Circadian regulation of the mouse *presenilin-2* gene: how is the molecular clockwork involved?

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TABLE OF CONTENT

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RÉSUMÉ

Plusieurs études indiquent que le gène de la *préséniline-2* est régulé de façon circadienne, notamment dans les noyaux suprachisamatiques (SCN), siège de l'horloge biologique principale, ainsi que dans le foie chez la souris. Nous avons entrepris de déterminer le mécanisme à l'origine de cette oscillation circadienne. CLOCK et BMAL1 sont les deux facteurs de transcription principaux de l'horloge circadienne. Nous avons démontré qu'ils exercent une certaine influence, toutefois partielle, sur l'expression du gène *préséniline-2 in vitro*. Nous avons déterminé par des études *in vivo* chez la souris que l'ARNm de *préséniline-2* n'oscille pas dans plusieurs tissus périphériques, notamment le muscle, la rate, le thymus, le cœur et le rein. Toutefois, nous avons démontré une oscillation dans le foie. Au niveau de l'oscillation de *préséniline-2* dans le foie, nous soupçonnons un autre mécanisme post-transcriptionnel d'agir en coopération avec le mécanisme transcriptionnel géré par CLOCK et BMAL1. En effet, nous avons détecté l'absence d'oscillation du transcrit entier, mais une oscillation évidente de formes écourtées de l'ARNm, probablement obtenues par épissage alternatif de l'ARNm.

ABSTRACT

Several evidence indicate that *presenilin-2* gene is rhythmically regulated in the mouse suprachiasmatic nuclei (SCN), which is the master circadian clock in mammals, as well as in the liver. We investigated the mechanism of circadian modulation behind *presenilin-2* oscillation. CLOCK and BMAL1 are the two major transcription factors for circadian gene expression. We demonstrated that CLOCK and BMAL1 partially modulate *presenilin-2* gene expression *in vitro*. *In vivo*, we showed that the gene does not oscillate in the muscle, spleen, thymus, heart and kidney. On the other hand, we demonstrated a circadian oscillation of *presenilin-2* in the liver. We suspect another posttranscriptional mechanism to act in cooperation with the transcriptional mechanism regulated by CLOCK/BMAL1 for the circadian modulation of presenilin-2. We showed that the full-length transcript of *presenilin-2* does not oscillate in the liver, but shorter transcripts, potentially issued from alternative spliced mRNA, do oscillate.

1. INTRODUCTION

1.1 Circadian rhythms

Circadian rhythms, which consist in physiological events that occur every 24 hours, are observed in many aspects of life (Dunlap et al., 2004). Behavioural events such as the sleep/wake cycle or the daily food intake, as well as basic cellular functions such as metabolism (Turek et al., 2005), neuron firing (Welsh et al., 1995) or hormone synthesis and release (Buijs and Kalsbeek, 2001) can be regulated in a circadian fashion. Circadian rhythms are generated in the brain by the suprachiasmatic nuclei (SCN) of the hypothalamus. These small brain structures are located bilaterally to the third ventricle and dorsally to the optic chiasms in mammals (Fig. 1). The SCN potentially act as a master clock or a pacemaker generating circadian rhythmicity for the whole organism as shown by lesion-graft experiments. Indeed, SCN graft in lesioned animals (Ralph et al., 1990) or various clock mutant animals (Sujino et al., 2003) restores the circadian behavioural rhythmicity as monitored by locomotor activity. Also, the restored rhythms are representative of the donor genotype, further giving credit to the hypothesis of the hierarchical dominance of the SCN on the peripheral oscillators (Sujino et al., 2003). Indeed, circadian clocks also exist in different organs such as the liver and the heart for example.

The SCN receive direct neural input from the retina through the retinohypothalamic tract (RHT) (Hendrickson et al., 1972; Moore and Lenn, 1972). The neural projections originate from a particular subset of retinal ganglion cells that have been described as the

circadian photoreceptors. These neurons produce a particular photopigment called melanopsin (Berson et al., 2002). Direct input of the light in the SCN resets and synchronizes the biological rhythms to the environment in order for the organism to adapt properly (Morse and Sassone-Corsi, 2002). The light/dark cycle is the most powerful synchronizer (or Zeitgeber) of the biological clock. Other non-photic Zeitgebers, like the temperature or the schedule of food intake, are also able to influence the circadian clock.

Monitoring the firing pattern in SCN neurons has shown that each cell is individually able to oscillate in a circadian manner (Welsh et al., 1995). Indeed, each neuron exhibits an independent circadian oscillation with its own period. The period is defined as the time between the beginning and the end of a cycle. Despite heterogeneity in the period of SCN neurons, the output of the SCN is homogenous because the SCN can be synchronized by the photic and non-photic Zeitgebers mentioned earlier (Yamaguchi et al., 2003). These studies demonstrated that SCN neurons are themselves autonomous circadian oscillators.

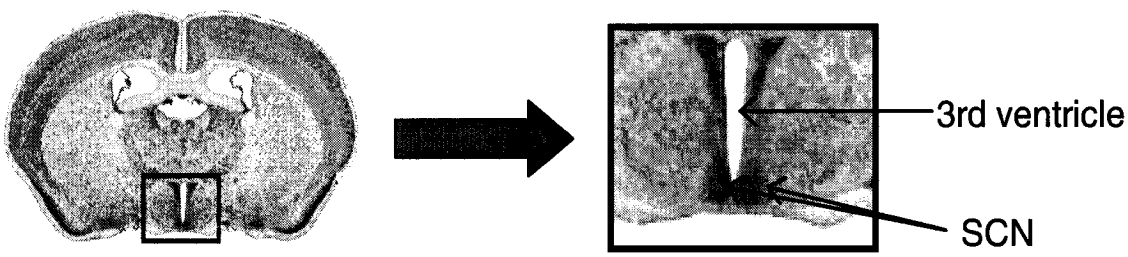

FIG.1: Suprachiasmatic nuclei (SCN) of a mouse brain. The two nuclei are part of the hypothalamus. They are located bilaterally to the third ventricle, between the two optic chiasms. These neurones receive direct neural projections from the retina. Thus, light perception is very important in the circadian rhythms and the SCN can be entrained or influenced by the environment. (SOURCE: High Resolution Mouse Atlas Brain, available on the web: http://www.hms.harvard.edu/research/brain/atlas.html)

1.2 Mammalian molecular circadian clockwork

In mammals, the molecular chronobiological research started when Takahashi and colleagues identified mice with abnormal circadian behaviour (Vitaterna et al., 1994). Indeed they identified, by random mutagenesis, a gene that was important for the endogenous period length and the persistence of the locomotor rhythmicity under constant conditions of darkness. This gene was named *Clock*. Further research aimed to characterize this novel gene and its product. The protein CLOCK was classified as a member of the bHLH-PAS transcription factor family (King et al., 1997). This kind of transcription factors is known to function with a partner, so the next step was to identify CLOCK's partner. This has been accomplished by Hogenesch and colleagues in 1998 when they showed that BMAL1 was not only able to heterodimerize with CLOCK, but also that the complex was transcriptionally active through the recognition of a specific DNA sequence called E-box, found in the promoter region of target genes (Hogenesch et al., 1998). Meanwhile, other important genes for the normal circadian behaviour in mice were identified, namely the *Period* genes: *Per1* (Tei et al., 1997), *Per2* (Albrecht et al., 1997; Shearman et al., 1997) and *Per3* (Takumi et al., 1998; Zylka et al., 1998). Interestingly, it was demonstrated that the heterodimer complex of CLOCK/BMAL1 could drive the rhythmic expression of *Per* genes expression (Gekakis et al., 1998). A molecular clockwork mechanism started to emerge after all these years in order to understand circadian regulation (Fig. 2). The discovery of two other clock genes, *Cryptochrome 1* (*Cry 1*) and *Cryptochrome 2* (*Cry 2*), added fresh information in the molecular clockwork (Kobayashi et al., 1998; Vitaterna et al., 1999). Mutants for *Cry* showed different circadian phenotypes. Indeed, Cry proteins were shown to be involved

in the maintenance of the period length and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ double mutants showed total loss of rhythmicity in constant darkness conditions, thus showing that these proteins are essential components for the molecular clockwork (van der Horst et al., 1999; Vitaterna et al., 1999). CRY proteins were then shown to be negative regulators of the biological clock (Kume et al., 1999). Indeed, they were shown to be potent inhibitors of CLOCK/BMAL1 transcriptional activity (without preventing the formation of the complex). Furthermore, CRYs were shown to mediate the subcellular translocation of PER proteins from the cytosol to the nucleus via the formation of heterodimer complexes (Kume et al., 1999; Yagita et al., 2000; Yagita et al., 2002). Another important gene for the mammalian circadian clockwork, *Rev-REBα*, was found to be a negative transcriptional regulator of *Bmal1* gene expression (Preitner et al., 2002). Together, PER, CRY and Rev-ERBα proteins constitute a negative regulatory feedback loop, which counteract the positive regulatory loop made of CLOCK and BMAL1 (Fig. 2). In summary, all the above-mentioned genes concretely interact together in complex intertwined regulatory feedback loops, stimulating or inhibiting each other expression (mechanism schematized and described in Fig. 2). This molecular clockwork is found in SCN neurons as well as in any other cell types (cells from the liver, the heart, fibroblasts, etc.).

Also important in the molecular clockwork are the posttranslational modifications of the clock proteins that help to generate the 24-hour oscillation. Indeed, phosphorylation is one major modification that is important for PER, CRY and BMAL1 proteins, regulating localisation and stability in the cytoplasm as well as in the nucleus (Lee et al., 2001; Eide

et al., 2002; Lee et al., 2004). Thus, kinases also play a critical role in the circadian rhythmicity and mutants in the phosphorylation mechanism show alteration in their period, both in mice and humans (Lowrey et al., 2000; Eide et al., 2005a; Xu et al., 2005).

In parallel, the specific function of each clock protein has been extensively studied. For example, the three *Period* genes were shown to have different functions within the molecular clockwork. The different mutants generated showed specific circadian phenotypes: *Per1* disruption leads to a shortened period (Cermakian et al., 2001; Zheng et al., 2001) whereas *Per2* disruption presented arrhythmicity (Zheng et al., 1999) and no particular phenotype in the circadian behaviour was observed in *Per3*-disrupted mutant mice (Shearman et al., 2000). Consistent with the variable severity of the gene disruption, the three PER were shown to have distinct functions: PER1 would be more likely implicated at the level of posttranscriptional modulation of other circadian proteins and PER2 would be a positive regulator of the positive limb of the oscillator. Finally, as suggested by its weak mutant phenotype, PER3 function would be more likely outside of the core molecular clockwork, functioning as an output signal of the circadian clock (Bae et al., 2001). Many other rhythmically expressed genes function as output signals of the circadian clock. They are designated as «clock-controlled genes» and they either can be directly regulated by CLOCK/BMAL1 through E-box mediated transcription (Jin et al., 1999) or indirectly by several other mechanisms as discussed below.

