on *Pparα* mRNA levels (Fig. 4A) ($F_{1,38}$=4.710, P<0.05), which were reduced in CMS rats (-15%, *P*<0.05 *vs* No Stress/Veh, Fischer's PLSD). Conversely, *Nampt* expression (Fig. 3B) was only influenced by lurasidone treatment ($F_{1,39}$=8.128, *P*<0.01). In particular, *Nampt* mRNA levels were increased when the drug was administered to CMS animals (+19%, *P*<0.01 *vs* Stress/Veh animals, Fischer's PLSD). No significant changes in the expression of *Bhlhe40* as a consequence of CMS or lurasidone treatment were found (Fig. 4C).

Analysis of mRNA levels in rat dorsal hippocampus

In the dorsal hippocampus (Table 3), most of the observed changes were driven by chronic exposure to lurasidone. Indeed, we observed a main effect of treatment for *Bmal1*, *Clock* and *Cry1* (respectively $F_{1,39}$=27.804, *P*<0.001, $F_{1,39}$=22.017, *P*<0.001 and $F_{1,39}$=15.562, *P*<0.001) independently from CMS exposure, as indicated by the lack of stress *x* drug interaction (respectively $F_{1,39}$=2.013, *P*>0.05, $F_{1,39}$=0.701, *P*>0.05 and $F_{1,39}$=0.173, *P*>0.05). Specifically, the expression of all these genes were significantly up-regulated by lurasidone administration in sham (*Bmal1*: +41%, *P*<0.001 *vs* No Stress/Veh; *Clock*: +33%, *P*<0.01 *vs* No

Stress/Veh; *Cry1*: +74%, *P*<0.01 *vs* No Stress/Veh, Fischer's PLSD) as well as in CMS rats (*Bmal1*: +20%, *P*=0.01 *vs* Stress/Veh; *Clock*: +46%, *P*<0.001 *vs* Stress/Veh; *Cry1*: +45%, *P*<0.05 *vs* Stress/Veh, Fischer's PLSD). Chronic lurasidone administration had a significant effect also on period genes (*Per1*: $F_{1,39}$=23.352, *P*<0.001; *Per2*: $F_{1,39}$=53.051, *P*<0.001). Specifically, we found a significant up-regulation of *Per1* and *Per2* mRNA levels in sham (respectively: +39%, *P*<0.001 *vs* No Stress /Veh; +45%, *P*<0.001 No Stress /Veh, Fischer's PLSD) as well as in CMS rats (respectively: +25%, *P*<0.01 *vs* Stress /Veh; +50%, *P*<0.001 Stress /Veh, Fischer's PLSD). Conversely, *Cry2* was the only gene whose expression were influenced by CMS exposure ($F_{1,39}$=15.187, *P*<0.001), with a significant increase of its mRNA levels (+57%, *P*<0.001 *vs* No Stress/Veh, Fischer's PLSD). *Rev-Erbα* were not modulated by the CMS ($F_{1,39}$=0.168, *P*>0.05) or by the pharmacological treatment ($F_{1,39}$=2.585, *P*>0.05), while we found a significant effect of the chronic lurasidone administration on *Rev-Erbβ* mRNA levels ($F_{1,39}$=15.148, *P*<0.001), whose expression was significantly increased only in CMS rats treated with lurasidone (+30%, *P*<0.001 Stress /Veh, Fischer's PLSD). *Nampt* expression was also modulated by drug treatment ($F_{1,39}$=13.932, *P*=0.001) without stress *x* treatment interaction ($F_{1,39}$=0.091, *P*>0.05). In fact, we observed a significant increased of its mRNA levels following lurasidone administration in sham (+27%, *P*<0.05 *vs* Stress /Veh, Fischer's PLSD) and in CMS rats (+31%, *P*<0.01 *vs* Stress /Veh, Fischer's PLSD). No significant changes were found for the expression of *Pparα* and *Bhlhe40* in dorsal hippocampus.

Analysis of mRNA levels in rat ventral hippocampus

Limited changes in the expression of clock genes were instead observed in the ventral region of hippocampus (Table 3). Indeed we found a significant effect of stress only on the expression of *Cry2* ($F_{1,37}$=18.214, *P*<0.001). In particular, its mRNA levels were increased in CMS rats (+30%, *P*<0.01 *vs* No Stress/Veh, Fischer's PLSD). On the contrary, the expression of *Per1* was modulated by the drug treatment ($F_{1,39}$=44.099, *P*=0.001) but not by CMS exposure ($F_{1,39}$=0.931, *P*>0.05). Indeed, *Per1* mRNA levels were markedly up-regulated following lurasidone treatment in both sham and CMS rats (respectively +87%, *P*<0.001 *vs* No Stress/Veh and +97%, *P*<0.001 *vs* Stress/Veh, Fischer's PLSD). Lastly, lurasidone was also able to modulate the expression of *Per2* selectively in CMS rats, as indicated by the significant stress *x* treatment interaction ($F_{1,39}$=10.263, *P*<0.01). Indeed, CMS rats chronically treated with lurasidone had higher levels of *Per2* (+45%, *P*<0.01 *vs* Stress/Veh, Fischer's PLSD) as compared to CMS rats treated with vehicle. We didn't find any significant effect on the expression of the other genes investigated in ventral hippocampus.

Analysis of clock genes protein levels in rat prefrontal cortex

Considering that the prefrontal cortex was the brain region where major changes were evident following CMS exposure and lurasidone treatment, we decided to establish if transcriptional changes were associated to similar modifications at translational levels. Hence, we investigated the protein levels of BMAL1, CLOCK, CRY1 and PER1 in the nuclear fraction of the prefrontal cortex (prototypical western blot analyses of these protein are shown in Figure 5).

As shown in Figure 6, we found a main effect of stress on BMAL1 ($F_{1,24}$=3.663, *P*=0.05) (Fig. 5A) and CLOCK ($F_{1,24}$=6.956, *P*<0.05) (Fig. 6B) protein levels, with a significant reduction in CMS rats (BMAL1: -33%, *P*<0.05 *vs* No Stress/Veh, Fischer's PLSD; CLOCK: -36%, *P*<0.05 *vs* No Stress/Veh, Fischer's PLSD). Chronic lurasidone treatment was able to normalize the changes of BMAL1 levels (+40%, *P*>0.05 *vs* Stress/Veh, Fischer's PLSD), without affecting the reduction of CLOCK levels produced by CMS exposure.

A significant effect of the CMS was also found for CRY1 levels (Fig. 6C) ($F_{1,24}$=6.526, *P*<0.05), with a significant stress X drug interaction ($F_{1,24}$=23.997, *P*<0.001). Indeed, CRY1 protein levels were markedly reduced in CMS rats (-47%, *P*<0.001 *vs* No Stress/Veh, Fischer's PLSD), an effect that was completely normalized by chronic lurasidone administration (+70%, *P*<0.001 *vs* Stress/Veh, Fischer's PLSD). Interestingly, lurasidone administration to sham rats significantly decreased CRY1 levels (-25%, *P*<0.05 *vs* No Stress/Veh animals, Fischer's PLSD). Conversely, while PER1 protein levels (Fig. 6D) were also significantly reduced in CMS rats (-40%, *P*<0.05 *vs* No Stress/Veh animals, Fischer's PLSD), these changes were not affected by chronic drug treatment.

Analysis of Per1 transcription regulators in rat prefrontal cortex

In addition to the CLOCK:BMAL1 E-box, the *Period1* gene promoter contains the glucocorticoid-responsive element (GRE) and the cAMP-responsive element (CRE), suggesting that other signaling pathways may control its expression (Fig. 7A). Hence, in order to establish the involvement of such systems on *Per1* gene expression changes, we investigated the protein levels of glucocorticoid receptors (GR) and of the transcription factor CREB in the nuclear extract from the prefrontal cortex (prototypical western blot analyses of these protein are shown in Figure 5).

We found a significant stress *x* treatment interaction ($F_{1,24}$=13.073, *P*<0.01) for GR levels (Fig. 7B). Indeed, in agreement with the modifications observed for Per1 expression (Fig. 1E), GR levels were significantly reduced following CMS exposure (-31%, *P*<0.01 *vs* No Stress/Veh, Fischer's PLSD), an effect that was normalized by lurasidone treatment (+41%, *P*<0.01 *vs* Stress/Veh, Fischer's PLSD). It is interesting to notice that GR levels were reduced in sham rats that were chronically treated with lurasidone (-20%, *P*<0.05 *vs* No Stress/Veh, Fischer's PLSD). On the contrary, we found a significant stress *x* drug interaction on CREB protein ($F_{1,23}$=7.053, *P*<0.05) (Fig. 7C). The levels of this transcription factor were indeed significantly up-regulated by lurasidone treatment, but only in sham rats (+103%, *P*<0.05 *vs* No Stress/Veh animals, Fischer's PLSD).

