The regulation of stress-induced changes in the expression of the circadian clock protein PERIOD1, in the mammalian limbic forebrain and hypothalamus

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A Thesis In the Department of Biology

Presented in Partial Fulfillment of the Requirement for the Degree of
Doctor of Philosophy (Biology) at Concordia University
Montreal, Quebec, Canada

March 2014

CONCORDIA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

This is to certify that the thesis prepared

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Entitled: The regulation of stress-induced changes in the expression of the circadian clock protein PERIOD1, in the mammalian limbic forebrain and hypothalamus

and submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy (Biology)

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ABSTRACT

The regulation of stress-induced changes in the expression of the circadian clock protein PERIOD1, in the mammalian limbic forebrain and hypothalamus.

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Concordia University, 2014

Most organisms have developed internal mechanisms, including the circadian and stress systems, to allow for anticipation of and adaption to regular and unpredictable changes in the environment. The circadian and stress systems communicate constantly with one another; the circadian control of the release of effectors of the stress system, such as glucocorticoid hormones, is well documented, but the processes that govern how stressful events disrupt circadian rhythmicity are less understood. Here, we sought to elucidate these cross-talk mechanisms, by demonstrating that the expression of the circadian clock protein PER1, in the mammalian forebrain is strongly modifiable by stress. Throughout our work, the light-sensitive master pacemaker, the suprachiasmatic nucleus (SCN), remained immune to the effects of all stress manipulations. We first established that categorically different acute stressors distinctively modulate the expression of PER1 and the neuronal activity marker FOS. Systemic stressors increased protein expression in the piriform cortex, paraventricular and dorsomedial nuclei, as well as in the central extended amygdala. Contrastingly,

processive stressors increased protein levels in all regions except for in the central extended amygdala, where protein expression was uniquely suppressed. Interestingly, the emotional state of fear, a complex processive stressor, increased PER1 expression in this region, an effect characteristic of systemic stress. Furthermore, we determined that the time of day and modality of stress exposure are vital factors that influence PER1 activity. We then explored the role of glucocorticoids and glucocorticoid receptors (GR) in the modulation of stress-induced PER1, using manipulations that included adrenalectomy and pharmacological blockade of GR. We found that stress-induced PER1 expression in all regions studied, aside from the piriform cortex and SCN, are dependent on glucocorticoid signaling. In summary, the results demonstrate that stress, through the modulatory action of glucocorticoids and GR, can alter circadian clock protein expression in select forebrain and hypothalamic nuclei, possibly leading to their functional dysregulation and subsequent disturbances in circadian physiology and behavior. Our findings allude to a novel functional role for the circadian protein PER1 as an intermediary between the circadian system and systems that dictate emotional states, in the mammalian brain.

ACKNOWLEDGEMENTS

First and foremost, I would like to sincerely thank my PhD supervisor, Dr. Shimon Amir, for his unparalleled encouragement and supervision throughout my time as his student. I am eternally grateful for the opportunity that only Dr. Amir gave me in pursuing my PhD studies, especially in light of my extracurricular undertakings and short venture away from scientific research. A guiding beacon over the last four years, Dr. Amir has contributed selflessly to my academic and personal achievements.

The findings presented in this thesis would have not been possible without the contributions and encouragement of my past and present labmates, CSBN colleagues and staff members (in no order): Suzanne Hood, Lauren Segall, Alex Gavrila, Valerie Harbour, Marie Branchaud, Spencer Rutherford, Arun Dayanandan, Jeff Anyan, Nuria de Zavalia, Ariana Frederick, Dan Madularu, Mayte Parada, Katuschia Germé, Tracey D'Cunha, Firas Sedki, Waqqas Shams, Heshmat Rajabi, Isabelle Bouvier and Andrea Jakob. Thank you for your unfaltering support and deep scientific discussions.

A very special acknowledgement goes to our lab manager Barry Robinson, who tremendously facilitated our work in the lab by juggling our numerous demands, maintaining order and teaching us proper lab etiquette and ethics.

I am especially indebted to my thesis committee members, Drs. Wayne Brake, Selvadurai Dayanandan, Jim Pfaus and Valdimir Titorenko, not only for their systematic guidance since my first month as a PhD student, but also for their intellectual contributions to my development as a scientist.

Finally, I would like to dedicate this thesis to my parents and sister. My mother, Sima, and my father, Rushdi, have been my inspiration and driving force from the very beginning of my educational and life journey. Thank you infinitely for giving me the strength and wisdom to pursue my dreams, and for your unselfish love and sacrifices. To my sister Aya, my best friend, my partner in crime, my better half, and my gift from God. You are the best sister anyone could wish for, thank you for teaching me how to live.

“A theory that explains everything, explains nothing.”

Karl Popper

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LIST OF ABBREVIATIONS

ACTH: Adrenocorticotropic hormone

ADX: Adrenalectomy

ANOVA: Analysis of variance

2DG: 2-Deoxy-D-glucose

AVP: Vasopressin

BMAL1, ***Bmal1***: Brain and Muscle Arnt-Like Protein-1 or *gene*

BNSTov: Oval nucleus of the bed nucleus of the stria terminalis

CEA: Central nucleus of the amygdala, lateral division

CLOCK, ***Clock***: Circadian locomotor output cycles kaput protein and *gene*

CORT: Corticosterone

CRE: cAMP/Ca^{2+} responsive element

CRH: Corticotrophin-releasing hormone

CRY, ***Cry***: Cryptochrome protein or *gene*

DMH: Dorsomedial hypothalamus

dsRNA: double-stranded RNA

FS: Forced swim

FOS, ***c-Fos***: FOS protein or *gene*

GABA: γ-amino-butyric acid

GR: Glucocorticoid receptors

GRE: Glucocorticoid responsive element

GRP: Gastrin-releasing peptide

GRX: Glucocorticoid receptor antagonism

HPA: Hypothalamic-pituitary-adrenal

IEG: Immediate-early gene

IL-1β: interleukin-1β

ipRGC: intrinsically photosensitive retinal ganglion cells

IR: Immunoreactive

LD: light/dark cycle

MAPK: Mitogen-activated protein kinase

mGR: Membrane glucocorticoid receptors

MR: Mineralocorticoid receptors

PACAP: Pituitary adenylate cyclase-activating polypeptide

PER, *Per*: Period protein or *gene*

Pi: Piriform cortex

PVN: Paraventricular nucleus of the hypothalamus

REV-ERB, ***Rev-Erb***: REV-ERB protein or *gene*

RHT: Retinohypothalamic tract

ROR, ***Ror***: RAR-related orphan receptor protein and *gene*

RORE*:* Retinoic acid-related orphan receptor responsive element

SCN: Suprachiasmatic nucleus

TTFL: Transcription-translation feedback loop

VIP: Vasoactive intestinal peptide

ZT: Zeitgeber time

GENERAL INTRODUCTION

THE CIRCADIAN SYSTEM

Responding to Predictable Environmental Stimuli

The Earth rotates around its axis over an approximate 24 h period, or day, resulting in predictable changes in the external environment for unicellular and multicellular organisms inhabiting the planet (Dickmeis et al., 2013). Tissues and organs within multicellular organisms form a hierarchically structured system, referred to as the circadian system, which has been optimized for survival and adaptation (Albrecht, 2012). In mammals, this biological timekeeping system is driven by rhythmic oscillations within circadian clocks scattered throughout the brain and periphery, translating into physiological and behavioral outputs that allow organisms to make predictions about daily recurring events. The cell-autonomous nature of these circadian oscillators has also been demonstrated in other multicellular organisms including the cyanobacterium *Synechococcus elongatus*, the filamentous fungus *Neurospora crassa*, the fruitfly *Drosophila melanogaster*, the plant *Arabidopsis thaliana*, and in the retinas and pineal glands of amphibians and birds (Bell-Pederson et al., 2005). Although circadian rhythms are thus endogenous by nature, they can be entrained to the local environment by an external cue, or zeitgeber (ZT), through input pathways. In mammals, light exposure during the daytime acts as the main ZT, and photic information is transmitted readily through the retina and retinohypothalamic tract (RHT) to the master pacemaker of the circadian system, the suprachiasmatic

nucleus of the hypothalamus (SCN) (Rusak and Zucker, 1979). Dysregulated timekeeping in mammals can lead to a host of pathological diseases, which include sleep and mood disorders, as well as changes in cognitive function and memory (Masri et al., 2012).

The Ticking of the Clock

On a molecular level, the SCN and all clocks located downstream in the brain and periphery are composed of an autoregulatory transcription-translation feedback loop (TTFL) that takes approximately 24 h to complete one cycle (Dickmeis et al., 2013). The positive transcriptional limb of the TTFL drives circadian gene transcription and is composed of the core transcription factors Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle Arnt-Like Protein-1 (BMAL1). These proteins are members of the basic-helix-loop-helix family of transcription factors and upon heterodimerization, bind to the E-box element in the promoter of the target circadian genes *Period (Per)* and *Cryptochrome (Cry)*, and initiate their transcription (Ye et al., 2011). Acting as the negative limb of the TTFL, the resulting cytoplasmic PER and CRY proteins heterodimerize and translocate back into the nucleus, where they inhibit their own transcription by binding to CLOCK/BMAL1 complexes (Kume et al., 1999). CLOCK/BMAL1 dimers also initiate the transcription of a secondary loop that acts in accordance with the core loop. This entails the transcription and translation of two retinoic acid-related orphan nuclear receptors, REV-ERBα/β and RAR-related Orphan Receptor (ROR) α/β, which subsequently compete to

bind Retinoic acid-related Orphan receptor Response Elements (RORE) present in the *Bmal1* promoter (Preitner et al., 2002). While RORs activate transcription of *Bmal1*, REV-ERBs repress the same transcription process (Preitner et al., 2002; Guillaumond et al., 2005). Hence, the circadian oscillation of *Bmal1* is both positively and negatively regulated by RORs and REV-ERBs.

Post-translational modifications determine the subcellular localization and stability of clock proteins, ultimately playing a major role in establishing the 24 h periodicity of the clock (Partch et al., 2006). These modifications include but are not limited to ubiquitin-mediated proteosomal degradation and phosphorylation with the Ser/Thr kinases casein kinase-1 and glycogen synthase kinase-3 (Cheong and Virshup, 2011). Ultimately, these post-translational processes contribute to a time delay between transcript and protein levels; in the case of *Per1* for example, there is a 4-6 h lag between the former and latter (Reppert and Weaver, 2002). Recently, post-transcriptional processes have emerged as a third layer of clock gene regulation. These mechanisms regulate the clock's transcriptome, and include alternative splicing, microRNA-mediated mRNA silencing, and global translational control (mRNA translation initiation) (Cheng et al., 2007; Sonenberg and Hinnebusch, 2009; Cao et al., 2011). Consequently, it can no longer be assumed that clock mRNA transcript levels always correlate with the levels of protein that they encode (O'Neill et al., 2013).

The Master Pacemaker

The master pacemaker of the circadian system is located in the SCN. Lesions in the SCN result in a loss of vital daily rhythms including sleep-wake, locomotor

activity, feeding, body temperature and hormone secretion (Stephan and Zucker, 1972; Meyer-Bernstein et al., 1999; Tahara et al., 2012). Introducing a SCN transplant can restore these rhythms (Ralph et al., 1990; Meyer-Bernstein et al., 1999). Although generally immune to the effects of non-photic cues, the SCN is highly sensitive to light and upon stimulation, signals a host of downstream pathways and multiple endocrine axes (Tonsfeldt and Chappell, 2012). Neuropeptides play a major role in synchronizing individual clock cells within the SCN to each other. The majority of SCN neurons contain γ-amino-butyric acid (GABA), which mediates most intra-SCN signaling (Moore and Speh, 1993). GABA signaling from the ventral SCN modulates the activity of neurons in the dorsal SCN, and through a feedback loop, dorsal neurons modulate ventral neurons in a reciprocal manner (Albus et al., 2005). Vasoactive Intestinal Peptide (VIP) is required to maintain the amplitude of rhythms in individual neurons, and for their synchronization (Granados-Fuentes and Herzog, 2013). Several other neuropeptides, including Arginine Vasopressin (AVP) and Gastrin-Releasing Peptide (GRP), are also expressed in abundance and deemed to be major contributors of SCN efferent signals (Moore et al., 2002; Tonsfeldt and Chappell, 2012).

Photic entrainment of the SCN entails the intrinsically photosensitive retinal ganglion cells (ipRGC) that express the photopigment melanopsin and form the RHT (Berson et al., 2002; Granados-Fuentes and Herzog, 2013). ipRGCs release glutamate and Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) onto SCN neurons, leading to an increase in intracellular Ca^{2+} influx

(Golombek et al., 2004), or in cAMP that activates the cAMP/Ca^{2+} Responsive Element (CRE) on the promoter of target genes. *Per1* and *Per2* are light-inducible as their promoters readily express functional CREs; this CRE-dependent mechanism, independent of the clock's TTFL, is an alternative manner in which some clock genes are rapidly expressed and can facilitate clock resetting or phase entrainment (Jenkins et al., 2007; Granados-Fuentes and Herzog, 2013).

Synchronizing Central and Peripheral Time

Rhythms of clock gene and protein expression have been observed downstream of the SCN, in nuclei, tissues and organs throughout the brain and periphery. Such downstream central and peripheral clocks, also known as subordinate oscillators, exhibit tissue-specific functions and directly contribute to regulating circadian-controlled behavioral and physiological outputs. Rhythms in peripheral clocks persist in culture, but in contrast to self-sustaining SCN neurons, these rhythms gradually dampen due to individual differences within cell rhythms, thus require synchronizing inputs from the SCN to achieve orchestrated circadian timing (Balsalobre et al., 1998; Nagoshi et al., 2004). The SCN employs both neural and hormonal signals, including glucocorticoids, to relay its messages to and coordinate downstream oscillators. Glucocorticoids, amongst other factors, contribute to the synchronization of peripheral clocks and interact with them to time physiological dynamics in target tissues across the 24 h period (Dickmeis et al., 2013). Accordingly, unlike the SCN, peripheral clocks are heavily subject to influence by non-photic cues, which include stress, drugs of abuse, feeding,

sound and social cues (Amir and Stewart, 1998; Balsalobre et al., 1998; Balsalobre et al., 2000b; Balsalobre et al., 2000a; Segall et al., 2006; Segall and Amir, 2010b). Exposure to these cues uncouples slave oscillators from the timing of the master pacemaker, and can result in a disruption of the phase or integrity of clock gene expression in these oscillators (Kalsbeek et al., 2010).

THE STRESS SYSTEM

Responding to Unpredictable Environmental Stimuli

In contrast to the circadian system, which enables animals to anticipate important events in the environment, the stress system is an adaptation that generates the 'fight-or-flight' response in organisms faced with unpredictable and irregular changes such as unexpected threats and challenges (Moore-Ede, 1986). Although stress can be beneficial to health and survival by increasing responsiveness and arousal, the dysregulation or chronic activation of the stress system is maladaptive and can result in a wide range of physical and psychological disorders. The activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis is traditionally viewed as one of the two main neuroendocrine systems involved in the integrated stress response in mammals (Meerlo and Turek, 2001).

Glucocorticoids and the Hypothalamic-Pituitary-Adrenal Axis

Under stressful circumstances, activation of the HPA axis readily takes place through a variety of sympathetic, parasympathetic and limbic circuits, ultimately

stimulating the parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus (Kolber et al., 2008) to release corticotrophin-releasing hormone (CRH) and AVP. These two neuropeptides in turn induce adrenocorticotropic hormone (ACTH) synthesis and secretion from the anterior pituitary (Chung et al., 2011). ACTH binds in the adrenal cortex and generates the systemic release of glucocorticoids, one of the major effectors of the stress system. This class of steroid hormones includes cortisol in humans and corticosterone (CORT) in rodents, and exerts an extensive range of effects in target tissues and organs throughout the brain and periphery. These actions are commonly recognized to elicit both rapid and delayed effects on physiological and behavioral responses (Tasker et al., 2006). Traditional genomic glucocorticoid responses are deemed to be slow acting, over several hours to days.

Glucocorticoid Receptors

Receptors for glucocorticoids are cytosolic in their unbound form, and are categorized as either glucocorticoid receptors (GR) or mineralocorticoid receptors (MR). The former receptor class exhibits a 10-fold lower affinity to its ligand, and consequently becomes fully occupied during stress and at the peak of the circadian glucocorticoid rhythm (Reul et al., 1987). Upon binding of the ligand to its receptor, a negative feedback loop is activated which results in the downregulation of HPA axis activity and the achievement of a steady state of homeostasis. Interestingly, both GR and MR show different localization patters in the brain (Groeneweg et al., 2012). Thus glucocorticoids serve to regulate two signaling pathways via GR and MR. Actions mediated by MR are proactive in the

maintenance of basal HPA activity. Contrastingly, GR-mediated responses include the suppression of increased excitability and the recovery from stress-induced activation (De Kloet et al., 1998). In an unbound form, both receptors reside in the cytoplasm. Upon binding, the ligand-receptor complex translocates into the nucleus, where it induces gene transactivation and transrepression (Datson et al., 2008) by directly binding to recognition sites in the DNA otherwise known as Glucocorticoid Responsive Elements (GRE) (Beato and Sanchez-Pacheco, 1996). More recently, there has been increasing evidence for the existence of putative membrane GR (mGR) coupled to downstream G protein-dependent signaling cascades, which may be potentially responsible for the rapid and nongenomic effects of glucocorticoids (Tasker et al., 2006).

Factors Affecting Stress Outcomes

The type of stress, time of day of exposure and mode of presentation of stressors play a major role in determining stress-induced effects on physiology and behavior in mammals. Stressful stimuli can be categorized as either processive (psychological) or systemic (physical), as they activate the HPA axis in a distinct manner (Dayas et al., 2001; Myers et al., 2013). Processive stressors do not constitute immediate threats to an organism's homeostatic regulation and first require cognitive processing through higher order limbic pathways. Conversely, systemic stressors represent direct physical and invasive challenges, disrupting internal homeostasis and relaying information directly to effector neurons in the hypothalamus (Emmert and Herman, 1999). In addition to the category, stress outcomes on an organism are contingent upon the time of day and mode of

stressor presentation. Stress exposure during the light (inactive) phase in nocturnal rodents generates more robust responses, since this is the time of day when basal glucocorticoids are at a nadir (Ulrich-Lai and Herman, 2009). Furthermore, in comparison with acute stress, repeated daily or chronic exposure produce episodic and cumulative increases in circulating corticosteroids (Herman et al., 2008), with chronic unpredictable stressors being significantly more aversive than predictable ones (Abbott et al., 1984).

CROSS-TALK BETWEEN BOTH SYSTEMS

Current Limitations

In brief, the circadian and stress systems are both important for an organism's adaptation to temporal features of the surrounding environment, and thus communicate with one another at multiple levels to adjust numerous physiologic activities. Dysregulation in either of these systems alters internal homeostasis and interestingly leads to similar pathologic conditions in all organs and tissues (Nader et al., 2010). Investigations of mutual interactions between the circadian and stress systems are recent and limited, and most of the mechanisms for their intercommunication remain to be examined.

Circadian Regulation of Glucocorticoid Secretion

One prime example of a better-understood aspect of the cross-talk between both systems is the circadian control of the HPA axis, specifically the daily ultradian release of glucocorticoids in mammals. Pulses of glucocorticoids, reflecting

changes in amplitude and frequency, are emitted non-homogenously across the light/dark (LD) cycle (Dickmeis et al., 2013), a phenomenon attributed primarily to the control by the SCN. Although the SCN is resilient to most non-photic cues, light signals entrain the rhythm of the SCN (Ishida et al., 2005), which then communicates through efferent connections with CRH/AVP-containing neurons of the PVN, ultimately producing regular diurnal secretions of glucocorticoids (Ishida et al., 2005; Nader et al., 2010). This SCN-mediated regulation is crucial for adjusting daily circadian behavioral and physiological activities. Interestingly, glucocorticoid secretion is also regulated by the SCN in another manner, one that is independent of the HPA axis and that entails altering the sensitivity of the adrenal cortex to ACTH through SCN-mediated activation of the autonomic nervous system (Ishida et al., 2005; Oster et al., 2006).

Stress and Glucocorticoids Modulate Central and Peripheral Clock Genes

In a reciprocal manner, the HPA axis, via release of glucocorticoids, influences clock function by phase-shifting the expression of core clock genes, a response that is particularly important during stressful circumstances. *In vitro* studies of this phenomenon have shown that treatment with the synthetic glucocorticoid analogue, dexamethasone, upregulates *Per1* expression in cultured human or rat-1 fibroblasts (Balsalobre et al., 2000b; Balsalobre et al., 2000a; Fukuoka et al., 2005). *In vivo*, acute systemic stress leads to the induction of *Per1* expression in the liver, kidney and heart of mice, an effect mediated by glucocorticoid actions on the GRE of *Per1* (Yamamoto et al., 2005). In addition, it has been shown that GR can respond to different levels of glucocorticoids in a

gene-specific manner; low doses of dexamethasone selectively induced *Per1*, possibly through the ability of a specific DNA sequence of the *Per1* GRE to act as a GR ligand (Reddy et al., 2009). The existence of a GRE on *Per2* has been recently investigated, implicating BMAL1 as a cofactor essential for glucocorticoid-induced *Per2* expression (So et al., 2009; Cheon et al., 2013).

Although a fair amount of evidence exists for the effects of stress and glucocorticoids on clocks in the periphery, significantly fewer studies address stress-induced changes in clock gene expression in centrally-located clocks that are downstream of the SCN (Takahashi et al., 2001; Amir et al., 2004; Lamont et al., 2005; Segall et al., 2006; Segall et al., 2009). The SCN is void of GR, and unlike downstream oscillators, its rhythms remain immune to internal and external stressors, in line with its function as the master pacemaker. Basal glucocorticoids are essential for the rhythmic expression of PER2 in select limbic forebrain nuclei; adrenalectomy (ADX) results in the abolishment of PER2 rhythms in the central extended amygdala (Amir et al., 2004; Lamont et al., 2005). Subcutaneous implantations of time-release CORT pellets in ADX animals fail to restore rhythmic expression of PER2 (Segall and Amir, 2010b). However, restoring CORT via the drinking water of rats establishes CORT nighttime peaks and mimics endogenous rhythms, consequently rescuing ablated PER2 rhythms (Segall et al., 2006). This regimen underscores the importance of *circadian* glucocorticoid signaling in basal rhythmic clock gene expression (Segall and Amir, 2010a). GR have also been implicated in the regulation of PER2 expression, in the central extended amygdala of mice containing an inactivation

of GR restricted to neural tissue (Segall et al., 2009). Lastly, categorically different stressors, including immobilization, forced swim and lipopolysaccharide injections have been shown to upregulate *Per1* but not *Per2* in the PVN, but not SCN or liver of mice (Takahashi et al., 2001), highlighting a gene- and region-specific effect of stress.

