

Thyroidectomy alters the daily pattern of expression of the clock protein, PER2,  
in the oval nucleus of the bed nucleus of the stria terminalis and central nucleus  
of the amygdala in rats

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## **Abstract**

A role for thyroid hormones in the regulation of the rhythmic expression of circadian clock genes is suggested by the finding that surgical removal of the thyroid gland alters circadian behavioral and endocrine rhythms in rodents. Virtually nothing is known about the role of thyroid hormones in the regulation of clock genes responsible for the generation of circadian rhythmicity. To study this issue, we assessed in rats the effect of thyroidectomy/parathyroidectomy (TPX) on the expression of the clock protein, PER2, in the suprachiasmatic nucleus (SCN), the master circadian clock, and in a number of key limbic forebrain structures, the oval nucleus of the bed nucleus of the stria terminalis (BNST-OV), the central nucleus of the amygdala (CEA), the basolateral amygdala (BLA) and the dentate gyrus (DG). TPX significantly altered the normal daily pattern of PER2 expression in the BNSTov and CEA, but had no effect on PER2 expression in the SCN, BLA and DG. Thus, although thyroid hormones modulate PER2 expression in the brain, the effect is tissue specific and therefore likely not to be mediated by a direct effect of the hormone on gene expression.

**Keywords:** Thyroid gland, Circadian rhythm, Clock genes, Suprachiasmatic nucleus, Basolateral amygdala, Dentate gyrus

Thyroid hormones appear to play a role in the regulation of behavioral and physiological circadian rhythms in mammals. This involvement is suggested by the evidence that surgical removal of the thyroid and parathyroid glands or chemical induction of hypothyroidism alters circadian locomotor activity rhythms and blunts the daily fluctuations in circulating corticosterone and prolactin levels in rodents [3, 18-21, 24]. How thyroid hormones might affect circadian rhythms is unknown. One possibility is that these hormones modulate the expression of clock genes implicated in the generation of circadian rhythms at the cellular level. To begin to study the involvement of thyroid hormones in the regulation of the expression of circadian clock genes, we examined the effect of thyroparathyroidectomy (TPX) on the rhythm of expression of the clock protein, PER2, in the suprachiasmatic nucleus (SCN), the master circadian clock, in rats. Furthermore, we assessed the effect of TPX on the expression of PER2 in several limbic forebrain structures shown to exhibit robust PER2 rhythms that are under the control of the SCN, the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), central nucleus of the amygdala (CEA), basolateral amygdala (BLA) and dentate gyrus (DG) [2, 17].

The experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee, Concordia University. A total of 12 thyroidectomized/parathyroidectomized (TPX) and 12 sham-operated (SHAM) male Sprague Dawley rats (Hsd:SD, 4 weeks old, 50-75g) were purchased from Harlan (Indianapolis, Indiana). They were housed two

per cage in a temperature- and humidity-controlled room under a normal 12h:12h light-dark (LD) cycle (300 lux at cage level) and had free access to food and water. Three weeks later they were transferred to individual cages housed in ventilated, sound and light tight isolation chambers and kept under a 12h:12h LD cycle with free access to food and water. One month later, the rats were injected with an overdose of sodium pentobarbital (~100 mg/kg) at one of two zeitgeber times (ZT1, ZT13; ZT0 denotes time of light on) and were perfused intracardially with 300 ml of cold saline (0.9% NaCl) followed by 300 ml of cold, 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3). ZT1 and ZT13 were chosen because they represent peak and trough expression times of PER2 protein in all brain regions studied. Following perfusion, brains were postfixed in 4% paraformaldehyde and stored at 4° C overnight. Serial coronal brain sections (50 µm) containing regions of interest (SCN, BNST, amygdala, hippocampus) were collected from each animal using a vibratome. Immunocytochemistry for PER2 was performed as previously described [17] using an affinity purified rabbit polyclonal antibody raised against PER2 (ADI, San Antonio, TX). Immunostaining was performed in two separate runs each containing brain sections from rats from the two time points (ZT1, ZT13) and treatment groups (TPX, Sham). Evidence for antibody specificity was obtained in blocking experiments performed previously in our laboratory by adding the PER2 peptide (1 mg/ml, diluted 1:100) to the primary incubation solution. The addition of the PER2 peptide prevented PER2 immunostaining staining in all regions of interest [2].