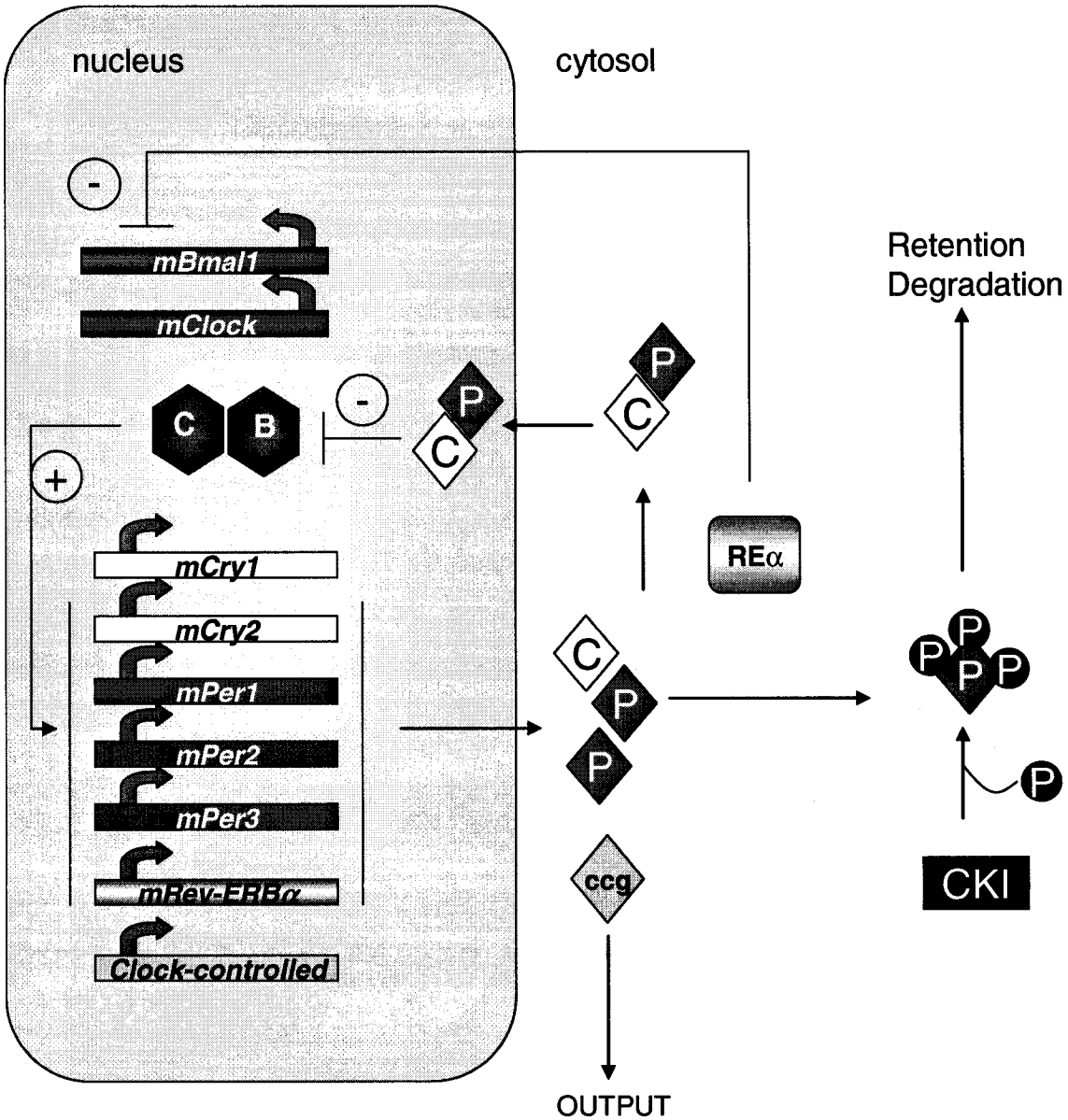

FIG.2: Molecular regulation of circadian rhythms in mammals. As describe in the text, CLOCK/BMAL1 heterodimer rhythmically induces the expression of clock genes, namely *Period 1, 2* and *3* (P, diamond), *Cryptochrome 1, 2* (C, diamond) and *Rev-ERBα* (REα, square). These genes generate regulatory feedback loops: *Pers* and *Crys* heterodimerize and repress their own transcription by inhibiting CLOCK/BMAL1 activity and Rev-ERBα represses *Bmal1* expression. All the steps shown above, including the posttranslational modification (phosphorypaltion (P, circles) by Casein Kinase I (CKI, square)) take approximately 24 hours to occur, thus completing a circadian oscillation. (Adapted from (Cermakian and Sassone-Corsi, 2000)

The molecular mechanism discussed above allows the setting of endogenous circadian rhythms. Indeed, when put under constant darkness conditions, mice still present clear and regular circadian behaviour with the slight difference that the period is a little less than 24 hours, which is representative of the endogenous free-running period (Fig. 3). As mentioned earlier, Zeitgebers such as light/dark cycle are important for the synchronization of the biological rhythms. With the discovery of the molecular clockwork, it was demonstrated that the Zeitgebers were important but not necessary for the good functioning of the circadian clock.

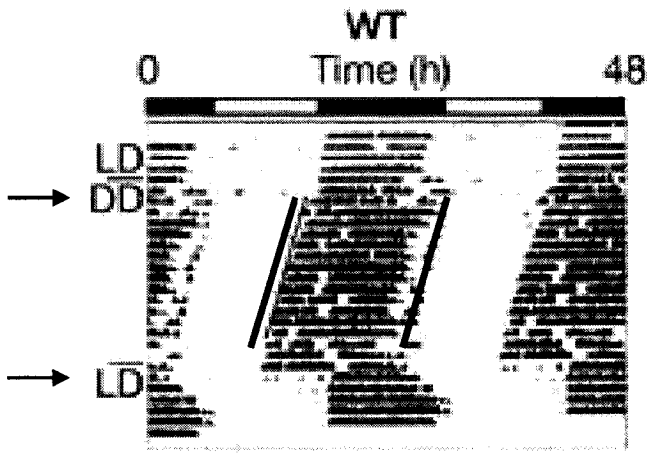

FIG. 3: Typical double-plotted actogram measuring circadian locomotor activity of a wild-type mouse. Mice are put in cages equipped with running wheels. Each line represents a day of recording and within each line, little black bars represent a bout of activity recorded. At the top of the figure, white and black bars represent respectively light phase (day) and dark phase (night) of the day. As shown, the activity is consolidated during the night, as mice are nocturnal animals. Also, their activity follows the environmental cues and their period is adapted to the environment. However, when put into constant darkness conditions (arrow, DD), they are in «free-run» and we can see that their endogenous period is slightly shorter than 24 hours, as they begin to be active a little bit earlier every day. When put back into light:dark conditions (LD), mice are re-entrained to the environment and their activity is consolidated again during the light-off phase. Wheel-running monitoring is a powerful tool to study the circadian behaviour of mutant mice.

1.3 Direct circadian modulation: transcriptional regulation

CLOCK and BMAL1 proteins are transcriptional activators. They are members of the bHLH-PAS protein family (Kewley et al., 2004). The bHLH motif and the PAS domain allow the two proteins to dimerize and bind DNA to activate the transcription of target genes. As shown in Figure 2, the CLOCK/BMAL1 complex induces the rhythmic expression of clock genes such as *Per1, 2* and *3, Cry 1* and *2, Rev-ERBα* and other genes called «clock-controlled genes». The induction of gene expression by CLOCK/BMAL1 is done via a specific DNA recognition sequence called an E-box. Three types of E-box exist: a canonical CACGTG E-box, which is preferred by the CLOCK/BMAL1 complex (Hogenesch et al., 1998) and two non-canonical E-boxes: 1- CACGTT, which was recently shown to drive *mPer2* circadian expression (Yoo et al., 2005) and 2- CANNTG, which is generally recognized by bHLH factors (Beltran et al., 2004). The circadian transcriptional regulation is very complex and not yet fully understood. In order to increase the specificity of E-box recognition by CLOCK/BMAL1, some have suggested that not only the E-box itself is important for circadian regulation, but also the sequences flanking the E-box are also important (Hogenesch et al., 1998). Specific base pairs strategically positioned around the E-box could enhance or decrease the binding affinity of bHLH proteins (Beltran et al., 2004). Despite all the findings and the numerous discussions about the E-box (Kyriacou and Rosato, 2000; Munoz et al., 2002; Hardin, 2004), no «enhanced E-box» nor specific flanking sequences apart from the canonical E-box have been identified so far to be ideal for CLOCK/BMAL1 binding and further circadian induction.

Clock-controlled genes are regulated in a circadian fashion but are not implicated in the molecular clockwork itself as the clock genes are. However, they oscillate in a circadian fashion in order to generate circadian behaviours or physiological events. They are, as suggested for PER3, outputs of the circadian clockwork. Products of clock-controlled genes include some transcription factors, metabolic enzymes, neuropeptides, signalling molecules and many other types of molecules. Some clock-controlled genes possess E-boxes in their 5'flanking region, but because an hexamer such as an E-box is statistically found every 4kb in the genome, it is impossible to conceive that they are all functional and responsive to CLOCK/BMAL1 (Munoz et al., 2002).

1.4 Indirect circadian modulation

The direct transcriptional circadian regulation by CLOCK/BMAL1, as discussed above, is not the only way of circadian modulation; indirect forms of regulation also exist. First, CLOCK/BMAL1 can cyclically induce the expression of other transcription factors. This way, the target genes of these clock-controlled transcription factors also oscillate in a circadian manner (Yamaguchi et al., 2000). Second, posttranscriptional modifications of mRNAs can generate oscillating transcripts, without any circadian transcriptional regulation of the gene (Harms et al., 2004). Among all the possible posttranscriptional modifications, alternative splicing is particularly interesting. Indeed, alternative splicing is known to produce tissue or cell-specific alternative proteins (Alberts et al., 2002). Together with circadian transcriptional regulation, alternative splicing could contribute to the tissue-specificity of circadian modulation observed in peripheral clocks. The effects of alternative splicing on protein functions go from very subtle modifications to total loss

of function or gain of new functions. Indeed, alternative splicing may affect the binding properties of a protein, its subcellular localization, its enzymatic activity or its stability (Stamm et al., 2005).

Finally, circadian rhythmicity can be generated by posttranslational modifications (Harms et al., 2004). Such posttranslational modifications include most importantly phosphorylation/dephosphorylation. Phosphorylation has been associated to the determination of subcellular localization and to the stability of clock proteins. In addition, phosphorylation is critical to the development of the 24-hour circuit of the molecular clockwork. Indeed, the core clock proteins, PERs, CLOCK and BMAL1 in the mouse liver for example, have been shown to be rhythmically phosphorylated (Lee et al., 2001).

1.5 The Presenilins

Presenilins were discovered in 1995 (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995). Two proteins belong to that family: presenilin-1 (PS1) and presenilin-2 (PS2). They share 63% of identity in their amino acid sequence (De Strooper et al., 1997). Presenilins are transmembrane proteins. However, the number of transmembrane domains and the membrane topology of the proteins still remain very controversial. Different experimental approaches (fusion protein, chimeric protein and immunofluorescence) with different levels of reliability were used to determine the membrane topology of presenilins. Based on these studies, several models containing 6

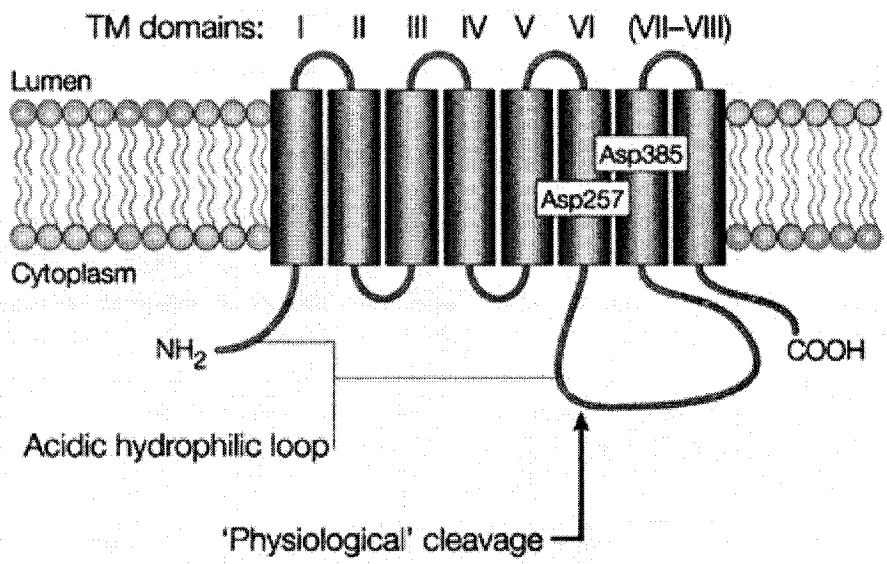

FIG. 4: Presenilin proteins topology. This represents the most widely accepted model of presenilin topology, with 8 transmembrane domains. Many mutations of *presenilin-1*, and a few mutations of *presenilin-2* are found in the transmembrane domains. They all lead to the early onset of Familial Alzheimer's Disease. Presenilins also perform «regulated intramembrane proteolysis», regulate calcium levels in neurons, participate to apoptosis and act as scaffold protein to facilitate phosphorylation of target proteins (Sisodia and St George-Hyslop, 2002).

(Doan et al., 1996; Lehmann et al., 1997), 7 (Dewji and Singer, 1997) or 8 (Doan et al., 1996) transmembrane domains have been proposed. Although the 8 transmembrane domains model is the most widely accepted model (Fig.4), the exact topology of presenilins has not been established yet.

Presenilins are polytopic proteins: they have multiple functions associated with many different cellular processes. The principal role of presenilin was proposed upon their discovery via genetic researchers whose aim was to determine the defective gene transmitted in familial cases of Alzheimer's Disease (FAD). More than 80 different point mutations in the amino acid sequence of Presenilin-1 and 7 mutations of Presenilin-2 lead to the early onset of FAD (Tandon and Fraser, 2002). At the molecular level, the consequence of these mutations is translated in an increase of $A\beta_{42}$ production (issued from the amyloid precursor protein (APP) cleavage) and deposition of this neurotoxic molecule into senile plaque in the brain tissue (Sisodia and St George-Hyslop, 2002). Presenilins are thought to be the core component of γ-secretase, the enzymatic complex that cleaves APP. Indeed, some studies demonstrated that presenilins intrinsically possess the γ-secretase activity (Wolfe et al., 1999; Edbauer et al., 2003). The presenilins have been described as a novel class of aspartyl proteases with the very uncommon characteristic of generating intramembraneous cleavage (Steiner and Haass, 2000). These studies led to the discovery of other presenilin substrates and broadened the field of action of these proteins. Indeed, it is now known that presenilins modulate intramembraneous cleavage of many Type I membrane proteins such as DCC, Notch, CD44, p75, N- and E-cadherin, etc. (Thinakaran and Parent, 2004). In the majority of the

cases, regulated intramembraneous proteolysis (RIP) by presenilins releases an intracellular domain (ICD) from the membrane proteins they cleave and these ICD then acts as signalling molecules.