THE PRESENT THESIS

Objectives and Rationale

Stressful circumstances, particularly over a long period, can result in physiological and behavioral disturbances that are also symptomatic of the dysfunction of the circadian system. Mechanisms underlying this disruption remain largely unknown, but one of the prominent hypotheses is that stress alters circadian clock protein expression in specific limbic and hypothalamic nuclei that are highly sensitive to stress and emotional state, leading to functional dysregulation of these nuclei and subsequent disruption of circadian-controlled variables. Here, we sought to elucidate some of the mechanisms that dictate how stress affects the expression of PER1 in these nuclei, focusing on the type of stress, the time of day and mode of stress exposure, and the role of glucocorticoids and their receptors. It is important to note that our work here did not focus on the changes in the *rhythms* of expression of *basal* PER1, but rather on the acute *stress-induced* changes in PER1 *levels* immediately subsequent to stress exposure.

Circadian Oscillators in Stress-Responsive Brain Nuclei

Firstly, we attempted to localize where the intercommunication between both systems may be taking place in the mammalian brain. We hypothesized that this cross-talk would be in brain regions that a) contain circadian oscillators and b) are heavily involved in the stress response system. In line with our two criteria, we chose to study two limbic forebrain regions, the oval nucleus of the bed nucleus of the stria terminalis (BNSTov) and lateral division of the central nucleus of the amygdala (CEA), two hypothalamic regions, the PVN and the dorsomedial hypothalamus (DMH) and one cortical structure, the piriform cortex (Pi) (Amir et al., 2004; Lamont et al., 2005; Segall et al., 2006; Harbour et al., 2013). While the former two represent brain areas that are critically involved in the emotional and behavioral regulation of stress pathways, the two hypothalamic structures integrate the autonomic and endocrine responses to stress and are also part of the principal region, the hypothalamus, where the circadian and stress systems anatomically and functionally converge (DiMicco et al., 2002). The Pi is the largest subdivision of the olfactory cortex (Suzuki and Bekkers, 2007), and the clock genes *Per1* and *Per2* are rhythmically expressed in this region (Matsui et al., 2005; Yamamoto et al., 2005). Although the function of the Pi is normally associated with the processing of olfactory information, it has a close reciprocal connection with the amygdala and hypothalamus, shows an abundance of GR expression (Ahima and Harlan, 1990), and is thus suggested to be a target of stress or stress-related hormones (Morgan et al., 1987; Badowska-Szalewska et al., 2004; Nacher et al., 2004). Lastly, we studied the SCN, the master

pacemaker; although the SCN has been previously reported to be resilient to the effects of acute stress (Challet, 2007), we were nevertheless interested in determining if any of the manipulations in the present investigation would affect PER1 expression in the SCN.

PER1: Bridging the Gap

The *Per* gene expresses three isoforms, *Per1*, *Per2*, and *Per3*, all of which contribute to maintaining rhythmic oscillations in the clock, as well as the functional integrity of the tissue or organ where each clock is located (Herzog et al., 1998; Albrecht and Oster, 2001). Unlike its two isoforms, a strong case for *Per3*, mainly localized in the SCN, as a clock gene has yet to be made, as current findings point to its redundant role in the circadian system (Shearman et al., 2000; Bae and Weaver, 2007; Hasan et al., 2011). Contrastingly, *Per1* and *Per2* are shown to have distinct and complementary roles in the mouse clock mechanism (Albrecht et al., 2001; Zheng et al., 2001). Furthermore, *Per1* exhibits several pleiotropic functions external to its role in the circadian system, including tumor suppression (Gery et al., 2007; Yang et al., 2009), cocaine sensitization (Akhisaroglu et al., 2004), alcohol drinking behavior (Dong et al., 2011) and important to our work, modulating behavioral responses to stressors (Zhang et al., 2011). The expression of *Per1* can be induced (Akashi and Nishida, 2000) through three independent mechanisms. These include the clock's oscillatory expression of *Per1*, *Per1*'s transient expression through the light-induced activation of its CRE, and lastly via glucocorticoid-induced activation of its GRE

(Yamamoto et al., 2005). Consequently, we hypothesized that PER1 may be a strong candidate in relaying stress-induced information, perhaps through glucocorticoid signaling, to clocks downstream of the SCN, upon which it can modulate circadian functioning within behaviorally important brain circuits.

Summary

Our work examines the role of several factors in the modulation of PER1 expression in centrally located clocks of limbic and hypothalamic brain regions deemed to be critical in stress, motivation and emotional regulation. Chapter 1 demonstrates that categorically different stressors, namely processive versus systemic, affect PER1 expression in a distinctive manner. Furthermore, this outcome is dependent upon the time of day of stress exposure, the modality of the stressor, and the brain region studied, where the central extended amygdala exclusively and repeatedly reveals its ability to 'distinguish' between different types of stress. Stress-induced changes in PER1 mirrored those of FOS, implicating *Per1* as an IEG possibly through a clock-independent mechanism. Chapter 2 investigates the effect of a complex processive stressor, the emotional state of fear, on PER1 and FOS expression in the forebrain, using a contextual fear conditioning paradigm. The results suggest that the response of PER1 to a complex processive stressor resembles its response to systemic stressors, further underscoring the vulnerability of clock genes to different types of stress. Chapter 3 characterizes the relationship between glucocorticoids, GR and PER1 in the brain, through the use of surgical (adrenalectomy) and pharmacological (GR antagonism) manipulations of glucocorticoid signaling. Collectively, our

findings presented in this thesis support a role for PER1 as an intermediary in integrating circadian and stress information, as well as highlight the susceptibility of clock genes to stress and fluctuations in glucocorticoid signaling. Our results shed light on how both systems may interact collectively to influence behavior and motivation, through processes that are perhaps external to the clock mechanism.

CHAPTER 1

Stress-induced changes in the expression of the clock protein PERIOD1 in the rat limbic forebrain and hypothalamus: role of stress type, time of day of exposure, and predictability

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Submitted to: PLOS ONE, March 2014

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ABSTRACT

Stressful events can disrupt circadian rhythmicity resulting in physiological and behavioral disturbances, but mechanisms underlying this disruption remain largely unknown. One hypothesis is that stress alters circadian clock protein expression in stress-responsive forebrain nuclei, leading to functional dysregulation of the brain circadian network and consequent disruption of circadian physiological and behavioral rhythms. To study this, we characterized the effects of several qualitatively different stressors on the expression of the stress-inducible core clock protein, PER1 and the neuronal activity marker, FOS in select forebrain and hypothalamic nuclei in rats. We found that acute exposure to processive stressors, restraint and forced swim, suppressed PER1 and FOS expression exclusively in the lateral division of the central nucleus of the amygdala and oval nucleus of the bed nucleus of the stria terminalis. Conversely, systemic stressors, interleukin-1β and 2-Deoxy-D-glucose, increased levels of PER1 and FOS in these structures. In all other regions studied, including the piriform cortex, paraventricular nucleus, and dorsomedial hypothalamus exposure to either processive or systemic stress elevated PER1 and FOS expression. Protein levels in the suprachiasmatic nucleus, the master pacemaker, were unaffected by any of the stress manipulations. Finally, the effect of stress on PER1 and FOS was modulated by time of day and, in the case of daily restraint, by stress predictability. Together, our results show that the expression of PER1 in the forebrain is strongly modulated by stress, consistent with the hypothesis that PER1 may serve as a link between stress and the brain

circadian network. Furthermore, the finding that the effect of stress on PER1 parallels the effect on FOS supports the idea that *Per1* serves as an immediate-early gene. Our observations shed light on a novel functional role for PER1 as a key player in the interface between stress and circadian rhythms.

INTRODUCTION

The circadian and stress systems are two interrelated regulatory systems crucial for allowing organisms to adapt to external environmental cues. While the former is responsible for adaptation to predictable aspects of the environment, mainly to the strong influence of daily light-dark cycles, the stress system, particularly its endocrine response governed by the hypothalamic-pituitary-adrenal (HPA) axis, is engaged for adaptation to unpredictable environmental challenges (Yamamoto et al., 2005; Nader et al., 2010). Although it is well established how the circadian system regulates the stress system, particularly the rhythmic secretion of glucocorticoids (Nader et al., 2010), not much is known about the mechanisms of action of stress on the circadian system. One hypothesis regarding this cross-talk entails stress-induced alterations in the expression of clock proteins within specific forebrain and hypothalamic nuclei, ultimately leading to a disruption in circadian-controlled variables.

On a molecular level, a conserved transcriptional–translational autoregulatory loop generates the oscillations that drive rhythmic expression patterns of core clock proteins, including PER1 and PER2, products of the *Period* gene (Reppert and Weaver, 2002; Lowrey and Takahashi, 2004). In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus harbors the central circadian clock that coordinates most aspects of physiology and behavior (Hastings and Herzog, 2004; Kriegsfeld and Silver, 2006). Clocks downstream of the SCN also exist and are located throughout the brain and in virtually all

peripheral organs and tissues. Their activity is synchronized to that of the master clock through both humoral and neural connections, which are not yet fully elucidated (Kalsbeek et al., 2006). Glucocorticoid signaling is implicated as one arm of the SCN responsible for the synchronization and regulation of clock gene expression in the brain and periphery (Nader et al., 2010). Recent evidence has shown that the temporal secretion of the glucocorticoid corticosterone (CORT), not just its presence, is crucial for the rhythmic expression of PER2 in select regions of the limbic forebrain (Lamont et al., 2005; Segall and Amir, 2010a). Glucocorticoids, which under normal circumstances fluctuate in a circadian fashion, affect clocks in numerous tissues but appear to spare the SCN, allowing it to maintain its intrinsic rhythm independently of internal and external stressors (Balsalobre et al., 2000b; Nader et al., 2010).

Potential brain sites for the cross-talk between the stress and circadian systems are stress-responsive hypothalamic and forebrain nuclei that contain clock machinery and exhibit daily rhythms in the expression of circadian proteins. These regions include the piriform cortex (Pi), the paraventricular nucleus (PVN), the dorsomedial hypothalamus (DMH), the oval nucleus of the bed nucleus of the stria terminalis (BNSTov) and the lateral division of the central nucleus of the amygdala (CEA) (Amir et al., 2004; Lamont et al., 2005; Harbour et al., 2013). One candidate that may well be involved in facilitating this cross-talk is the clock protein PER1 (Takahashi et al., 2001; Paladino et al., 2010; Zhang et al., 2011; Koresh et al., 2012; O'Callaghan et al., 2012; Spencer et al., 2013). PER1 is rapidly induced by stress in peripheral organs through a glucocorticoid response

element (GRE) on the promoter region of its gene (Yamamoto et al., 2005). Furthermore, it is induced by photic cues, such as light at night in the SCN, through its cAMP response element (Shigeyoshi et al., 1997). Notably, *Per1* has also been implicated in behavioral processes such as cocaine sensitization (Akhisaroglu et al., 2004) and alcohol drinking behavior in mice (Dong et al., 2011), indicating that it also plays a role outside the circadian clock machinery.

Stressors can be categorized contingent upon which central stress-integrative circuit they activate (Myers et al., 2013). Processive stressors, such as restraint and fear, are defined as stressors that have no inherent physical cause, activating the HPA axis through corticolimbic regions implicated in regulating motivation and emotion (Fuchs and Flugge, 2003). Conversely, systemic stressors, such as interleukin-1beta (IL-1ß) or 2-Deoxy-D-glucose (2DG) treatments, represent direct physical and invasive threats, disrupting internal homeostasis and relaying information directly to effector neurons in the hypothalamus (Miller et al., 1998; Emmert and Herman, 1999). Furthermore, stressors exert their effects in a phase-dependent manner (Braga et al., 2002; Kario et al., 2002) and the mode of presentation of a stressor, acute versus repeated daily and predictable versus unpredictable, plays a major role in determining subsequent physiological and behavioral effects on an organism (Araujo et al., 2003; Hammen et al., 2009).

Here, our objective was to study the acute effects of qualitatively different stressors, categorized as either processive or systemic (Day et al., 2004), on the

expression of PER1 in the aforementioned stress-responsive rat forebrain and hypothalamic nuclei. In addition, we investigated the effects of daytime versus nighttime stress, as well as repeated daily predictable versus unpredictable stress, on PER1 expression. Lastly, in all experiments we assessed the expression of FOS, the protein product of the immediate-early gene (IEG), *c-Fos*, and an indirect marker of cellular activity in the brain. Our results established that stress-induced changes in PER1 expression in these select brain nuclei are strongly subject to influence by the category of stress, the brain region studied, the time of day of stressor administration, and the mode of stressor presentation. Stress-induced alterations in PER1 levels mirrored those of FOS, implicating *Per1* as an IEG (Akashi and Nishida, 2000), possibly through mechanisms independent of its clock functions.

MATERIALS AND METHODS

Animals and Housing

All experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of Concordia University. Adult male Wistar rats (126-150 g) were purchased from Charles River Canada (St. Constant, QC, Canada). The rats were individually housed in clear plastic cages, with free access to food and water, and kept under a 12h light/dark (LD) schedule for approximately 2 weeks until they were fully entrained. The ventilated cages were equipped with a running wheel and housed in light and sound-attenuated isolation boxes. Running wheel data were collected by

VitalView software (Mini-Mitter, Sunriver, OR, USA). Actograms were acquired and analyzed using Actiview Biological Rhythm Analysis software (Mini-Mitter). Wheel running recordings and actograms were used to ensure that all rats were stably entrained to the 12:12 LD cycle.

Stressors

Processive

Rats were exposed to one of two processive stressors, restraint, consisting of 30 min in custom-designed ventilated Plexiglas tubes (7 mm thick, internal diameter of 75 mm, adjustable in length from 130-180 mm), or forced swim (FS) consisting of 15 min in 25 cm of 22°C water in a 40 cm-high and 20 cm-wide Plexiglas tube. Control rats were handled only.

Systemic

Rats were exposed to an immune challenge, an intraperitoneal injection of 5 µg/kg human IL-1ß (IL-1ß, Cell Guidance Systems, Carlsbad, CA, USA) reconstituted in sterile water at a concentration of 0.1 mg/ml, or a metabolic challenge, a subcutaneous injection of 250 mg/kg 2DG (2DG, Sigma-Aldrich, Oakville, ON, Canada) in 0.9% saline. Control rats were injected with vehicle only.

Plasma CORT Collection and Analysis

Rats were wrapped and restrained in a towel, then tail-clipped with a razor for rapid blood collection using capillary tubes (0.5 ml). Samples were centrifuged at 4°C, 13,000 r.p.m. for 10 min, and the plasma was extracted and stored at -80°C.

Plasma CORT levels were assessed in duplicates using a CORT Enzyme Immunoassay (EIA) kit (Enzo Lifesciences, Farmingdale, NY, USA), as previously described (Segall et al., 2006).

Tissue Preparation & Immunohistochemistry

Rats were deeply anesthetized with sodium pentobarbital (100 mg/kg) and perfused intracardially with 300 ml of cold 0.9% saline, followed by 300 ml of cold 4% paraformaldehyde. Brains were removed and post-fixed for 24h in 4% paraformaldehyde and stored at 4°C overnight. They were then sliced in 50 µm serial coronal sections on a vibratome, and stored at −20°C in Watson's Cryoprotectant. Immunohistochemistry for PER1 and FOS was performed as previously described (Verwey et al., 2009) using an affinity-purified rabbit polyclonal antibody, raised against PER1 (1:24,000 - R1177, EMD-Millipore) and FOS (1:100,000 - Calbiochem, Gibbstown, NJ, USA).

Microscopy & Data Analysis

Stained sections were mounted onto gel-coated slides and dehydrated in a series of alcohols and Citrisolv (Fisher), then coverslipped. The sections were examined under a light microscope (Leica, DMR) and images were captured with a Sony XC-77 video camera, Scion LG-3 frame grabber with a 400x400 µM template, and Image SXM software (http://www.ImageSXM.org.uk1 v1.95, S.D. Barret). The mean number of PER1 and FOS immunoreactive (IR) cells per region was then calculated for each animal from the counts of six unilateral images showing the highest number of labeled nuclei, as previously described

(Amir et al., 2004). Differences between groups and time-points were revealed using a two-way analysis of variance (ANOVA), a confidence level (α) set at 0.05 and a Bonferroni post-hoc test. Differences between groups for the time-of-day effects of stress only were revealed using a one-way ANOVA, a confidence level (α) set at 0.05 and a Dunnett's post-hoc analysis.

Experimental Protocol

Qualitatively different stressors (processive versus systemic)

Rats (*n*=4 per group) were acutely exposed to either processive (restraint or FS), or systemic (IL-1ß or 2DG) stressors at ZT2 (ZT0 denotes the onset of the light phase), a time of day when endogenous basal levels of CORT are at a nadir. Levels of PER1, FOS and CORT were analyzed 1, 3 and 6 h post-stress onset, corresponding to ZT3, 5 and 8. These time points were selected in order to reflect post-stress changes in PER1, FOS and CORT levels in the short, medium and long-term respectively. In subsequent experiments, brains and blood were only collected in the short-term, 1 h post-stress onset, as this was the time when the most robust effects of stress on protein levels were observed.

Time of day of exposure (day versus night stress)

PER1, FOS and CORT expression is rhythmic over a 24h period, with levels varying depending on the time of day (Sage et al., 2001; Matsui et al., 2005). To assess the importance of time of day and analyze the phase-dependent effects of stress, rats (*n*=4 per group) were exposed to restraint or 2DG at ZT14, 2 h after the onset of the dark phase, with subsequent analyses at ZT15.

Mode of presentation (repeated daily predictable versus unpredictable stress)

Rats (*n*=4 per group) were assigned to one of four groups: predictable day, predictable night, unpredictable day and unpredictable night. Predictable day rats were exposed to restraint stress (30 min) at ZT2 once a day, for 10 days, and were killed at ZT3 on Day 10. Predictable night rats were restrained once a day at ZT14, and were killed at ZT15 on Day 10. Unpredictable day rats were restrained once a day for 10 days at a randomly selected ZT during the day, except on Day 10, when they were restrained at ZT2 then killed at ZT3. This was done in order to control for the effect of time of day on levels of PER1, FOS and CORT. Unpredictable night rats were restrained once a day at a randomly selected ZT during the night, except for Day 10, when they were restrained at ZT14 and killed at ZT15.

RESULTS

PER1, FOS expression increases in Pi, PVN, DMH following acute processive stressors, but decreases exclusively in BNSTov, CEA

Restraint

As expected, exposure to a 30-min restraint stressor at ZT2 led to a robust activation of the HPA axis (Fig. 1a). Plasma CORT levels peaked 1 h after the onset of the stressor, at ZT3 ($F_{1,12}$ = 55.98, p<0.001) and returned to baseline 6 h later at ZT8 ($F_{2,12}$ = 28.53, p<0.001). The effect of restraint stress on PER1 and FOS expression is shown in Figs. 2 and 3, and statistical analyses in Table 1.

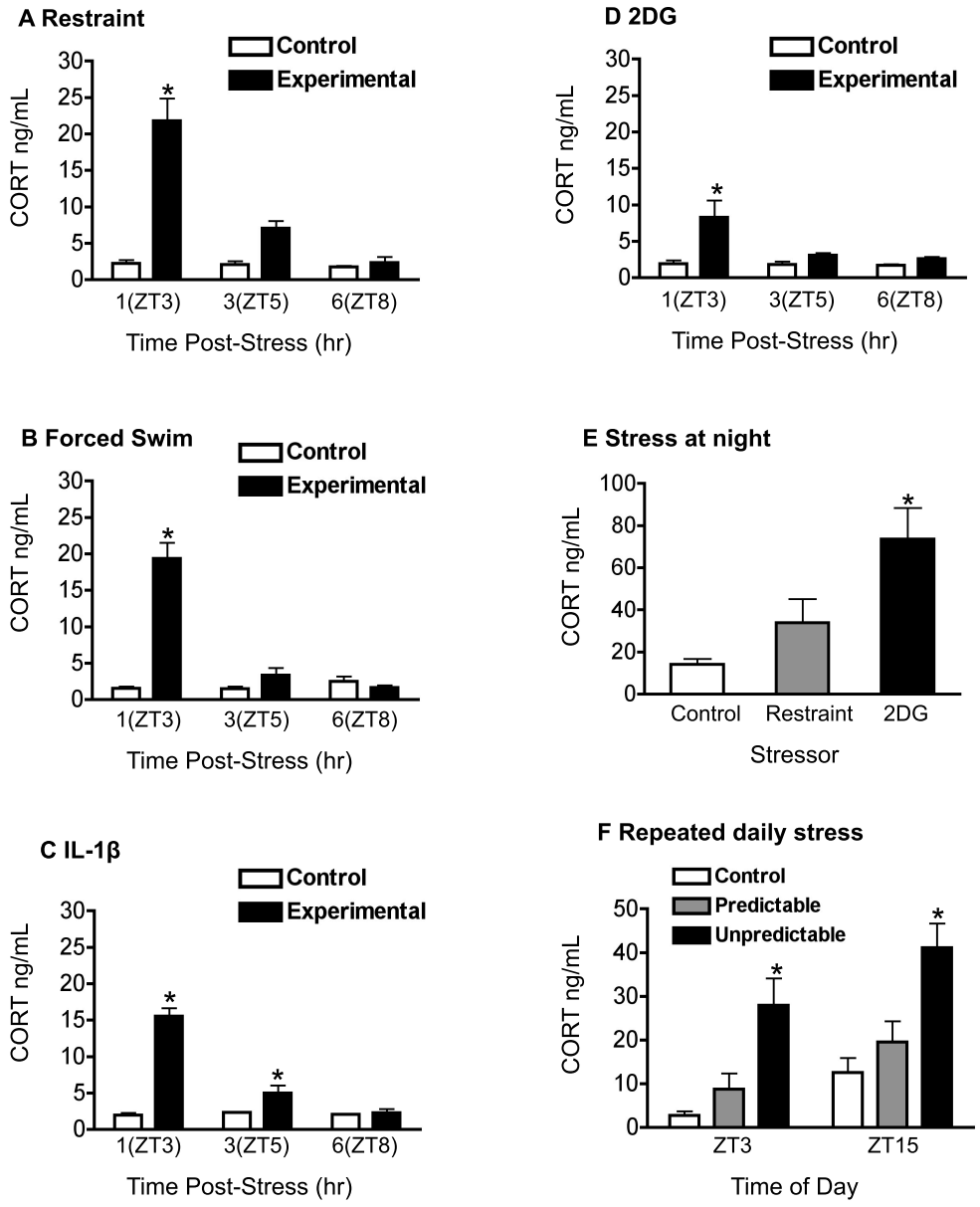

Figure 1
Plasma corticosterone (CORT) levels post-stress. Stress-induced elevations in plasma CORT decline as a function of zeitgeber time (ZT) in control and stressed (experimental) rats, following **a)** 30 min restraint **b)** 15 min forced swim **c)** treatment with 5 μg/kg IL-1β **d)** treatment with 250 mg/kg 2DG. Plasma CORT levels are modulated by **e)** 30 min restraint stress or treatment with 250 mg/kg 2DG administered during the nighttime only at ZT14 **f)** repeated daily predictable vs. unpredictable 30 min restraint stress, administered at either ZT2 or ZT14. Means ± SEM are shown, n=4 per group; * significant difference from corresponding control group, p<0.05.