PER2-stained brain sections were examined under a light microscope and images were captured using a Sony XC-77 video camera, a Scion LG-3 frame grabber, and Image SXM software (v1.6, S D Barrett, <http://www.ImageSXM.org.uk>). All cells exhibiting strong nuclear PER2 immunostaining were counted manually on the captured images using a 400 x 400- $\mu\text{m}$  template for the SCN, BNSTov, CEA, BLA and 200 x 400- $\mu\text{m}$  template for the DG. For analysis, the mean number of PER2 immunoreactive cells per region was calculated for each animal from the counts of 6 unilateral images showing the highest number of labeled nuclei. Differences between groups were revealed with analysis of variance (ANOVA). Alpha level was set at 0.05 for all analyses.

Examples of PER2 expression in the SCN, BNSTov, CEA, BLA and DG of SHAM rats killed at ZT1 or ZT13, and graphs showing the effect of TPX on the levels of PER2 in each of these regions are shown in Fig. 1. In the SCN PER2 expression was high at ZT13 ( $F[1,20]=1552.6$ ,  $p<.0001$ ) and TPX had no effect on the overall level of expression ( $F[1,20]=1.76$ ,  $p=.198$ ). Similarly, as shown in Fig. 1, in both the BNSTov and CEA PER2 expression was high at ZT13 (BNSTov:  $F[1,20]=57.6$ ,  $p<.0001$ ; CEA:  $F[1,20]=36.63$ ,  $p<.0001$ ) and no significant main effect of treatment on the level of expression was noted (BNSTov:  $F[1,20]=.47$ ,  $p=.50$ ; CEA:  $F[1,20]=1.7$ ,  $p=.19$ ). However, a significant Treatment x Time interaction was observed in both the BNSTov ( $F[1,20]=11.07$ ,  $p<.005$ ) and CEA ( $F[1,20]=11.39$ ,  $p<.005$ ). Post-hoc analysis revealed a

significant increase in PER2 expression in the BNSTov and CEA of TPX rats at ZT1 (Tukey,  $p < .05$ ). In the BLA and DG PER2 expression peaked at ZT1 (BLA:  $F[1,20]=78.7$ ,  $p < .0001$ ; DG:  $F[1,20]=227.5$ ,  $p < .0001$ ). Furthermore, TPX had no effect on PER2 expression in BLA ( $F[1,20]=.4$ ,  $p = .534$ ) and DG ( $F[1,20]=.093$ ,  $p = .764$ ) and no significant Time x Treatment interaction was noted.

The results show that surgical removal of the thyroid and parathyroid glands alters the normal daily pattern of expression of the clock protein, PER2 in the BNSTov and CEA in rats. Specifically, TPX led to a significant increase in PER2 expression at ZT1, when PER2 expression in the BNSTov and CEA is normally low, and to a small decrease in expression at ZT13, when PER2 expression is normally high in these areas. These changes in PER2 expression may reflect a blunting effect of TPX on the rhythm of PER2 expression in these areas [2, 17]. Alternatively, it is possible that TPX changed the phase of peak expression of PER2 and/or the waveform of expression (e.g., duration of peak expression) without affecting the amplitude of expression. The research design employed in this study in which the expression of PER2 was assessed only at two time points representing the normal peak and trough of PER2 expression (i.e., ZT1 and ZT13) does not allow for a definitive distinction between these alternatives. Contrary to the change in PER2 expression in the BNSTov and CEA, the expression of PER2 in the SCN, BLA and DG was not affected, demonstrating that the effect of removal of the thyroid and parathyroid glands on PER2 expression is region specific.