Many other functions for presenilins have been proposed. First, mutations of presenilin are associated with a dysregulation of intracellular calcium homeostasis (Leissring et al., 1999; Leissring et al., 2000; Leissring et al., 2001; Akbari et al., 2004). As we know, calcium equilibrium is crucial for many biological basic functions, particularly in neurons. Second, the presenilins are thought to regulate to some extent programmed cell death in neurons (Xie et al., 2004). Indeed, endoproteolytic cleavage of Presenilin-2 generates a C-terminal fragment known to be anti-apoptotic. However, on a FAD mutation background, the C-terminal fragment has pro-apoptotic properties (Popescu and Ankarcrona, 2004). Finally, it was shown that presenilins facilitate β-catenin protein turnover by acting as a scaffold protein between β-catenin and GSK-3β kinase (Kang et al., 2002). This represents an interesting function for the circadian field of research because phosphorylation is very important for clock protein stability and subcellular localization. We could easily speculate that presenilins could potentially couple clock proteins and kinases to facilitate phosphorylation as they do for β-catenin.

2. RATIONALE

The project presented here focuses on *presenilin-2*. We decided to work specifically on that particular gene for many reasons. First, one group detected a circadian oscillation of *presenilin-2* mRNA in the mouse liver (Kornmann et al., 2001). One year later, three other research groups detected *presenilin-2* circadian oscillation by microarray analysis in the mouse SCN and liver (Panda et al., 2002b; Storch et al., 2002; Ueda et al., 2002). From web-based data associated with Panda *et al.* study, we were able to reconstitute *presenilin-2* circadian curve (Fig. 5) and we discovered that *presenilin-2* in the mouse SCN oscillates with a phase similar to that of *Per1*, a known clock gene. This suggested that both genes might be regulated by the same machinery, that is, directly through CLOCK/BMAL1 induction. Moreover, by analysing the 5'-flanking region of mouse *presenilin-2* gene, we found many E-boxes including one canonical E-box (-205 to -199 from the initiation codon), the DNA recognition element of CLOCK/BMAL1 complex, further suggesting that *presenilin-2* might be directly regulated by the circadian clock. The aim of this project was to investigate the mechanism of circadian modulation of *presenilin-2* in the mouse SCN and peripheral tissues.

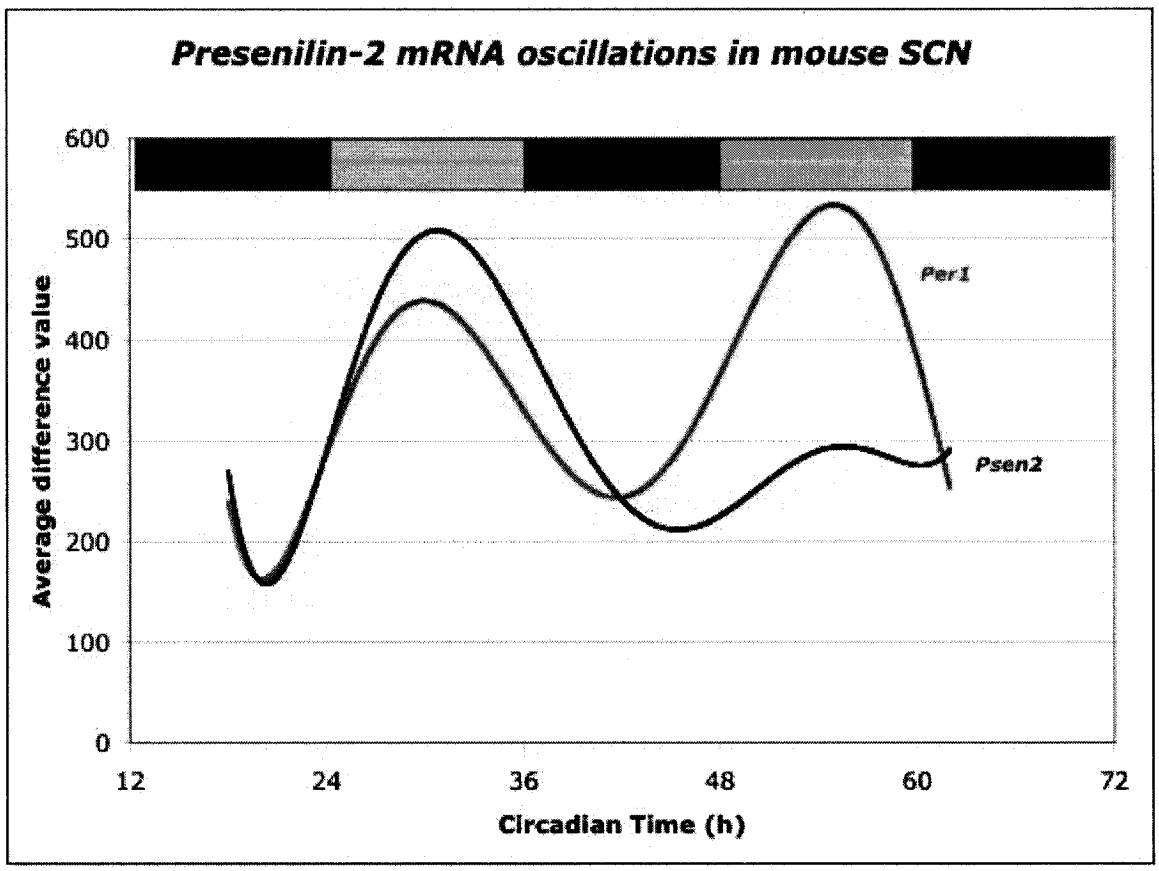

FIG. 5: Circadian oscillation of *presenilin-2* mRNA in the mouse SCN. According to Panda and colleagues web database, *presenilin-2* mRNA level peaks at the same circadian time as *Period 1*. This suggests that both genes might be regulated by the same machinery, namely directly by the core-clock transcription factors CLOCK/BMAL1, at least in the SCN ((Panda et al., 2002b), http://expression.gnf.org/cgi-bin/circadian/index.cgi).

3. MATERIAL AND METHODS

3.1 Cell culture

COS-7 cells were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin (Gibco), 100 µg/µL streptomycin (Gibco) and 2 mM L-glutamine (Gibco). Plates were kept in an environment containing 5% CO_2 at 37°C.

3.2 Whole-cell protein extraction

Cells were plated in 6-well plates. When they reached 90-95% confluence, they were transiently transfected with 4 µg of FLAG-tagged *Clock* and/or MYC-tagged *Bmal1* expression vectors (Travnickova-Bendova et al., 2002) using Lipofectamine 2000 (Invitrogen). PBluescript vector (Invitrogen) was co-transfected with the above-mentioned vectors acting as carrier DNA to facilitate transfection. Approximately 48 hours later, cells were washed with cold 1X PBS and resuspended in 1 mL of cold 1X PBS. Cells were transferred in microtubes and centrifuged at 13 000 rpm for 15 seconds. 300 µL of lysis buffer (stock lysis buffer diluted 3 times (stock lysis buffer 3X: 30 mM HEPES, 0.3 mM EDTA, 150 mM NaCl, 150 mM KCl, 15 mM $MgCl_2$, 12 mM spermidine, 6 mM DTT and 52.5%glycerol), 1/100 volume of NP-40 (Amersham), 0.5 mM PMSF (Roche) and anti-protease cocktail (complete-mini) 0.1X (Roche)) were added to the cell pellet. Cells were lysed for 30 minutes on ice, vortexed for 10 seconds and centrifuged for 2 minutes at 13 000 rpm. The supernatant was stored at -80°C.

3.3 Gel Shift assay

DNA probes were designed to represent the consensus E-box of the 5'-flanking region of the mouse *presenilin-2* gene and its surrounding region (position -210 to -194 from the initiation codon). Equimolar quantities of two complementary DNA oligonucleotides were mixed and heated for 5 minutes at 85°C and then cooled down to room temperature to allow the annealing of the oligonucleotides. The solution was then adjusted to 5 pmol/µL. The double-stranded DNA probes were then radiolabeled by phosphorylation of the 5' extremities using [γ^{32}P]ATP (Amersham) and the Invitrogen T4 pol kinase kit (10 Units for a 25 µL reaction volume). After one hour at 37°C, the phosphorylation reaction was stopped by the ordered addition of 0.5M EDTA pH 8.0, glycogen (10 mg/mL) (USB Corporation), 3M sodium acetate pH 5.2 and 2.5 volume of cold 100% ethanol. DNA precipitated for 30 minutes at -80°C. After 10 minutes of centrifugation (13 000 rpm) at room temperature, the pellet was washed and resuspended in pure distilled water to a final concentration of 0.05 pmol/µL.

The samples were prepared as following: 200 000 cpm of radiolabeled probe were mixed with 1 µL of poly dI-dC (1µg/µL) (Amersham), 8 µL of 3X buffer (30 mM HEPES pH 8.0, 0.3 mM EDTA pH 8.0, 150 mM NaCl, 150 mM KCl, 15 mM $MgCl_2$, 12 mM spermidine, 6 mM DTT and 52.5% glycerol), 0.5 µg of protein extracts and completed to 16 µL with water. Some samples contained competitors like the non-radiolabeled probe or the *Per1* E-box in 10 or 100-fold excess. The sample mixes were incubated 30 minutes at room temperature. Meanwhile, the 4% polyacrylamide gel was pre-run at 50V with inverted polarities. Samples were then loaded on the gel and migrated for 15 minutes at

240 V and 90 minutes at 150 V. The gel was dehydrated 20 minutes in a bath of 10% methanol, 10% acetic acid and dried under vacuum at 90°C. Autoradiography was visualized using a phosphor screen and the Personal Molecular Image FX (Bio-Rad).

For the Antibody SuperShift Assay, the samples were prepared as for the gel shift assay except that the radiolabeled probe was only added after a 20 minutes incubation on ice of the protein extracts, poly dI-dC, 3X buffer and specific antibodies (here anti-FLAG and anti-MYC antibodies 0.4 mg/mL - Sigma). Then, the radiolabeled probe is added (200 000 cpm) and the samples are incubated for 30 minutes at room temperature. The following steps are also identical to the gel shift assay.

3.4 Amplification of *presenilin-2* 5'flanking region

The 5'flanking region of the *presenilin-2* gene (GeneBank accession number AC132436, subsequence from 201531 to 205921) was amplified by PCR using mouse tail genomic DNA. Several lengths of this region were amplified (Table 1). The PCR mixture contained a total volume of 25 µL (50 ng of DNA, 1X PCR buffer, 0.8 µM of each primer, 0.4 mM of each dNTPs, 2mM of $MgSO_4$ and 1.25 units of Platinum Taq HiFi DNA Polymerase (Invitrogen)). The PCR cycling conditions were: denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and elongation at 68°C for 1 minute to 9 minutes, depending on the length of the amplified fragment.

Length	Primer	5'-sequence-3'
4.3 kb	REV	5'-CAAGGATCCACAGGGACAGGCATAGGCAG-3'
	FFW	5'-CTCCTCGAGTTTCCCAGGCCAAGCAGGTG-3'
2.5 kb	REV	5'-CAAGGATCCACAGGGACAGGCATAGGCAG-3'
	FFW	5'-CTGCTCGAGGCTACATCCATTCCTCGGTC-3'
0.8 kb	REV	5'-CACACAGATCTACAGGGACAGGCATAGGCAG-3'
	FFW	5'-CACCCAGGTACCCCTACCCTTAATGG-3'

Table 1: Summary of the primers used for the 5'flanking region of *presenilin-2* gene amplification. PS2(4.3), PS2(2.5) and PS2(0.8) reporter plasmids were generated by cloning amplified fragments in pGL2-Promoter reporter vector expressing luciferase.

Thirty cycles were performed in each case. Visualization of the PCR products was done using agarose electrophoresis and DNA was stained with bromide ethidium, allowing UV visualization in Bio-Rad ChemiDoc XRS apparatus. PCR products were purified by agarose-gel extraction (QIAGEN Gel extraction kit) following proper restriction enzyme digestion of extremities (10 μL digestion reactions contained 2 units of each restriction enzymes) to allow the cloning of the fragments into pGL2-Promoter vector (Promega) (see section 3.4 below).

Overlapping PCR (Sambrook, 2001) was used to mutate specifically the E-boxes found in a 0.25 kb 5'-flanking region of the mouse *presenilin-2* gene fragment, which was generated by enzymatic cleavage of the 0.8 kb fragment previously amplified. This 0.25 kb fragment contained two E-boxes: a CACGTG consensus E-box and a CANNTG non-consensus E-box. Primers, in which mismatch base pairs were included, were designed in order to mutate the E-boxes (Table 2).

E-box	Primer	5'-sequence-3'
CACGTG	REV	5'-GAACTCCTGT**CTCGAG**GAGGAAA -3'
	FFW	5'-TTTCCTC**CTCGAG**ACAGGAGTTC -3'
CAACTG	REV	5'-GTCAAA**CTACAG**CTCAAAACCG -3'
	FFW	5'-CGGTTTTGAG**CTGTAG**TTTCAG -3'

Table 2: Summary of the primers used for directed mutagenesis of the E-boxes found in the 0.25kb 5'flanking region of *presenilin-2* gene. Mismatch base pairs are underlined. Bold region represent the disrupted E-boxes.

Again, PCR products were purified using QIAquick PCR Purification kit (QIAGEN), and fragments were gel-extracted (QIAGEN QIAquick Gel extraction kit) following proper restriction enzyme digestion of extremities to allow the cloning of the fragments in pGL2-Promoter vector (Promega). Mutations were confirmed by sequencing.