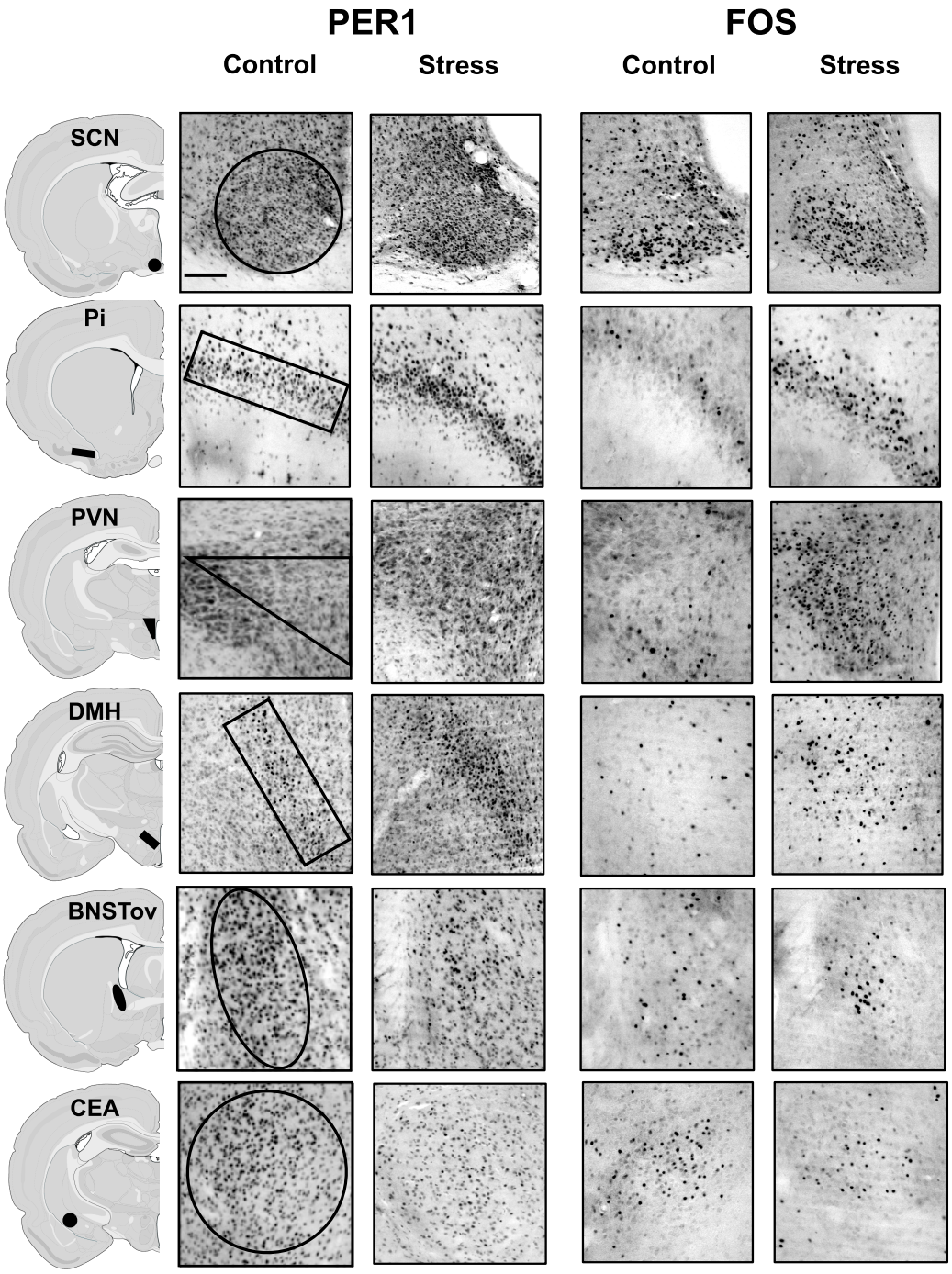

Figure 2
Examples of PER1 and FOS expression in different forebrain and hypothalamic regions. Experimental rats were stressed with a 30 min restraint session at ZT2, and protein subsequently collected at ZT3 (scale bar: 100 μm).

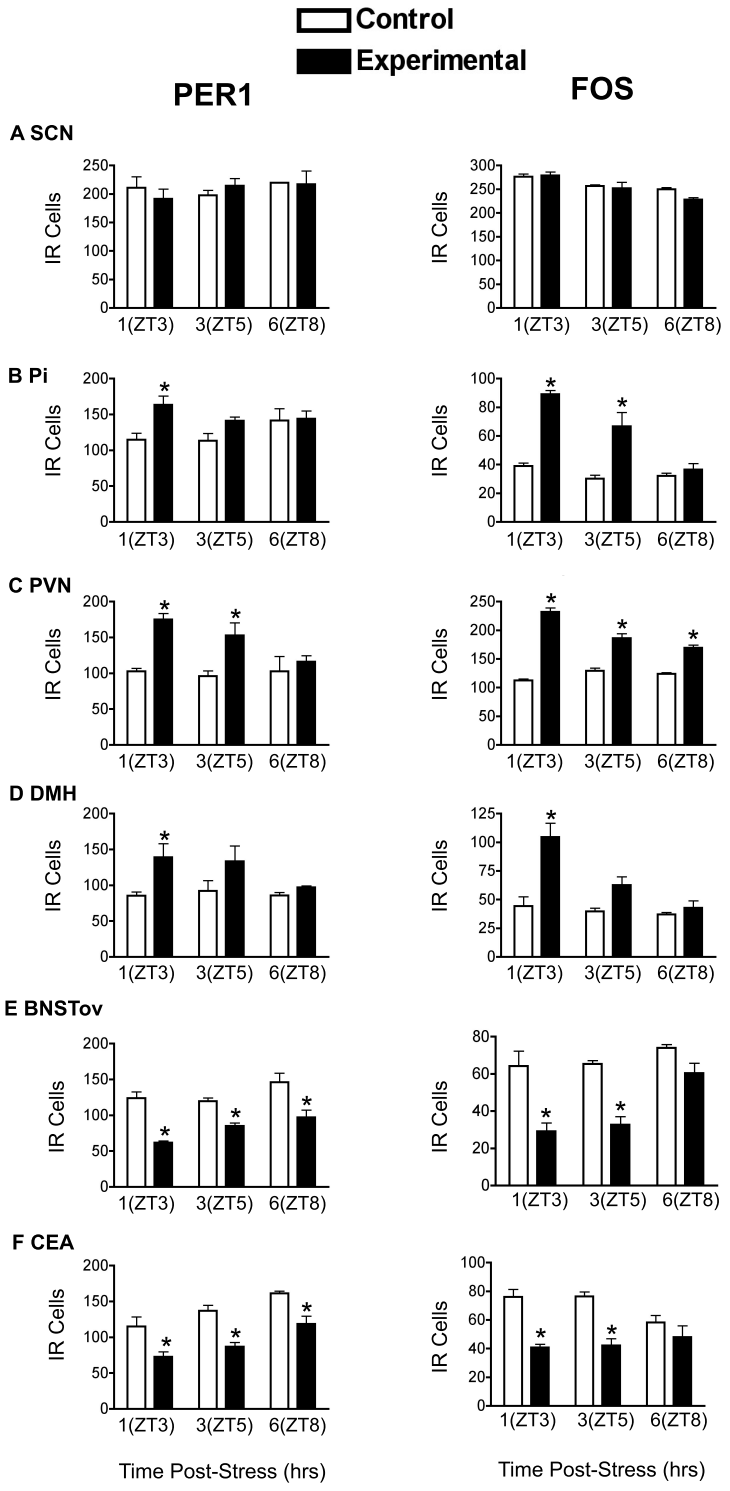

Figure 3
Number of PER1 and FOS immunoreactive (IR) cells following restraint, in different forebrain and hypothalamic regions. IR cells are shown as a function of ZT in control and restraint-stressed rats. Means ± SEM are shown, *n*=4 per group; * significant difference from corresponding control group, $p<0.05$.

Table 1
Acute restraint stress and time post-stress onset (ZT) modulate stress-induced PER1 and FOS expression in limbic and hypothalamic brain regions.

Structure/ Protein	Restraint	ZT	Restraint x ZT
SCN/			
PER1	F(1,13) = 0.01, ns	F(2,13) = 0.44, ns	F(2,13) = 0.5, ns
FOS	F(1,17) = 1.65, ns	F(2,17) = 14.52, *p*<0.001	F(2,17) = 1.49, ns
Pi/			
PER1	F(1,18) = 8.11, *p*=0.01	F(2,18) = 1.01, ns	F(2,18) = 2.12, ns
FOS	F(1,17) = 54.52, *p*<0.0001	F(2,17) = 17.21, *p*<0.0001	F(2,17) = 10.44, *p*<0.01
PVN/			
PER	F(1,15) = 23.88, *p*<0.001	F(2,15) = 2.8, ns	F(2,15) = 2.92, ns
FOS	F(1,17) = 245.94, *p*<0.0001	F(2,17) = 9.83, *p*<0.01	F(2,17) = 24.2, *p*<0.0001
DMH/			
PER1	F(1,14) = 10.48, *p*<0.01	F(2,14) = 1.54, ns	F(2,14) = 1.25, ns
FOS	F(1,16) = 26.46, *p*<0.0001	F(2,16) = 11.83, *p*<0.0001	F(2,16) = 7.36, *p*<0.01
BNSTov/			
PER1	F(1,16) = 54.87, *p*<0.0001	F(2,16) = 6.74, *p*<0.01	F(2,16) = 1.48, ns
FOS	F(1,17) = 42.82, *p*<0.0001	F(2,17) = 9.31, *p*<0.01	F(2,17) = 2.54, ns
CEA/			
PER1	F(1,17) = 35.74, *p*<0.0001	F(2,17) = 12.31, *p*<0.001	F(2,17) = 0.12, ns
FOS	F(1,16) = 40.03, *p*<0.0001	F(2,16) = 0.82, ns	F(2,16) = 3.69, *p*<0.05

ns, not significant

PER1 and FOS levels in the SCN were unaffected by restraint at all 3 time points (Fig. 3a), consistent with previous findings (Takahashi et al., 2001). In the Pi, PVN and DMH the levels of both proteins increased significantly post-stress, most noticeably 1 h after restraint onset, at ZT3 (Figs. 3b-d). In contrast, restraint induced an opposite effect in the BNSTov and CEA, where a significant suppression of both PER1 and FOS was observed in the short-term, 1 h after stress onset at ZT3 (Figs. 3e,f). The levels of PER1 and FOS in these two brain regions increased over the next 6 h and reached control levels by ZT8 (Figs. 3e,f).

Forced Swim

FS significantly elevated plasma CORT levels ($F_{1,18}$ = 53.83, p<0.0001) 1 h post-stress at ZT3 (Fig. 1b), with levels returning to baseline 6 h later, by ZT8 ($F_{2,18}$ = 40.88, p<0.0001). The effect of FS stress on PER1 and FOS expression is shown in Fig. 4, with statistical analyses in Table 2. PER1 and FOS levels in the SCN were unaffected by FS at all time points assessed (Fig. 4a). Acute exposure to FS significantly increased the expression of PER1 and FOS in the Pi, PVN and DMH. This effect was most pronounced in the short-term 1 h post-FS onset, at ZT3 (Figs. 4b-d), with levels of both proteins returning to baseline 6 h post-stress, by ZT8. Significantly, as with restraint, exposure to FS stress transiently suppressed PER1 and FOS levels in the BNSTov and CEA (Figs. 4e,f), highlighting a brain region-specific effect of processive stress on PER1 and FOS expression.

PER1, FOS expression increases in Pi, PVN, DMH, BNSTov and CEA following acute systemic stressors

IL-1β

Plasma CORT levels were significantly elevated ($F_{1,18}$ = 100.77, p<0.0001) 1 h following exposure to IL-1β (Fig. 1c), and returned to control levels 5 h later, by ZT8 ($F_{2,18}$ = 53.14, p<0.0001). Fig. 5 shows the effects of IL-1β on PER1 and FOS levels, with corresponding statistical analyses in Table 3. IL-1β had no effect on PER1 and FOS levels in the SCN (Fig. 5a). Furthermore, as with restraint and FS stress, acute treatment with IL-1β significantly elevated PER1 and FOS in the Pi, PVN and DMH (Figs. 5b-d). In contrast to processive stressors, however, acute exposure to IL-1β also increased the expression of PER1 and FOS in the BNSTov and CEA (Figs. 5e,f).

2DG

Treatment with 2DG also significantly elevated plasma CORT ($F_{1,18}$ = 12.36, p<0.01) in the short-term at ZT3 (Fig. 1d), with levels returning to baseline by ZT8 ($F_{2,18}$ = 5.25, p<0.05). The effects of 2DG on PER1 and FOS are shown in Fig. 6 and statistical analyses in Table 4. Once again, no changes in PER1 and FOS expression were seen in the SCN post-2DG (Fig. 6a). Acute exposure to 2DG significantly increased levels of both proteins in the short-term at ZT3, in all other brain regions studied, including the Pi, PVN, DMH, BNSTov and CEA (Figs. 6b-f).

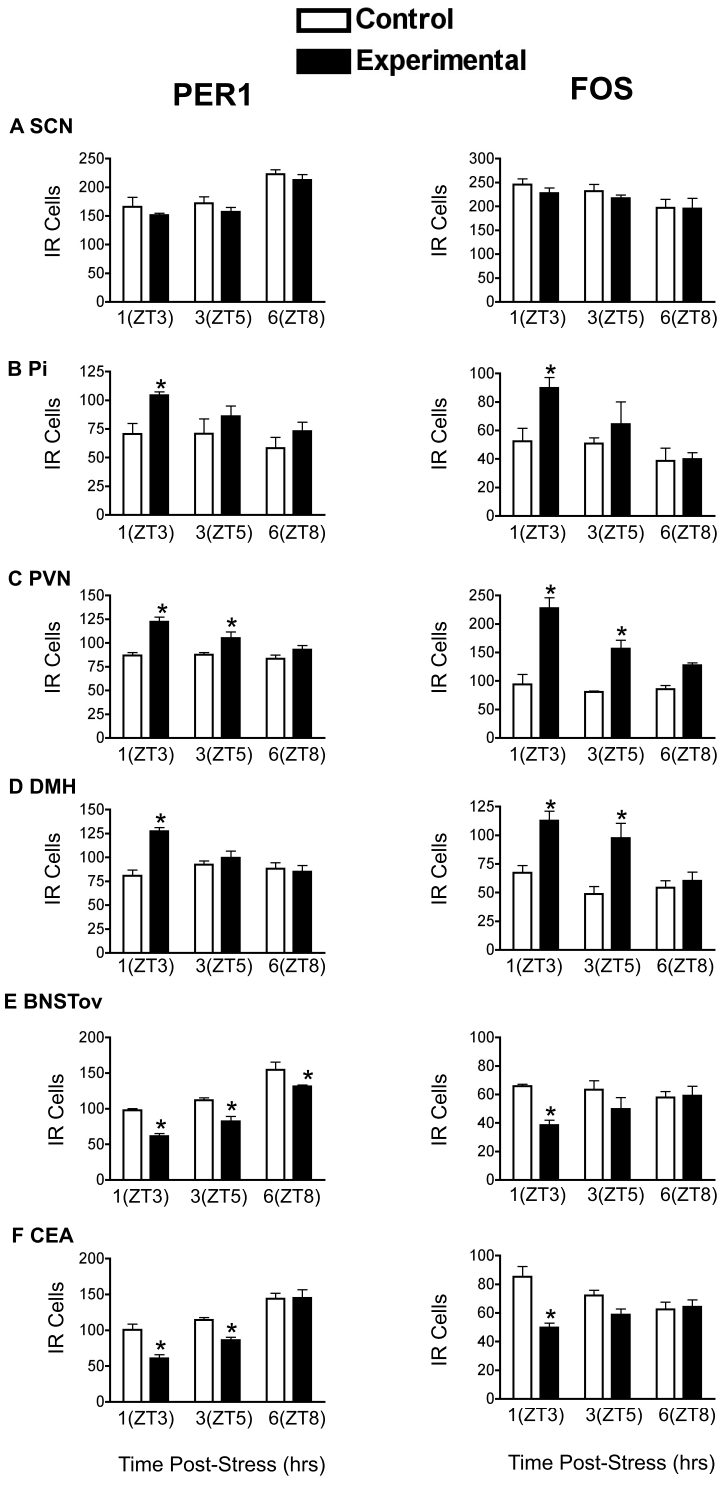

Figure 4
Number of PER1 and FOS immunoreactive (IR) cells following forced-swim, in different forebrain and hypothalamic regions. IR cells are shown as a function of ZT in control and forced swim-stressed rats. Means ± SEM are shown, *n*=4 per group; * significant difference from corresponding control group, $p<0.05$.

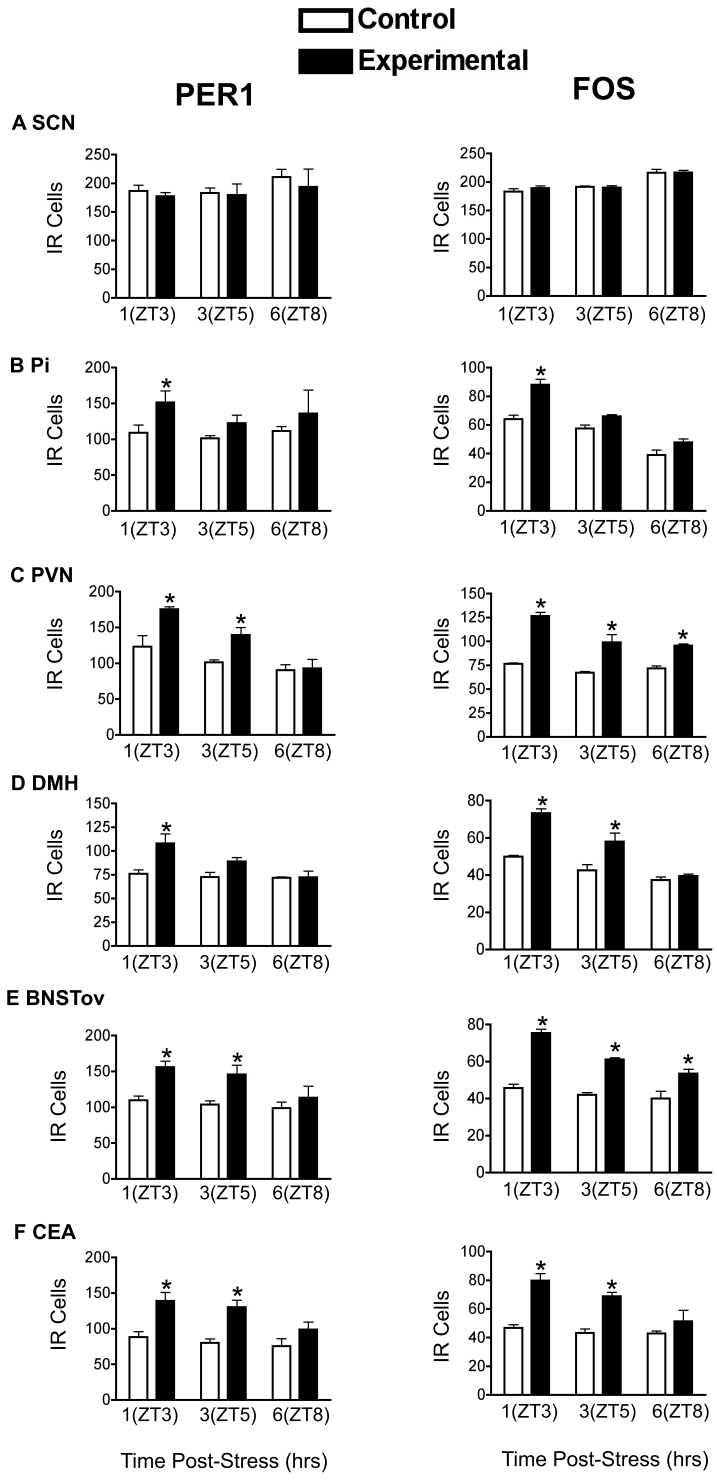

Figure 5
Number of PER1 and FOS immunoreactive (IR) cells following IL-1β, in different forebrain and hypothalamic regions. IR cells are shown as a function of ZT in control rats and animals exposed to 5 μg/kg IL-1β. Means ± SEM are shown, *n*=4 per group; * significant difference from corresponding control group, $p<0.05$.

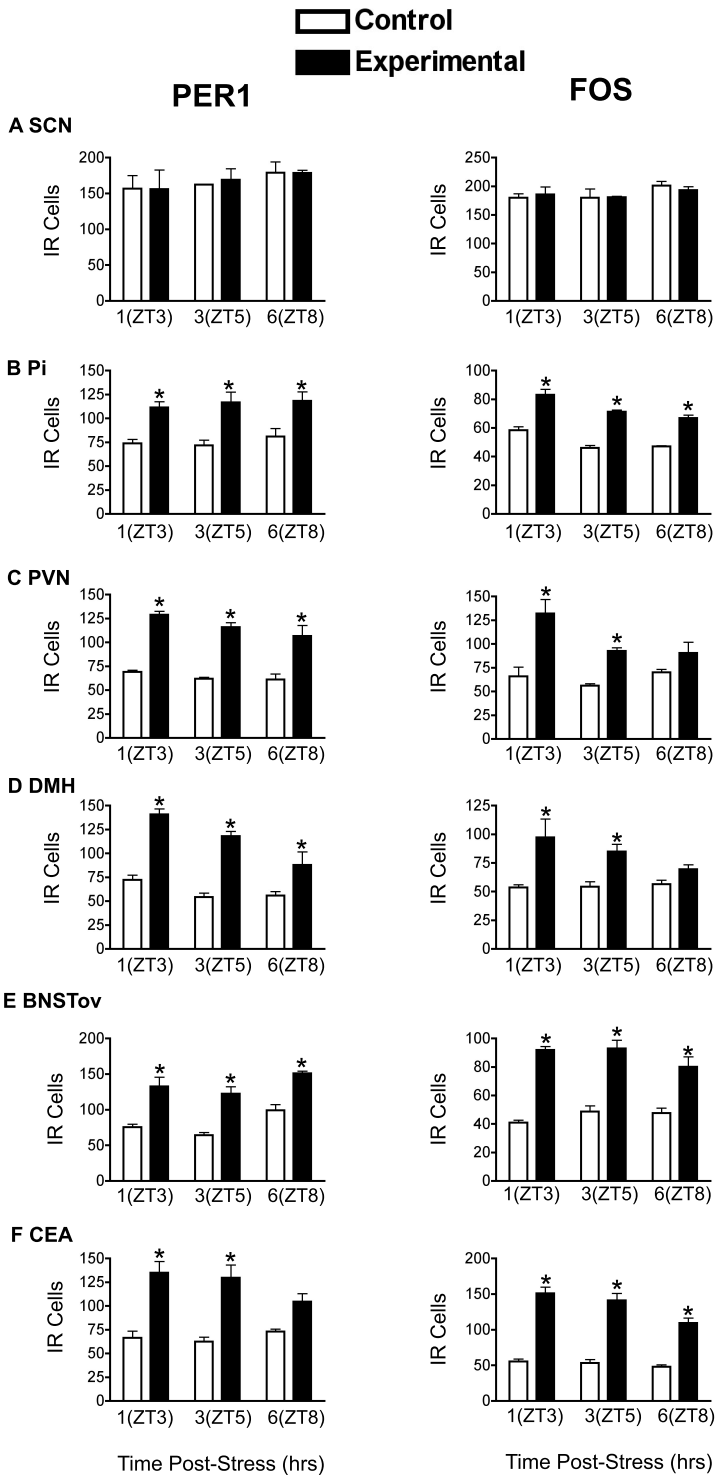

Figure 6
Number of PER1 and FOS immunoreactive (IR) cells following 2DG, in different forebrain and hypothalamic regions. IR cells are shown as a function of ZT in control rats and animals exposed to 250 mg/kg 2DG. Means ± SEM are shown, *n*=4 per group; * significant difference from corresponding control group, $p<0.05$.