Discussion

In this study, we provide evidence that animals exposed to chronic stress show alterations of the clock gene machinery, which is essential for maintaining the circadian rhythms. The changes produced by CMS, which produces a depressive-like phenotype, are suggestive of altered transcription and translation of clock genes primarily in the prefrontal cortex. Interestingly, we demonstrate that chronic treatment with the multi receptor drug lurasidone is able to normalize some of these alterations (Fig. 8).

While the circadian clock has been investigated in many non-brain tissues, such as the liver, where it plays a major role in metabolism, less is known about the alterations of this system, outside the SCN, within key brain regions, which play an important role in different neuropsychiatric conditions. Furthermore, to our knowledge, no studies have investigated the influence of a pharmacological intervention on these mechanisms under a "pathological situation", as represented by the CMS paradigm. Our results showed that clock gene expression is modulated by stress exposure primarily in the prefrontal cortex and that most of these changes can be normalized by chronic lurasidone treatment, which is also able to regulate the clock gene machinery in the hippocampus.

Under physiological conditions, the heterodimer CLOCK:BMAL1 drives clock-controlled gene (CCG) expression of both core and interlocked feedback loop genes. Even if, being a cycle it is difficult to establish a temporal consecution of the changes observed, in the prefrontal cortex, the reduction of BMAL1 and CLOCK protein levels in the nuclear compartment after CMS exposure may be responsible for the reduced expression of *Rev-Erbβ*, *Cry2*, *Per1* and *Per2*. Interestingly, since REV-ERBs bind the RRE present in the promoter of *Bmal1* and repress its expression (6), the low levels of *Rev-Erbβ* transcripts could lead to a dysfunction of the interlocked feedback loop and, in turn, to a decrease of its repressive activity on *Bmal1* gene, which indeed was up-regulated in CMS rats. As mentioned before, also the reduction of *Cry2*, *Per1* and *Per2* mRNA levels following chronic stress exposure may be a consequence of the decreased CLOCK:BMAL1 activity, an alteration that was paralleled by a significant reduction of CRY1 and PER1 protein levels in the nucleus. Furthermore, the dysregulation of *mTor* pathways that is observed in CMS rats (12) may also contribute to the modulation of *Per1/2* gene expression (14).

One potential link between stress exposure and the dysfunction in clock gene machinery is represented by changes of HPA axis function. First, plasma concentration of corticosterone displays a time-dependent variation either in diurnal and nocturnal animals (15), a phenomenon that is controlled by both the master clock in the SCN and the peripheral clock within the adrenal gland (16, 17). The activity of this hormone is mediated by glucocorticoid receptors (GRs) that, after binding to their endogenous ligand, enter the nucleus, interact with specific DNA responsive elements (GRE), leading to transcriptional regulation of different genes. We showed that GR protein levels were significantly reduced in the nuclear fraction of the prefrontal cortex from CMS rats, an effect that was normalized by lurasidone treatment. Since a canonical GRE is present in the promoter region of the *Per1* gene, while an intronic GR-binding sequence confers GC responsiveness to *Per2* (18), the effects observed on GR protein levels may contribute to the alterations found in *Per's* mRNA levels. Accordingly, it has been recently shown that the expression of *Per2* is also mediated by

BMAL1-dependent binding of GR to the overlapping GRE/E-box in the upstream region of the *Per2* gene (19). Moreover, since the binding of CRY1/2 with GR alters the transcriptional response to glucocorticoids (20), the reduction of CRY1 protein levels combined with GR impairment found in our model may affect the expression of a wide variety of genes, extending the mechanisms potentially affected by chronic stress exposure.

Interestingly, it has been demonstrated that an acute restraint stress causes a rapid increase in the level of *Period 1* mRNA (21), in line with the idea that, while prolonged stress has negative outcomes and may lead to the development of pathological phenotypes, brief stressors may produce an activation of protective mechanisms that may help the organism in coping with the adverse condition. Furthermore, it has been demonstrated that 7 days of stress did not influence the protein levels of PER2 in hippocampus and in the prefrontal cortex, while decreased it in the SCN (22). As mentioned before most studies have focused on the SCN, where it has been shown that 4 weeks of chronic unpredictable stress reduced the amplitude of *Per2*, but not *Per1,* oscillations, an effect that was normalized by desipramine treatment (23).

Only recently the effect of prolonged stress exposure on the expression of clock genes was analyzed in brain regions such as the hippocampus, the amygdala and the nucleus accumbens. Indeed, it has been shown that CLOCK protein levels were modulated in the hippocampus, but not in the SCN, of stressed rats (24). Moreover, a disturbed diurnal oscillation of the expression of *Clock*, *Cry2*, *Per1*, *Per3*, *Id2*, *Rev-erbα*, *Ror-β*, *Ror-γ* was observed in the mouse amygdala (25), and alteration of *Per1*, *Per2*, *Clock* and *Bmal1* rhythms were also reported in the nucleus accumbens (7).

One interesting finding of our study is the ability of chronic treatment with lurasidone, a multi-receptor modulator, with the highest affinity for 5-HT_7 receptors, followed by D_2, 5-$HT2_A$ and 5-$HT1_A$ receptors, to regulate clock gene expression under CMS as well as in normal rats, an effect that, as previously demonstrated (12), was paralleled by antidepressant effect at behavioral levels. With this study, we demonstrated that chronic lurasidone administration was able to normalize the reduction of the protein levels of BMAL1, but not of CLOCK, in the nuclear compartment of CMS rats. Given that the mRNA levels of all the genes controlled by the heterodimer CLOCK:BMAL1 were restored after the pharmacological treatment, we infer that the effects on BMAL1 may be sufficient to restore the basal transcriptional levels of *Rev-Erbβ*, *Cry*s and *Per*s. However, as described above, it is feasible that other pathways may contribute to the observed effects, since we have also demonstrated that GR protein levels were modulated by lurasidone administration to CMS rats.

In the ventral hippocampus, which participates in emotional regulation and anxiety-related behaviors, we found limited effects of stress exposure as well as of lurasidone administration. Conversely, in the dorsal part of the hippocampus, which is preferentially involved in learning and memory, we found major effects of the pharmacological treatment that significantly increased the expression of *Bmal1*, *Clock*, *Cry1*, *Per1* and *Per2*, independently from the stress exposure. These results are in agreement with data from Uz and colleagues showing that chronic fluoxetine treatment produced a significant increase of *Clock*, *Bmal1*, *Per2*, *Cry2* gene expression in the hippocampus of normal rats (26). Moreover, the results of the present study demonstrate that, while lurasidone has

some modulatory activity in normal animals, as previously demonstrated (27-30), its effects are more pronounced in CMS rats than in normal rats, suggesting a greater effectiveness of the pharmacological treatment under pathological conditions. Since lurasidone is characterized by multi receptor profile, it is difficult to establish which mechanism may contribute to its modulatory activity on clock gene in sham as well as in stressed rats. One feasible candidate is represented by 5-HT7 receptors, towards which the drug has the highest affinity. Indeed, it has been reported that the 5-HT7 receptors are implicated in the circadian rhythms phase resetting and also in the REM sleep in human as well as in rodents (31, 32). Moreover, the activity of lurasidone in prefrontal cortex and hippocampus is consistent with the modulatory activity of GABA – glutamate function through the blockade of 5-HT7 receptors located on GABAergic interneurons (33).

In order to investigate possible changes downstream from the clock gene machinery, we have also investigated the expression of genes that, containing the E-box, are controlled by the CLOCK:BMAL1 heterodimer, even if they do not belong to the aforementioned loops. In particular, we found that the expression of *Pparα* was specifically decreased in the prefrontal cortex of stressed animals, while *Nampt* transcription was significantly increased after lurasidone treatment in prefrontal cortex and in the dorsal hippocampus.

The role of *Pparα* has not been extensively investigated, but it may be involved in the control of the food intake and in metabolic processes (34). Since one of the most profound effects of stress exposure is weight loss, often due to a reduction of food intake, it may be inferred that the reduction of *Pparα* expression in CMS rats may, at least in part, contribute to dysmetabolic changes brought about by stress exposure. Since it has been demonstrated that *Nampt* plays a fundamental role in neuronal survival and in cognitive function (35), the increase of *Nampt* expression after lurasidone treatment may contribute to the protective and procognitive effects of this multi receptor antipsychotic drug (10, 11).

There are two major limitations in the present study. First, we performed our analyses at a single time point, which does not allow us to assess a potential shift of the peak expression in these genes or a loss of rhythmicity. Nevertheless we believe we are providing compelling evidence that clock gene expression are dys-regulated as a consequence of CMS exposure and/or pharmacological intervention, and that such changes do occur in brain regions outside the SCN. Second, while CMS rats show a clear anhedonic phenotype, we do not have any information about possible alterations of biological rhythms (i.e. analysis of activities or of sleep). However, although further studies are needed to clarify these issues, for the first time, our study provides a clear indication that a pharmacological treatment is able to modulate clock gene expression in an animal model of depressive disorder within brain regions different from the SCN. We think that the changes observed in the prefrontal cortex that play a fundamental role in the onset and development of psychiatric diseases may help to clarify the link between disruption of circadian rhythms and these pathologic conditions.

