# Brain glucocorticoid receptors are necessary for the rhythmic expression of the clock protein, PERIOD2, in the central extended amygdala in mice

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Acknowledgement
Supported by Fonds de la recherche en santé Québec (FRSQ), Canadian Institutes of Health Research (CIHR), the Natural Sciences and Engineering Research Council of Canada (NSERC), the Concordia University Research Chairs Program (CRUC), Centre National de la Recherche Scientifique, Agence Nationale de la Recherche (project GRADA) and European Union Framework Program 6 (STREP PheCOMP, NovelTune).

Abstract

The adrenal glucocorticoid, corticosterone, induces changes in gene expression in both neural and non-neural tissues. The rhythmic release of corticosterone has been shown in rats to be necessary for the rhythmic expression of the clock protein PERIOD2 (PER2) in select regions of the limbic forebrain. The mechanisms mediating the effects of glucocorticoids on changes in gene expression have been linked to the transcriptional activity of the low affinity glucocorticoid receptor, GR. We examined the patterns of PER2 expression in the brains of mice containing a inactivation of GR gene restricted to neural tissues ($GR^{NesCre}$ mice). We found that central deletion of the GR gene blunts the daily pattern of PER2 expression in the oval nucleus of the bed nucleus of the stria terminalis (BNSTov) and central nucleus of the amygdala (CEA) both of which make up the central extended amygdala, but not in the suprachiasmatic nucleus (SCN), basolateral amygdala (BLA) or dentate gyrus of the hippocampus (DG). These results implicate brain GR receptors in the regulation of PER2 expression in the BNSTov and CEA and are consistent with our previous findings that the rhythmic expression of PER2 in these areas is selectively sensitive to fluctuations in circulating corticosterone.

Keywords: basolateral amygdala, central nucleus of the amygdala, circadian rhythms, dentate gyrus, oval nucleus of the bed nucleus of the stria terminalis, suprachiasmatic nucleus

The master circadian clock, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, entrains circadian rhythms in behaviour and physiology through mechanisms that are still poorly understood. Secretion of the adrenal glucocorticoid hormone corticosterone is rhythmically driven by the SCN, and has proven to be a potent inducer and synchronizer of clock gene expression in peripheral tissues as well as in the brain [3, 9, 12]. We previously reported that adrenalectomy abolishes the daily rhythm of expression of the clock protein, PERIOD2 (PER2) in select regions of the limbic forebrain that are sensitive to glucocorticoids, the oval nucleus of the bed nucleus of the stria terminalis (BNSTov) and central nucleus of the amygdala (CEA) [12]. The diurnal patterns of PER2 expression in the SCN as well as other regions of the limbic forebrain, such as the basolateral amygdala (BLA), and dentate gyrus (DG), remain undisturbed. Rhythmic hormone replacement via the drinking water but not replacement via constant release pellets restores PER2 rhythmicity to the BNSTov and CEA [2, 7, 12]. We have also shown that perturbing the daily profile of circulating corticosterone in intact rats via acute or chronic corticosterone injections dampens and even attenuates clock protein rhythms in the BNSTov and CEA [11]. These findings underscore the importance of rhythmic glucocorticoid release, and emphasize how external factors that influence the daily variations in hormone signaling may affect the rhythms, and presumably the functional output of these oscillators.

The effects of circulating corticosterone on behavior and physiology are known to be mediated by two distinct receptors, a high affinity mineralocorticoid receptor and a low affinity glucocorticoid receptor [10]. Both belong to the nuclear receptor family and act as transcription factors. To investigate the role of central GR in the regulation of PER2 rhythmicity in the BNSTov and CEA we examined the expression of PER2 in the limbic forebrain of mice bearing a whole-brain mutation of *Nr3c1* gene, encoding GR, generated using the Cre*loxP* system [13]. Our findings indicate that brain GR are necessary for the rhythmic expression of PER2 in the BNSTov and CEA, but not in the SCN, BLA, or DG.

All experimental procedures followed the guidelines set out by the Canadian Council on Animal Care (http://www.ccac.ca/) and were approved by the Animal Care Committee at Concordia University (Montreal, QC, Canada). Mutant ($GR^{LoxP/LoxP}$ Tg(NesCre*kln*), thereafter denominated $GR^{NesCre}$) and control ($GR^{LoxP/LoxP}$) mice were generated and genotyped as described previously [13]. All mice were kept on a 12:12 hour light/dark schedule and had free access to food and water. $GR^{NesCre}$ (n=14) and control littermates (n=14) were anesthetized and perfused intracardially as previously described [2] at one of two time points: Zeitgeber 1 (ZT1, one hr after lights on), or ZT13 (1 hr after lights off). Brains were removed and post fixed overnight at 4 °C in paraformaldehyde, serial coronal sections (50 µm) were taken on a vibratome and then stored in Watson's Cryoprotectant at –20 °C until processing.