3.5 Cloning

PCR products and plasmids were all digested using 2 units of respective restriction enzymes from Invitrogen. Ligations of 5'-flanking region of mouse *presenilin-2* fragments with pGL2-Promoter vector were all conducted using 400 units of T4 ligase enzyme (New England Biolabs) in 20µL total volume reactions. Ligations were performed for 1 hour at room temperature (23-25°C). Ligase was then inactivated at 65°C for 10 minutes. 1 µL of the ligation reaction was transformed by electroporation in XL1-Blue electrocompetent bacteria (5.7 ms, 2.5 eV). Preparation of plasmid was done as described in QIAGEN plasmid purification handbook using QIAGEN midi- or maxi-preps kits. All plasmids were sequenced by the Service de séquençage de l'Université Laval in Québec City.

3.6 Luciferase assays

COS-7 cells were plated in 24-well plates and grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin (Gibco), 100 µg/µL streptomycin (Gibco) and 2 mM L-glutamine (Gibco). Plates were kept in an environment containing 5% CO_2 at 37°C. When cells reached 90-95% confluence (approximately 24 hours after plating), they were transfected using 1.5 µL of Lipofectamine 2000 (Invitrogen) with a total of 600 ng of DNA (25 ng of luciferase reporter gene (Per1-Luc (Travnickova-Bendova et al., 2002) as positive control, PS2(4.3), PS2(2.5), PS2(0.8), PS2-2E, PS2-1E or PS2-0E), 200 ng of mouse CLOCK expressing vector (Travnickova-Bendova et al., 2002) or negative control vector (pSG5 – Stratagene), 200 ng of mouse BMAL1 expressing vector (Travnickova-Bendova et al., 2002) or the corresponding negative control vector (pCS2+MTK), 25 ng of pCR-3-LacZ reporter vector (Invitrogen) and 150 ng of pBleuscript vector (Invitrogen) as a carrier DNA for transfection. 48 hours after transfection, cells were lysed using 120 µL of the 5X lysis buffer (125 mM Tris-Phosphate, 10 mM EDTA, 5 mM DTT, 50% Glycerol, 5% Triton X-100) diluted 5 times. Lysis was conducted for 5 minutes with soft hand shaking. Then, lysates were centrifuged (13 000 rpm) 1 min at room temperature. 75 µL of lysate were transferred in a luminometer microplate and 25 µL of luciferin buffer (Luciferase buffer 1X, 470µM D-luciferine (BD Biosciences), 270 µM Coenzyme-A (Sigma) and 530 µM ATP (Amersham)) were injected automatically using the Orion II Microplate Luminometer (Berthold Detection System). The luciferase buffer is composed of 40 mM Tris-Phosphate, 2.14 mM $MgCl_2$, 5.4 mM $MgSO_4$, 0.2 mM EDTA and 66.6 mM DTT in its 2X stock solution version. In each well, light emission was measured and quantified

for 5 seconds (100 data recorded per well) using the Simplicity 4.0 program. Average value of light emission was then calculated and this value was taken as the Relative Light Unit (RLU) for data analysis. Of the remaining lysate, 20 µL were used to quantify the protein amount using BioRad DC Protein Assay (according to the manufacturer protocol) and 25 µL were used to quantify the transfection efficacy using β-galactosidase assay. The β-gal assay can be briefly described as following: 750 µL of Buffer Z (60 mM $Na_2HPO_47H_2O$, 40mM $NaH_2PO_4H_2O$, 10 mM KCl, 1 mM $MgSO_47H_2O$ and 50 mM β-mercaptoethanol) were added to the lysate. After 5 min at 37°C, 150 µL of substrate ONPG (4 mg/mL) (Sigma) was added (ONPG was dissolved in Buffer Z without β-mercaptoethanol). Then, the reaction was incubated at 37°C until the appearance of a yellow color (approximately 5 minutes). The reaction was then stopped by the addition on 375 µL of Na_2CO_3 1M and OD_{420nm} was taken.

3.7 Animal housing and tissue processing

Animal care and treatment procedures were done according to the guidelines of the Canadian Council for Animal Care. Male C57BL/6N mice (Charles River) of 6 weeks old were housed on a 12 hours light/12 hours dark schedule (LD) for two weeks. Then, mice were put in constant darkness conditions (DD) and were sacrificed by decapitation on the second day of constant darkness. Six animals were sacrificed every 4 hours during 24 constitutive hours. Brain, kidney, liver, heart, spleen, muscle and thymus were immediately extracted and frozen on dry ice, thus providing brains and tissues at circadian times (CT) 2, 6, 10, 14, 18 and 22 (CT2 being 2 hours after onset of subjective day). During all the housing period, mice were fed *ad libitum*.

Total RNA from peripheral tissues (kidney, liver, heart, spleen, muscle and thymus) were extracted using Trizol reagent (Invitrogen), according to the manufacturer's instructions. Briefly, 3 mL of Trizol reagent were added to each tissue for homogenization. The RNA was isolated with chloroform and then precipitated with isopropanol. Then, the RNA was dissolved in DEPC treated water and quantified by spectrometry. RNA integrity was verified on agarose gel stained with ethidium bromide. Concentration of all individual extracts was adjusted to 1 µg/µL (except for liver extracts, which were adjusted to 2 µg/µL). Then, pool solutions were made by mixing equal volume of adjusted RNA extracts of a given tissue from all animals sacrificed at a common circadian time. RNA extracts were stored at -80°C. 10 µm coronal sections were made from frozen brain using a Cryostat (Microm Heidelberg) at -20°C. The slices were then used for *in situ* hybridization (see below).

3.8 Ribonucleases Protection Assay (RPA)

A part of the mouse *presenilin-2* coding sequence (GeneBank accession number NM_011183) was amplified by PCR using the following primers: 5'-GCAGTGGGCATGGTGTGC-'3 and 5'-CCTGGGTAGCCAGGCAG-'3. The amplified fragment was cloned in pBluescript vector. Riboprobes were synthesized from linearized plasmid DNA template by *in vitro* transcription using the Promega Riboprobe system T7. [α^{32}P]dUTP (PerkinElmer, 10µCi/µL) was incorporated in the probe during *in vitro* transcription. DNA template was eliminated by DNase digestion. *Presenilin-2* probe was purified through NucAway spin column (Ambion) and level of radioactivity incorporation was estimated by counting 1µL on a Beckman Coulter LS6500

Multipurpose scintillation counter. Hybridation and RNase digestion procedures were performed using the Ambion RPA III^{TM} kit with slight modifications. Briefly, 15 µg of extracted RNA from mouse peripheral tissues (kidney, liver, heart, spleen, muscle and thymus) were mixed with 1.7 X 10^5 cpm and co-precipitated using ammonium acetate 5M and cold ethanol. Pellets were resuspended in 10 µL of Hybridization Buffer III and samples were incubated at 42°C overnight. RNase digestion of unhybridized RNA was done using a mixture of T1 RNase and RNase A, diluted 100 times in RNase Digestion Buffer III. The reaction was done at 37°C and stopped after 30 minutes by RNAse Inactivation Buffer III. Yeast RNA was added to help precipitation of protected RNA. Pellets were resuspended in 6 µL of Gel Loading Buffer II and samples were separated in a 6% polyacrylamide gel (acryl-bis 38:2) containing 8M of urea. Detection of protected fragments was possible using a phosphor screen and the Personal Molecular Images FX (Bio-Rad). Quantification of the specific protected fragments was possible using the Quantity One analysis program (Bio-Rad).

3.9 *In Situ* hybridization

Probe synthesis: Sense and anti-sense mouse *presenilin-2* and anti-sense *arginine-vasopressin* (AVP used as a positive control for SCN labelling) riboprobes were synthesized using the Promega *in vitro* transcription kit and radiolabeled with [^{35}S]αUTP (PerkinElmer). *In vitro* transcription reactions were incubated for 90 minutes at 37°C. Then, the DNA templates were degraded by DNAse enzymatic digestion and the remaining riboprobes were purified on Ambion NucAway purification columns. Level of

radioactivity of the probes was quantified using Beckman Coulter LS6500 Multipurpose scintillation counter.

Pre-hybridization: Slides containing coronal section of mouse brain (see section 3.6) were incubated 15 minutes in 4% paraformaldehyde, washed 2 minutes in 1X PBS and 2 minutes in 2X SSC (20 X SSC stock solution: 3M NaCl, 0.3M sodium citrate, pH adjusted to 7.0). Slides were treated 10 minutes in 0.1 M TEA containing acetic anhydride (Sigma) and then washed again 2 minutes in 2X SSC and 2 minutes in 1X PBS. The slides were incubated 30 minutes in Tris-Glycine (Fisher) (0.1M each) solution with stirring and then washed as previously. Finally, slides were dried with 1 minute incubation in 70%, 90%, 95% and 100% ethanol bath.

Hybridization: Riboprobes were denatured by heating 5 minutes at 95°C. Hybridization buffer (50% deionised formamide, 0.1% (w/v) dextran sulphate, 2X SSC, 0.01M DTT, 1X Denhardt's solution, 1mg/ml salmon sperm DNA and 0.2 mg/ml tRNA) was added in order to obtain 450 pM riboprobe solutions. 90 µl of the riboprobe solution were added on the slides, which were covered with coverslips and incubated overnight at 54°C.

Post-hybridization: The coverslips were removed and the slides were washed twice in 2X SSC for 5 minutes. Then, unhybridized RNAs were degraded for 30 minutes at 37°C in RNAse buffer (0.1M Tris, 0.01M EDTA pH 8.0, 5M NaCl) containing 20 µg/mL of RNAse A (QIAGEN). Slides were transferred for 5 minutes in RNAse buffer only and then washed as following: twice in 4X SSC for 10 minutes, twice in 2X SSC for 10

minutes, 15 minutes in 0.5X SSC at 53°C and 30 minutes in 0.2X SSC at 62°C. Finally, slides were dried as described previously in ethanol baths. Slides were exposed on a Kodak film for 2 weeks at -80°C.

4. RESULTS

4.1 CLOCK and BMAL1 recognize and bind to *presenilin-2* upstream region

The first aim of that study was to determine whether the CLOCK/BMAL1 transcriptional complex was able to recognize and bind to the upstream region of *presenilin-2* gene in order to induce its transcription. To this end, we performed a gel shift assay, which allows the study of the interaction between DNA-binding proteins and their target DNA sequence. The upstream region of *presenilin-2* gene was screened up to 4.3 kb and was found to contain many DNA response elements, among which many E-boxes were present. E-boxes are the response element of the bHLH-PAS transcription factors protein family. However, only one canonical E-box (CACGTG), the type of E-box preferred by CLOCK/BMAL1 heterodimer complex, was found at position -205 to -199 from the transcription initiation site (+1). First, for the gel shift assay, FLAG-tagged CLOCK and MYC-tagged BMAL1 expressing vectors (Travnickova-Bendova et al., 2002) were co-transfected into COS-7 cells to allow the expression of the proteins. Then, whole-cell protein extraction was performed and protein content was quantified using the Lowry assay. Control protein extracts were also made: one expressing only the endogenous COS-7 cells proteins, one expressing only CLOCK and finally one expressing only BMAL1. For the purpose of the gel shift assay, the region between -210 to -194 was tested. The double-stranded oligonucleotide probe, radiolabeled with [γ-^{32}P]ATP, was incubated with COS-7 whole-cell protein extracts containing CLOCK and BMAL1. The samples were run through a polyacrylamide gel and the appearance of a specific band was observed (Fig. 6, compare lane 4 to lanes 1-3). This particular band was absent in conditions of protein extracts either expressing none or only one of the two DNA-binding

proteins tested here. To be able to attribute this interaction to the E-box itself, competition assays were performed with a 10-fold excess of identical unlabeled DNA probe or another probe already known to interact with CLOCK/BMAL1, the *Per1* E-box; both clearly displaced the interaction and weakened the intensity of the specific band (Fig. 6, lanes 5 and 7). A 100-fold excess of these same competitors totally abolished the interaction (data not shown). On the other hand, competition with a mutated form of the E-box did not affect the intensity of the band, thus showing no interaction between the proteins and this DNA sequence (Fig. 6, lane 6). Another proteins-DNA complex was formed in the gel shift experiment (see Fig. 6, open arrow head). Indeed, we used COS-7 whole-cell protein extracts and found that endogenous COS-7 cell proteins specifically bind to *presenilin-2* E-box as this complex was competed the same way as the specific complex by the cold probe, the *Per1* probe and the mutated E-box probe.