To conclude, the alterations in clock genes expression due to CMS exposure may underline the development of symptoms correlated with circadian abnormalities. Moreover, the ability of chronic lurasidone treatment to modulate clock genes expression

together with the normalization of the anhedonic phenotype suggest a potential role of this drug in ameliorating the disruption of circadian rhythms observed in subject affected by major depression and stress-related disorders (11, 12, 27, 28, 36, 37).

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References

1. Turek FW. From circadian rhythms to clock genes in depression. International clinical psychopharmacology. 2007;22 Suppl 2:S1-8.
2. Emens J, Lewy A, Kinzie JM, Arntz D, Rough J. Circadian misalignment in major depressive disorder. Psychiatry research. 2009;168(3):259-261.
3. Mendlewicz J. Disruption of the circadian timing systems: molecular mechanisms in mood disorders. CNS drugs. 2009;23 Suppl 2:15-26.
4. Gibson EM, Wang C, Tjho S, Khattar N, Kriegsfeld LJ. Experimental 'jet lag' inhibits adult neurogenesis and produces long-term cognitive deficits in female hamsters. PloS one. 2010;5(12):e15267.
5. Li JZ, Bunney BG, Meng F, Hagenauer MH, Walsh DM, Vawter MP, Evans SJ, Choudary PV, Cartagena P, Barchas JD, Schatzberg AF, Jones EG, Myers RM, Watson SJ, Jr., Akil H, Bunney WE. Circadian patterns of gene expression in the human brain and disruption in major depressive disorder. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(24):9950-9955.
6. Lowrey PL, Takahashi JS. Genetics of circadian rhythms in Mammalian model organisms. Advances in genetics. 2011;74:175-230.
7. Logan RW, Edgar N, Gillman AG, Hoffman D, Zhu X, McClung CA. Chronic Stress Induces Brain Region-Specific Alterations of Molecular Rhythms that Correlate with Depression-like Behavior in Mice. Biological psychiatry. 2015;78(4):249-258.
8. Ishibashi T, Horisawa T, Tokuda K, Ishiyama T, Ogasa M, Tagashira R, Matsumoto K, Nishikawa H, Ueda Y, Toma S, Oki H, Tanno N, Saji I, Ito A, Ohno Y, Nakamura M. Pharmacological profile of lurasidone, a novel antipsychotic agent with potent 5-hydroxytryptamine 7 (5-HT7) and 5-HT1A receptor activity. The Journal of pharmacology and experimental therapeutics. 2010;334(1):171-181.
9. Cates LN, Roberts AJ, Huitron-Resendiz S, Hedlund PB. Effects of lurasidone in behavioral models of depression. Role of the 5-HT(7) receptor subtype. Neuropharmacology. 2013;70:211-217.
10. Horisawa T, Nishikawa H, Toma S, Ikeda A, Horiguchi M, Ono M, Ishiyama T, Taiji M. The role of 5-HT7 receptor antagonism in the amelioration of MK-801-induced learning and memory deficits by the novel atypical antipsychotic drug lurasidone. Behavioural brain research. 2013;244:66-69.
11. Tarazi FI, Riva MA. The preclinical profile of lurasidone: clinical relevance for the treatment of schizophrenia. Expert opinion on drug discovery. 2013;8(10):1297-1307.
12. Luoni A, Macchi F, Papp M, Molteni R, Riva MA. Lurasidone Exerts Antidepressant Properties in the Chronic Mild Stress Model through the Regulation of Synaptic and Neuroplastic Mechanisms in the Rat Prefrontal Cortex. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum. 2014.
13. Guidotti G, Calabrese F, Anacker C, Racagni G, Pariante CM, Riva MA. Glucocorticoid receptor and FKBP5 expression is altered following exposure to chronic stress: modulation by antidepressant treatment. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2013;38(4):616-627.
14. Cao R, Robinson B, Xu H, Gkogkas C, Khoutorsky A, Alain T, Yanagiya A, Nevarko T, Liu AC, Amir S, Sonenberg N. Translational control of entrainment and synchrony of the suprachiasmatic circadian clock by mTOR/4E-BP1 signaling. Neuron. 2013;79(4):712-724.
15. Buijs RM, Kalsbeek A. Hypothalamic integration of central and peripheral clocks. Nature reviews Neuroscience. 2001;2(7):521-526.
16. Son GH, Chung S, Choe HK, Kim HD, Baik SM, Lee H, Lee HW, Choi S, Sun W, Kim H, Cho S, Lee KH, Kim K. Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(52):20970-20975.
17. Nader N, Chrousos GP, Kino T. Interactions of the circadian CLOCK system and the HPA axis. Trends in endocrinology and metabolism: TEM. 2010;21(5):277-286.
18. So AY, Bernal TU, Pillsbury ML, Yamamoto KR, Feldman BJ. Glucocorticoid regulation of the circadian clock modulates glucose homeostasis. Proceedings of the National Academy of Sciences of the United States of America. 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 research. 2013;41(12):6161-6174.
20. Lamia KA, Papp SJ, Yu RT, Barish GD, Uhlenhaut NH, Jonker JW, Downes M, Evans RM. Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. Nature. 2011;480(7378):552-556.
21. Yamamoto T, Nakahata Y, Tanaka M, Yoshida M, Soma H, Shinohara K, Yasuda A, Mamine T, Takumi T. Acute physical stress elevates mouse period1 mRNA expression in mouse peripheral tissues via a glucocorticoid-responsive element. The Journal of biological chemistry. 2005;280(51):42036-42043.
22. Kinoshita C, Miyazaki K, Ishida N. Chronic stress affects PERIOD2 expression through glycogen synthase kinase-3beta phosphorylation in the central clock. Neuroreport. 2012;23(2):98-102.
23. Jiang WG, Li SX, Zhou SJ, Sun Y, Shi J, Lu L. Chronic unpredictable stress induces a reversible change of PER2 rhythm in the suprachiasmatic nucleus. Brain research. 2011;1399:25-32.

24. Jiang WG, Li SX, Liu JF, Sun Y, Zhou SJ, Zhu WL, Shi J, Lu L. Hippocampal CLOCK protein participates in the persistence of depressive-like behavior induced by chronic unpredictable stress. Psychopharmacology. 2013;227(1):79-92.
25. Savalli G, Diao W, Schulz S, Todtova K, Pollak DD. Diurnal oscillation of amygdala clock gene expression and loss of synchrony in a mouse model of depression. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum. 2015;18(5).
26. Uz T, Ahmed R, Akhisaroglu M, Kurtuncu M, Imbesi M, Dirim Arslan A, Manev H. Effect of fluoxetine and cocaine on the expression of clock genes in the mouse hippocampus and striatum. Neuroscience. 2005;134(4):1309-1316.
27. Calabrese F, Luoni A, Guidotti G, Racagni G, Fumagalli F, Riva MA. Modulation of neuronal plasticity following chronic concomitant administration of the novel antipsychotic lurasidone with the mood stabilizer valproic acid. Psychopharmacology. 2013;226(1):101-112.
28. Fumagalli F, Calabrese F, Luoni A, Bolis F, Racagni G, Riva MA. Modulation of BDNF expression by repeated treatment with the novel antipsychotic lurasidone under basal condition and in response to acute stress. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum. 2012;15(2):235-246.
29. Luoni A, Rocha FF, Riva MA. Anatomical specificity in the modulation of activity-regulated genes after acute or chronic lurasidone treatment. Progress in neuro-psychopharmacology & biological psychiatry. 2014;50:94-101.
30. Stan TL, Sousa VC, Zhang X, Ono M, Svenningsson P. Lurasidone and fluoxetine reduce novelty-induced hypophagia and NMDA receptor subunit and PSD-95 expression in mouse brain. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology. 2015;25(10):1714-1722.
31. Hedlund PB, Huitron-Resendiz S, Henriksen SJ, Sutcliffe JG. 5-HT7 receptor inhibition and inactivation induce antidepressantlike behavior and sleep pattern. Biological psychiatry. 2005;58(10):831-837.
32. Thomas DR, Melotto S, Massagrande M, Gribble AD, Jeffrey P, Stevens AJ, Deeks NJ, Eddershaw PJ, Fenwick SH, Riley G, Stean T, Scott CM, Hill MJ, Middlemiss DN, Hagan JJ, Price GW, Forbes IT. SB-656104-A, a novel selective 5-HT7 receptor antagonist, modulates REM sleep in rats. British journal of pharmacology. 2003;139(4):705-714.
33. Tokarski K, Bobula B, Grzegorzewska-Hiczwa M, Kusek M, Hess G. Stress- and antidepressant treatment-induced modifications of 5-HT(7) receptor functions in the rat brain. Pharmacological reports : PR. 2012;64(6):1305-1315.
34. Chakravarthy MV, Zhu Y, Lopez M, Yin L, Wozniak DF, Coleman T, Hu Z, Wolfgang M, Vidal-Puig A, Lane MD, Semenkovich CF. Brain fatty acid synthase activates PPARalpha to maintain energy homeostasis. The Journal of clinical investigation. 2007;117(9):2539-2552.
35. Stein LR, Wozniak DF, Dearborn JT, Kubota S, Apte RS, Izumi Y, Zorumski CF, Imai S. Expression of Nampt in hippocampal and cortical excitatory neurons is critical for cognitive function. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2014;34(17):5800-5815.
36. Luoni A, Berry A, Calabrese F, Capoccia S, Bellisario V, Gass P, Cirulli F, Riva MA. Delayed BDNF alterations in the prefrontal cortex of rats exposed to prenatal stress: preventive effect of lurasidone treatment during adolescence. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology. 2014;24(6):986-995.
37. Luoni A, Hulsken S, Cazzaniga G, Racagni G, Homberg JR, Riva MA. Behavioural and neuroplastic properties of chronic lurasidone treatment in serotonin transporter knockout rats. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum. 2013;16(6):1319-1330.