The mechanism whereby thyroid hormones might selectively affect PER2 in the BNSTov and CEA remains to be determined. Thyroid hormones such as thyroxine (T4) and triiodothyronine (T3) might affect PER2 expression by directly or indirectly modulating the transcription of the Per2 gene. Such a mechanism, however, is unlikely to explain the lack of effect of TPX on PER2 expression in the SCN, BLA and DG. Rather, the selective effect of TPX on PER2 expression in the BNSTov and CEA may be related to findings that TPX blunts the daily rhythm of plasma corticosterone levels [11, 20, 21, 26]. Indeed, we have shown previously that the rhythm of expression of PER2 in the BNSTov and CEA, but not in the SCN, BLA and DG is controlled by the glucocorticoid hormone, corticosterone. Specifically, we found that surgical removal of the adrenal glands selectively abolished the daily rhythm of PER2 expression in the BNSTov and CEA [2, 17] and that treatment that restored the daily rhythm of circulating corticosterone restored PER2 rhythms in these brain regions [25]. Finally, an effect of TPX on neurotransmitters and peptides such as dopamine and corticotropin-releasing hormone, that might be involved in the regulation of PER2 expression in the BNSTov and CEA could be envisioned [22, 23, 27, 29].

The release of thyroid hormones into the circulation exhibits a circadian rhythm [13, 14], and previous studies have shown that surgical removal of the thyroid and parathyroid glands shortens the free running period of locomotor activity rhythms in rats [18, 19]. Such results support the hypothesis that fluctuating levels of thyroid hormones modulate circadian rhythmicity by

modulating the expression of clock genes in the SCN. The present finding that TPX had no effect on the rhythm of expression of PER2 in the SCN suggests that the effect of circulating thyroid hormones on circadian period is independent of an effect on the expression of clock genes in the SCN and is more likely mediated downstream from the SCN oscillator.

Finally, the present results show that TPX affects PER2 expression in two limbic forebrain structures, the BNST-OV and CEA, known to play key roles in regulation of emotional states and the control of physiological and endocrine responses to stress [1, 4, 5, 8, 10]. These results may be relevant, therefore, to the observation that, in humans, abnormalities of thyroid function are often associated with emotional disturbances and depressive illness [6, 7, 9, 12], and with the observation that such affective disorders are commonly associated with disruptions of circadian rhythms [15, 16, 28].



## References

- 1 Alheid, G.F., Extended amygdala and basal forebrain, *Ann N Y Acad Sci*, 985 (2003) 185-205.
- 2 Amir, S., Lamont, E.W., Robinson, B. and Stewart, J., A circadian rhythm in the expression of PERIOD2 protein reveals a novel SCN-controlled oscillator in the oval nucleus of the bed nucleus of the stria terminalis, *J Neurosci*, 24 (2004) 781-90.
- 3 Beasley, L.J. and Nelson, R.J., Thyroid gland influences the period of hamster circadian oscillations, *Experientia*, 38 (1982) 870-1.
- 4 Cardinal, R.N., Parkinson, J.A., Hall, J. and Everitt, B.J., Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex, *Neurosci Biobehav Rev*, 26 (2002) 321-52.
- 5 Cassell, M.D., Freedman, L.J. and Shi, C., The intrinsic organization of the central extended amygdala, *Ann N Y Acad Sci*, 877 (1999) 217-41.
- 6 Chakrabarti, K., Singh, P.M. and Joshi, S.P., Thyroid function in depression, *Nepal Med Coll J*, 8 (2006) 47-8.
- 7 Cowdry, R.W., Wehr, T.A., Zis, A.P. and Goodwin, F.K., Thyroid abnormalities associated with rapid-cycling bipolar illness, *Arch Gen Psychiatry*, 40 (1983) 414-20.
- 8 Davis, M. and Shi, C., The extended amygdala: are the central nucleus of the amygdala and the bed nucleus of the stria terminalis differentially involved in fear versus anxiety?, *Ann N Y Acad Sci*, 877 (1999) 281-91.