Table 2
Acute forced swim stress and time post-stress onset (ZT) modulate stress-induced PER1 and FOS expression in limbic and hypothalamic brain regions.

Structure/ Protein	Forced Swim	ZT	Forced Swim x ZT
SCN/			
PER1	F(1,16) = 2.86, ns	F(2,16) = 24.8, p<0.0001	F(2,16) = 0.04, ns
FOS	F(1,18) = 0.94, ns	F(2,18) = 4.01, p<0.05	F(2,18) = 1.49, ns
Pi/			
PER1	F(1,16) = 8.78, p<0.01	F(2,16) = 3.4, ns	F(2,16) = 0.8, ns
FOS	F(1,17) = 6.4, p<0.05	F(2,17) = 7.6, p<0.01	F(2,17) = 2.49, ns
PVN/			
PER1	F(1,18) = 31.61, p<0.0001	F(2,18) = 6.5, p<0.01	F(2,18) = 4.36, p<0.05
FOS	F(1,17) = 245.94, p<0.0001	F(2,17) = 9.83, p<0.01	F(2,17) = 24.2, p<0.0001
DMH/			
PER1	F(1,17) = 12.49, p<0.01	F(2,17) = 4.27, p<0.05	F(2,17) = 10.04, p<0.01
FOS	F(1,16) = 25.47, p=0.0001	F(2,16) = 8.4, p<0.001	F(2,16) = 4.53, p<0.05
BNSTov/			
PER1	F(1,18) = 37.6, p<0.0001	F(2,18) = 60.81, p<0.0001	F(2,18) = 0.65, ns
FOS	F(1,15) = 8.66, p=0.01	F(2,15) = 0.74, ns	F(2,15) = 3.66, ns
CEA/			
PER1	F(1,18) = 13.79, p<0.01	F(2,18) = 39.24, p<0.001	F(2,18) = 4.0, p<0.05
FOS	F(1,18) = 15.25, p=0.001	F(2,18) = 0.35, ns	F(2,18) = 7.39, p<0.01

ns, not significant

Table 3
Acute IL-1β challenge and time post-stress onset (ZT) modulate stress-induced PER1 and FOS expression in limbic and hypothalamic brain regions.

Structure/ Protein	IL-1β	ZT	IL-1β x ZT
SCN/			
PER1	F(1,16) = 0.59, ns	F(2,16) = 1.09, ns	F(2,16) = 0.09, ns
FOS	F(1,16) = 0.21, ns	F(2,16) = 30.06, *p*<0.001	F(2,16) = 0.34, ns
Pi/			
PER1	F(1,18) = 4.64, *p*<0.05	F(2,18) = 0.62, ns	F(2,18) = 0.23, ns
FOS	F(1,16) = 31.85, *p*<0.001	F(2,16) = 60.62, *p*<0.001	F(2,16) = 4.63, *p*<0.05
PVN/			
PER1	F(1,16) = 14.39, *p*<0.01	F(2,16) = 16.03, *p*<0.001	F(2,16) = 3.27, ns
FOS	F(1,17) = 106.23, *p*<0.0001	F(2,17) = 11.97, *p*<0.001	F(2,17) = 4.91, *p*<0.05
DMH/			
PER1	F(1,16) = 13.22, *p*<0.01	F(2,16) = 6.77, *p*<0.01	F(2,16) = 4.33, *p*<0.05
FOS	F(1,16) = 34.80, *p*<0.0001	F(2,16) = 32.25, *p*<0.0001	F(2,16) = 7.19, *p*<0.01
BNSTov/			
PER1	F(1,16) = 14.29, *p*<0.01	F(2,16) = 2.93, ns	F(2,16) = 1.17, ns
FOS	F(1,16) = 137.24, *p*<0.0001	F(2,16) = 19.71, *p*<0.0001	F(2,160 = 6.82, *p*<0.01
CEA/			
PER1	F(1,17) = 26.79, *p*<0.01	F(2,17) = 3.74, *p*<0.05	F(2,17) = 1.32, ns
FOS	F(1,17) = 42.43, *p*<0.0001	F(2,17) = 7.26, *p*<0.01	F(2,17) = 4.42, *p*<0.05

ns, not significant

Table 4
Acute 2DG challenge and time post-stress onset (ZT) modulate stress-induced PER1 and FOS expression in limbic and hypothalamic brain regions.

Structure/ Protein	2DG	ZT	2DG x ZT
SCN/			
PER1	F(1,15) = 0.02, ns	F(2,15) = 0.98, ns	F(2,15) = 0.03, ns
FOS	F(1,16) = 0, ns	F(2,16) = 1.36, ns	F(2,16) = 0.22, ns
Pi/			
PER1	F(1,18) = 39.3 $p<0.0001$	F(2,18) = 0.44, ns	F(2,18) = 0.16, ns
FOS	F(1,18) = 154.44, $p<0.0001$	F(2,18) = 21.65, $p<0.0001$	F(2,18) = 0.82, ns
PVN/			
PER1	F(1,18) = 123.67, $p<0.0001$	F(2,18) = 3.45, ns	F(2,18) = 0.75, ns
FOS	F(1,18) = 33.13, $p<0.0001$	F(2,18) = 4.36, $p<0.05$	F(2,18) = 3.53, ns
DMH/			
PER1	F(1,18) = 86.6, $p<0.0001$	F(2,18) = 11.69, $p<0.001$	F(2,18) = 3.79, $p<0.05$
FOS	F(1,17 = 30.25, $p<0.0001$	F(2,17) = 1.78, ns	F(2,17) = 2.76, ns
BNSTov/			
PER1	F(1,17) = 73.73, $p<0.0001$	F(2,17) = 7.91, $p<0.01$	F(2,17) = 0.1, ns
FOS	F(1,18) = 133.18, $p<0.0001$	F(2,18) = 1.23, ns	F(2,18) = 2.16, ns
CEA/			
PER1	F(1,18) = 60.05, $p<0.0001$	F(2,18) = 0.94, ns	F(2,18) = 2.81, ns
FOS	F(1,18) = 220.0, $p<0.0001$	F(2,18) = 7.15, $p<0.01$	F(2,18) = 3.62, $p<0.05$

ns, not significant

Taken together, the observed effects of processive and systemic stressors on PER1 and FOS expression indicate not only a brain region-specific effect, but also a stress-specific role, where the category of stressor itself appears to differentially modulate the expression of both proteins in select brain regions, namely the BNSTov and CEA (Fig. 7). Furthermore, all stressors increased plasma CORT levels despite having differential effects on PER1 and FOS expression in the BNSTov and CEA, implicating CORT as a potential modulator within these brain regions.

Daily variation in stress-induced changes in PER1 and FOS expression in the limbic forebrain and hypothalamus

The experiments described above were conducted during the light phase of the LD cycle, with stressors introduced at ZT2 and the most robust effects on PER1, FOS and CORT seen in the short-term 1 h after the onset of the stressor. Because the effect of stress might be influenced by the time of exposure (Ulrich-Lai and Herman, 2009), and because basal levels of PER1 and FOS expression and of circulating CORT follow a diurnal rhythm (Herzog et al., 1998; Nunez et al., 1999), we sought to determine whether acute stress at night would differentially affect protein expression in the short-term, compared to daytime stress. PER1, FOS and CORT were analyzed at ZT15, 1 h after the onset of either a processive (restraint) or systemic (2DG) stressor.

Plasma CORT varied across all 3 groups (Fig. 1e, $F_{2,9} = 7.68$, $p<0.05$). CORT levels were significantly higher following the nighttime 2DG challenge, though not after restraint, compared to control levels (Fig. 1e, $p<0.05$). Nighttime restraint or 2DG treatment had no effect on PER1 or FOS expression in the SCN (Fig. 8a). PER1 and FOS expression were influenced by stress in the Pi (Fig. 8b, $F_{2,9} = 25.93$, $p<0.01$ and $F_{2,9} = 65.44$, $p<0.0001$, respectively), and in the DMH (Fig. 8d, $F_{2,9} = 7.38$, $p<0.05$ and $F_{2,9} = 9.13$, $p<0.01$, respectively). Restraint and 2DG elicited significant increases in PER1 and FOS levels in both these regions (Figs. 8b,d, $p<0.05$). PER1 expression in the PVN did not significantly differ across the groups, but FOS levels did (Fig. 8c, $F_{2,8} = 8.11$, $p<0.01$). From both stressors, only restraint significantly increased FOS expression in the PVN (Fig. 8c, $p<0.05$). Stress affected PER1 expression in the CEA (Fig. 8f, $F_{2,9} = 7.05$, $p<0.01$), but not in the BNSTov (Fig. 8e). Nighttime restraint significantly suppressed PER1 levels in the CEA (Fig. 8f, $p<0.05$), whereas nighttime 2DG had no effect on PER1 in this region. FOS expression varied significantly across all groups in the BNSTov (Fig. 8e, $F_{2,9} = 21.07$, $p<0.01$) and CEA (Fig. 8f, $F_{2,9} = 51.57$, $p<0.01$). Both regions exhibited an increase in FOS expression following exposure to 2DG (Figs. 8e,f, $p<0.05$).

Taken together, the results show that although, in the nuclei studied, the changes in PER1 and FOS expression post-nighttime stress resemble those seen following daytime stress, daytime stress induces more robust changes, highlighting the time-of-day effects of stress on PER1 and FOS in these regions.

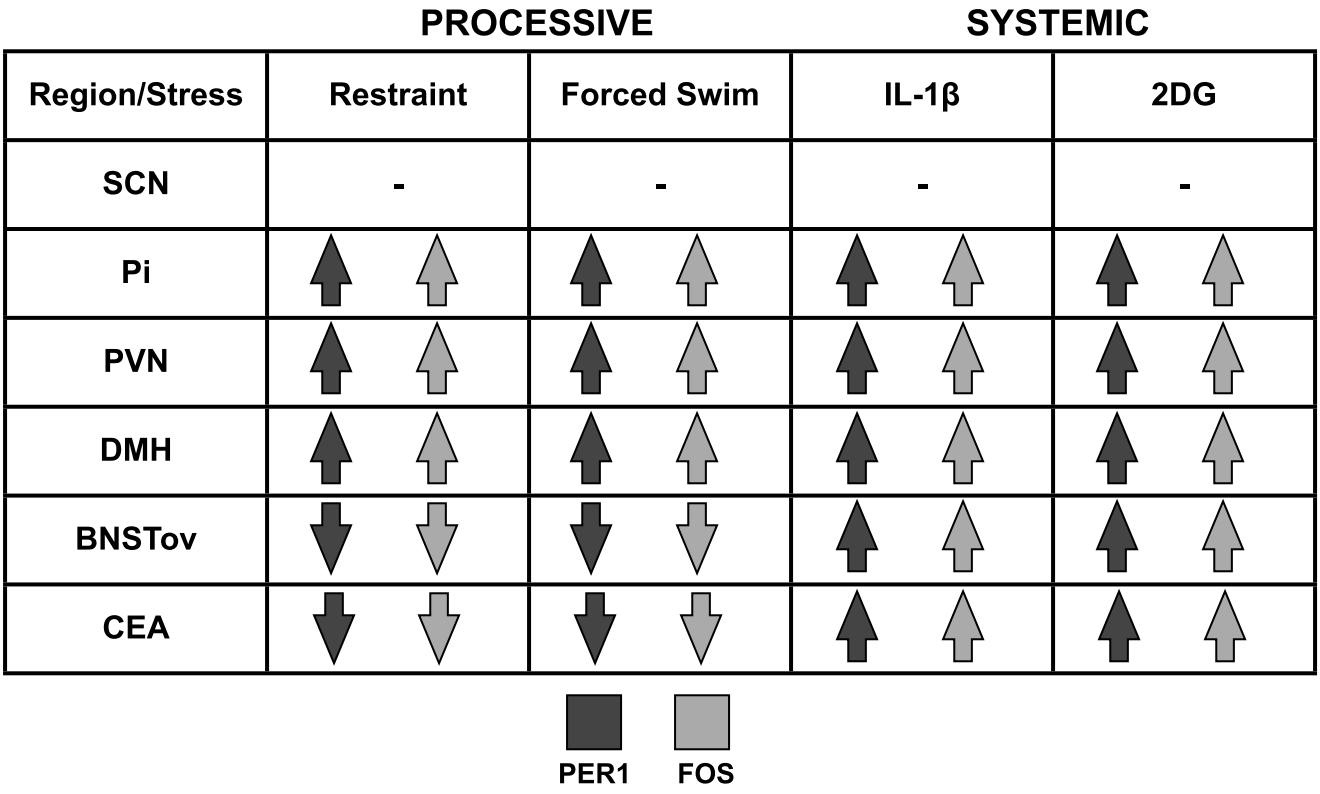

Figure 7
Effects of processive versus systemic stress on PER1, FOS levels. Summary of distinct effects of categorically different stressors, processive versus systemic, on PER1 and FOS expression in different forebrain and hypothalamic nuclei.

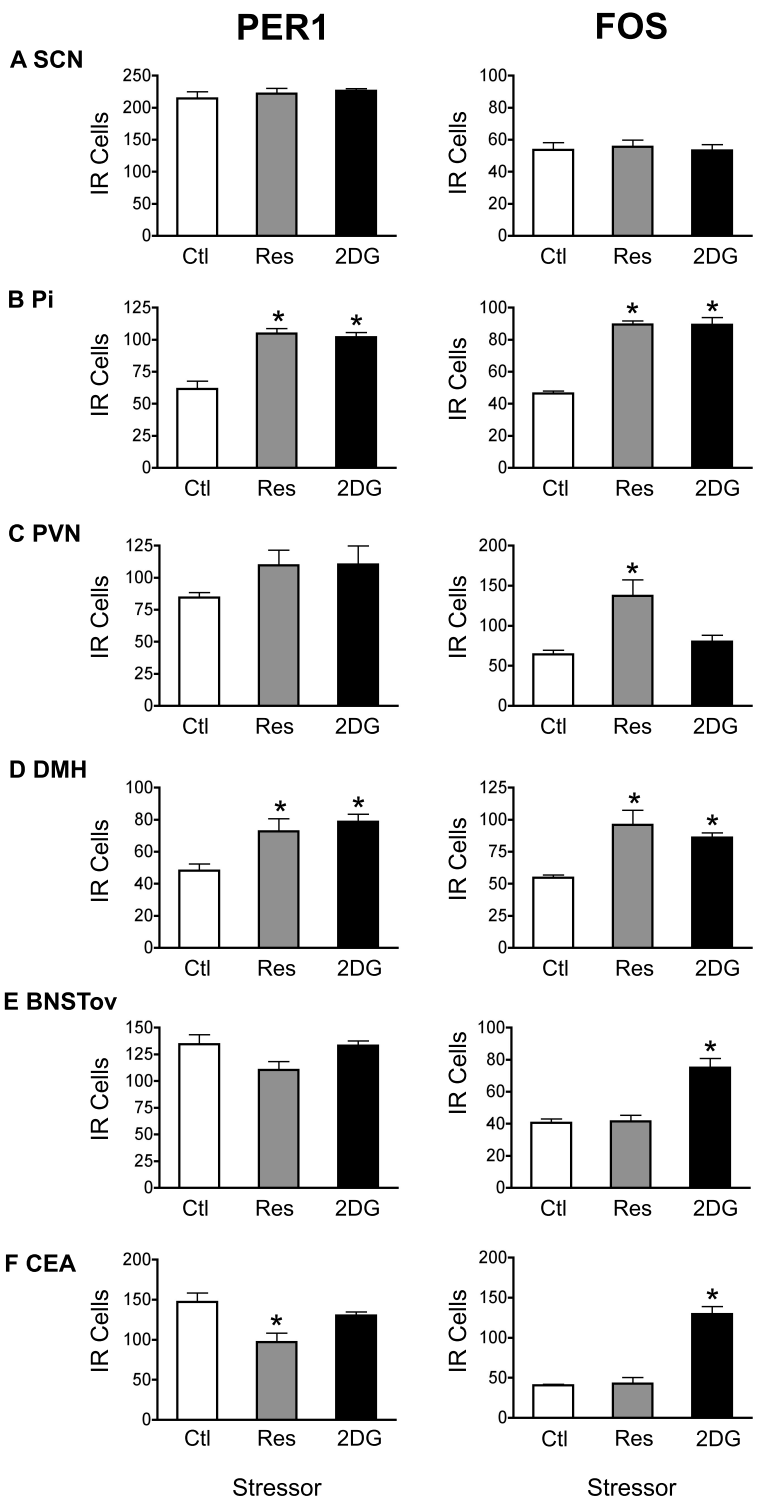

Figure 8
Number of nighttime PER1 and FOS immunoreactive (IR) cells following restraint or 2DG, in different forebrain and hypothalamic regions. IR cells are shown following 30 min restraint stress or treatment with 250 mg/kg 2DG administered during the nighttime only at ZT14. Means ± SEM are shown, *n*=4 per group; * significant difference from corresponding control group, $p<0.05$.

Repeated daily unpredictable, not predictable, restraint stress mimics the effect of acute processive stress on PER1 and FOS expression

The effect of stress on behavior and physiology are known to depend on factors such as chronicity (acute versus repeated daily stressors) (Strausbaugh et al., 1999) and predictability (predictable versus unpredictable stressors) (Zucchi et al., 2009). To assess the importance of the mode of presentation of a stressor on PER1 and FOS expression and CORT, we investigated the effect of daily predictable versus unpredictable restraint stress during the light and dark phases, for 10 days.

The light-entrained locomotor activity rhythms were unaffected by daytime or nighttime exposure to predictable or unpredictable restraint stress (data not shown), supporting the notion that clock machinery in the SCN is resistant to stress perturbations (Takahashi et al., 2001). Plasma CORT levels were significantly elevated on Day 10 following exposure to daily unpredictable restraint stress only (Fig. 1f, $F_{2,18} = 19.86$, $p<0.0001$), with CORT levels being higher during the nighttime at ZT15 than at ZT3 (Fig. 1f, $F_{1,18} = 9.61$, $p<0.01$). The effect of repeated daily unpredictable and predictable restraint stress on PER1 and FOS expression is shown in Fig. 9, with statistical analyses in Table 5. Repeated daily restraint had no effect on PER1 or FOS levels in the SCN at ZT3 and ZT15 (Fig. 9a). Both modes of restraint significantly increased protein expression in the Pi and DMH during the light and dark phase compared to control levels (Figs. 9b,d), but only at ZT3 in the PVN (Fig. 9c).

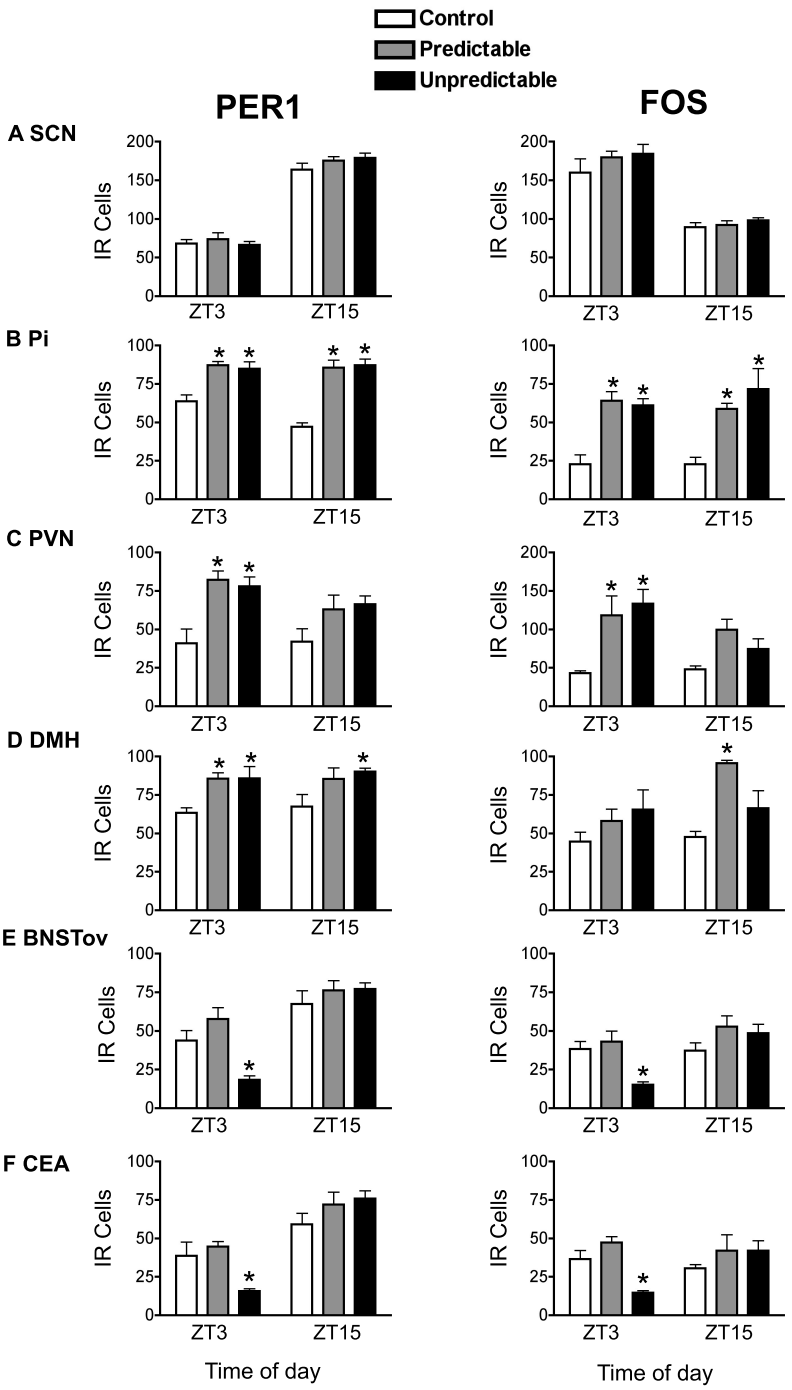

Figure 9
Number of PER1 and FOS immunoreactive (IR) cells following repeated daily predictable or unpredictable restraint, in different forebrain and hypothalamic regions. IR cells are shown following repeated daily predictable vs. unpredictable 30 min restraint stress, administered at either ZT2 or ZT14 with subsequent PER1 and FOS collection at ZT3 or ZT15. Means ± SEM are shown, n=4 per group; * significant difference from corresponding control group, p<0.05.