Figure legends

Figure 1: Analysis of clock genes expression in the prefrontal cortex of rats exposed to CMS: effect of lurasidone treatment. The mRNA levels of *Bmal1* (A), *Clock* (B), *Cry1* (C), *Cry2* (D), *Per1* (E) and *Per2* (F) were measured in the prefrontal cortex of non-stressed or chronically stressed rats, treated for 5 weeks with vehicle or lurasidone and killed 24 h after the last administration. The data, expressed as a percentage of No Stress/Vehicle (set at 100%), are the mean ± SEM of at least 9 independent determinations. *p<0.05, ** p<0.01, ***p<0.001 vs. No Stress/Vehicle; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. Stress/Vehicle (two-way ANOVA with PLSD).

Figure 2: Correlation analyses between *Bmal*1 (A), *Per*1 (B), *Per*2 (C) and sucrose preference in the prefrontal cortex of rats exposed to stress. Analyses by Pearson's product–moment correlation (r). The data derived from 19 independent determinations.

Figure 3: Analysis of Rev-Erbα, Rev-Erbβ expression in the prefrontal cortex of rats exposed to CMS: effect of lurasidone treatment. The mRNA levels of *Rev-Erbα* (A), *Rev-Erbβ* (B) were measured in the prefrontal cortex of non-stressed or chronically stressed rats, treated for 5 weeks with vehicle or lurasidone and killed 24 h after the last administration. The data, expressed as a percentage of No Stress/Vehicle (set at 100%), are the mean ± SEM of at least 9 independent determinations. *p<0.05 vs. No Stress/Vehicle; $^{\#}$p<0.05 vs. Stress/Vehicle (two-way ANOVA with PLSD).

Figure 4: Analysis of CLOCK:BMAL1 heterodimer regulated-genes expression in the prefrontal cortex of rats exposed to CMS: effect of lurasidone treatment. The mRNA levels of *Pparα* (A), *Nampt* (B) and *Bhlhe40* (C) were measured in the prefrontal cortex of non-stressed or chronically stressed rats, treated for 5 weeks with vehicle or lurasidone and killed 24 h after the last administration. The data, expressed as a percentage of No Stress/Vehicle (set at 100%), are the mean ± SEM of at least 9 independent determinations. *p<0.05 vs. No Stress/Vehicle; $^{\#\#}$p<0.01 vs. Stress/Vehicle (two-way ANOVA with PLSD).

Figure 5: Representative western blot analyses of the BMAL1, CLOCK, CRY1, PER1, GR, CREB and β-[illegible]. Experimental conditions are described in Materials and methods.

Figure 6: Analysis of BMAL1, CLOCK, CRY1 and PER1 protein levels in the prefrontal cortex of rats exposed to CMS: effect of lurasidone treatment. The protein levels of BMAL1 (A), CLOCK (B), CRY1 (C) and PER1 (D) were measured in the nucleus of prefrontal cortex of non-stressed or chronically stressed rats, treated for 5 weeks with vehicle or lurasidone and killed 24 h after the last administration. The data, expressed as a percentage of No Stress/Vehicle (set at 100%), are the mean ± SEM of at least 6 independent determinations. *p<0.05, ***p<0.001 vs. No Stress/Vehicle; $^{\#\#\#}$p<0.001 vs. Stress/Vehicle (two-way ANOVA with PLSD).

Figure 7: Analysis of GR and CREB protein levels in the prefrontal cortex of rats exposed to CMS: effect of lurasidone treatment. The protein levels of GR (B) and CREB (C) were measured in the nucleus of prefrontal cortex of non-stressed or chronically stressed rats, treated for 5 weeks with vehicle or lurasidone and killed 24 h after the last administration. (A) Schematic representation of the *Per1* promoter. The data, expressed as a percentage of No Stress/Vehicle (set at 100%), are the mean ± SEM of at least 6 independent determinations. *p<0.05, **p<0.01 vs. No Stress/Vehicle; $^{\#}$p<0.05 vs. Stress/Vehicle (two-way ANOVA with PLSD).

Figure 8: Schematic representation of the effect of CMS and lurasidone treatment on the clock genes expression. CMS exposure modulated transcription and translation of clock genes primarily in the prefrontal cortex, and chronic treatment with antipsychotic lurasidone is able to normalize some of these alterations. Indeed, compare to the basal condition (Panel A), stress exposure (Panel B) reduced the protein levels of BMAL1, CLOCK and GR in the nuclear compartments producing a decrease in the expression of the *Rev-Erb*s, *Per*s and *Cry*s genes. In turn the reduction of *Rev-Erb*β and of *Hdac* mRNA levels may underlie the decreased transcription of *Bmal1.* Moreover, the effect on *Per*s and *Cry*s gene was paralleled by a similar alteration of the protein levels. On the other side, chronic treatment with lurasidone (Panel C) was able to restore the normal protein levels BMAL1 and GR with consequent normalization of the expression of the target genes. Moreover, here, the expression of *Bmal1* was similar to those observed in basal condition (Panel A) probably because also *Rev-Erb*± and of *Hdac* mRNA levels were normalized by the pharmacological treatment. Finally, lurasidone was able to correct only the alteration due to the CMS of CRY1.

Table 1 Sequences of Forward and Reverse Primers and Probe used in Real-time PCR Analyses

Gene	*Forward primer*	*Reverse primer*	*Probe*
Bmal1	ATCCTGAGCACGGTGAGTTT	AAGAGGCGTCGGGACAAAAT	CAACATGCAATGCGATGTCC
Clock	ATCTTTGTCGGCGTTGAGGA	AAAGGTTCGATCACAGCCCA	CAGAAGCTCAAGAAAGTCCTCG
Cry1	TCAATCCACGGAAAGCCTGT	CCACAAACAACCCACGCTTT	GGAACCCCATCTGTGTTCAA
Cry2	TAGTCCACGCCAATGATGCA	TGCCCAAACTGAAAGGCTTC	TCTATGAGCCCTGGAATGCT
Per1	AGAGCTGAGTCCTTGCCATT	TGGCTGATGACACTGATGCA	AGCGGAGTTCTCACAGTTCA
Per2	TTGTGCCTCCCGATGATGAA	AGTGGGCAGCCTTTCGATTA	GTACATCACACTGGACACTAGC
Rev-Erbα	ACGTCCCCACACACTTTACA	ACAAGTGGCCATGGAAGACA	GGCACCAGCAACATTACCAA
Rev-Erbβ	ACGGATGAGTGTTTCCTGCA	AGCGACGAGGAAATGAGCTT	TTCTGGTGTCTGCAGATCGA
Pparα	TGCATTGTGTGACATCCCGA	TGGCGTACGACAAGTGTGAT	GAACCGGAACAAATGCCAGT
NAMPT	GGACGGAGTGGATATCAATAC	GAGGTCTCTGGTTAACTTCTG	AATGTCTCCTTTGGTTCTGG
BHLHE40	GGACGGAGTGGATATCAATAC	GAGGTCTCTGGTTAACTTCTG	AATGTCTCCTTTGGTTCTGG
36B4	TCAGTGCCTCACTCCATCAT	AGGAAGGCCTTGACCTTTTC	TGGATACAAAAGGGTCCTGG