- 9 Denicoff, K.D., Joffe, R.T., Lakshmanan, M.C., Robbins, J. and Rubinow, D.R., Neuropsychiatric manifestations of altered thyroid state, *Am J Psychiatry*, 147 (1990) 94-9.
- 10 Dong, H.W., Petrovich, G.D., Watts, A.G. and Swanson, L.W., Basic organization of projections from the oval and fusiform nuclei of the bed nuclei of the stria terminalis in adult rat brain, *J Comp Neurol*, 436 (2001) 430-55.
- 11 Dunn, J.D. and Hess, M.E., Pituitary-adrenal function in thyroparathyroidectomized male and female rats, *J Endocrinol*, 71 (1976) 47-50.
- 12 Jackson, I.M., The thyroid axis and depression, *Thyroid*, 8 (1998) 951-6.
- 13 Kalsbeek, A., Buijs, R.M., van Schaik, R., Kaptein, E., Visser, T.J., Doulabi, B.Z. and Fliers, E., Daily variations in type II iodothyronine deiodinase activity in the rat brain as controlled by the biological clock, *Endocrinology*, 146 (2005) 1418-27.
- 14 Kalsbeek, A., Fliers, E., Franke, A.N., Wortel, J. and Buijs, R.M., Functional connections between the suprachiasmatic nucleus and the thyroid gland as revealed by lesioning and viral tracing techniques in the rat, *Endocrinology*, 141 (2000) 3832-41.
- 15 Kramer, B.A. and Katz, J.L., Circadian temperature variation and depressive illness, *J Clin Psychiatry*, 39 (1978) 439-44.
- 16 Kripke, D.F., Mullaney, D.J., Atkinson, M. and Wolf, S., Circadian rhythm disorders in manic-depressives, *Biol Psychiatry*, 13 (1978) 335-51.

- 17 Lamont, E.W., Robinson, B., Stewart, J. and Amir, S., The central and basolateral nuclei of the amygdala exhibit opposite diurnal rhythms of expression of the clock protein Period2, *Proc Natl Acad Sci U S A*, 102 (2005) 4180-4.
- 18 McEachron, D.L., Lauchlan, C.L. and Midgley, D.E., Effects of thyroxine and thyroparathyroidectomy on circadian wheel running in rats, *Pharmacol Biochem Behav*, 46 (1993) 243-9.
- 19 McEachron, D.L., Levine, J. and Adler, N.T., Evidence that the pacemaker controlling activity rhythms is shortened in male thyroparathyroidectomized (TPX) rats: similarities to the effects of estradiol in females, *Prog Clin Biol Res*, 341B (1990) 13-24.
- 20 Murakami, N., Hayafuji, C. and Takahashi, K., Thyroid hormone maintains normal circadian rhythm of blood corticosterone levels in the rat by restoring the release and synthesis of ACTH after thyroidectomy, *Acta Endocrinol (Copenh)*, 107 (1984) 519-24.
- 21 Ottenweller, J.E. and Hedge, G.A., Thyroid hormones are required for daily rhythms of plasma corticosterone and prolactin concentration, *Life Sci*, 28 (1981) 1033-40.
- 22 Peterson, A.L., Gilman, T.L., Banks, M.L. and Sprague, J.E., Hypothyroidism alters striatal dopamine release mediated by 3,4-methylenedioxymethamphetamine (MDMA, ecstasy), *Synapse*, 59 (2006) 317-9.