Table 5
Repeated daily predictable vs. unpredictable restraint stress and time of day (ZT) modulate PER1 and FOS expression in limbic and hypothalamic brain regions.

Structure/ Protein	Predictability	ZT	Predictability x ZT
SCN/			
PER1	F(2,18) = 1.03, ns	F(1,18) = 379.79, p<0.0001	F(2,18) = 0.86, ns
FOS	F(2,18) 1.37, ns	F(1,18) = 97.48, p<0.0001	F(2,18) = 0.44, ns
Pi/			
PER1	F(2,18) = 40.76, p<0.0001	F(1,18) = 2.72, ns	F(2,18) = 3.25, ns
FOS	F(1,17) = 28.17, p<0.0001	F(2,17) = 0.12, ns	F(2,17) = 0.75, ns
PVN/			
PER1	F(2,17) = 10.81, p<0.001	F(1,17) = 2.67, ns	F(2,17) = 0.9, p<0.01
FOS	F(2,17) = 9.18, p<0.01	F(1,17) = 3.44, ns	F(2,17) = 1.99, ns
DMH/			
PER1	F(2,18) = 8.81, p<0.01	F(1,18) = 0.32, ns	F(2,18) = 0.09, ns
FOS	F(2,16) = 7.66, p<0.01	F(1,16) = 4.45, ns	F(2,16) = 3.46, ns
BNSTov/			
PER1	F(2,18) = 4.86, p<0.05	F(1,18) = 44.1, p<0.0001	F(2,18) = 6.14, p<0.01
FOS	F(2,18) = 4.25, p<0.05	F(2,18) = 9.42, p<0.01	F(2,18) = 4.92, p<0.05
CEA/			
PER1	F(2,18) = 2.11, ns	F(1,18) = 48.51, p<0.0001	F(2,18) = 5.53, p<0.05
FOS	F(2,18) = 4.02, p<0.05	F(1,180 = 1.24, ns	F(2,18) = 5.22, p<0.05

ns, not significant

The BNSTov and CEA exhibited a significant reduction in PER1 and FOS expression only at ZT3 and exclusively following repeated unpredictable, not predictable, restraint stress (Figs. 9e,f), underscoring the particular importance of both time of day and mode of stress presentation in the regulation of PER1 and FOS expression in these nuclei.

DISCUSSION

Although there is growing evidence supporting the integration of the circadian and stress systems, little is known about the molecular mechanisms that regulate this cross-talk (Nader et al., 2010). The circadian protein PER1 is not only a crucial component of the clock machinery, but also modulates behavioral responses to stressful circumstances (Dong et al., 2011; Zhang et al., 2011). Here, we sought to determine the role of PER1 as a possible intermediary between the circadian and stress systems. We examined stress-induced changes in PER1 expression, by assessing the effects of several variables that included the type, time of exposure and mode of presentation of the stressor. We found that processive versus systemic stressors have differential effects on PER1 expression, contingent upon the brain region studied, the time of day of stressor administration and the predictability of the stressor. Overall, our findings strongly demonstrate that clock proteins in the brain are modifiable by stress, as previously suggested (Lamont et al., 2005; Segall et al., 2006; Segall and Amir, 2010b), and are consistent with the idea that PER1 plays an important role in mediating the effect of stress on circadian molecular rhythms within behaviorally

important brain circuits.

Role of qualitatively different stressors on PER1 expression

We found that categorically different stressors, namely processive versus systemic stressors, have distinct effects on PER1 expression in the forebrain. Specifically, we found that exposure to systemic stressors such as IL-1β and 2-DG increased PER1 expression in all nuclei studied, including the Pi, PVN, DMH, BNSTov and CEA. In contrast, exposure to processive stressors such as restraint and FS increased PER1 expression in the Pi, PVN and DMH but strongly suppressed PER1 expression in the BNSTov and CEA, demonstrating that the change in PER1 expression in these two nuclei, but not in the Pi, PVN and DMH, depends on the nature of the stressor.

The BNSTov and CEA, referred to as the central extended amygdala, are highly interconnected forebrain structures that share similar morphology and chemical architecture, and have overlapping connections with brain areas that coordinate behavioral responses to stressors (Bienkowski and Rinaman, 2013). Processive stress involves a higher order cortical processing that frequently engages the BNSTov and CEA through the activation of limbic-hypothalamic circuitry (Dayas et al., 2001). Exposure to processive stress results in the suppression of GABAergic projections from these two regions, leading to a disinhibition that increases overall amygdaloid output and facilitates autonomic and neuroendocrine responses of the central extended amygdala (Day et al., 2005). Our finding that processive stressors have an opposite effect on PER1

expression in these nuclei, compared to systemic stress, suggests a mechanism whereby different stressful events lead to complex local disruptions in circadian rhythms of clock gene expression that, in turn, alter behavior and physiology.

Phase-dependent effect of stress on PER1 expression

It is well known that the effects of different stress manipulations on an organism are dependent on time of day (Gattermann and Weinandy, 1996; Kudielka et al., 2004; Roeser et al., 2012). Accordingly, we compared the effect of daytime versus nighttime exposure to processive and systemic stress on PER1 expression in order to determine whether the effect of these stress manipulations on PER1 is temporally regulated. Stress-induced increases in plasma CORT levels were much higher during the night, when basal levels of CORT peak in rodents (van der Spek et al., 2012). Changes in PER1 levels post-nighttime stress matched those of post-daytime stress, however the latter were much more pronounced. This may be due to the already high levels of basal PER1 during the dark phase, or to reduced sensitivity of the target structures to stress stimuli during this time of day.

Role of mode of stressor presentation on PER1 expression

Repeated daily exposure to either predictable or unpredictable restraint stress had no effect on PER1 expression in the SCN or on locomotor activity rhythms, but differentially affected PER1 levels in the forebrain. Specifically, daily unpredictable stress increased PER1 expression in the Pi, PVN and DMH and suppressed expression in the BNSTov and CEA, replicating the effects of acute

restraint stress exposure in these nuclei. Daily predictable stress also increased PER1 in the Pi, PVN and DMH. However, contrary to the effect of unpredictable stress, predictable exposure to restraint had no effect on PER1 expression in the BNSTov and CEA. The finding that the effect of restraint on PER1 in the BNSTov and CEA habituates following repeated exposure to predictable, but not unpredictable, restraint stress emphasizes the ability of these nuclei to differentiate not only between the type of stress administered, but also between the mode of presentation of the stressor.

Consistent with this, there is ample evidence supporting habituation of HPA secretory responses to repeated daily predictable versus acute stress (Sawchenko, 1998). Furthermore, repeated daily unpredictable, not predictable, stress has been shown to produce enhanced basal glucocorticoid levels, with no indication of habituation over a long-term stress paradigm (Haile et al., 2001). Interestingly, we saw evidence of habituation of PER1 that matched that of CORT trends in response to repeated daily predictable stress in the BNSTov and CEA, underscoring a region-specific role for glucocorticoids in the modulation of stress-induced PER1 and accordingly, the susceptibility of clock genes to fluctuations in glucocorticoid signaling.

Per1 **as an IEG**

In all our experiments we compared the effect of stress on PER1 to that on FOS in order to study the hypothesis that PER1 functions as an IEG in the forebrain. FOS expression in the forebrain is known to be strongly and rapidly modulated

by stress (Hoffman et al., 1993; Daskalakis et al., 2014). We found that in all cases, the stress-induced changes in PER1 expression closely mirrored those in FOS, including the distinct suppressive effect of processive stressors in the BNSTov and CEA. This homology supports the idea that like *c-Fos*, *Per1* may be functioning as an IEG, and suggests a mechanism whereby stress can rapidly and transiently disrupt the functioning of circadian oscillators within specific forebrain nuclei.

Modulation of stress-induced PER1 expression by glucocorticoid-dependent mechanisms

Glucocorticoid hormones have been implicated in the regulation of clock gene rhythms in peripheral tissues and in the forebrain of rats. For example, we have shown that loss of endogenous glucocorticoids via adrenalectomy or loss of glucocorticoid signaling via genetic deletion of brain GR blunt the rhythms of expression of PER2 in the BNSTov and CEA (Amir et al., 2004; Lamont et al., 2005; Segall et al., 2006; Segall et al., 2009). Furthermore, a role for glucocorticoid signaling is supported by the presence of a GRE on the promoter region of *Per2* (Cheon et al., 2013). In the present study, all stressors elevated plasma CORT levels, and this was mirrored by the increase in PER1 expression in all regions studied except the BNSTov and CEA, where exposure to processive stressors suppressed PER1 expression despite the high levels of circulating CORT. It remains to be determined whether the stress-induced increases in CORT and in PER1 levels are linked functionally. A possible mechanism for the induction of PER1 via glucocorticoid signaling is a rapid

CORT-induced transcription of *Per1* mediated by the GRE on the *Per1* promoter, as has been demonstrated in the liver (Yamamoto et al., 2005). The unique suppressive effects of processive stressors on PER1 levels in the BNSTov and CEA may be attributed to neuronally mediated mechanisms as described above, or glucocorticoid-dependent ones, although it is probable that these two systems are not mutually exclusive. Interestingly, acute daytime processive stressors elevated plasma CORT to levels higher than systemic ones. Such high levels of CORT may suppress rather than increase PER1 expression in the BNSTov and CEA.

Conclusion

The functional significance of clock outputs downstream of the SCN in stress-responsive forebrain and hypothalamic nuclei is largely unknown. It remains to be determined whether stress-induced alterations in PER1 observed throughout our work affect clock machinery and function, and whether they play a critical role in modulating the activity of the stress system. In conclusion, the data presented in the current study show that the mechanism that controls the expression of PER1 in the forebrain and hypothalamus is vulnerable to stress, and support the hypothesis that PER1 may integrate circadian and stress information by acting as an interface between stress and circadian oscillators in the brain.

Acknowledgements

This study was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), le Fonds de la recherche en santé Québec (FRSQ) and the Concordia University Research Chair Program.

CHAPTER 2

A complex processive stressor, conditioned fear, elicits systemic stressor effects on the expression of the circadian clock protein PERIOD1 in the rat limbic forebrain and hypothalamus

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Submitted to: PLOS ONE, March 2014

Author contributions:

- Designed research: Sherin Al-Safadi, Shimon Amir
- Performed research: Sherin Al-Safadi, assisted by Marie Branchaud, Arun Dayanandan, Spencer Rutherford, Barry Robinson
- Analyzed data: Sherin Al-Safadi
- Wrote manuscript: Sherin Al-Safadi, editing by Shimon Amir

ABSTRACT

Stressful events that affect motivational and emotional states can also result in disturbances of circadian rhythms. It is thought that these effects are brought about by stress-induced changes in circadian clock protein expression, in mammalian brain regions that are vulnerable to stress and anxiety. We recently found that categorically different stressors can distinctively modulate the expression of the core clock protein, PER1 in the forebrain. Systemic stressors increased the expression of PER1, and the neuronal activity marker FOS, in the piriform cortex, paraventricular and dorsomedial hypothalamic nuclei, and in the central extended amygdala. Processive stressors increased protein levels in all regions except for the central extended amygdala, where levels of PER1 and FOS were uniquely suppressed. Here, we studied the effect of a complex processive stressor, the emotional state of fear, on PER1 and FOS expression in the brain. We used a contextual fear conditioning paradigm to elicit a fear response, whereby rats were exposed to a context previously paired with footshock, a stressor that exhibits processive and systemic characteristics. We also examined the effects of acute and repeated daily footshock stress on PER1 and FOS expression. Protein levels in the suprachiasmatic nucleus, the master pacemaker, were unaffected by all manipulations. Interestingly, we found that the emotional state of fear elicited an increase in PER1 levels in the central extended amygdala, an effect characteristic of systemic stressors. However, FOS expression in this region was suppressed in response to conditioned fear. These findings constitute the first evidence in which both proteins exhibit contrasting

trends of expression in response to the same stressor. Together, our data demonstrate that the response of PER1 to a complex processive stressor resembles its response to systemic stressors, further underscoring the vulnerability of clock genes to motivational and emotional states.

INTRODUCTION

Circadian rhythms are evolutionary conserved processes that allow organisms to adapt to predictable challenges in their cyclic environment (Dickmeis et al., 2013). In mammals, circadian rhythms are disrupted by circumstances that impede homeostasis, including stressful events that affect motivation and emotion. However, the process that defines exactly how emotional states influence circadian rhythmicity is not well defined. We have shown that the emotional state of fear, induced by exposure to a context previously paired with footshock, attenuates the effect of light on FOS expression in the master pacemaker, the suprachiasmatic nucleus, and on circadian locomotor activity rhythms in rats (Amir and Stewart, 1998, 1999a, b). In the present study, we report that alongside the SCN, conditioned fear can also affect subordinate oscillators located in forebrain regions that are important in regulating stress and emotions.

Optimal synchronization of the internal milieu to the external cyclic environment depends on the coordination of the light-sensitive master pacemaker with subordinate oscillators elsewhere in the brain and periphery. Using the core clock proteins, PER1 and PER2 as functional markers, we previously identified subordinate oscillators in the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), and the lateral division of the central nucleus of the amygdala (CEA), regions of the limbic forebrain important in regulating stress and emotion in rats (Amir et al., 2004; Lamont et al., 2005; Amir and Stewart,

2009). Moreover, we found that the expression of PER1 in these nuclei is strongly modulated by stress; exposure to processive stressors such as restraint, which trigger characteristic affective responses, suppressed PER1 expression in the BNSTov and CEA (Chapter 1). In contrast, we observed that exposure to systemic stressors such as 2-Deoxy-D-glucose, which affect homeostasis without inducing an acute emotional response, elevated PER1 expression (Chapter 1). Interestingly, PER1 levels in other stress-related nuclei such as the piriform cortex (Pi), paraventricular (PVN) and dorsomedial (DMH) hypothalamic nuclei were increased in response to both types of stressors (Chapter 1). Furthermore, immobilization stress, forced swim and lipopolysaccharide injections have been shown to upregulate *Per1*, not *Per2*, in the paraventricular nucleus (PVN) but not SCN, of mice (Takahashi et al., 2001). Together, these results demonstrate that the expression of PER1 in select nuclei of the forebrain is modifiable by stress, and suggest that the suppression of PER1 expression in the BNSTov and CEA might be a distinct molecular signature of stressful stimuli that exhibit an emotional component.

Here, to further explore this idea, we investigated the effect of the emotional state of fear on changes in the expression of PER1 and FOS, a neuronal activity marker (Milanovic et al., 1998), in the forebrain of rats. Fear was induced by exposing rats to a context previously paired with footshock, a stressor determined to have both processive (Amir and Stewart, 1998; Day et al., 2008) and systemic qualities (Amano et al., 2007; Brevet et al., 2010). We also assessed the effects of acute and repeated daily footshock stress on protein

expression. Contrary to our expectation, we found that the emotional state of fear elicits an effect on clock protein expression that is in fact characteristic of systemic, not processive, stress, highlighting the complex nature of stress circuitry on clock gene expression.

MATERIALS AND METHODS

Animals and Housing

All experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of Concordia University. Adult male Wistar rats (126-150 g) were purchased from Charles River Canada (St. Constant, QC, Canada). The rats were individually housed in clear plastic cages, with free access to food and water, and kept under a 12h light/dark (LD) schedule for approximately 2 weeks until they were fully entrained. The cages were equipped with a running wheel and housed in ventilated and light- and sound-attenuated isolation boxes. Running wheel data were collected by VitalView software (Mini-Mitter, Sunriver, OR, USA). Actograms were acquired and analyzed using Actiview Biological Rhythm Analysis software (Mini-Mitter). Wheel running recordings and actograms were used to verify that all rats were stably entrained to the 12:12 LD cycle.

Footshock

Footshock was administered using a 24x26x34 cm box fitted with a stainless steel grid floor connected to a shock source and scrambler (Med Associates Inc.

Georgia, VT, USA), as previously described (Amir and Stewart, 1998). Each footshock session took place over a 5 min period and consisted of 15 randomized shocks (1 s, 1 mA). Cumulative defecation and freezing time, defined as the lack of movement except for respiration, were assessed as behavioral measures for fear during all footshock sessions.

Plasma CORT Collection and Analysis

Rats were restrained in a towel and tail-clipped with a razor. Blood was collected using 0.5 ml capillary tubes. Samples were centrifuged at 4°C, 13,000 r.p.m. for 10 min and plasma was extracted and stored at -80°C. Plasma CORT levels were assessed in duplicates using a CORT Enzyme Immunoassay (EIA) kit (Enzo Lifesciences, Farmingdale, NY, USA), as previously described (Segall et al., 2006).

Immunohistochemistry

Rats were anesthetized with sodium pentobarbital (100 mg/kg) and perfused intracardially with 300 ml of cold 0.9% saline, followed by 300 ml of cold 4% paraformaldehyde. Brains were removed and post-fixed for 24 h in 4% paraformaldehyde and stored at 4°C overnight, then sliced in 50 µm serial coronal sections on a vibratome, and stored at −20°C in Watson's Cryoprotectant. Immunohistochemistry for PER1 and FOS was performed as previously described (Verwey et al., 2009) using an affinity-purified rabbit polyclonal antibody, raised against PER1 (1:24,000 - R1177, EMD-Millipore) and FOS (1:100,000 - Calbiochem, Gibbstown, NJ, USA).

Microscopy and Data Analysis

Stained sections were mounted onto gel-coated slides and dehydrated in a series of alcohols and Citrisolv (Fisher), then coverslipped. The sections were examined under a light microscope (Leica, DMR) and images were captured with a Sony XC-77 video camera, Scion LG-3 frame grabber with a 400x400 μM template, and Image SXM software (http://www.ImageSXM.org.uk1 v1.95, S.D. Barret). The mean number of PER1 and FOS immunoreactive (IR) cells per region was then calculated for each animal from the counts of six unilateral images showing the highest number of labeled nuclei, as previously described (Amir et al., 2004). Differences between groups were revealed using a one-way analysis of variance (ANOVA), a confidence level (α) set at 0.05 and a Tukey's post-hoc analysis.

Fear Conditioning Protocol

A contextual fear conditioning protocol, in which a distinct context is paired with footshock (Amir and Stewart, 1998), was utilized in order to investigate the effect of the emotional state of fear, as well as acute and repeated daily footshock stress, on changes in PER1 and FOS levels. Rats (*n*=4 per group) were assigned to one of six groups (Fig. 1, groups 1-6). During Days 1-4 of the experiment, the conditioning phase, groups 2, 4 and 6 were removed from their home cage at ZT2, and placed in the conditioning box. The rats were footshocked over a 5 min period, and then returned to their home cages. ZT2 was selected based on the finding that clock proteins are robustly expressed during the light phase of the LD cycle in response to stress (see Chapter 1 and(Pantazopoulos et al., 2011). Rats

in groups 1, 3 and 5 were placed in the conditioning box at ZT2 but not shocked, and then returned to their home cages. On Day 1 only, all rats were given a 2 min acclimation period in the conditioning boxes prior to the start of the 5 min footshock period. On Day 5, the test day, rats in groups 1 and 2 were placed in the conditioning box but not shocked, rats in groups 3 and 4 were placed in a distinct novel context and not shocked, and those in groups 5 and 6 were brought to the conditioning box and footshocked. All rats were killed at ZT3, 1 h after the onset of the stressor.

RESULTS

Effects of conditioned fear - A comparison between rats that were footshocked on Days 1-4 then exposed to the conditioning box but not shocked on the test day, and rats that were footshocked on Days 1-4 then placed in a second novel context on the test day without being shocked, allow for the assessment of the effects of the conditioned fear response (Fig. 1, groups 2 vs. 4).

Effects of acute footshock stress - A comparison between rats that were only shocked once on the test day, and rats that were never exposed to footshock, allow for the assessment of the effects of acute footshock stress (Fig. 1, groups 5 vs. 1).

Effects of repeated daily footshock stress - A comparison between rats that were exposed to footshock on all 5 days, and rats that were only shocked once on the

test day, allow for the assessment of the effects of repeated daily footshock stress (Fig. 1, groups 5 vs. 6).

Conditioned fear evokes learned fear behavior and activates the HPA axis

Daily locomotor activity rhythms were unaffected in all groups (data not shown). Freezing time was significantly different across the 6 groups (Fig. 2a, $F_{5,18}$ = 16.94, p<0.0001). Rats that were exposed to a context previously paired with footshock exhibited increased freezing behavior, compared to rats that were footshocked daily then placed in a distinct context on the test day but not shocked (Fig. 2a, groups 2 vs. 4, p<0.05). Rats that were repeatedly footshocked on all 5 days also exhibited an increase in freezing time, compared to those that were only footshocked once on the test day (Fig. 2a, groups 6 vs. 5, p<0.05). There were no significant differences in freezing time between rats that were footshocked only once on the test day, and rats that were never footshocked (Fig. 2a, groups 5 vs. 1).

Cumulative defecation varied significantly across the 6 groups (Fig. 2b, $F_{5,18}$ = 10.78, p<0.0001). Rats that were exposed to a context previously paired with footshock defecated significantly more than those that were footshocked daily but exposed to a distinct context without shock on the test day (Fig. 2b, groups 2 vs. 4, p<0.05).

Group/Day	1-4	5
1	No Shock	Same Context/ No Shock
2	Shock	Same Context/ No Shock
3	No Shock	New Context/ No Shock
4	Shock	New Context/ No Shock
5	No Shock	Same Context/ Shock
6	Shock	Same Context/ Shock

Figure 1
Protocol for classical contextual fear conditioning. Rats (*n*=4 per group) in groups 2, 4 and 6 were footshocked daily on Days 1-4, while those in groups 1, 3 and 5 were not footshocked. On Day 5, rats in groups 1 and 2 were placed in the conditioning box but not shocked, rats in groups 3 and 4 were placed in a second novel context and not shocked, and rats in groups 5 and 6 were footshocked in the conditioning box.