Table 2 Effect of CMS and Lurasidone treatment on Sucrose intake

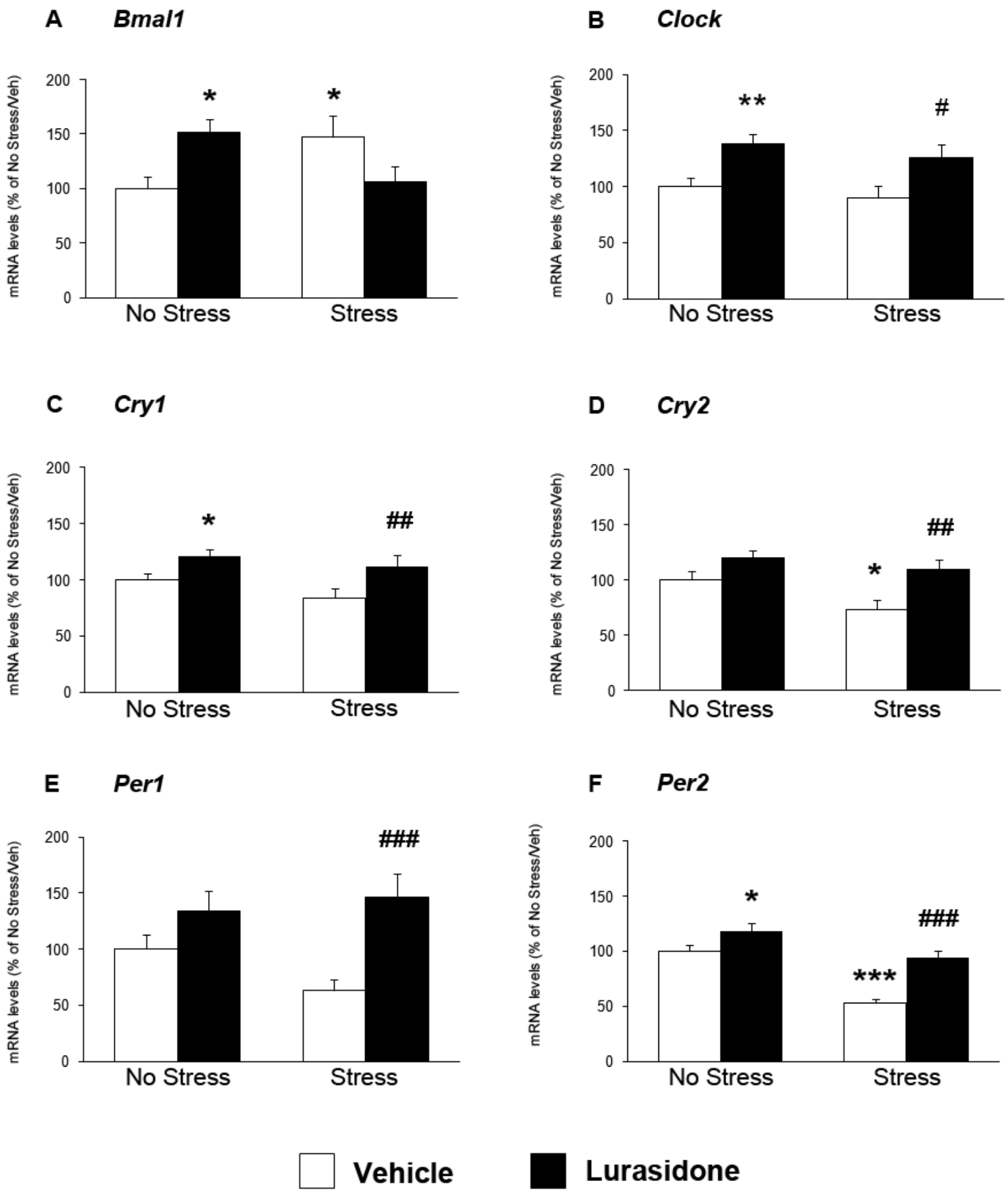
Experimental groups	*Baseline*	*Week 2*	*Experimental groups*	*Week 7*
No stress	11.6 ± 0.9	11.2 ± 0.5	No stress/Vehicle	11.0 ± 0.8
			No stress/Lurasidone	12.7 ± 0.9
Stress	11.8 ± 0.4	6.7 ± 0.5***	Stress/Vehicle	6.0 ± 0.9°°°
			Stress/Lurasidone	12.1 ± 0.9###

2 weeks of CMS induced a significant reduction of the sucrose consumption, effect that was already present after 7 weeks of stress, but was normalized by 5 weeks of concomitant treatment with lurasidone. The data, expressed as grams (g) of sucrose intake, are the mean ± SEM of at least 9 independent determinations. ***p<0.001 vs No stress (Student's t test); °°°p<0.001 vs No stress/Vehicle, ###p<0.001 vs Stress/Vehicle (two-way ANOVA with Fisher PLSD).

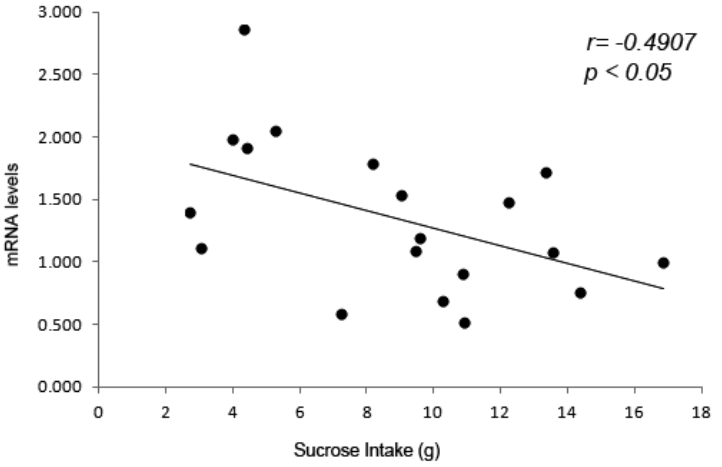
Table 3: **Analysis of clock and CCGs genes expression in the hippocampus of rats exposed to CMS: effect of lurasidone treatment**

	No Stress/Vehicle	No Stress/Lurasidone	Stress/Vehicle	Stress/Lurasidone
Dorsal Hippocampus				
Bmal1	1.008	1.420***	1.191	1.428$^{\#\#}$
Clock	1.009	1.343**	1.026	1.506$^{\#\#\#}$
Cry1	1.047	1.826**	1.381	2.011$^{\#}$
Cry2	1.050	1.229	1.648***	1.482
Per1	1.022	1.417***	1.217	1.519$^{\#\#}$
Per2	1.013	1.467***	1.139	1.698$^{\#\#\#}$
Rev-Erbα	1.013	1.112	1.031	1.150
Rev-Erbβ	1.010	1.126	1.055	1.364$^{\#\#\#}$
Pparα	1.013	1.064	1.016	1.033
Nampt	1.027	1.303*	1.012	1.337$^{\#\#}$
Bhlhe40	1.037	1.089	1.153	1.156
Ventral Hippocampus				
Bmal1	1.062	1.146	1.085	1.181
Clock	0.956	0.909	1.038	0.990
Cry1	1.016	1.126	1.151	1.145
Cry2	0.945	0.968	1.228**	1.283$^{\#\#\#}$
Per1	1.018	1.905***	1.079	2.124$^{\#\#}$
Per2	1.026	0.891	0.849	1.229
Rev-Erbα	1.022	1.028	0.886	0.967
Rev-Erbβ	1.016	1.071	1.116	1.158
Pparα	1.011	1.029	0.968	0.977
Nampt	1.015	1.171	1.089	1.297
Bhlhe40	1.020	1.000	1.058	1.085

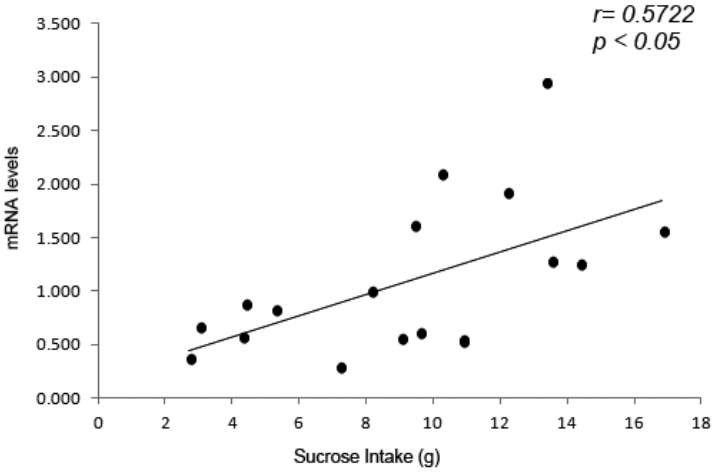
The mRNA levels of *Bmal1*, *Clock*, *Cry1*, *Cry2*, *Per1, Per2, Rev-Erbα, Rev-Erbβ*, *Pparα*, *NAMPT* and *BHLHE40* were measured in the dorsal and ventral hippocampus of non-stressed or chronically stressed rats, treated for 5 weeks with vehicle or lurasidone and killed 24 h after the last administration. The data, expressed as fold change, are the mean ± SEM of at least 9 independent determinations. ** p<0.01, ***p<0.001 vs. No Stress/Vehicle; $^{\#}$ p<0.05, $^{\#\#}$ p<0.01, $^{\#\#\#}$p<0.001 vs. Stress/Vehicle (two-way ANOVA with SCPHT).