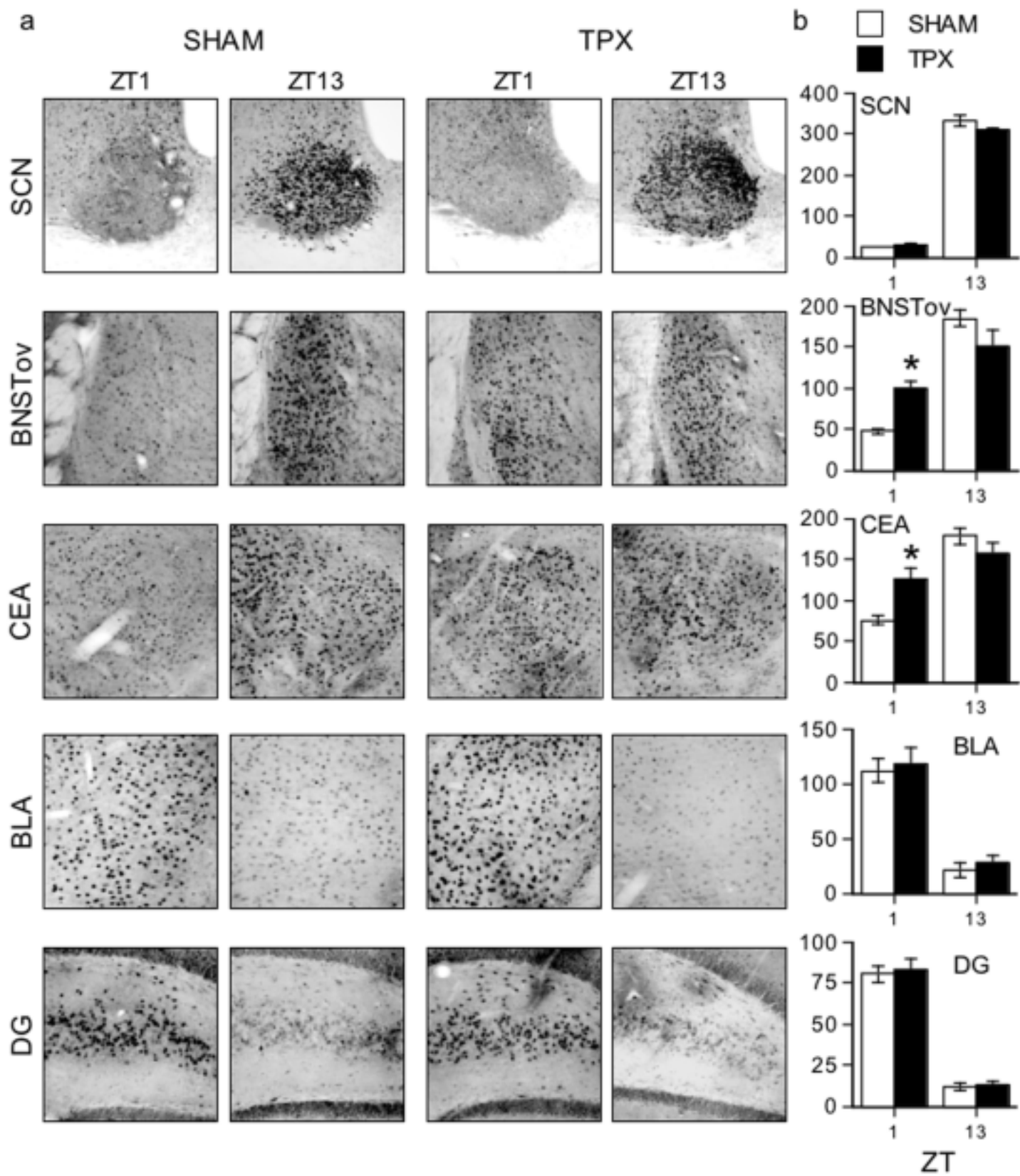
- 23 Reymond, M.J., Benotto, W. and Lemarchand-Beraud, T., The secretory activity of the tuberoinfundibular dopaminergic neurons is modulated by the thyroid status in the adult rat: consequence on prolactin secretion, *Neuroendocrinology*, 46 (1987) 62-8.
- 24 Schull, J., McEachron, D.L., Adler, N.T., Fiedler, L., Horvitz, J., Noyes, A., Olson, M. and Shack, J., Effects of thyroidectomy, parathyroidectomy and lithium on circadian wheelrunning in rats, *Physiol Behav*, 42 (1988) 33-9.
- 25 Segall, L.A., Perrin, J.S., Walker, C.D., Stewart, J. and Amir, S., Glucocorticoid rhythms control the rhythm of expression of the clock protein, *Period2*, in oval nucleus of the bed nucleus of the stria terminalis and central nucleus of the amygdala in rats, *Neuroscience*, 140 (2006) 753-7.
- 26 Takeuchi, A., Suzuki, M. and Tsuchiya, S., Effect of thyroidectomy on the secretory profiles of growth hormone, thyrotropin and corticosterone in the rat, *Endocrinol Jpn*, 25 (1978) 381-90.
- 27 Wang, P.S., Huang, S.W., Tung, Y.F., Pu, H.F., Tsai, S.C., Lau, C.P., Chien, E.J. and Chien, C.H., Interrelationship between thyroxine and estradiol on the secretion of thyrotropin-releasing hormone and dopamine into hypophysial portal blood in ovariectomized-thyroidectomized rats, *Neuroendocrinology*, 59 (1994) 202-7.
- 28 Wehr, T.A., Sack, D., Rosenthal, N., Duncan, W. and Gillin, J.C., Circadian rhythm disturbances in manic-depressive illness, *Fed Proc*, 42 (1983) 2809-14.