To ascertain that CLOCK and BMAL1 were indeed present in the specific complex (Fig. 6, black arrow head), an antibody supershift assay was performed. The addition of specific antibodies directed against either CLOCK (anti-FLAG antibody) or BMAL1 (anti-MYC antibody) in the same type of samples as described previously for the gel shift assay should further shift the migration of the protein-DNA complex because of the formation of a higher molecular weight complex. That is indeed what we observed, as shown in Figure 7 (compare lane 5 to lanes 6-9). The specific band was displaced by either of the two antibodies, thus confirming that both proteins were present in the complex. Increasing concentrations of antibodies were tested, but even the lowest concentration was sufficient to supershift the complex. It is noteworthy to mention that

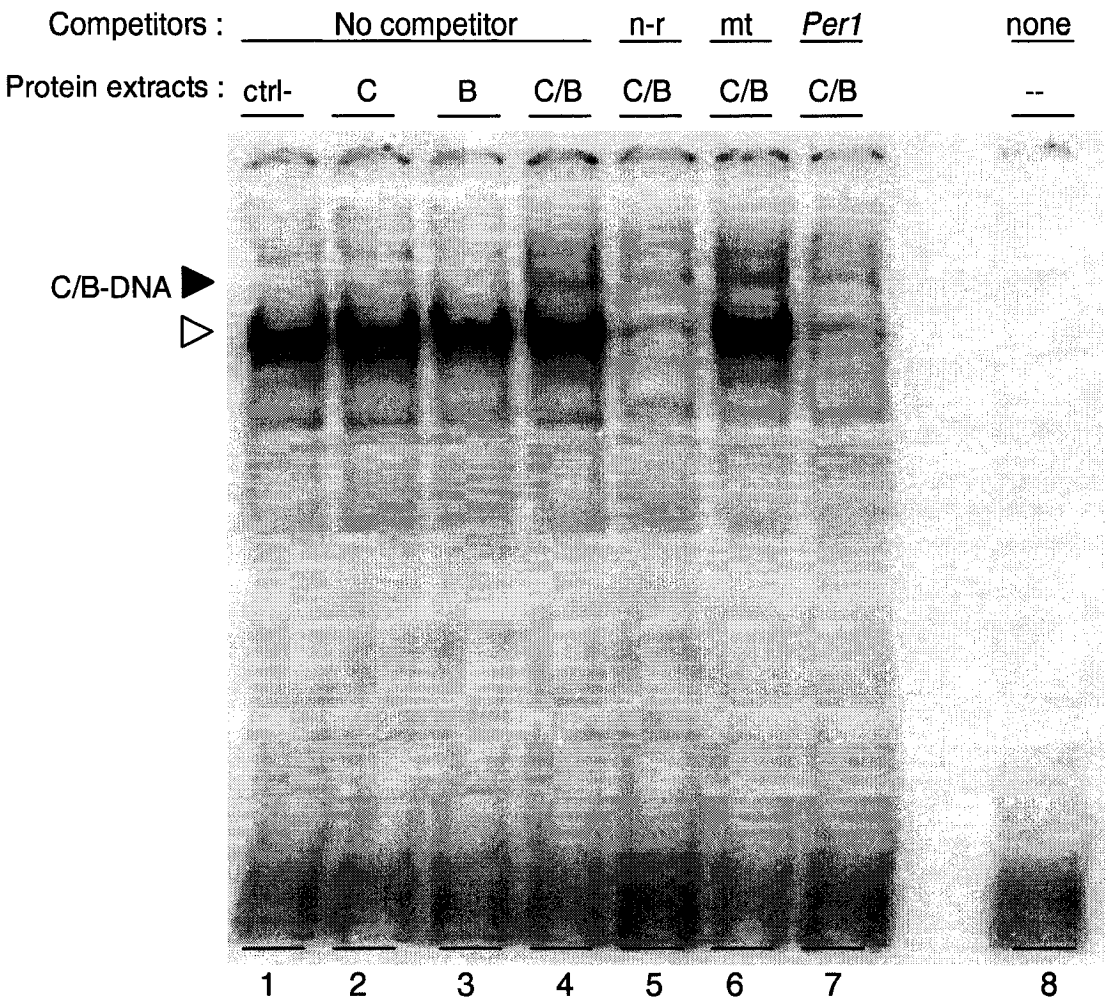

FIG. 6: Specific recognition of *presenilin-2* canonical E-box by CLOCK/BMAL1. Gel shift assay was performed using a probe representing *presenilin-2* canonical E-box (CACGTG) and its flanking base pairs (region -210 to -194 from the initiation codon). Whole-cell (COS-7 cells) protein extracts were incubated with the probe. The black arrow head represents the specific shifted band. Competition assays with a 10-fold excess of an unlabeled identical probe (n-r) or the *Per1* probe, known to interact with CLOCK/BMAL1 (C/B), displaced the binding as observed by the decrease in the intensity of the specific band. Competition with a mutated probe (mt), that is with a disrupted E-box, showed no affinity for C/B, thus confirming that the interaction is specifically due to the E-box itself. The open arrow head represents a secondary complex formed by COS-7 endogenously expressed proteins and the *presenilin-2* E-box probe.

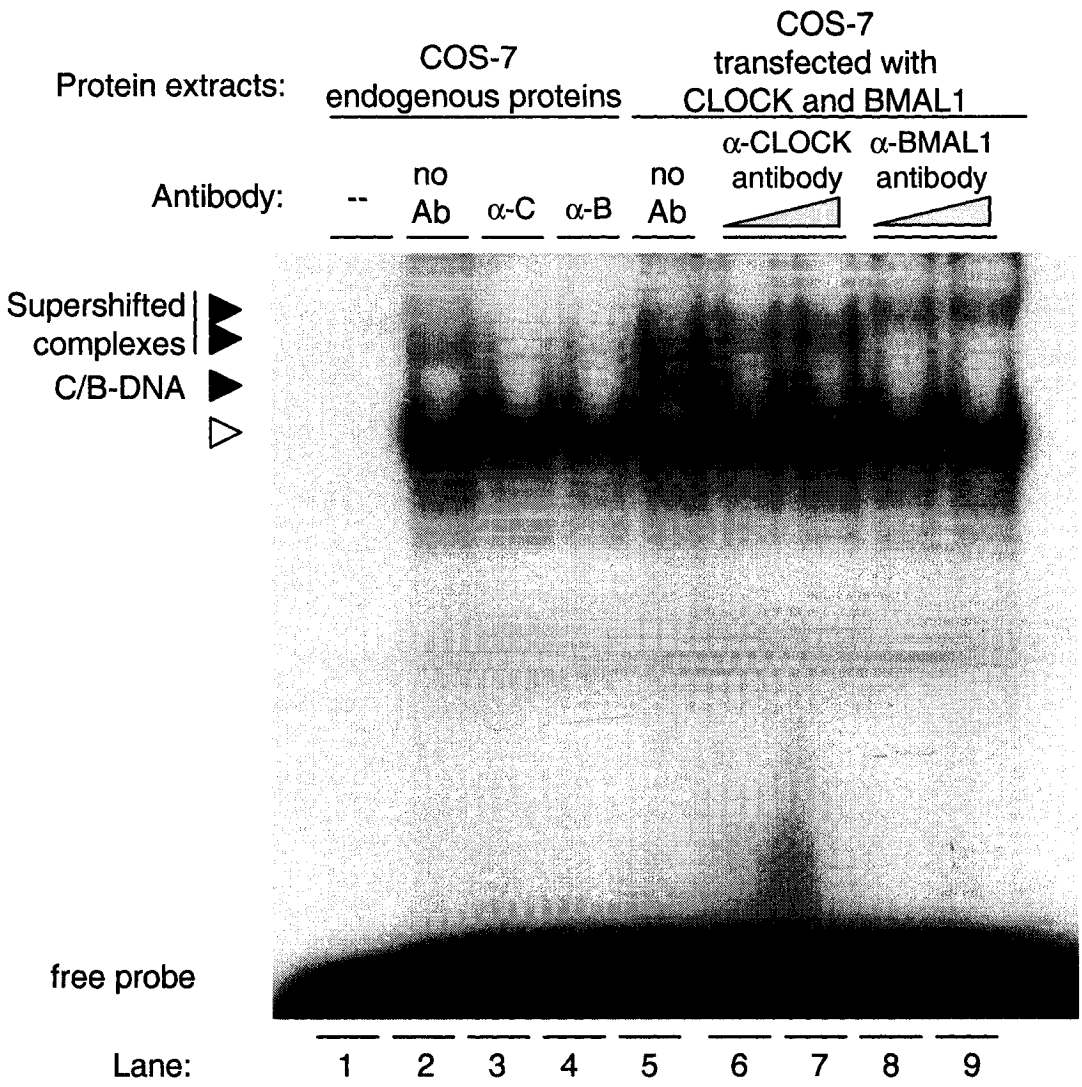

FIG. 7: Confirmation of the presence of CLOCK and BMAL1 in the supershifted complex. Antibody supershift assay was performed to confirm that the initial shifted band (C/B-DNA: black arrow head) was really formed by a complex made of DNA-CLOCK-BMAL1. Specific antibodies directed against either CLOCK or BMAL1 were incubated with the probes or the protein extracts. Supershifted bands confirmed that both CLOCK and BMAL1 were present in the shifted complex. The other complex formed of endogenous COS-7 proteins with presenilin-2 E-box DNA sequence (open arrow head) was not supershifted by the specific antibodies (α-CLOCK and α-BMAL1).

the other complex formed by the endogenous COS-7 proteins was not displaced by the specific antibodies (Fig. 7, open arrow head). This further confirms our initial thoughts about the specific CLOCK/BMAL1-DNA complex. Overall, these results showed that the CLOCK/BMAL1 transcription complex specifically recognizes and binds to *presenilin-2* canonical E-box.

4.2 CLOCK/BMAL1 complex is able to induce *presenilin-2* gene expression

To determine whether the interaction between CLOCK/BMAL1 transcription complex and *presenilin-2* upstream region was functional or not, we performed luciferase assays. As previously mentioned, many E-boxes were found in the *presenilin-2* gene upstream region. Indeed, up to 19 E-boxes were identified within a 4.3 kb region upstream of the transcription initiation site. This upstream region was amplified by PCR using mouse genomic tail DNA as template (plasmid PS2(4.3)). In parallel, 2.5 kb (PS2(2.5)) and 0.8 kb (PS2(0.8)) upstream regions were also amplified in order to try to determine the minimal promoter region. For the luciferase assays, these regions were cloned upstream the luciferase gene in the pGL2-Promoter reporter vector. Co-transfection of these reporter vectors, together with CLOCK and BMAL1 expression vectors in COS-7 cells, allowed us to assay for the transcriptional activation by CLOCK/BMAL1. In this type of assay, if the interaction of CLOCK/BMAL1 with *presenilin-2* «promoter region» is functional, luciferase protein will be produced. After cell lysis, the amount of luciferase produced can be quantified by an enzymatic reaction which produces light. The more luciferase protein produced, the more light emitted by the enzymatic reaction. When assayed, these regions showed clear and similar activation of the luciferase gene (Fig. 8).

In order to be able to confirm that the induction observed was due to the direct interaction of CLOCK/BMAL1 with its DNA response element (E-box), we further shortened the upstream region of *presenilin-2* gene to only 0.25 kb. This fragment was generated by enzymatic digestion of the 0.8 kb fragment previously amplified by PCR and verified by sequencing. This small region contained only two E-boxes, including the canonical one (CACGTG). Again, induction of luciferase expression with this small region was observed as previously with the 4.3 kb, 2.5 kb and 0.8 kb regions (Fig. 9, PS2-2E).

By overlapping PCR, the non-canonical E-box was mutated (see Table 2) and mutation was confirmed by sequencing. Following this modification, a slight decrease in the activation by CLOCK/BMAL1 was observed (Fig. 9). The canonical E-box was then also mutated by the same strategy (see Table 2). When both canonical and non-canonical E-boxes were disrupted, approximately half of the activation was lost, but a 7-fold induction still remained. Total abolition of induction of gene expression by CLOCK/BMAL1 was expected, as no DNA response elements for CLOCK/BMAL1 were present anymore. The remaining induction may be due to another transcription factor that is itself induced by the CLOCK/BMAL1 complex in the COS-7 cells. Since, CLOCK and BMAL1 were overexpressed in this assay, the proposed secondary mechanism may have overridden the effect of CLOCK/BMAL1; alternatively, this could indicate cooperativity in the gene expression mechanisms. The difference in the level of induction observed in the two luciferase assay experiments (Fig. 8 compared to Fig. 9) can be explain by two reasons: the technical reason is the variability between experiments or between DNA preparations

and the biological reason is that an inhibitory or a repressive element in the promoter region may have been removed between the 0.8 kb and the 0.25 kb promoters. However, we did not search for such element in the *presenilin-2* promoter region.