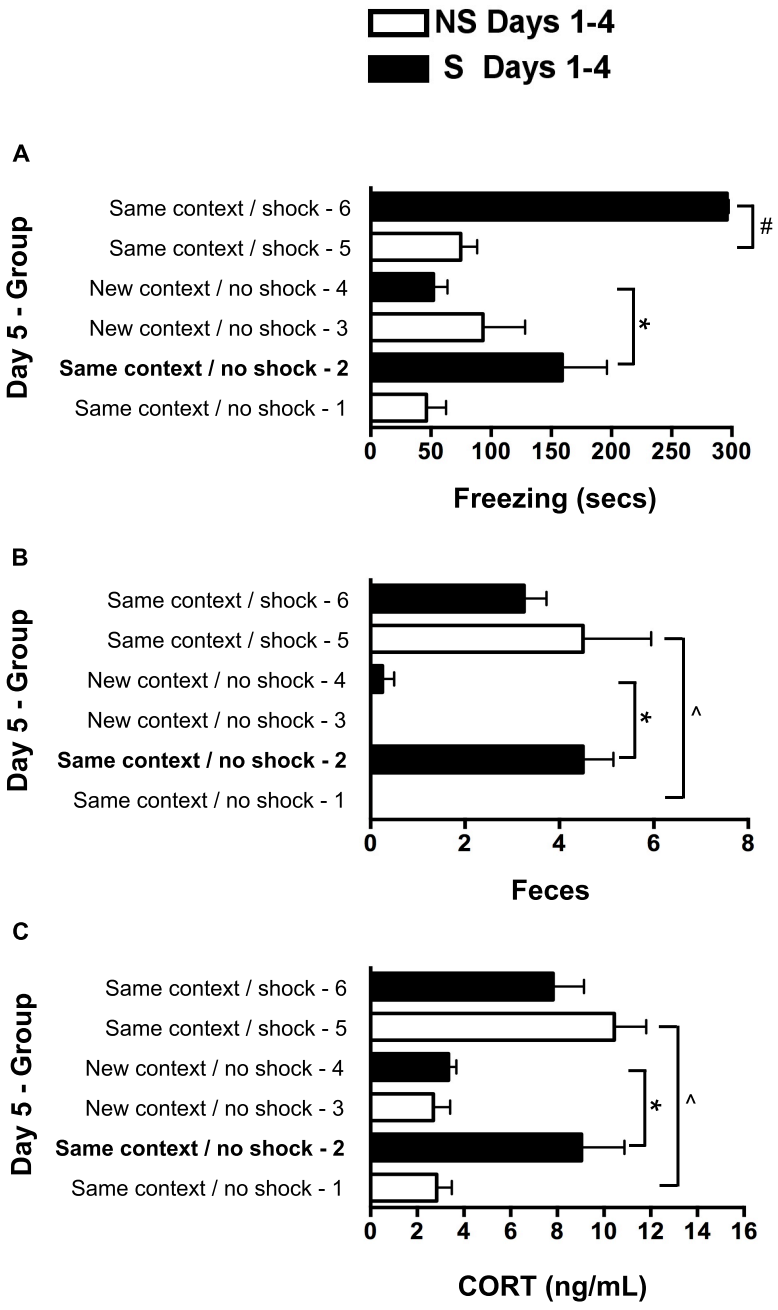

Figure 2
Conditioned fear evokes learned fear behavior and activates the HPA axis. Contextual fear conditioning modulates **a)** freezing time, **b)** cumulative defecation, and **c)** plasma CORT levels (see Fig. 1 for grouping details). Rats in groups 2, 4 and 6 were footshocked daily on Days 1-4, while those in groups 1, 3 and 5 were not footshocked. On Day 5, rats in groups 1 and 2 were placed in the conditioning box but not shocked, rats in groups 3 and 4 were placed in a second novel context and not shocked, and rats in groups 5 and 6 were footshocked in the conditioning box. The fear-conditioned rats (group 2) are shown in bold. Means ± SEM are shown, n=4 per group; significant effects (p<0.05) of * conditioned fear (groups 2 vs. 4), ^ acute footshock (groups 5 vs. 1), $^{\#}$ repeated daily footshock (groups 5 vs. 6).

The mean number of feces in rats that were exposed to footshock stress only once was significantly greater than in those that were never exposed to any footshock (Fig. 2b, groups 5 vs. 1, $p<0.05$). There were no significant differences in cumulative defecation between rats that were footshocked on all 5 days, and rats that were acutely footshocked on the test day (Fig. 2b, groups 5 vs. 6).

Plasma CORT levels varied significantly across all 6 groups (Fig. 2c, $F_{5,18} = 8.95$, $p<0.001$). Fear-conditioned rats exhibited significantly elevated levels of plasma CORT, compared to rats that were shocked daily then placed into a second novel context on the test day and not shocked (Fig. 2c, groups 2 vs. 4, $p<0.05$). Circulating CORT levels were also greater in rats that were shocked on the test day only, compared to those that were never exposed to footshock (Fig. 2c, groups 5 vs. 1, $p<0.05$). There were no differences in CORT levels between rats that were footshocked daily on all 5 days, and rats that were footshocked only once on the test day (Fig. 2c, groups 5 vs. 6). Taken together, these results highlight the robust effect of the conditioned fear response on select behavioral and physiological measures of fear.

The emotional state of fear exhibits both processive and systemic stressor qualities on PER1 and FOS expression

The effects of the emotional state of fear, acute and repeated daily footshock on PER1 and FOS expression are shown in Fig. 3. There were no differences in protein expression in the SCN of rats in all 6 groups (Fig. 3a). PER1 expression

varied across all 6 groups in the BNSTov (Fig. 3b, $F_{5,18} = 7.14$, $p<0.001$) and CEA (Fig. 3c, $F_{5,18} = 13.56$, $p<0.0001$). Levels of PER1 in both regions were significantly elevated in rats that were exposed to a context previously paired with footshock, compared to rats that were footshocked daily then placed in a distinct context without shock on the test day (Figs. 3b,c, groups 2 vs. 4, $p<0.05$). FOS levels also varied across the 6 groups in the BNSTov (Fig. 3b, $F_{5,17} = 4.52$, $p<0.01$) and CEA (Fig. 3c, $F_{5,16} = 7.31$, $p<0.001$). FOS expression in these two regions was significantly suppressed in the fear-conditioned rats, compared to rats that were footshocked daily then placed in a novel context on the test day without being shocked (Figs. 3b,c, groups 2 vs. 4, $p<0.05$).

PER1 and FOS levels in the PVN were significantly different across the 6 groups (Fig. 3d, $F_{5,18} = 9.16$, $p<0.001$ and $F_{5,18} = 49.07$, $p<0.0001$, respectively). We observed no significant change between PER1 levels in the PVN of fear-conditioned rats, and rats that were footshocked during the conditioning phase then placed in a distinct context without shock on Day 5 (Fig. 3d, groups 2 vs. 4). However, conditioned fear had a strong effect on FOS expression in rats that were exposed to a context previously paired with footshock, compared to rats that were footshocked daily then placed in a distinct context without shock on the test day (Fig. 3d, groups 2 vs. 4, $p<0.05$). PER1 levels in the DMH varied significantly across all 6 groups (Fig. 3e, $F_{5,16} = 16.29$, $p<0.0001$).

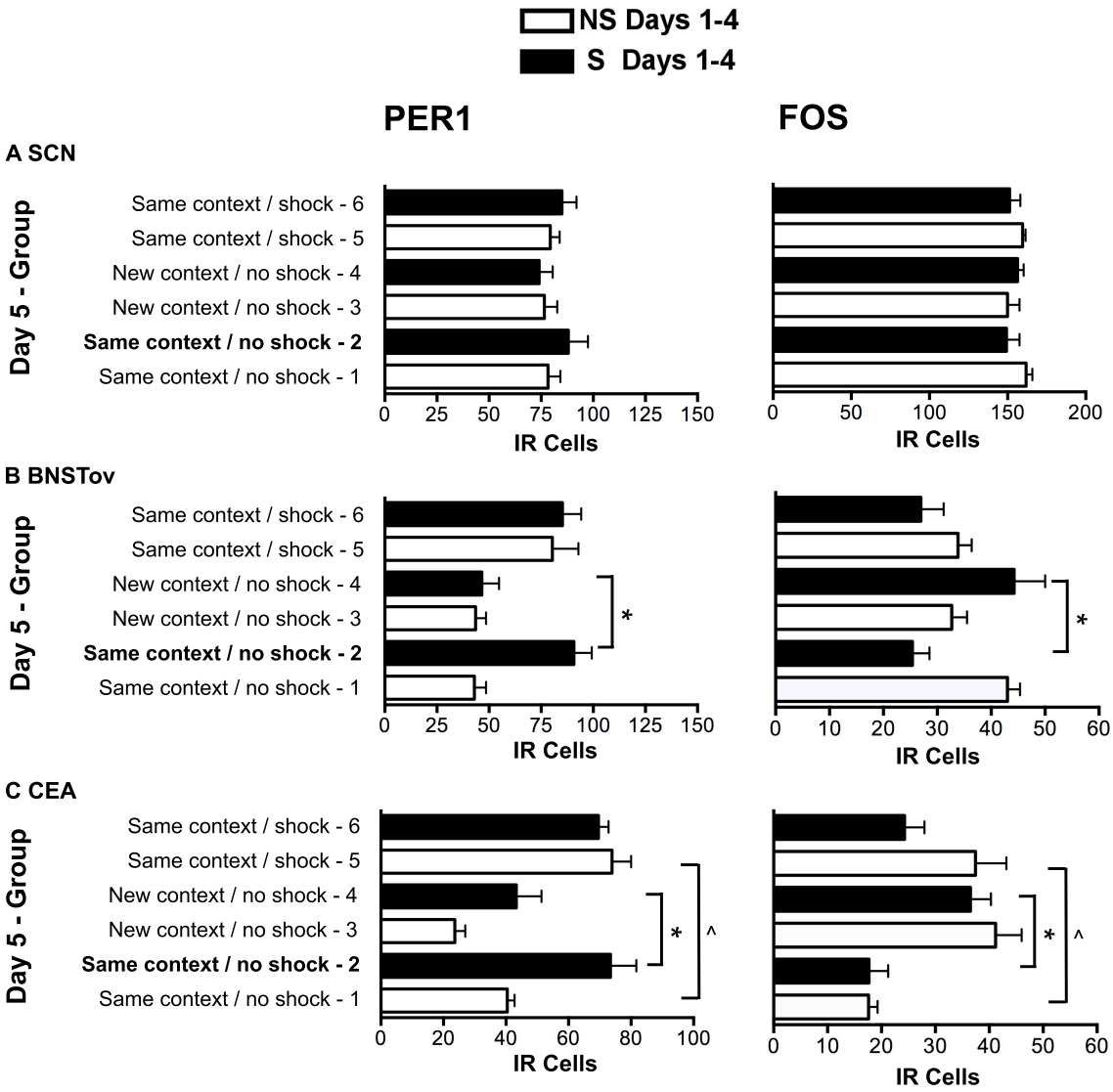

Figure 3
Conditioned fear modulates PER1 and FOS expression. The number of PER1 and FOS immunoreactive (IR) cells in the **a)** SCN, **b)** BNSTov, **c)** CEA, on Day 5 following contextual fear conditioning using footshock stress administered at ZT2 (see Fig. 1 for grouping details). Rats in groups 2, 4 and 6 were footshocked daily on Days 1-4, while those in groups 1, 3 and 5 were not footshocked. On Day 5, rats in groups 1 and 2 were placed in the conditioning box but not shocked, rats in groups 3 and 4 were placed in a second novel context and not shocked, and rats in groups 5 and 6 were footshocked in the conditioning box. The fear-conditioned rats (group 2) are shown in bold. Means ± SEM are shown, n=4 per group; significant effects (p<0.05) of * conditioned fear (groups 2 vs. 4), ^ acute footshock (groups 5 vs. 1), $^{\#}$ repeated daily footshock (groups 5 vs. 6).

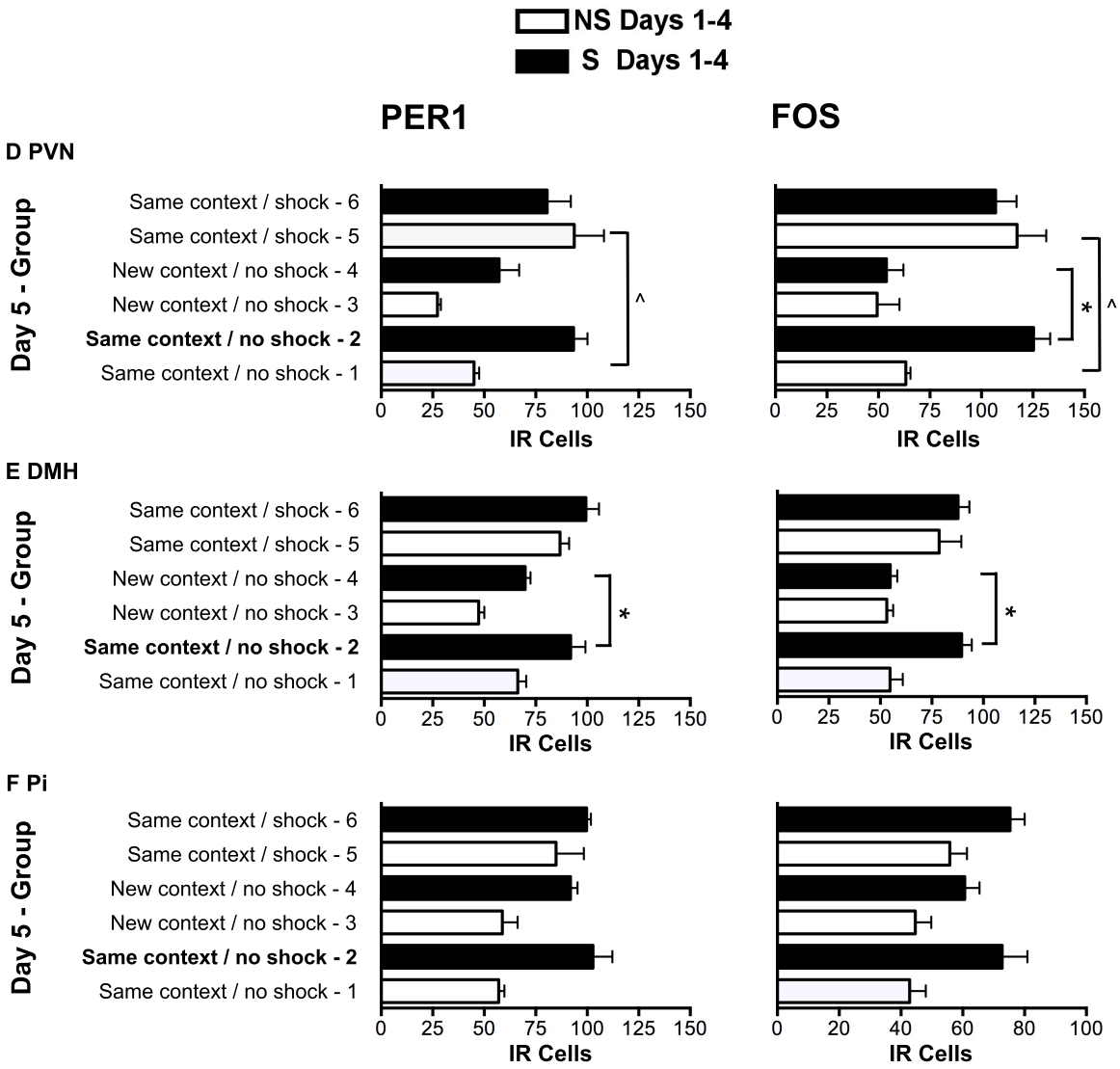

Figure 3 (continued)
Conditioned fear modulates PER1 and FOS expression. The number of PER1 and FOS immunoreactive (IR) cells in the d**)** PVN, **e)** DMH and **f)** Pi, on Day 5 following contextual fear conditioning using footshock stress administered at ZT2 (see Fig. 1 for grouping details). Rats in groups 2, 4 and 6 were footshocked daily on Days 1-4, while those in groups 1, 3 and 5 were not footshocked. On Day 5, rats in groups 1 and 2 were placed in the conditioning box but not shocked, rats in groups 3 and 4 were placed in a second novel context and not shocked, and rats in groups 5 and 6 were footshocked in the conditioning box. The fear-conditioned rats (group 2) are shown in bold. Means ± SEM are shown, n=4 per group; significant effects (p<0.05) of * conditioned fear (groups 2 vs. 4), ^ acute footshock (groups 5 vs. 1), $^{\#}$ repeated daily footshock (groups 5 vs. 6).

PER1 expression was significantly elevated in the DMH of fear-conditioned rats (Fig. 3e, groups 2 vs. 4, $p<0.05$). FOS expression was also affected in this region (Fig. 3e, $F_{5,16} = 6.87$, $p<0.01$). FOS levels were elevated in rats that were exposed to a context previously paired with footshock, compared to rats that were footshocked on Days 1-4 and placed in a second novel context on the test day (Figs. 3e, groups 2 vs. 4, $p<0.05$). Lastly, although there were variations in PER1 (Fig. 3f, $F_{5,17} = 6.05$, $p<0.001$) and FOS (Fig. 3f, $F_{5,18} = 5.73$, $p<0.01$) levels in the Pi of rats in all 6 groups, these differences were not attributed to the effects of conditioned fear, acute or repeated daily footshock.

We also assessed the effect of acute footshock stress on PER1 and FOS levels in the forebrain. We found that the expression of both proteins was significantly greater in the CEA and PVN of rats that were exposed to footshock only once on the test day, compared to rats that were never exposed to footshock (Figs. 3c,d, groups 5 vs. 1). Interestingly, there were no significant differences of repeated daily footshock in any region on PER1 and FOS expression (Figs. 3a-f, groups 5 vs. 6).

Taken together, our results demonstrate that the effects of the emotional state of fear, a complex processive stressor, on PER1 expression in the BNSTov and CEA are reminiscent of the stimulatory effect of systemic stressors on PER1 expression in these two regions. We also observed a suppression of FOS levels in the BNSTov and CEA following a conditioned fear response; this observation

is consistent with previous findings that FOS-expressing neurons in the central extended amygdala are inhibited by conditioned fear or acute (unconditioned) processive stress (Day et al., 2008). The present results demonstrate that induction of a state of fear is associated with a unique molecular signature in nuclei of the central extended amygdala, which combine processive and systemic stress elements (a decrease in FOS expression and an increase in PER1, respectively).

DISCUSSION

Stress can have debilitating consequences on motivation and emotion, in part attributed to a disruption of circadian rhythms. There is increasing evidence that circadian clock genes may be integrating circadian and stress information by acting as an interface between both systems in the brain. Here, we used a contextual fear conditioning protocol to study the effect of a complex processive stressor, the emotional state of fear, on the expression of the circadian clock protein, PER1 in the forebrain. Based on our findings in Chapter 1, we hypothesized that the induction of a state of fear would result in the suppression of PER1 expression in the BNSTov and CEA. Instead, we found that this manipulation led to an elevation in PER1 in most brain regions studied, including the BNSTov and CEA, a response characteristic of systemic, not processive stress. Our data demonstrate that the PER1 response to a complex processive stressor that combines conditioned (fear) and neurogenic (footshock) components resembles the PER1 response to systemic stressors.

The mechanism that mediates the effect of the emotional state of fear on PER1 expression and which sets it apart from the effect of other processive stressors remains to be defined. It is possible that the systemic-like effects of footshock stress, the aversive stimulus in our paradigm, may be overriding the processive effects of fear on PER1 expression. It is also possible that the effect of conditioned fear on protein levels is subject to the subcategories of processive stressors themselves; whether they have physically aversive properties (neurogenic), are of psychological origin (psychogenic) or have a learned component (fear-conditioned) (Hayley et al., 2001; Figueiredo et al., 2003; Anisman et al., 2005).

Our present findings suggest that footshock stress acts as a systemic stressor by increasing the expression of PER1 in the forebrain. The effects of acute footshock on PER1 were significant in the PVN and CEA only, underscoring a region-specific functional role for PER1. Interestingly, the effect of acute stress on PER1 was not analogous in the BNSTov and CEA, in contrast to our observations in Chapter 1. Despite being densely interconnected and functionally-related, the BNSTov and CEA do indeed exhibit disparities in their function; the former responds to signals more akin to anxiety, while the latter is critical for fear conditioning using explicit cues (Davis and Shi, 1999).

Furthermore, we established that there were no significant effects of repeated daily footshock stress on PER1 levels at ZT3, in any brain region studied. It has recently been shown that repeated exposure to a fear-inducing odor can alter the

rhythms of expression of PER2 (Pantazopoulos et al., 2011); hence it is possible that repeated exposure to footshock stress does not affect PER1 expression acutely, but rather changes the rhythms of PER1 expression over a 24 h period. This is consistent with the idea that stressors can uncouple subordinate oscillators from the master clock, resulting in a disruption of circadian rhythmicity.

Our manipulations had no effect on PER1 expression in the SCN, not a surprising finding in light of the master pacemaker's resilience to non-photic cues (Tonsfeldt and Chappell, 2012). In addition, we found that exposure to a context previously paired with footshock evoked robust behavioral and physiological responses associated with fear, as indicated by increased freezing time and cumulative feces, as well as elevated levels of circulating plasma CORT. It remains to be determined whether the stress-induced increases in CORT and PER1 levels in the forebrain are linked functionally, possibly by the glucocorticoid responsive element (GRE) on the *Per1* promoter, as has been observed in the mouse liver (Yamamoto et al., 2005).

Interestingly, contrary to the analogous effects of the different processive and systemic stressors on PER1 and FOS expression in the BNSTov and CEA (Chapter 1), the responses to fear in these nuclei revealed an asymmetrical change consisting of both a suppression of FOS expression and an induction of the expression of PER1. These responses are perhaps mediated by distinctive but overlapping mechanisms that play a key role in the modulation of amygdaloid output. Our data provide evidence for contrasting trends of PER1 and FOS

expression in response to the same stressor. Furthermore, our results support the finding that c-*Fos* expression in the central extended amygdala is inhibited by conditioned fear (Day et al., 2005; Day et al., 2008).

Conclusion

We have shown that the emotional state of fear represents a unique form of stress, which combines the suppressive effect of processive stress on FOS expression with the stimulating effect of systemic stress on PER1 expression. However, how conditioned fear comes to elicit such disparate effects remains to be determined. Our results suggest that conditioned fear can disrupt the expression of the circadian clock protein PER1 in select regions of the forebrain that are highly sensitive to stress, possibly resulting in the desynchrony of clock gene oscillations in neural circuits that are important in regulating motivational and behavioral states.

Acknowledgements

This study was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), le Fonds de la recherche en santé Québec (FRSQ) and the Concordia University Research Chair Program.

CHAPTER 3

Glucocorticoids modulate stress-induced changes in the expression of the circadian clock protein PERIOD1 in the rat forebrain

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Submitted to: Journal of Neuroscience, March 2014

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- Designed research: Sherin Al-Safadi, Shimon Amir
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- Analyzed data: Sherin Al-Safadi
- Wrote manuscript: Sherin Al-Safadi, editing by Shimon Amir

ABSTRACT

The temporal secretion of glucocorticoids in mammals is under circadian control, but glucocorticoids themselves are also implicated in modulating circadian clock gene expression in the brain and periphery. We have shown that the expression of the circadian clock protein PER1 in the forebrain is modulated by stress, and that this effect is associated with changes in plasma corticosterone levels, suggesting a possible role for glucocorticoids in the mediation of stress-induced changes in the expression of PER1 in the brain. To study this hypothesis, we assessed the effects of acute exposure to a processive stressor, restraint, and a systemic stressor, 2-Deoxy-D-glucose, on PER1 expression in adrenalectomized and sham-operated rats. We found that adrenalectomy reversed the effects of both stressors on PER1 expression in the oval nucleus of the bed nucleus of the stria terminalis and central nucleus of the amygdala, collectively known as the central extended amygdala, and in the dorsomedial hypothalamus. In contrast, adrenalectomy enhanced the effect of restraint and 2-Deoxy-D-glucose on PER1 levels in the paraventricular nucleus. Interestingly, although both stressors increased PER1 expression in the piriform cortex, the changes observed were independent of glucocorticoid signaling. The suprachiasmatic nucleus, the master circadian clock, was unaffected by any manipulation. Lastly, pretreatment of intact rats with the glucocorticoid receptor antagonist, mifepristone, affected stress-induced PER1 expression in a manner similar to adrenalectomy. Together, the results demonstrate a key role for glucocorticoid signaling in the modulation of stress-induced changes in PER1 expression in the brain.