A
Bmal1
B
Clock
C
Cry1
D
Cry2
E
Per1
F
Per2
mRNA levels (% of No Stress/Veh)
200
150
100
50
0
No Stress
Stress
*
**
#
##
###
***
Vehicle
Lurasidone

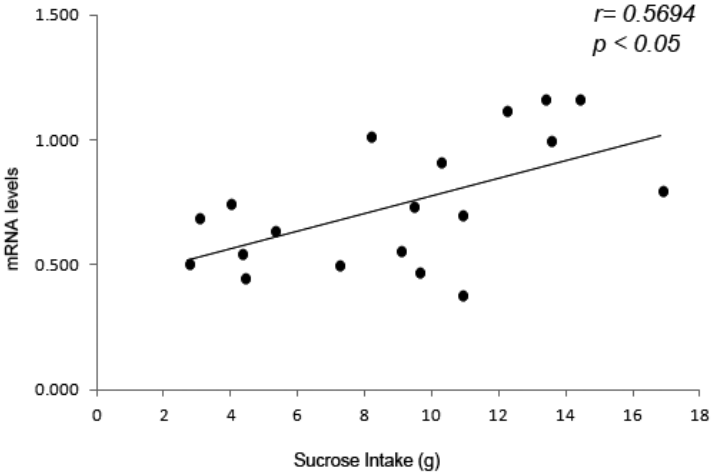
A Bmal1

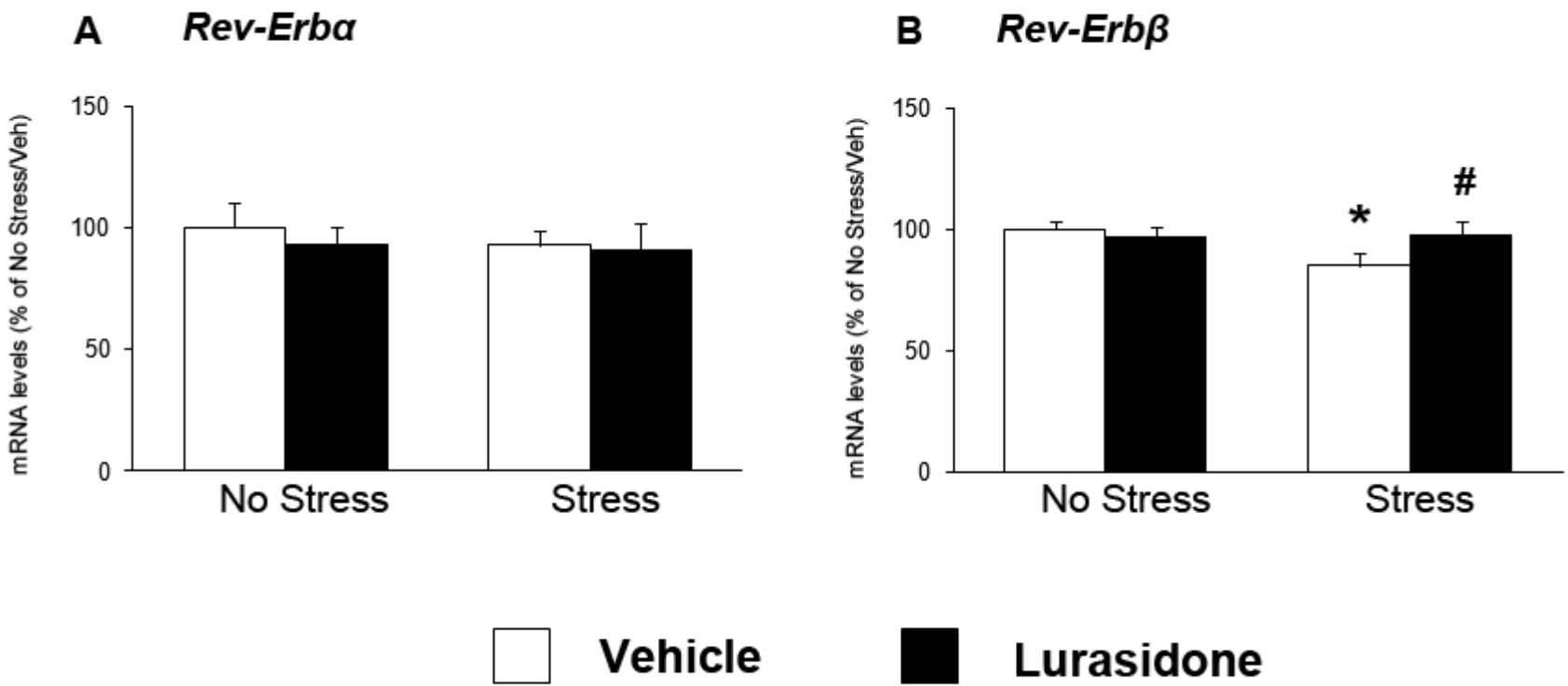


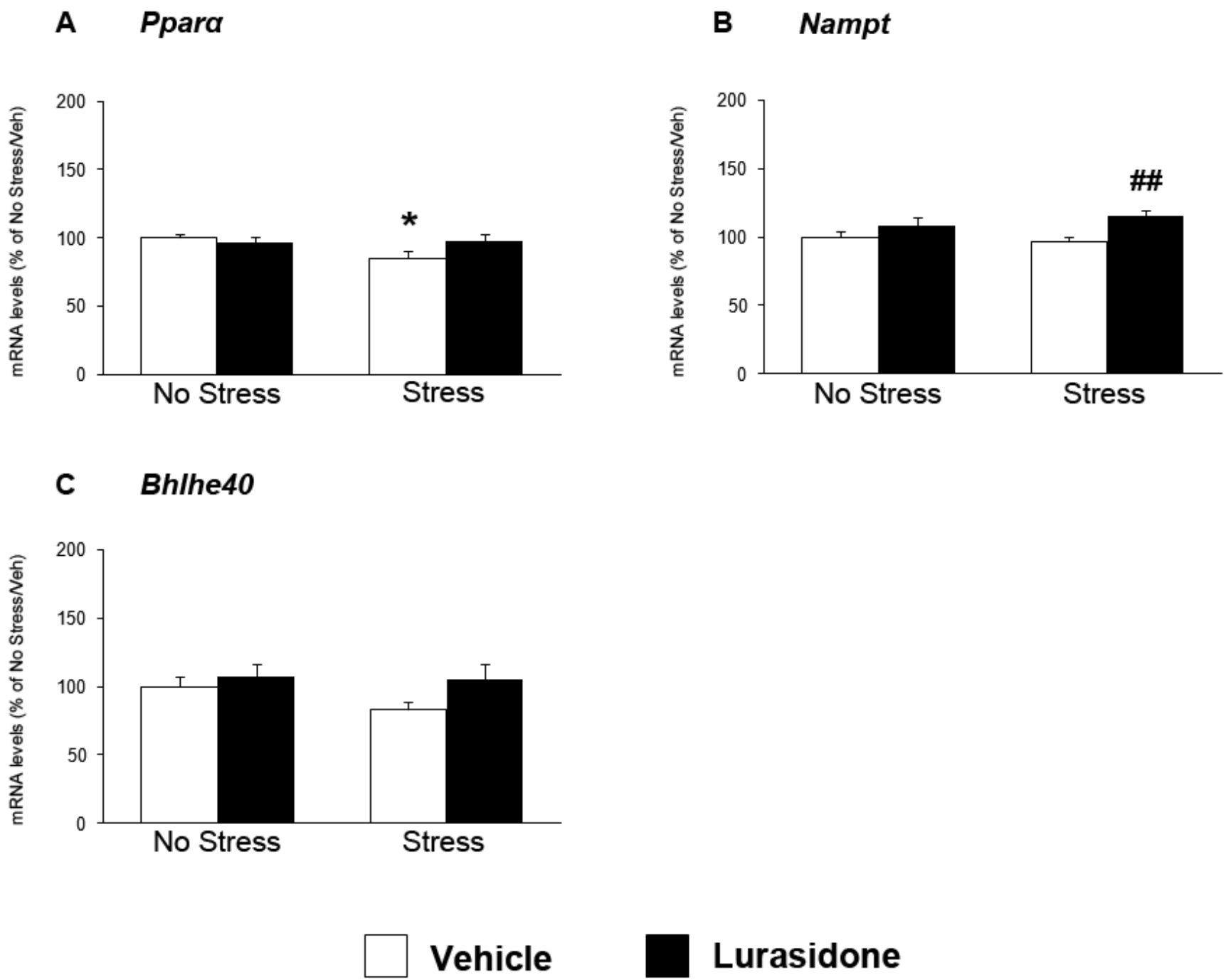
B Per1

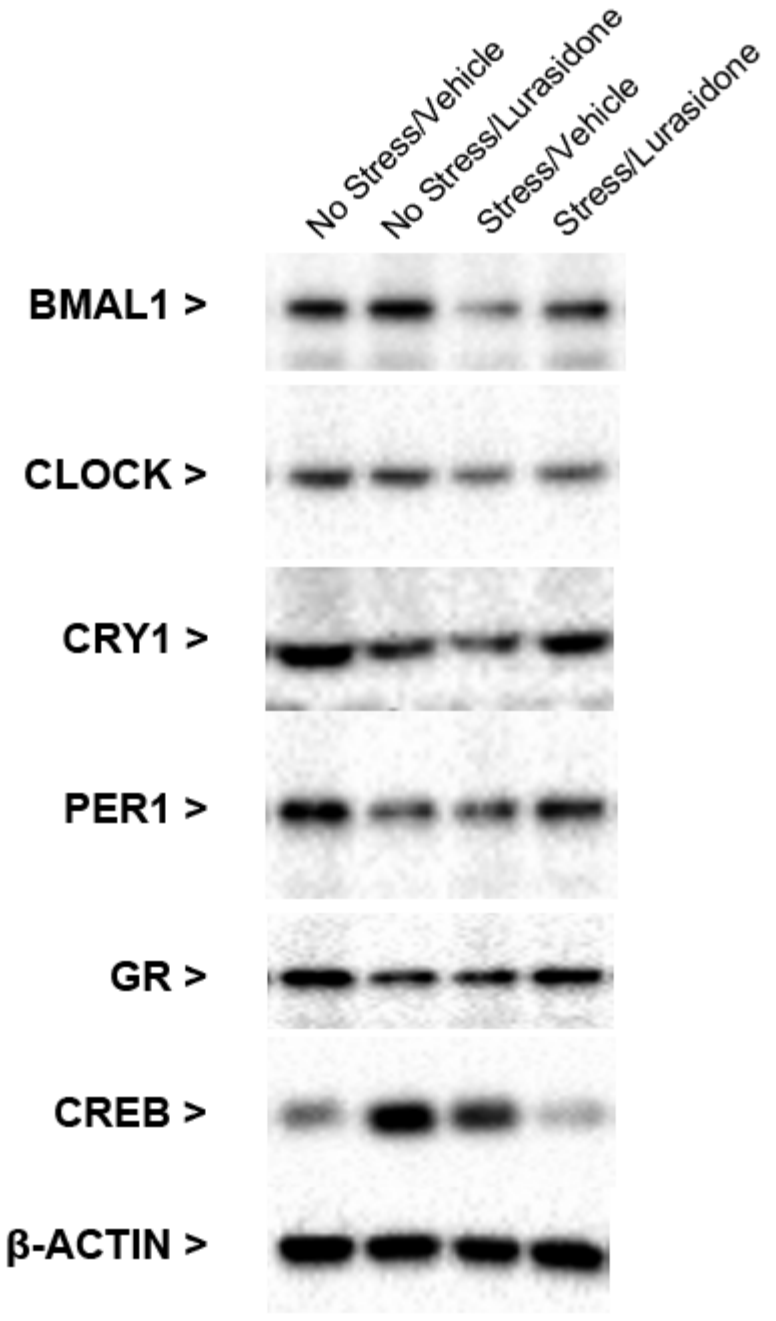


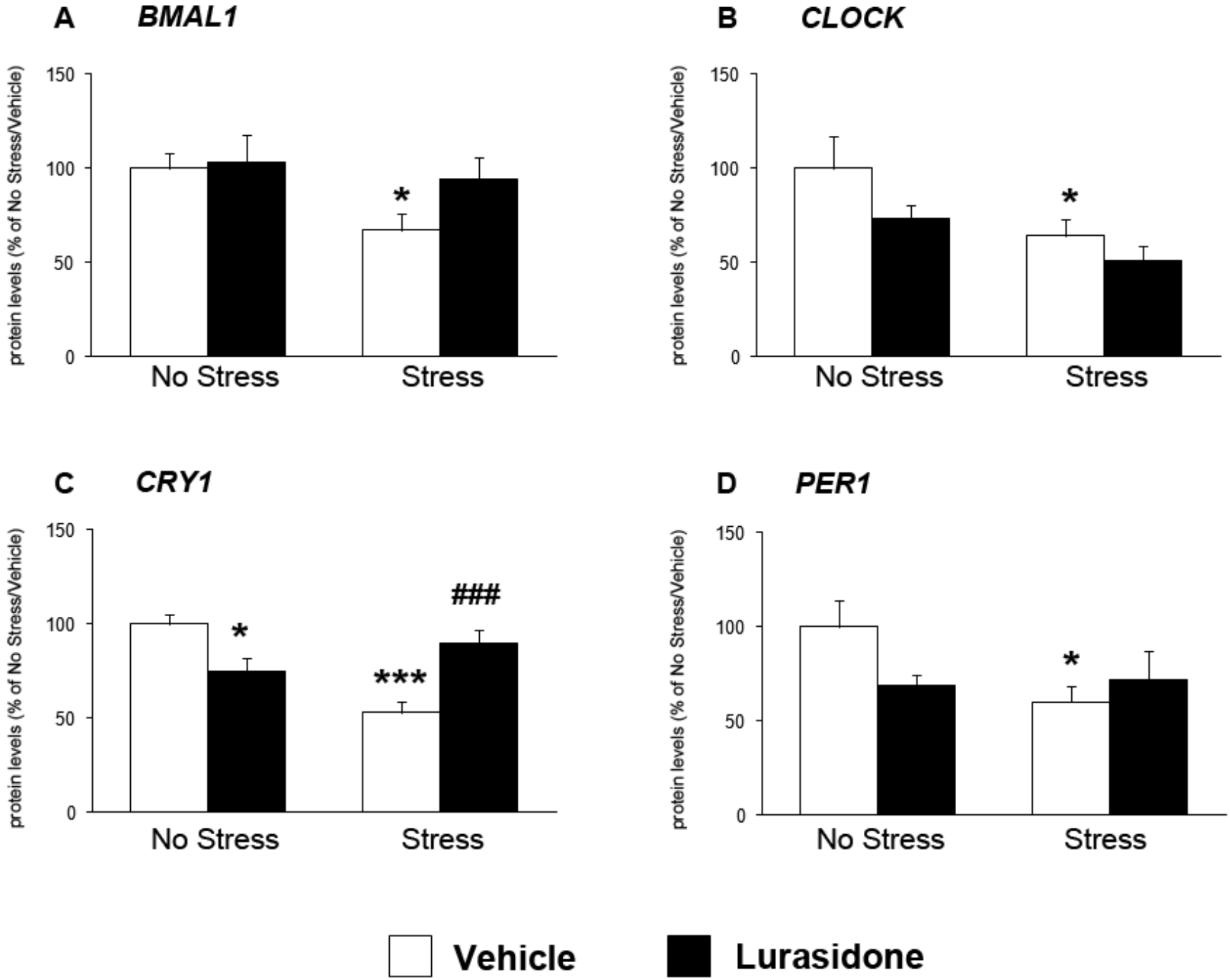
C Per2



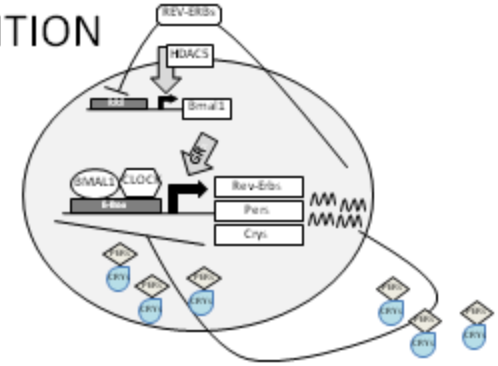

A
Rev-Erbα
mRNA levels (% of No Stress/Veh)
150
100
50
0
No Stress
Stress
B
Rev-Erbβ
mRNA levels (% of No Stress/Veh)
150
100
50
0
*
#
No Stress
Stress
Vehicle
Lurasidone


A
Pparα
mRNA levels (% of No Stress/Veh)
200
150
100
50
0
*
No Stress
Stress
B
Nampt
mRNA levels (% of No Stress/Veh)
200
150
100
50
0
##
No Stress
Stress
C
Bhlhe40
mRNA levels (% of No Stress/Veh)
200
150
100
50
0
No Stress
Stress
Vehicle
Lurasidone