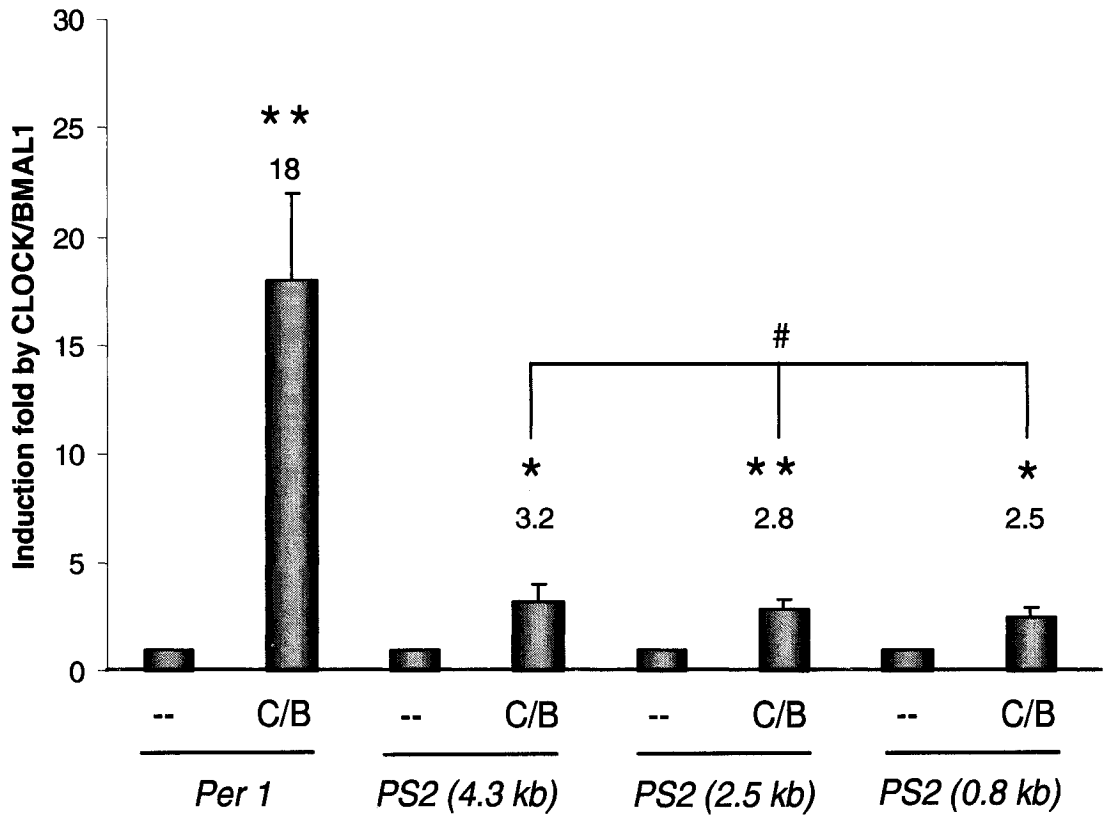

FIG. 8: Induction of *presenilin-2* expression by CLOCK/BMAL1 transcription factors *in vitro*. Luciferase assays showed that CLOCK and BMAL1 are able to induce *presenilin-2* gene expression. Here are shown the results obtained with the 4.3 kb, 2.5 kb and 0.8 kb upstream region of mouse *presenilin-2* gene. In all cases, an approximate 2-fold induction of luciferase expression was detected in presence of CLOCK/BMAL1 comparatively to when neither of the transcription factors are present. All the activations were significant using the one-tailed Student t-test * $p \leq 0.01$ and ** $p \leq 0.006$. The effect of the length of the promoter region on the activation by CLOCK/BMAL1 was non-significant (#) according to the factorial ANOVA test ($F_{(2,48)} = 0.22$, $p = 0.80$). *Per1* promoter was used as a positive control for CLOCK/BMAL1 activation.

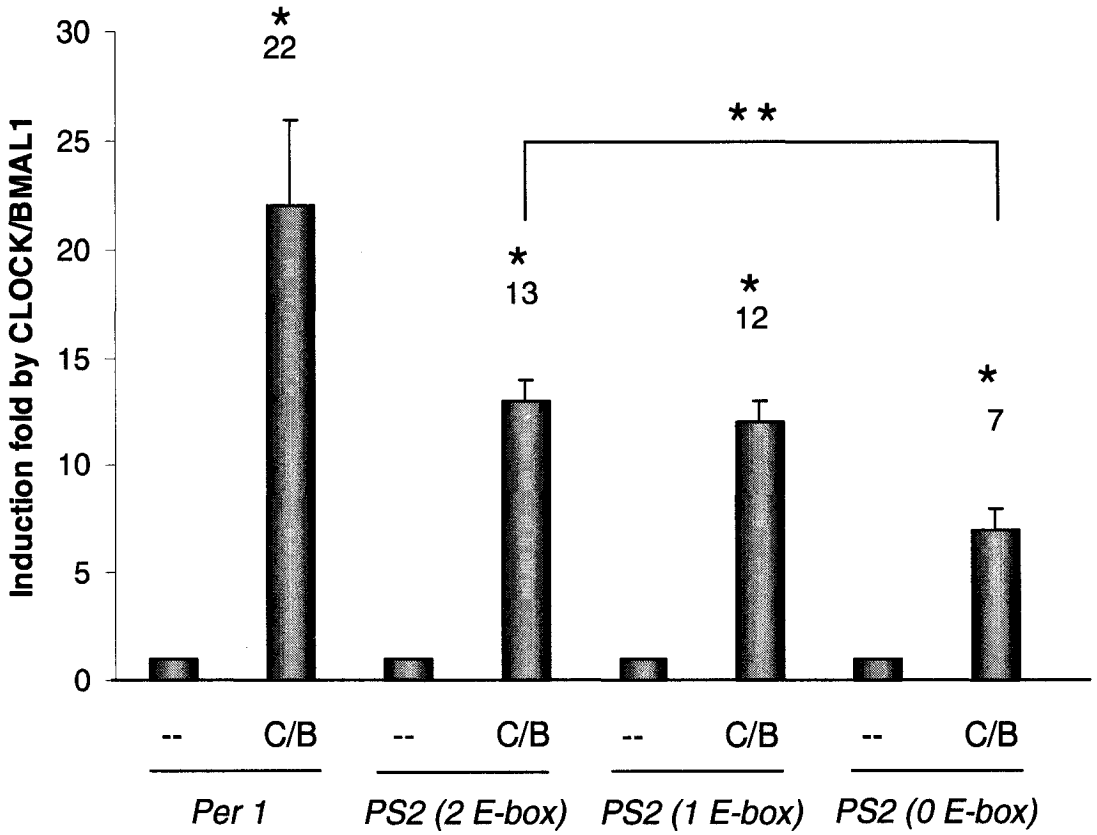

FIG. 9: Effect of the mutation of *presenilin-2* E-boxes on the transcriptional induction *in vitro*. Luciferase assays were performed in order to investigate the *presenilin-2* gene expression by CLOCK/BMAL1 transcription complex. The assay was performed using a 0.25 kb upstream region containing its initial two E-boxes (PS2-2E). Disruption of the non-canonical E-box (PS2-1E) results in a small decrease in the induction. Disruption of the canonical E-box (PS2-0E) had a stronger decrease effect, statistically significant compared to the PS2-2E. All mutations were confirmed by sequencing. Control assay with the empty reporter vector showed no induction of luciferase. *Per1* promoter was taken as a positive control for CLOCK/BMAL1 activation. * All activations were statistically significant according to the t-test, $p \leq 0.008$. Also, the number of E-box was statistically significant according to the factorial ANOVA test ($F_{(2,12)} = 9.48$, $p = 0.003$). ** Post-Hoc Tukey HSD test also revealed a significant difference between the activation with 2 E-boxes as compared to the activation with no E-box: $p = 0.0003$.

4.3 *Presenilin-2* does not oscillate in all peripheral tissues

The expression of *presenilin-2 in vivo* was investigated in order to study the circadian mechanism in a more physiological context. We wanted to confirm the oscillation of *presenilin-2* mRNA levels in the SCN and in the liver, as seen in the literature. However, since circadian clocks exist throughout the body, other peripheral organs, such as the muscle, the spleen, the thymus, the heart and the kidney were tested for *presenilin-2* circadian expression. First, mice were entrained on a 12-hour light: 12-hour dark schedule (LD) to allow synchronization of their endogenous clocks. Then the mice were put into constant darkness condition (DD) and on the second day, 6 mice were decapitated every 4 hours for 24 consecutive hours. The reason why we put the mice in constant darkness condition is to avoid influence of external environmental cues on the endogenous clock. The organs extracted at different circadian times (CT2, CT6, CT10, CT14, CT18 and CT22) allowed us to study the endogenous oscillation of mouse *presenilin-2* mRNA. In the absence of light/dark cycling, the mice don't know whether it is the day or the night, so we are working with their *subjective* day and night rather than real day and night. Brain, muscle, spleen, thymus, heart, kidney and liver were extracted, immediately frozen on dry ice and conserved at -80°C until used. Total RNA was extracted from each organs (except the brain) using the Trizol reagent. RNA from each organ was quantified by spectrophotometry. Then, concentrations were adjusted to 1µg/µl for all tissues except for the liver, which was adjusted to 2µg/µl due to high concentration of RNA in this tissue. Integrity of the RNA was then verified on agarose gel and equal amounts of RNAs from animals killed at the same circadian time were pooled together. So, each timepoint in the experiment is representative of 6 animals.

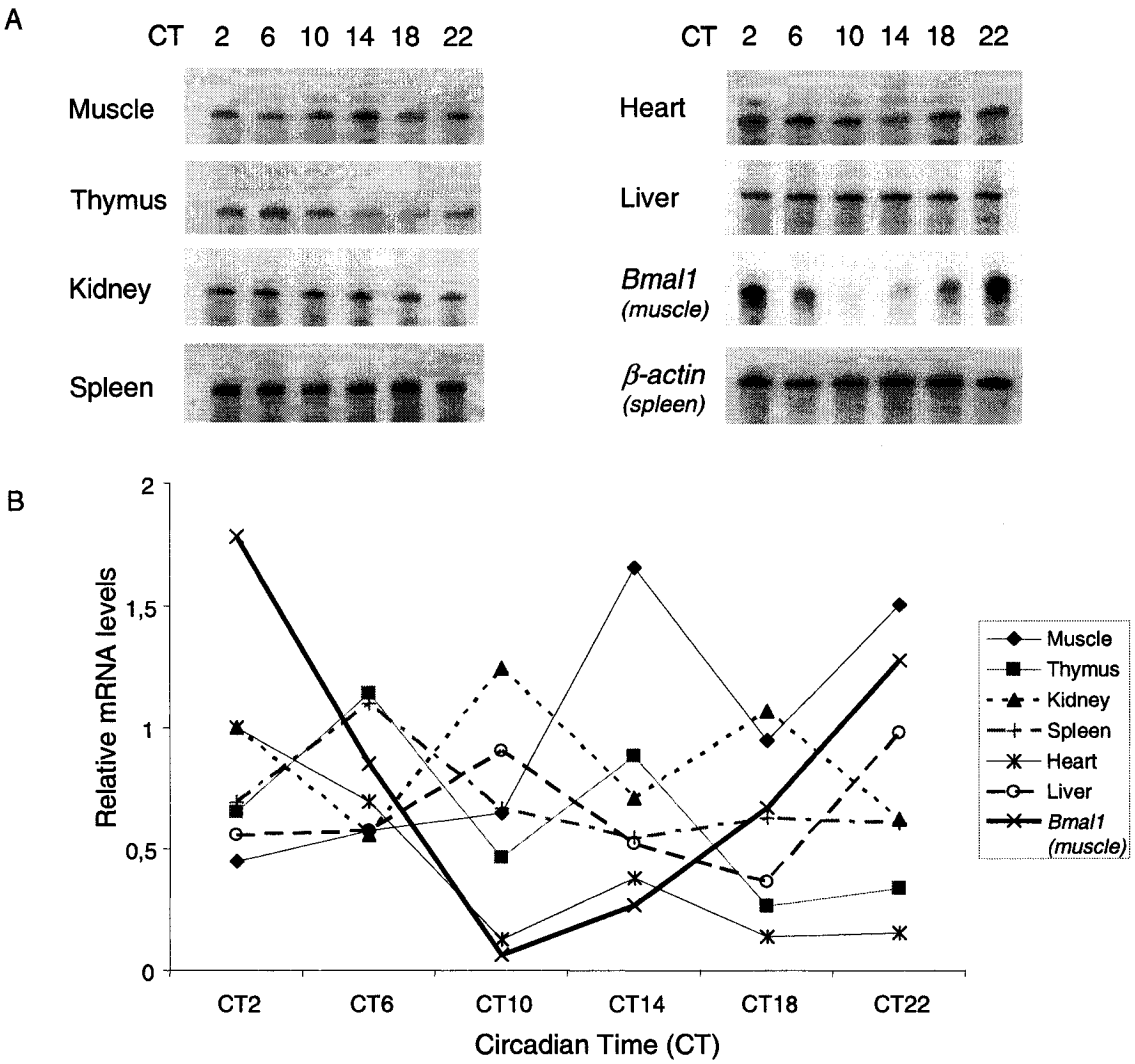

FIG. 10. 24-hour quantification of *presenilin-2* mRNA levels *in vivo*. RPA allowed the quantification of *presenilin-2* mRNA levels in time. A) Representative daily profiles of *presenilin-2* mRNA in the muscle, spleen, thymus, heart, kidney and liver. B-C) Quantifications by densitometry were performed on the RPA bands in order to assay for circadian oscillation of the mRNA. No clear circadian rhythms were detected. The riboprobe used here was complementary to exons 6 to 9 (see Fig. 11A). *β-actin* gene was used as a negative control (non-oscillating transcript) and to normalize mRNA quantity at each timepoints in each tissues. Only one representative β-actin RPA is shown here. *Bmal1* gene was used as a positive control (oscillating transcript) (Guillaumond et al., 2005).