INTRODUCTION

Circadian rhythms allow organisms to adapt to temporal features of the external environment, and can be disrupted by stressful events in mammals. Such disruptions are associated with several physiological and behavioral changes, but the mechanism that dictates how stress affects circadian rhythmicity is not well understood. Increasing evidence suggests that glucocorticoid hormones, primary effectors of the stress system, might contribute to this process by influencing circadian clock gene expression in the brain and periphery (Yamamoto et al., 2005; Segall and Amir, 2010a). Recently, we found that the expression of the core clock protein PER1 is modulated by stress in select forebrain nuclei, but the light-entrainable master pacemaker, the suprachiasmatic nucleus (SCN), remained immune to these effects. Importantly, the category of stress, time of day, and mode of exposure all played an important role in determining stress outcomes on PER1 expression (Chapter 1). Here, we investigate a role for glucocorticoids in the modulation of stress-induced changes in PER1 expression in the forebrain.

Mammalian circadian rhythms are governed by the SCN, and by subordinate clocks located in other brain regions and in the periphery. At the molecular level, circadian clocks consist of transcriptional and translational feedback loops driven by a small set of core clock proteins (Challet, 2007). These include PER1, a key negative clock regulator that is responsive to select stressors in rodents, and to manipulations of glucocorticoid signaling (Balsalobre et al., 2000b; Takahashi et al., 2001; Yamamoto et al., 2005). Here, we are studying the hypothesis that

stress can modify the expression of PER1 in forebrain regions that are important in motivation and emotion, and that this effect is dependent on adrenal glucocorticoid hormones. We have previously shown that depletion of endogenous glucocorticoids via adrenalectomy (ADX), or selective genetic deletion of brain glucocorticoid receptors (GR), blunt the rhythm of expression of the clock protein, PER2 in nuclei of the central extended amygdala, namely the oval nucleus of the bed nucleus of the stria terminalis (BNSTov) and lateral division of the central nucleus of the amygdala (CEA), without affecting the rhythms of the SCN in rats (Shieh, 2003; Amir et al., 2004; Lamont et al., 2005; Segall et al., 2006; Nader et al., 2010; Segall and Amir, 2010b).

In the present study, we show that the loss of endogenous glucocorticoids, through either ADX or pharmacological blockade of GR (GRX), mitigates the effects of categorically different stressors on PER1 expression. Our results suggest that the effect of stress on PER1 in select regions of the forebrain depends on glucocorticoid signaling, and lend further support to the hypothesis that stress-induced dysregulation of the circadian system is linked to glucocorticoid-modulated changes in circadian clock protein expression in the brain.

MATERIALS AND METHODS

Animals and Housing

All procedures followed guidelines outlined by the Canadian Council on Animal Care and were approved by the Animal Care Committee of Concordia University.

Intact, ADX and sham-operated male Wistar rats weighing 126-150 g were purchased from Charles River Canada (St. Constant, QC, Canada). Rats were housed in clear plastic cages individually, with free access to food and water, and kept under a 12:12h light/dark (LD) schedule for approximately 2 weeks until they were fully entrained. The cages were placed in ventilated, light and sound-attenuated isolation boxes and were equipped with a running wheel. Running wheel data were collected by VitalView software (Mini-Mitter, Sunriver, OR, USA). Actograms were acquired and analyzed using Actiview Biological Rhythm Analysis software (Mini-Mitter). The actograms were used to confirm that rats were stably entrained to the 12:12h LD cycle. During the entrainment period, CORT was reintroduced in ADX rats through their drinking water; this regimen mimics endogenous CORT rhythms, which are crucial for the rhythmic expression of clock proteins (Segall et al., 2006; Segall and Amir, 2010a, b). CORT (Sigma-Aldrich, Oakville, ON, Canada) was dissolved in 0.9% saline, at a concentration of 25 mg/L as previously described (Segall et al., 2006). Sham rats were provided with 0.9% saline only. On the morning of the test day, prior to stress exposure, the drinking fluid of ADX rats was replaced with 0.9% saline only.

Treatment

The non-selective GR antagonist mifepristone (Sigma-Aldrich, Oakville, ON, Canada) was reconstituted in 0.9% saline containing 5% dimethyl sulfoxide (Sigma-Aldrich, Oakville, ON, Canada) and 1% Tween 20 (Fischer Scientific, Ottawa, ON, Canada). The suspension was re-vortexed 1 min prior to injecting,

and as needed throughout the dosing. The drug was then administered intraperitoneally at a concentration of 40 mg/kg. A similar dose was shown to attenuate the stress-induced HPA axis response in rats (Zalachoras et al., 2013). Control rats received vehicle or mifepristone but were not exposed to stress.

Stressors

Processive

Rats were exposed to a 30 min restraint challenge in custom-designed ventilated Plexiglas tubes (7 mm thick, internal diameter of 75 mm, adjustable in length from 130-180 mm), as previously described (Chapter 1). Control rats were handled only.

Systemic

Rats were exposed to a metabolic challenge, a subcutaneous injection of 250 mg/kg 2DG (2DG, Sigma-Aldrich, Oakville, ON, Canada) in 0.9% saline, as previously described (Chapter 1). Control rats were injected with vehicle only.

Plasma CORT Collection and Analysis

Rats were placed in restraining devices, then tail-clipped with a razor for rapid blood collection using capillary tubes (0.5 ml). Samples were centrifuged for 10 min at 4°C, 13,000 r.p.m. and the plasma was extracted and stored at -80°C. Plasma CORT levels were assessed in duplicates using a CORT Enzyme Immunoassay (EIA) kit (Enzo Lifesciences, Farmingdale, NY, USA), as previously described (Segall et al., 2006).

Tissue Preparation and Immunohistochemistry

Rats were given an overdose of sodium pentobarbital (100 mg/kg) at zeitgeber time 3 (ZT3, where ZT0 denotes lights on and ZT12 denotes lights off), and perfused intracardially with 300 ml of cold 0.9% saline, followed by 300 ml of cold 4% paraformaldehyde. Brains were extracted and kept in 4% paraformaldehyde at 4°C overnight. They were then sliced in 50 µm serial coronal sections on a vibratome, and stored at −20°C in Watson's Cryoprotectant.

Immunohistochemistry for PER1 was performed as previously described (Verwey et al., 2009) using an affinity-purified rabbit polyclonal antibody raised against PER1 (1:24,000 - R1177, EMD-Millipore).

Microscopy and Data Analysis

Stained sections were mounted onto gel-coated slides and dehydrated in a series of alcohols and Citrisolv (Fisher), then coverslipped. The sections were examined under a light microscope (Leica, DMR) and images were captured with a Sony XC-77 video camera, Scion LG-3 frame grabber with a 400x400 µM template, and Image SXM software (http://www.ImageSXM.org.uk1 v1.95, S.D. Barret). The mean number of PER1 immunoreactive (IR) cells per region was then calculated for each animal from the counts of six unilateral images showing the highest number of labeled nuclei, as previously described (Amir et al., 2004). Differences between groups and time-points were revealed using a two-way analysis of variance (ANOVA), a confidence level (α) set at 0.05 and a Bonferroni post-hoc test.

Experimental Protocol

Adrenalectomy

To study the role of glucocorticoids in the stress-induced changes in PER1 expression in the brain, we exposed ADX or sham-operated rats (*n*=4 per group) to a processive stressor, restraint, or a systemic stressor, 2DG, at ZT2. Control rats for restraint were handled only and those for 2DG were injected with vehicle. Rats were killed 1 h after stress onset, at ZT3 for analysis of PER1 expression and plasma CORT. These time points were selected based on previous findings (Chapter 1), which indicate that stress-induced PER1 is robustly expressed during the light phase of the LD cycle, 1 h after the onset of the stressor.

Pharmacological Blockade of Glucocorticoid Receptors

In order to assess the importance of GR in mediating the effect of categorically different stressors on the expression of PER1, we administered the GR antagonist mifepristone systemically, 1 h prior to the onset of the stressors, at ZT1. Rats (*n*=4 per group) were then exposed to either restraint or 2DG at ZT2, and consequently killed in the short-term at ZT3 for the analysis of PER1 and plasma CORT. Two control groups, which were not subjected to stress, received either vehicle or mifepristone only.

RESULTS

Adrenalectomy enhances PER1 levels in the PVN following acute stress, but mitigates stress-induced PER1 expression in the DMH, BNSTov and CEA

Acute processive and systemic stress, restraint and 2DG, respectively, significantly elevated plasma CORT (Fig. 1a, $F_{2,18} = 37.48$, $p<0.0001$). The effect of ADX on stress-induced PER1 expression in the forebrain is shown in Figs. 2 and 3, and statistical analyses in Table 1. PER1 levels in the SCN remained unaffected by either stressor or ADX (Fig. 3a). Both restraint and 2DG increased PER1 expression in the Pi of shams, as previously observed in Chapter 1 (Fig. 3b). Interestingly however, PER1 expression was unaffected by ADX, indicating that the stress-induced increase in PER1 in the Pi was independent of CORT (Fig. 3b). ADX significantly enhanced the effect of 2DG on PER1 levels in the PVN (Fig. 3c). Contrastingly, ADX reversed the stress-induced changes on PER1 in the DMH, BNSTov and CEA (Figs. 3d-f). Specifically, ADX blocked 2DG-induced PER1 expression in the DMH (Fig. 3d). Exposure to the processive stressor, restraint suppressed PER1 expression in the BNSTov and CEA of sham rats (Figs. 3e,f), as previously shown in Chapter 1. ADX significantly attenuated this suppression in both regions (Figs. 3e,f). Conversely, treatment with the systemic stressor 2DG increased PER1 levels in the BNSTov and CEA of sham-operated rats; ADX reversed this increase in the CEA (Figs. 3e,f).

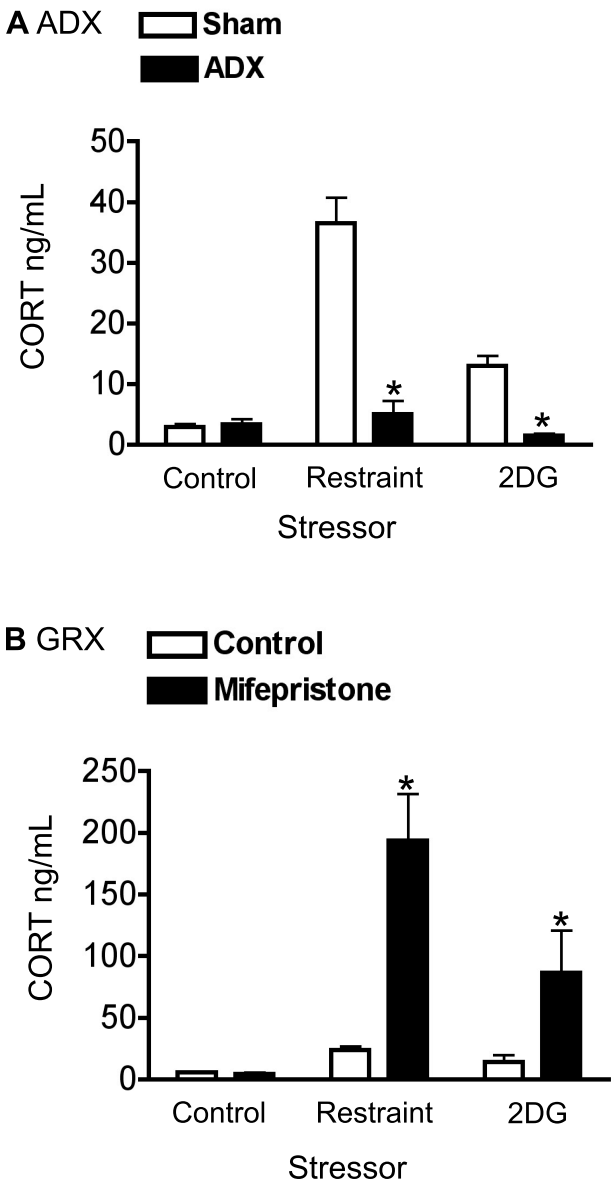

Figure 1
ZT3 plasma corticosterone (CORT) levels as a function of the category of stress. a) Sham-operated (sham) and adrenalectomized (ADX) rats were exposed to restraint or 2DG at ZT2. **b)** Treated rats were injected with mifepristone (GRX) at ZT1, 1 h prior to stress exposure. Control (control) and treated (mifepristone) rats were then exposed to restraint or 2DG at ZT2. Means ± SEM are shown, n=4 per group; * significant difference from corresponding control group, p<0.05.

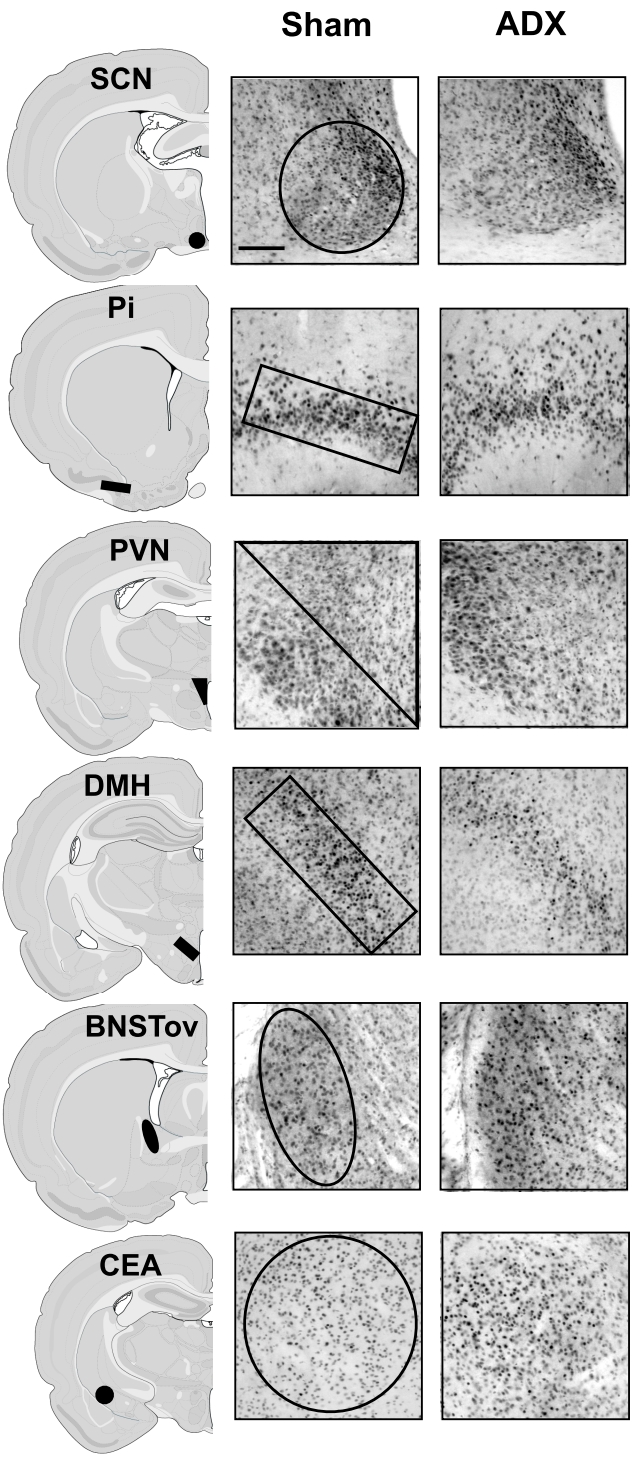

Figure 2
Examples of PER1 expression in different forebrain and hypothalamic nuclei at ZT3. Sham or adrenalectomized (ADX) rats were exposed to a 30 min restraint session at ZT2, and then killed at ZT3 (scale bar: 100µm).

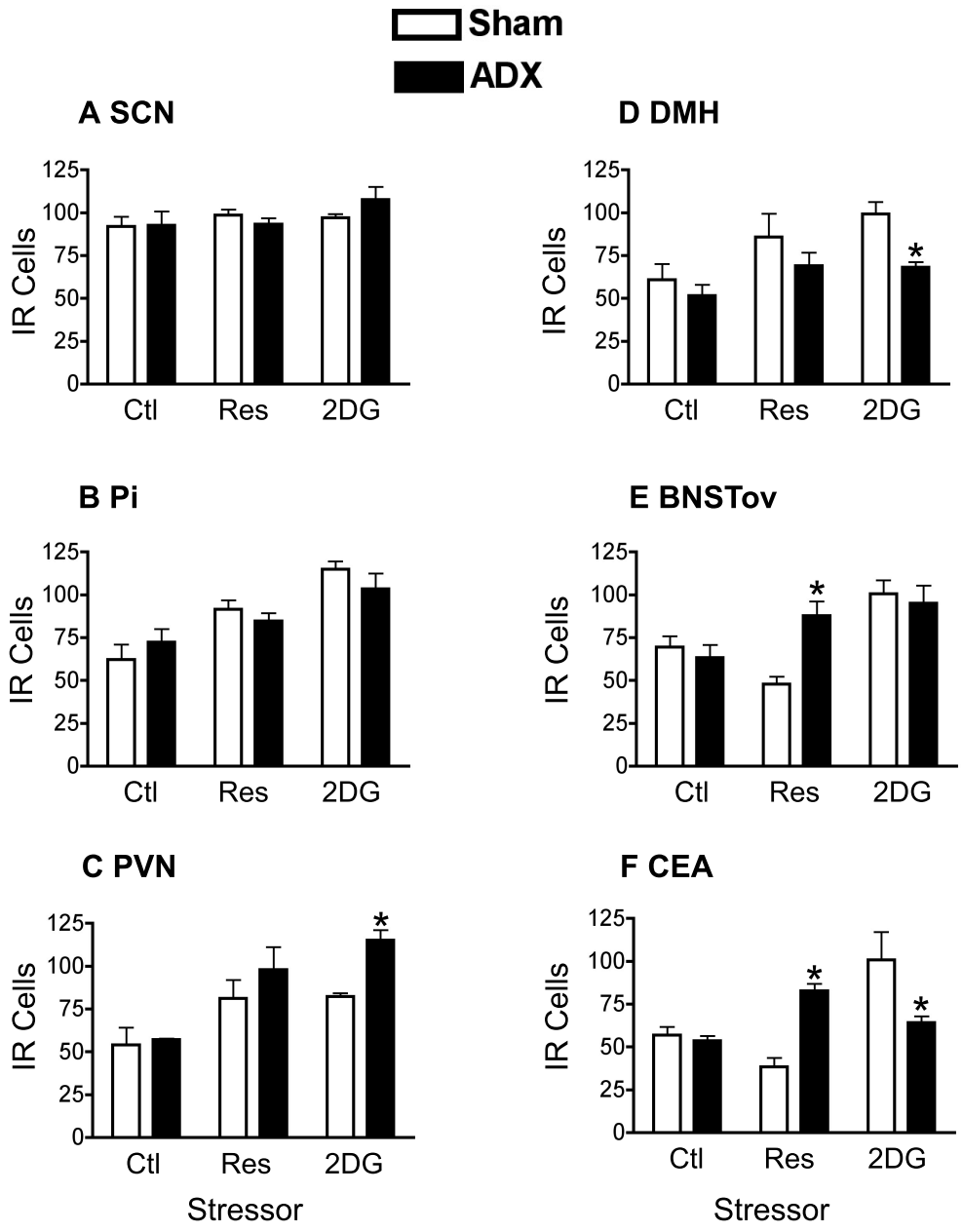

Figure 3
Number of PER1 immunoreactive (IR) cells in different forebrain and hypothalamic nuclei at ZT3. IR cells are shown as a function of the category of stress in sham-operated (sham) and adrenalectomized (ADX) rats. Means ± SEM are shown, n=4 per group; * significant difference from corresponding sham group, p<0.05.

Table 1
Adrenalectomy (ADX) and the category of stressor modulate stress-induced PER1 expression in limbic and hypothalamic brain regions.

Structure	ADX	Stressor	ADX x Stressor
SCN	F(1,15) = 0.17, ns	F(2,15) = 1.4, ns	F(2,15) = 0.86, ns
Pi	F(1,18) = 0.24, ns	F(2,18) = 18.03, $p<0.0001$	F(2,18) = 1.33, ns
PVN	F(1,18) = 6.41, $p<0.05$	F(2,18) = 14.37, $p<0.001$	F(2,18) = 1.57, ns
DMH	F(1,18) = 8.84, $p<0.01$	F(2,18) = 5.0, $p<0.05$	F(2,18) = 1.34, ns
BNSTov	F(1,18) = 2.26, ns	F(2,18) = 10.75, $p<0.001$	F(2,18) = 5.99, $p<0.05$
CEA	F(1,18) = 0.06, ns	F(2,18) = 7.03, $p<0.01$	F(2,18) = 13.78, $p<0.001$

ns, not significant

Glucocorticoid receptor blockade mimics the effects of adrenalectomy on stress-induced PER1 expression in the PVN, DMH, BNSTov and CEA

Plasma CORT levels increased following exposure to restraint stress or 2DG at ZT2 (Fig. 1b, $F_{1,17} = 34.75$, $p<0.0001$). Treatment with mifepristone, a GR antagonist, further increased circulating CORT levels (Fig. 1b, $F_{2,17} = 17.48$, $p<0.0001$). The synergistic effect of mifepristone on CORT was dependent upon the category of stressor, processive (restraint) or systemic (2DG) (Fig. 1b, $F_{2,17} = 12.15$, $p<0.001$). The effect of GR blockade on stress-induced PER1 expression is shown in Figs. 4 and 5, and statistical analyses in Table 2.

Acute stress exposure had no effect on PER1 levels in the SCN of control and mifepristone-treated animals (Fig. 5a). Mifepristone treatment had no effect on restraint and 2DG-induced PER1 expression in the Pi (Fig. 5b). As with adrenalectomy, blockade of GR further accentuated the stress-induced increase in PER1 expression in the PVN (Fig. 5c). In addition, mifepristone attenuated the effect of both stressors on PER1 levels in the DMH, maintaining PER1 expression post-stress at baseline levels (Fig. 5d). This effect was also observed in the BNSTov and CEA, where the administration of mifepristone mitigated the restraint-induced PER1 suppression, as well as the 2DG-induced increase in PER1 levels (Figs. 5e,f).