- 29 Yilmazer-Hanke, D.M., Hantsch, M., Hanke, J., Schulz, C., Faber-Zuschratter, H. and Schwegler, H., Neonatal thyroxine treatment: changes in the number of corticotropin-releasing-factor (CRF) and neuropeptide Y (NPY) containing neurons and density of tyrosine hydroxylase positive fibers (TH) in the amygdala correlate with anxiety-related behavior of wistar rats, *Neuroscience*, 124 (2004) 283-97.

Fig. 1

a: Photomicrographs showing examples of PER2 expression in the SCN, BNSTov, CEA, BLA and DG of sham-operated (SHAM) and thyroidectomized/parathyroidectomized (TPX) rats killed at ZT1 or ZT13 (magnification=x20). b: Graphs showing the mean ( $\pm$ s.e.m.) number of PER2-immunoreactive nuclei as a function of zeitgeber time (ZT) in each of these regions in SHAM (n=6/time point) and TPX (n=6/time point) rats. Asterisks indicate significant difference from the corresponding SHAM group ( $p < .05$ ).

Figure



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Thyroidectomy alters the daily pattern of expression of the clock protein, PER2, in the oval nucleus of the bed nucleus of the stria terminalis and central nucleus of the amygdala in rats

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Details of revision

### **Reviewer #1**

We agree with the reviewer that the argument concerning the involvement of REV-ERB and ROR as possible targets of thyroid hormones' effect on clock gene expression is not sufficiently supported in the literature, and we thank him for pointing out the source of the confusion. In our revised manuscript we have taken out all the text on this issue (from the abstract, introduction and discussion sections).

### **Reviewer#2**

We addressed all the comments of this reviewer by adding the appropriate information. Specifically, in response to comment about antibody specificity we include a statement on our published 'blocking experiments' using the PER2 peptide; furthermore, we indicate in the methods sections that each of the two immunocytochemical assays we performed contained brain sections from rats from the two time points and conditions (Sham, TPX); we elaborate more on the counting procedure (all cells strongly immunoreactive to PER2 within region of interest are counted using a fixed size templates); finally, as recommended by the reviewer, in Fig. 1 we add a set of images from each of the regions under study taken from TPX rats killed at ZT1 or ZT13.