Ribonuclease Protection Assay (RPA) was performed to quantify the variation of *presenilin-2* mRNA level through time in different organs. The riboprobe used in this assay was complementary to exons 6-7-8-9 of the *presenilin-2* gene (Fig. 11A, probe I). No circadian rhythm of the *presenilin-2* mRNA level was detected in the skeletal muscle, the spleen, the thymus, the heart, the kidney and the liver (Fig. 10). The results for the liver were unexpected as four other studies had detected circadian oscillation of *presenilin-2* in this tissue (Kornmann et al., 2001; Panda et al., 2002b; Storch et al., 2002; Ueda et al., 2002). Interestingly, as opposed to all of the other tissues (not shown), in the liver three protected bands were observed instead of only one band (Fig.11B, left). Closer observation and quantification of the second and third major bands resulted in a clear circadian rhythm with a minimum around the end of the subjective day and the beginning of the subjective night (CT10-CT14) (Fig. 11C).

We proposed that these protected probe fragments with lower molecular weights were in reality due to alternatively spliced forms of the *presenilin-2* mRNA. Indeed, alternative splicing has already been shown for *presenilin-2*: exon-5 spliced form leads to a sporadic form of Alzheimer's disease (Sato et al., 2001) and an exon-8 spliced form of *presenilin-2* has been found to be expressed in the mouse liver (GenBank, accession number Q61144). According to this information, a second probe for RPA, complementary to exons 8-9 (fig.10A, probe II), was designed in order to investigate whether this exon-8 spliced form was responsible for the pattern of bands observed in the liver. The results obtained with this new probe were identical to those with the longer probe tested previously, (Fig. 11B) although the second band with probe II migrated slightly lower

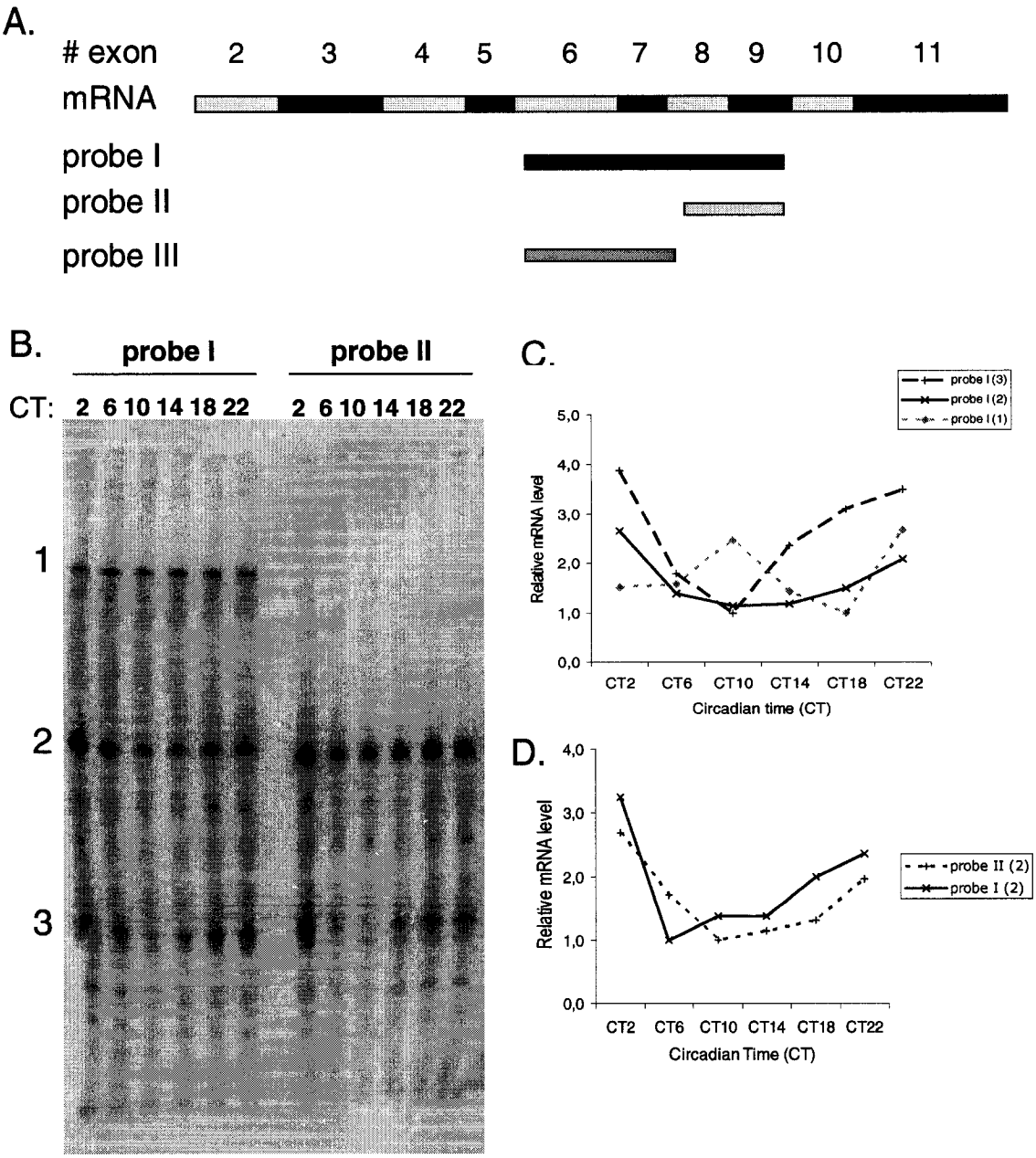

FIG. 11. Rhythmic expression of *presenilin-2* in the mouse liver. RPA were performed with RNA extracted from mouse liver to determine the daily *presenilin-2* mRNA level. The experimental design is presented in A. RPA with probe I resulted in a particular pattern of protected fragments that was not observed in any other tissue tested here (panel B, left side). The second (2) and third (3) bands were quantified and the mRNA abundance showed a clear circadian oscillation with a minimum around CT10-CT14 (C). The other RPA probe (II) tested showed the same pattern of protected fragments (panel B, right side). Quantification of both the second and third bands resulted in the same circadian oscillation as compared to the corresponding fragment with probe III (D). Here are shown only the quantification of the second band.

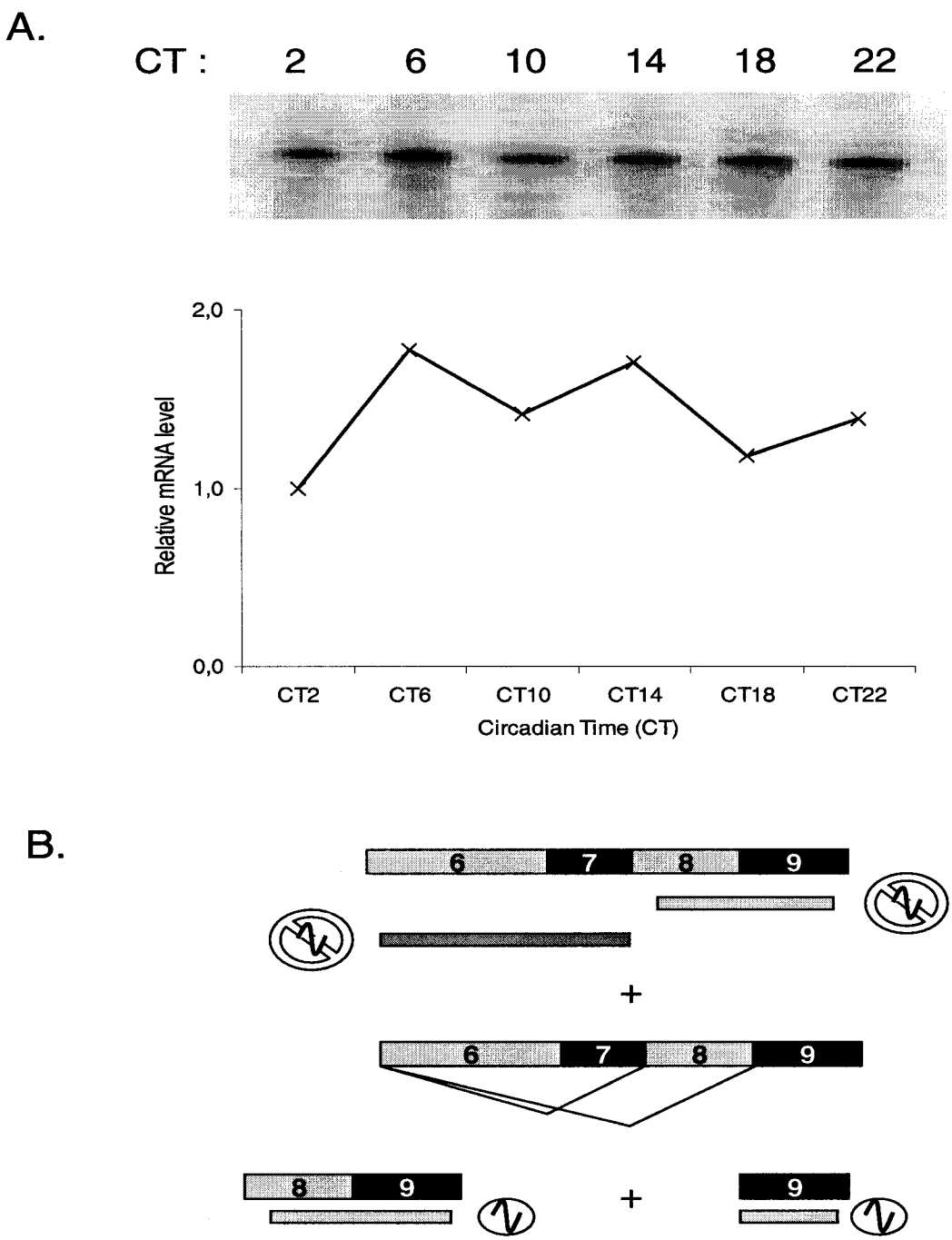

FIG. 12: Investigation of the rhythmic expression of *presenilin-2* in the mouse liver. RPA performed with probe III, which is complementary to exons 6-7 (Fig. 11A). According to these results, it hybridized only to the full-length non-oscillating variant (A). We concluded that there might be several spliced variant of *presenilin-2* expressed in the mouse liver, namely the non-oscillating full-length, one variant lacking exons 6-7 and one lacking exons 6-7-8 (B), the latter two being rhythmic in abundance.

than when protected by probe I. Indeed, probe II did not protected the entire exon 8 as opposed to probe I, thus generating a slight difference in the molecular weight. The circadian oscillations of the second and third bands were identical in both protection conditions (quantification of band 3 not shown) (Fig. 11D). Complete analysis of the results (Fig. 11 and 12) lead to two possible explanations (Fig. 12B). First, there could be a mixture of the full-length transcripts and an alternatively spliced variant lacking exons 7-8. Second, there could be a mixture of the full-length form and two other spliced forms, one lacking exons 6-7 and one lacking exons 6-7-8.

In order to distinguish between the two hypotheses, a third RPA probe, complementary to exons 6-7, was designed (Fig 11A, probe III). The results obtained here allowed the disqualification of the first hypothesis as only hybridization of the probe to the non-oscillating full-length transcript was detected (Fig. 12). Indeed, there was no circadian oscillation of the protected fragment and there was no alternative smaller fragments protected, suggesting that exons 6-7 are either spliced together or not at all.

4.4 *Presenilin-2* oscillation in the mouse SCN

It was of great interest to confirm *presenilin-2* oscillation in the mouse suprachiasmatic nuclei (SCN) as reported by Panda and colleagues in their microarray study (Panda et al., 2002b).To this end, we used *in situ* hybridization. First, mouse brains were extracted every 4-hours for 24 constitutive hours (n=6 per circadian time point) and immediately frozen on dry ice. Coronal sections of frozen brains were put on microscope slides. Then, a probe in the region of exons 6 to 9 of the mRNA was designed, as previously reported

in the literature (Lee et al., 1996). *In situ* hybridization was performed on brains at CT2, 6, 10, 14, 18, and 22. Unfortunately the results obtained were not conclusive, as no clear signal was detected in the SCN (data not shown). Also, we tested our probe on the hippocampus region of the brain, a region which highly expresses *presenilin-2* (Lee et al., 1996), and we did not observe the expected high signal. Thus, another probe, complementary to exons 6-7, was designed and tested, but again no signal was detected (data not shown). However, the positive control for SCN labelling, arginin-vasopressin (AVP), perfectly worked.

5. DISCUSSION

The present study aimed to determine the mechanism of circadian regulation of the mouse *presenilin-2* gene. Indeed, it was previously showed by other research groups that the *presenilin-2* transcript oscillates in the mouse suprachiasmatic nuclei (SCN) and liver by microarray studies. However, microarray studies only provide indicative information that needs to be confirmed more accurately. Moreover, these studies did not investigate the mechanism behind this; they only differentiated the oscillating versus the non-oscillating transcripts. When we analyzed Panda's data, we found that in the mouse SCN, *presenilin-2* mRNA oscillates with the same phase as *Period 1* (*Per1*), a known clock gene (Panda et al., 2002b). The expression of both genes peaks at the same time of the day: in the middle of the day. This suggested that both genes might be regulated by the same mechanism. It is already well established that *Per1* gene expression is initiated by the CLOCK/BMAL1 transcription complex (Gekakis et al., 1998). This transcription complex requires a specific DNA response element called an E-box, which we have also found in the 5'flanking region of mouse *presenilin-2* gene. All together, these pieces of evidence prompted us to investigate whether *presenilin-2* is controlled by the mammalian biological clock.