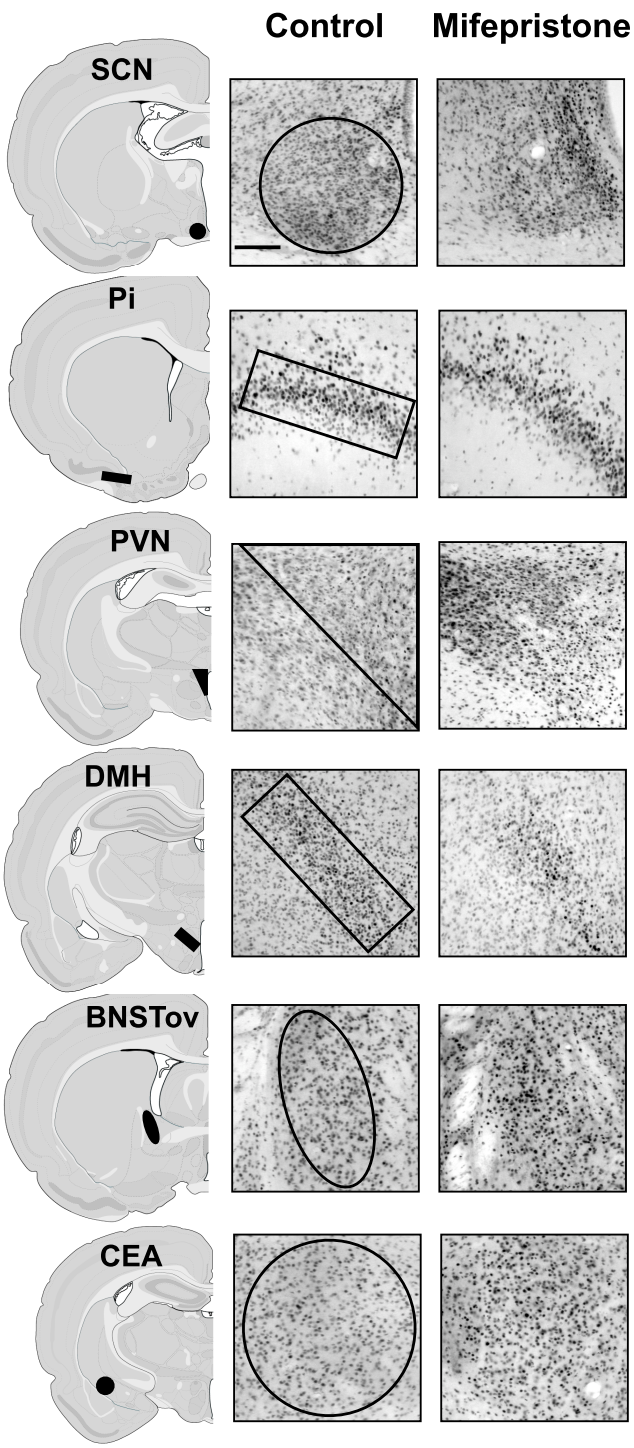

Figure 4
Examples of PER1 expression in different forebrain and hypothalamic nuclei at ZT3. Rats were injected with vehicle or mifepristone at ZT1, exposed to a 30 min restraint session at ZT2, and then killed at ZT3 (scale bar: 100 µm).

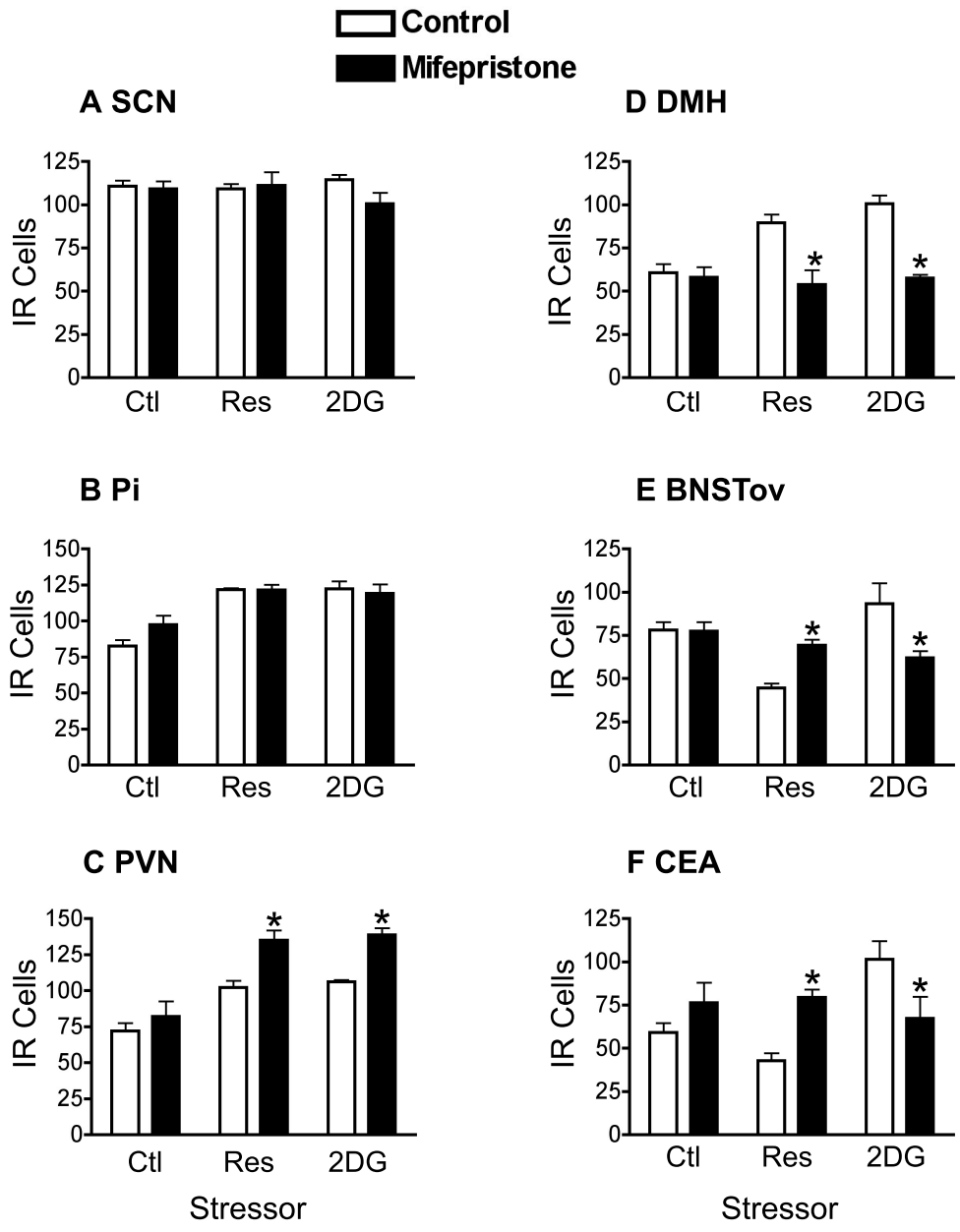

Figure 5
Number of PER1 immunoreactive (IR) cells in different forebrain and hypothalamic nuclei at ZT3. IR cells are shown as a function of category of stress in control and mifepristone-treated (GRX) rats. Means ± SEM are shown, *n*=4 per group; * significant difference from corresponding sham group, $p<0.05$.

Table 2
Pharmacological blockade of glucocorticoid receptors (GRX) and the category of stressor modulate stress-induced PER1 expression in limbic and hypothalamic brain regions.

Structure	GRX	Stressor	GRX x Stressor
SCN	F(1,17) = 1.12, ns	F(2,17) = 0.16, ns	F(2,17) = 1.34, ns
Pi	F(1,18) = 0.97, ns	F(2,18) = 27.41, p<0.0001	F(2,18) = 1.95, ns
PVN	F(1,18) = 24.78, p<0.0001	F(2,18) = 33.05, p<0.0001	F(2,18) = 2.26, ns
DMH	F(1,17) = 39.89, p<0.0001	F(2,17) = 7.55, p<0.01	F(2,17) = 8.7, p<0.01
BNSTov	F(1,18) = 0.22, ns	F(2,18) = 7.79, p<0.01	F(2,18) = 10.65, p<0.001
CEA	F(1,18) = 0.84, ns	F(2,18) = 3.62, p<0.05	F(2,18) = 8.44, p<0.01

ns, not significant

Taken together, the results show that the loss of endogenous glucocorticoids, through ADX or pharmacological blockade of GR, enhances the stress-induced expression of PER1 in the PVN, but mitigates it in the DMH, BNSTov and CEA.

DISCUSSION

In mammals, the rhythmic secretion of glucocorticoids is governed by the SCN clock (Dickmeis et al., 2013). Glucocorticoids have also been shown to play a role in the modulation of clock genes in subordinate oscillators downstream of the SCN (Balsalobre et al., 2000b; Amir and Robinson, 2006; Segall and Amir, 2010a; Kalsbeek et al., 2012). Here, we used ADX and the GR blocker, mifepristone to investigate the role of glucocorticoids and their receptors in stress-induced changes in the expression of the clock protein, PER1 in the brain. We found that both treatments could alter the effect of stress on PER1 and, furthermore, that the effect of these manipulations was contingent upon the type of stress and brain region studied. These findings demonstrate a complex role for glucocorticoid signaling in stress-induced changes in PER1 expression in the brain, and suggest a mechanism for how stress interacts with circadian clocks in specific brain regions to influence behavior and physiology.

In the present study, we found that the elimination of endogenous glucocorticoids via ADX reversed the suppressive effect of restraint but also the stimulatory effect of 2DG on PER1 expression in the BNSTov and CEA. This finding demonstrates that the effect of stress on PER1 is modulated by glucocorticoids but also depends on the category of stress; namely, glucocorticoids modulate the

suppressive effect of processive stress and the stimulatory effect of systemic stress on PER1 expression in the BNSTov and CEA. Furthermore, in the DMH, ADX attenuated the stimulatory effect of both stressors on PER1 expression, pointing to a permissive role for glucocorticoids in this region. In contrast, ADX enhanced the effect of stress on PER1 expression in the PVN, a nucleus that receives major afferent inputs from the DMH (Keim and Shekhar, 1996; Li and Sawchenko, 1998; Bailey and Dimicco, 2001), pointing to an inhibitory role for glucocorticoids on PER1 expression in this region. Similar inhibitory effects of glucocorticoids on a variety of peptides in the PVN have been previously reported (Tasker et al., 2005). ADX had no effect on stress-induced PER1 expression in the Pi, indicating that the regulation of PER1 expression in this region by stress is independent of glucocorticoid signaling. Other possible mediators of the effect of stress on PER1 expression in the Pi include the neurotransmitter dopamine; dopaminergic systems are linked to stress-related behaviors (Sim et al., 2013), endogenous dopamine has been implicated in the regulation of PER1 expression in the brain (Hood et al., 2010), and dopamine receptors are richly expressed in the Pi (Fremeau et al., 1991). Finally, PER1 expression in the SCN was unaffected by manipulations in glucocorticoid signaling, as the SCN is void of GR, and unlike its downstream oscillators, is immune to the effects of internal and external stressors (Balsalobre et al., 2000b).

Importantly, blockade of GR mimicked the effect of ADX, underscoring the importance of glucocorticoid signaling via GR in stress-induced changes in PER1

expression in the forebrain. Further studies are required to determine whether GR and the putative Glucocorticoid Responsive Element (GRE) on *Per1* (Yamamoto et al., 2005) collectively mediate not only the stimulatory effect of stress, but also the unique suppressive effects of processive stressors on PER1 in the BNSTov and CEA. Nuclear glucocorticoid-GR complexes generate a slow genomic response through a process that takes several hours to days. We found, however, that stress-induced changes in PER1 expression were most robust in the short-term, 1 h post stress onset. This suggests that stress-induced, glucocorticoid-dependent changes in PER1 expression are mediated by rapid nongenomic mechanisms. One possibility entails the activation of a membrane-bound GR (mGR) (Tasker et al., 2006; Strehl et al., 2011; Vernocchi et al., 2013). The mGR signaling cascade may provide a rapid mechanism for *Per1* transcriptional regulation that runs in parallel to the intracellular GR-mediated regulation of gene transcription (Chen and Qiu, 2001; Tasker et al., 2006).

We have previously shown that basal glucocorticoids are essential for the rhythmic expression of PER2 in the BNSTov and CEA, as ADX results in the abolishment of PER2 rhythms in these two regions exclusively (Amir et al., 2004; Lamont et al., 2005). Ablated PER2 rhythms are rescued by introducing CORT via the drinking water of rats, a regimen that mimics endogenous CORT rhythms and underscores the importance of circadian glucocorticoid signaling in basal rhythmic clock gene expression (Segall et al., 2006; Segall et al., 2009; Segall and Amir, 2010a). Here, our novel data extends these previous findings by

demonstrating that stress-induced glucocorticoid signaling can transiently modulate the expression of PER1 not only in the BNSTov and CEA, but also in the DMH and PVN, two hypothalamic nuclei that are critical in the stress response. Moreover, we have shown that stress-induced glucocorticoids can elicit a second wave of *Per1* expression, which may impede basal clock gene rhythms. It is possible that glucocorticoid signaling in the brain is directly affecting PER1 expression in enkephalin-producing (ENK) neurons, as stress increases *c-Fos* expression in ENK neurons (Day et al., 1999; Kozicz, 2002), glucocorticoids have been shown to modulate the expression of enkephalin (Honkaniemi et al., 1992; Pompei et al., 1995), and clock genes are localized in this subpopulation of cells (Amir et al., 2004; Lamont et al., 2005).

In summary, we have demonstrated that categorically different acute stressors, through glucocorticoid signaling, can modulate PER1 expression in a region-specific manner in brain nuclei that are important in stress and motivation. Moreover, the previous finding that different stressors can elicit a disturbance in CORT levels that is associated with the stress-induced expression of *Per1* but not *Per2*, in the PVN but not in the SCN or liver (Takahashi et al., 2001), further supports the present results. It is possible that stress-induced disruptions in PER1 can reset local clocks in the brain, leading to transient misalignment between the stress-resilient master clock and downstream brain oscillators, and ultimately to disruptions in circadian-controlled behavioral changes. Alternatively, stress-induced changes in PER1 may have implications outside the clock; aside

from its canonical circadian role, PER1 has been shown to regulate stress-induced grooming and nociceptive behaviors (Zhang et al., 2011), as well as anxiety-related behavior in the nucleus accumbens (Spencer et al., 2013). The use of RNA interference to selectively suppress PER1 within specific brain regions will allow us to further assess the functional role of PER1 in stress-induced changes in motivated and affective behaviors.

Acknowledgements

This study was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), les Fonds de la recherche en santé Québec (FRSQ) and the Concordia University Research Chair Program. The authors declare no competing financial interests.

GENERAL DISCUSSION

The findings presented in this thesis strongly reinforce the notion that stress, primarily through glucocorticoid signaling, modifies circadian clock protein expression in regions of the mammalian brain that are vulnerable to, and play a role in, stress and anxiety. We have demonstrated that categorically different stressors, processive versus systemic, regulate the expression of the circadian clock protein PER1 in a distinct fashion. Processive stressors, which trigger characteristic affective responses, suppressed PER1 expression in the BNSTov and CEA. In contrast, systemic stressors, which affect homeostasis without inducing an acute emotional response, elevated PER1 expression in these two regions. Interestingly, we also demonstrated that the PER1 response to the emotional state of fear, a complex processive stressor that combines conditioned and neurogenic components, resembles the PER1 response to systemic stressors. We next determined that the modality of the stressor, repeated daily predictable versus unpredictable, also modulates PER1 expression, with evidence of habituation of the PER1 response to repeated daily predictable stress exclusively in the BNSTov and CEA. Furthermore, we have shown that there is a narrow window of time for the most robust effects of stress on PER1 expression, namely during the subjective day or inactive phase in rodents, and immediately after the onset of the stressor in the short-term, implicating *Per1* as an IEG. Lastly, we established that the stress-induced effects on PER1 expression in select regions of the forebrain are modulated by glucocorticoids and their receptors. In summary, the present work underscores the sensitivity of

clock genes to fluctuations in glucocorticoid signaling in select forebrain regions. Consequently, stress-induced changes in clock protein expression in these nuclei may lead to disruptions in clock gene oscillations, in neural circuits that are important in regulating behavior and motivation.

Our work shines a light on the BNSTov and CEA, two regions that exhibit a distinct molecular signature of PER1 suppression in response to stressors with an emotional component. Despite being densely interconnected and functionally related, (Cassell et al., 1986; Day et al., 1999; Dong et al., 2001), the BNSTov and CEA do indeed exhibit disparities in their function; the former responds to signals more akin to anxiety, while the latter is critical for fear conditioning using explicit cues (Davis and Shi, 1999). Unraveling the inputs, subnuclear connectivity and projections of the cells that express PER1 in the central extended amygdala will allow us to better understand the function of PER1 in this region.

Another interesting finding in our work was the effect of a complex processive stressor, the emotional state of fear, on PER1 expression. We hypothesized that the state of fear, induced by exposure to a context previously paired with footshock, would affect PER1 levels in a manner similar to processive stressors such as restraint. Instead, we found that the change in PER1 expression following the induction of fear in fact resembled the change seen in response to a systemic stressor. In Chapter 2, we attributed this outcome to the systemic-like effects of footshock stress. Although this stressor has previously been reported

to exhibit both processive and systemic qualities, we found in fact that acute footshock stress affected PER1 expression in a manner reminiscent of systemic stressors. Furthermore, we alluded to distinctive circuitry for subcategories of processive stressors that may be playing a role in the PER1 response to conditioned fear.

We also examined the importance of the mode of stressor presentation, by studying the effects of repeated daily predictable versus unpredictable processive stress. We were unable to study the effects of systemic stress in this context, as repeated exposure to physiological insults would have resulted in severe health problems and the likely loss of animals. Accordingly, we studied the effect of processive stress only; this circumstance in fact realistically parallels the human experience, whereby the more damaging effects of chronic stress are a result of prolonged exposure to emotional pressure, rather than physiological disruptions in homeostasis.

The window of time in which stress exerted its most robust effects on PER1 was during the inactive phase of the day, most likely due to the exclusion of conflicting variables such as general activity and feeding behavior. In addition, the effect of stress was most pronounced in the short-term, 1 hour following the onset of a stressor. The fact that stress can induce protein synthesis within this small timeframe is highly indicative of a role for *Per1* as an IEG. It is possible that PER1 itself may then be affecting a distinct second wave of gene expression (Loebrich and Nedivi, 2009) in, or perhaps external to, the clock mechanism. In

all, our results support previous literature that indicates that *Per1* is an IEG (Akashi and Nishida, 2000), and support our hypothesis that PER1 is transiently integrating circadian and stress information in the brain, by acting as an interface between subordinate oscillators and stressful stimuli.

Although scarce, evidence for associations between glucocorticoid signaling and clock gene expression currently exists (Balsalobre et al., 2000b; Balsalobre et al., 2000a; Takahashi et al., 2001; Amir et al., 2004; Fukuoka et al., 2005; Lamont et al., 2005; Segall et al., 2009). Accordingly, we sought to demonstrate that stress-induced changes in PER1 are dependent on glucocorticoids and their receptors. Interestingly, a stress-induced elevation in circulating glucocorticoids did not always translate into an increase in PER1 expression; processive stressors, which incidentally generated the highest levels of CORT, suppressed PER1 levels in the BNSTov and CEA. This strongly underscores a role for glucocorticoids as modulators rather than inducers of clock protein expression. Furthermore, although all stressors throughout our work consistently elevated plasma CORT, the stress-induced response of PER1 was not always glucocorticoid dependent; for example, the Pi expresses GR and is a stress-responsive brain region, but we failed to observe any effect of hormone manipulation on PER1 in this region. Our results emphasize the selective nature of glucocorticoid regulation of clock gene expression. It should be added, however, that other effectors of the stress system, including dopamine or CRH, might also be mediating the effect of stress on PER1 expression.

We have demonstrated that glucocorticoid signaling modulates stress-induced changes in PER1 expression. In an effort to assess the involvement of GR, we utilized systemic injections of the non-selective GR blocker mifepristone. In order to further validate this finding and evade any unwanted global effects of GR antagonism, it would be beneficial to study the effects of microinfusions of a more selective GR antagonist on PER1 in the brain regions of interest. Alternatively, it is possible to utilize double-stranded RNA-mediated (dsRNA) interference to transiently suppress GR expression within these select brain nuclei. In Chapter 3, we proposed a nongenomic mGR-dependent alternative mechanism for the regulation of stress-induced PER1 expression. Although these putative membrane receptors have not yet been isolated, there is increasing evidence that such receptors exist and that the rapid downstream effects of activation of these receptors depend on G-protein signaling mechanisms (Tasker et al., 2006; Strehl et al., 2011; Vernocchi et al., 2013). If the stress-induced changes in PER1 are indeed mediated by mGR signaling and consequent epigenetic modifications, this would imply that the GRE on *Per1* serves a function primarily in the presence of basal levels of glucocorticoids, and in the context of the genomic regulation of clock-related PER1 functions.

While the SCN functions as a master pacemaker and orchestrator of circadian-controlled outputs, subordinate clocks in the brain and periphery are responsible for temporally compartmentalizing distinct processes to optimize physiology and behavior. In the present thesis, we found that stressful stimuli can transiently affect PER1 expression in subordinate oscillators located in nuclei that are

important in the regulation of motivation and emotiona. It is possible that stress-induced changes in PER1 levels are impeding the timing of circadian oscillations within these brain regions, and uncoupling them from the rhythms of the SCN. Alternatively, it is possible that the PER1 response to stress is involved in systems outside the clock; a pleiotropic role for PER1 is supported by evidence that implicates it in tumor suppression, anxiety-related behavior, cocaine sensitization and alcohol drinking behavior in mice (Akhisaroglu et al., 2004; Dong et al., 2011; Spencer et al., 2013). In fact, it is most likely that the aforementioned scenarios function in parallel and perhaps even synergistically, as they both lead to changes in circadian behavior and physiology, as well as regulate the response to stressful stimuli.

While elucidating the functional role of clocks in the periphery remains relatively straightforward, determining this role in the brain is far more complex. Future studies pertaining to the function of clock genes in stress-responsive brain nuclei entail suppressing PER1 using localized injections of dsRNA, and examining the effect on physiological and behavioral measures in chronically stressed animals. One such measure is the elevated plus-maze, a well-characterized model of anxiety in rodents (Raught et al., 2001) that would not only shed light on the stress-induced role of PER1, but also the effect of PER1 suppression on the circadian variation in behavior.

The circadian system has provided an attractive model with which to study how the brain interacts with the environment to control physiology and behavior. And,

although much has been learned in recent years about the molecular and neural mechanisms underlying this system, it continues to present major conceptual and methodological challenges. In humans, disorders of the circadian system have been associated with conditions such as jet lag, shift work, aging, depression, Alzheimer's disease, post-traumatic stress disorder and even diabetes and obesity (Son et al., 2011). Although altered glucocorticoid rhythms are a prominent feature of the aforementioned disorders, it remains to be determined whether this is a symptom of circadian dysregulation, or whether it is a trigger for disturbances in chronobiology. The findings in this thesis assist in the elucidation of novel molecular mechanisms through which stress can modify the expression of clock genes in the forebrain and hypothalamus, and bring us one step closer to understanding the nature and functional significance of PER1 oscillations within these brain regions. We have shown that stress, primarily but not exclusively through the action of glucocorticoid signaling, can modify clock gene expression in brain oscillators that play an important role in the interface between stress and the circadian systems. Given the profound effect that both systems have on health, a deeper understanding of their cross-talk may provide new targets for pharmacological interventions and rational therapies for existing disorders and diseases.

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