Immunohistochemistry for PER2 was performed as previously described [2] using an affinity purified rabbit polyclonal antibody raised against PER2 (1:800, ADI, San Antonio, TX). PER2-stained brain sections were examined under a light microscope and images were captured using a Sony XC-77 video camera, a Scion LG-3 frame grabber, and Image SXM software (v1.8, S D Barrett, http://www.ImageSXM.org.uk). Cells immunopositive for PER2 were counted in the regions studied using the captured images. For analysis, the mean number of PER2-immunoreactive cells per region was calculated for each animal from the counts of 6 unilateral images showing the highest number of labeled nuclei, as previously described [2]. Differences between groups were revealed with analyses of variance (ANOVA). Alpha level was set at 0.05 for all analyses.

Examples of PER2 expression in the SCN and limbic forebrain of wild-type mice perfused at ZT1 or ZT13 and graphs showing mean PER2 expression in each area in wild type and mutant mice as a function of time are shown in Fig 1. It can be seen that in wild-type mice PER2 expression in the SCN, BNSTov, and CEA is low in the morning and high in the evening, whereas expression in the BLA and DG is diametrically opposite, peaking in the morning and tapering off in the evening, as previously shown in rats [2, 7]. Compared with control littermates, the rhythm of PER2 expression in the BNSTov and CEA of $GR^{NesCre}$ mice was abolished,similar to that seen in adrenalectomized rats [2, 7, 12]. ANOVA

revealed a significant group x time interaction for both the BNSTov and CEA (BNSTov; F[1, 23]=24.3, *P*<0.0001; CEA; F[1, 22]=38.8, *P*<0.0001). PER2 expression in the SCN, BLA and DG was rhythmic in both wild type and mutant mice and no significant group difference or interaction between genotype and time was noted for any of the regions.

We show here in mice that a brain specific inactivation of GR gene selectively blunts the daily fluctuations in expression of the clock protein PER2 in the BNSTov and CEA, two functionally and structurally homologous regions commonly grouped into a superstructure referred to as the central extended amygdala [1]. PER2 expression in the SCN, BLA and DG remains unaffected, in spite of the presence of GR in the latter two regions [4, 5], mirroring previous results seen after adrenalectomy in rats. The present results extend our previous findings by demonstrating the importance of glucocorticoid signaling via brain GR in the regulation of the rhythmic expression of PER2 [2, 7, 12].

Glucocorticoids regulate a wide variety of functions, including immunological, metabolic, and developmental processes. In the mammalian brain, glucocorticoids modulate synaptic plasticity, HPA axis activation, and behavior such as conditional fear learning, and sensitization to drugs of abuse. These hormones can induce changes in physiology through indirect interactions, for example influencing cell membrane permeability, as well as neurohormone and neurotransmitter release. The effects of glucocorticoids on gene expression

are mediated by their binding to two steroid hormone receptors: the high affinity mineralocorticoid receptor, and the low affinity glucocorticoid receptor. Both are members of the nuclear hormone receptor super family and act as transcriptional regulators.

Activated receptors can bind to GREs (glucocorticoid responsive elements) and nGREs (negative GREs) present in the promoter or enhancer of target genes and activate or repress transcription. GR can also regulate transcription by modulating the activity of other transcription factors through protein-protein interactions. In this manner, GR can repress AP-1 and NFkb transcriptional activity or potentiate Stat5 activity [6].

The molecular mechanism whereby glucocorticoid hormones regulate the expression of PER2 in the BNSTov and CEA remains to be determined. In cell culture, the expression of the clock gene *per1,* but not *per2,* is activated by a glucocorticoids agonist [3]. *In vivo*, in the periphery, *per1* is up-regulated in a hormone dependent manner in response to restraint stress through direct binding of GR with a GRE situated distal to the promoter [14]. Although the *per2* promoter does not contain a functional GRE site, the *per1* gene contains two, of which the distal site is functional and has been shown through chromatin immunoprecipitation to bind the GR [14]. It is possible that GR modulates PER2 expression indirectly through transcriptional activation of the *per1* gene. The role of the *per1* gene has been poorly characterized; it appears to be functionally distinct from *per2* and plays a significant part in photic entrainment of the

circadian clock, whereas *per2* is more important in behavioral output [8]. *Per1* has been shown in the SCN to influence the expression of PER2, thereby affecting the rate at which the protein degrades, which is a crucial step in driving the transcriptional/translational feedback loop [15]. Further examination of the physical interaction between GR and clock genes may help to understand the interface between hormone signaling and changes in clock gene expression.

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Figure 1.

A) Photomicrographs showing examples of PER2 expression in the SCN, BNSTov, CEA, BLA, and DG of control and $GR^{NesCre}$ (KO) mice killed in the morning (ZT1) or evening (ZT13). Areas of interest are demarcated with a circle. B) Graphs showing means±sem of PER2-immunoreactive cells in all five regions of control and $GR^{NesCre}$ (KO) mice at ZT1 and ZT13 (n= 6-7/group/timepoint). Asterisks indicate significant difference from the corresponding ZT1 values. Deletion of brain GR abolished the daily variations in PER2 in the BNSTov and CEA, but not the SCN, BLA, or DG.

**Figure 1**

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