No Stress/Vehicle
No Stress/Lurasidone
Stress/Vehicle
Stress/Lurasidone
BMAL1 >
CLOCK >
CRY1 >
PER1 >
GR >
CREB >
β-ACTIN >

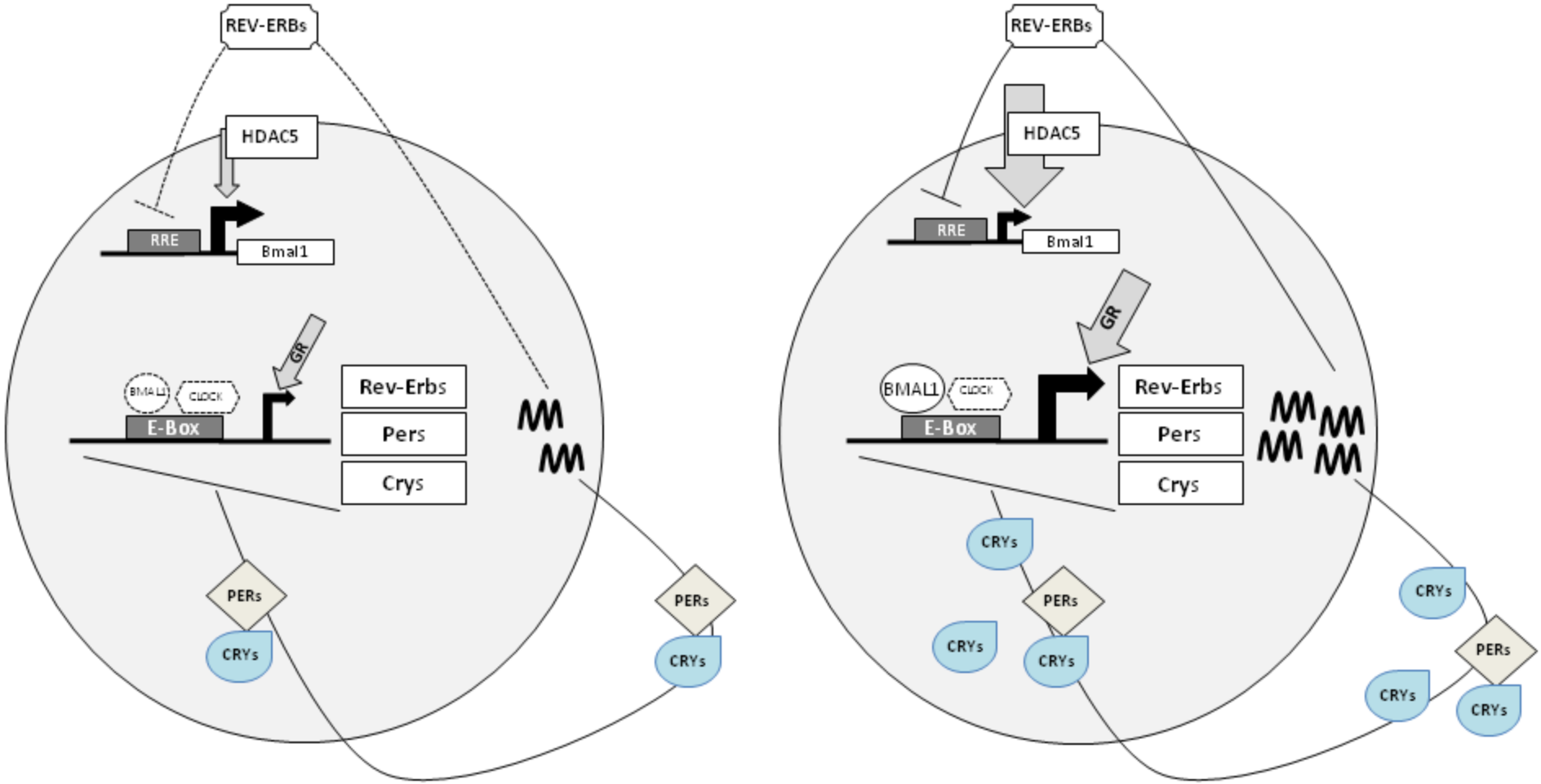

A
BMAL1
protein levels (% of No Stress/Vehicle)
150
100
50
0
*
No Stress
Stress
B
CLOCK
protein levels (% of No Stress/Vehicle)
150
100
50
0
*
No Stress
Stress
C
CRY1
protein levels (% of No Stress/Vehicle)
150
100
50
0
*
***
###
No Stress
Stress
D
PER1
protein levels (% of No Stress/Vehicle)
150
100
50
0
*
No Stress
Stress
Vehicle
Lurasidone

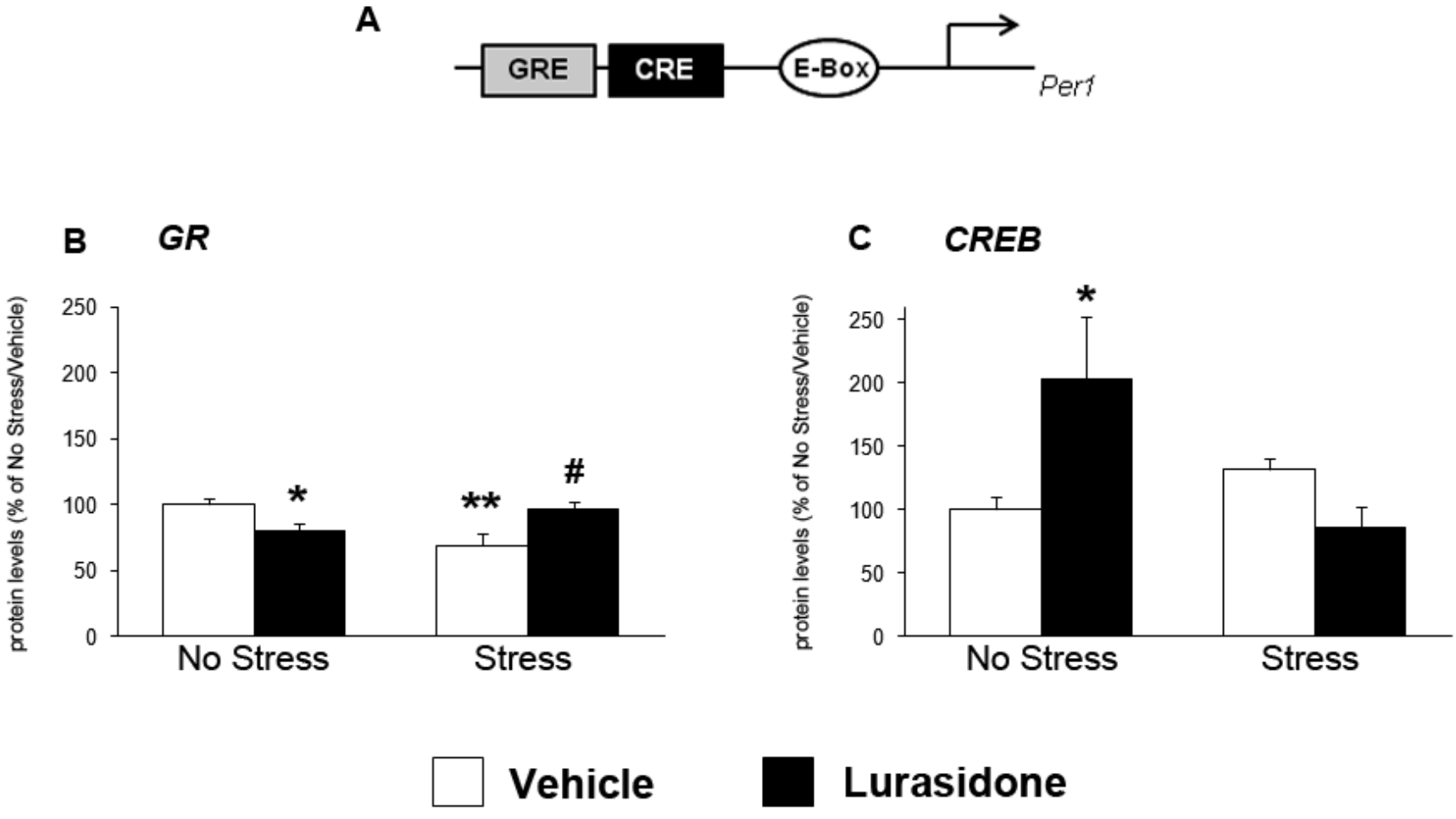
A: BASAL CONDITION



B: CMS EXPOSURE

C: LURASIDONE + CMS EXPOSURE




A
GRE
CRE
E-Box
Per1
B
GR
protein levels (% of No Stress/Vehicle)
250
200
150
100
50
0
*
**
#
No Stress
Stress
C
CREB
protein levels (% of No Stress/Vehicle)
250
200
150
100
50
0
*
No Stress
Stress
Vehicle
Lurasidone