5.1 Is *presenilin-2* a clock-controlled gene?

5.1.1 Recognition of *presenilin-2* E-box by CLOCK and BMAL1

We first wanted to know whether the CLOCK/BMAL1 transcription complex was able to recognize and bind to *presenilin-2* canonical E-box. With a gel shift assay and an

antibody supershift assay, we determined that CLOCK and BMAL1 both recognize and bind to *presenilin-2* E-box (Fig. 6-7). Moreover, with competition assay, we determined that this interaction is specifically due to the E-box itself and not only to DNA in general. Results from these assays also told us that there is another protein, endogenously expressed in COS-7 cells, that interacts specifically with the E-box. Indeed, as shown in Figure 6, the competition assays gave the same results with this unknown endogenous protein as with CLOCK/BMAL1. We did not investigate the nature of that protein, but it is likely to be a member of the bHLH family of transcriptional regulators (Kewley et al., 2004). Another important thing to consider is that we have only tested the canonical E-box in the gel shift and the antibody supershift assays. Thus, we don't know from these results whether CLOCK and BMAL1 also recognize other E-boxes (non-canonical) in the *presenilin-2* upstream region. There are great chances that if we would have tested the other E-boxes, we would have found the same results in this *in vitro* context. Overall, we have demonstrated here that the CLOCK/BMAL1 heterodimer complex specifically recognizes and binds to *presenilin-2* canonical E-box.

5.1.2 Transcriptional regulation of mouse *presenilin-2* gene

The next step in this investigation was to determine whether this interaction between CLOCK/BMAL1 and the *presenilin-2* canonical E-box was functional. In other words, we wanted to know if the CLOCK/BMAL1 complex induces *presenilin-2* expression upon interaction with the canonical E-box. We must consider that a DNA-response element such as an E-box of 6 base pairs is statistically found every 4.0 kb and that it is impossible that they all serve as circadian regulatory elements (Kyriacou and Rosato,

2000). We performed luciferase assays, which consist of a reporter gene transactivation assay in transfected cells, to determine the functionality of the interaction. First, we tested a 4.3 kb 5'flanking region of *presenilin-2* gene and obtained an approximately 2-fold induction of luciferase gene expression (Fig. 8) In order to determine what was the minimal region necessary for CLOCK/BMAL1 induction of gene expression, we shortened the tested region to 2.5 kb and then to 0.8 kb. The activation by CLOCK/BMAL1 observed with any of the three promoter length was similar (no significant difference). This indicated that the major circadian DNA response element in *presenilin-2* promoter is found within 800 base pairs from the transcription initiation site.

To be consistent with our previous experiments (gel shift and supershift assays), we wanted to confirm that this gene induction was really due to the interaction of CLOCK/BMAL1 with the canonical E-box of *presenilin-2*. A way to reach that goal was to disrupt all the E-boxes found in *presenilin-2* gene upstream region and to investigate gene expression. However, the 0.8 kb contained as much as 6 E-boxes. So, we further shortened the tested region to 0.25 kb and confirmed that the «intact» 0.25 kb region was sufficient to CLOCK/BMAL1 activation (Fig. 9). We disrupted the two E-boxes present in that region (one canonical and one non-canonical). The first mutation disrupted the non-canonical E-box and we observed a very small, but significant decrease in the induction of luciferase gene expression. When we disrupted the remaining canonical E-box, we did not obtain the expected results. Indeed, the induction of gene expression decreased, but we were expecting a total abolition of gene activation as no more DNA

response element for CLOCK/BMAL1 were present anymore. However, a significant induction still remained (Fig. 9).

There are a few possible explanations for the remaining transactivation of gene expression in the luciferase assays with the reporter vector containing no more E-box. First, the overexpression of CLOCK/BMAL1 in the COS-7 cells could have induced the expression of another transcription factor, which possesses a recognition site (other than an E-box) in the *presenilin-2* 5'flanking region. This would explain the remaining activation via an indirect circadian regulatory mechanism. Also, we cannot exclude the possibility that both mechanisms could regulate *presenilin-2* expression.

Another possible explanation concerns the E-box itself. Although it is widely accepted that CLOCK/BMAL1 complex bind to CACGTG type of E-box, several research groups (Hogenesch et al., 1998; Beltran et al., 2004) have tried to extend the recognition sequence in order to enhance its specificity. Indeed, flanking base pairs of the E-box could positively or negatively influence the binding of CLOCK/BMAL1 complex. This could suggest that other DNA elements are important in addition to the E-box. It is noteworthy to mention that the mutation of the E-box generated in the luciferase assays is different than the one generated for the gel shift assay, so we cannot compare the two results. Finally, another type of E-box has been identified to be recognized by CLOCK/BMAL1 in the mouse *Per 2* promoter: CACGTT (Yoo et al., 2005). We did not find any E-box of that kind in the mutated *presenilin-2* upstream region tested in our luciferase assays. Overall, we demonstrated that CLOCK/BMAL1 transcription complex

is able to induce *presenilin-2* gene expression. However, this mechanism could be overridden by another indirect mechanism present in the *in vitro* context, when CLOCK and BMAL1 proteins are overexpressed in COS-7 cells.

5.2 Circadian expression of *presenilin-2* in vivo

5.2.1 *Presenilin-2* expression in peripheral tissues

Circadian oscillators exist outside of the SCN. In addition to non-SCN brain regions, circadian oscillators are found in peripheral organs such as the liver and the heart for example. This prompted us to investigate the oscillation of *presenilin-2* in these peripheral organs, in addition to the muscle, the spleen, the thymus and the kidney. Also, we wanted to confirm the oscillation of *presenilin-2* mRNA in the liver, as reported by many microarray studies (Kornmann et al., 2001; Panda et al., 2002a; Storch et al., 2002; Ueda et al., 2002). To this end, we designed an *in vivo* experiment with mice. As we showed in Fig. 10, no circadian rhythms were detected in the muscle, the spleen, the thymus, the heart, the kidney, but were in the liver. Indeed, we confirmed *presenilin-2* oscillation in the liver (Kornmann et al., 2001; Panda et al., 2002a; Storch et al., 2002; Ueda et al., 2002). Moreover, the phase of *presenilin-2* oscillation found here was consistent with the one described in Panda's study, with a minimum of expression around CT10-CT14 (end of the subjective day-beginning of the subjective night). All RPA experiments were performed at least two times. The fact that we pooled RNA extracts from a common tissue of all mice killed at the same timepoint (n=6 per timepoints) did not allow us to do statistical analysis on the data presented in Fig. 10. However, we

believe that these results are relevant to draw conclusions on the circadian rhythm of *presenilin-2* mRNA.

5.2.2 What is happening in the liver?

The results obtained with the RPA in the liver started a novel chapter of this research project. Indeed, we started studying alternative splicing possibilities for *presenilin-2*. The observations made with the three tested probes (Fig. 11A) suggested the presence of novel alternatively spliced variants of *presenilin-2* in the mouse liver, different than the ones identified so far in the literature (GenBank Accession number: Q61144; (Sato et al., 2001), we found a spliced variant lacking exon 8. Our results suggested that either exons 7-8 are missing or two other different spliced variants are expressed at the same time: one is lacking exons 6-7 and one is lacking exons 6-7-8. Among the two possibilities, we were able to disqualify the first one, as we showed that exons 6-7 are either present or spliced together, inseparably (Fig. 12). We would like to confirm these results. To this end, we will use the 5'RACE (Rapid Amplification of cDNA Ends) technique, which consists on amplifying a sequence from an mRNA template between a known region (here exon 9) and the 5' end of the mRNA. With this technique, we will be able to clone all the *presenilin-2* variants expressed in the mouse liver *in vivo*. Then, sequencing of the amplified fragments will answer the question of whether exons 6, 7 and 8 are present or not. This work is in progress. What is particularly interesting here is that the «full-length» *presenilin-2* RNA (the one detected with the entire hybridization of probe I – see Fig. 11A) does *not* oscillate while shorter RNAs do.

The big question is: Could alternative splicing be rhythmically regulated for *presenilin-2*? To my knowledge, nothing concerning circadian regulation of alternative splicing has been reported. However, there are interesting possibilities that could support the results of circadian regulation of *presenilin-2* in the liver. First, the most obvious one could be the circadian regulation of a protein involved in the recognition of the splicing sites in *presenilin-2* pre-mRNA and/or that is part of the spliceosome (machinery that carries out the splicing reaction). This particular protein could be exclusively expressed in the liver or rhythmically expressed only in the liver and not in other tissues (tissue-specificity) (Akhtar et al., 2002; Panda et al., 2002b).

Another mechanism could involve *presenilin-2* promoter itself. Indeed, promoter structure (or the presence of enhancers in the promoter) has been suggested to be important for alternative splicing (see references in (Kornblihtt et al., 2004). Interestingly, it has recently been found that exon 5 of *presenilin-2* mRNA possesses a sequence that modulate the splicing of the same exon depending on the conditions (hypoxic stress conditions) (Higashide et al., 2004). Overall, this kind of *cis*-acting elements could couple transcriptional regulation and alternative splicing through a complex combination of proteins-DNA and proteins-proteins interactions (Kornblihtt et al., 2004). The complexity of the transcriptional-splicing machinery could also account for the cell-type specificity of the phenomenon. Moreover, transcriptional coregulators (such as steroid hormones (Auboeuf et al., 2002)) are known to help the regulation of alternative splicing. It is interesting to speculate that alternative splicing could refine transcriptional circadian regulation of *presenilin-2* in the liver. The transcriptional activators CLOCK/BMAL1

could act in cooperation with the alternative splicing machinery recruitment in this particular tissue.

A third explanation for the circadian oscillation of shorter *presenilin-2* transcripts in the mouse liver could be the differential stability of the different mRNA forms. Also, proteins important for RNA stability could be rhythmically regulated. As a result, their target RNA molecules could also be rhythmically modulated. Indeed, equilibrium between synthesis and degradation of mRNA can generate circadian rhythms (reviewed in (Shu and Hong-Hui, 2004). Thus, degradation of mRNA (decrease of messenger levels) could possibly stimulate the expression of new transcripts (Fig. 13).

Finally, we can integrate all the possibilities together and get the following scenario: *presenilin-2* gene expression is induced by CLOCK/BMAL1 and alternatively spliced. Then, the alternative transcripts are unstable species and fluctuate in a circadian fashion, whereas the «full-length» transcript is more stable (Fig. 13).

5.2.3 *Presenilin-2* expression in the SCN

In order to investigate the circadian modulation of *presenilin-2* in a more physiological context, we first tried to confirm its circadian expression in the mouse SCN, as reported in Panda's study (Panda et al., 2002b). To this end, we used *in situ* hybridization on coronal sections of frozen mouse brains taken at 6 different timepoints over the day. The first probe we tested was designed like what had been used and shown to work in the literature (Lee et al., 1996). Unfortunately, this probe did not work in our hands. We tried

a second probe but obtained the same disappointing results. However, our positive control, a probe designed to recognize AVP, a neuropeptide expressed in the SCN, worked in both cases (data not shown). We were expecting to detect at least the expression of *presenilin-2* in the mouse SCN, but we did not. Thus, we can not conclude whether *presenilin-2* oscillates or not in the mouse SCN. However, it is noteworthy to mention that although Panda and colleagues (Panda et al., 2002b) detected oscillation of *presenilin-2* in the mouse SCN, Ueda and co-workers did *not*, using the same technique (Affimetrix mouse high-density oligonucleotide probe array) (Ueda et al., 2002). Despite the great efforts that are put into standardization of microarray studies, comparison between two such studies remains difficult because of the variability. Indeed, false-positive as well as false-negative results can emerge from very subtle differences between two studies. In the present case, the determination of the threshold between oscillating versus non-oscillating transcripts may have been different for both groups (Panda et al., 2002b; Ueda et al., 2002). Thus, we cannot confirm either of the studies with our results. We could have used another strategy to determine whether *presenilin-2* mRNA oscillates in the mouse SCN. For example, we could have isolated the SCN from the brains by taking small punches around the region of the SCN, extracted RNA from them and performed RNase protection assays, as we know that the probe works with this technique. However, despite the sensitivity of the RNase protection assay, a large amount of starting RNA is required and this could have caused some limitations. On the other hand, taking RNA from the whole brain would not have answered our initial question because, first, *presenilin-2* is expressed in many different brain structures (Lee et al., 1996) and second, other circadian oscillators exist in other brain areas. These other oscillators, even within

the brain, do not necessarily have the same circadian phase. As a result, it would not have been indicative of what happens in the SCN.

Actually, the possibility of *presenilin-2* circadian oscillation in other brain structures is also of great interest. Indeed, as we know, *presenilin-2* is expressed in the hippocampus for example. This brain structure is greatly affected in cases of Alzheimer's Disease (AD). We could ask whether *presenilin-2* is rhythmically regulated in the hippocampus and whether this could have an impact on the development of Alzheimer's Disease or not. The circadian oscillation of *presenilin-2* could influence the APP (amyloid precursor protein) processing and have an impact on therapy.

5.2.4 Why would *presenilin-2* oscillate?

In this project, we investigated the mechanism behind the circadian modulation of *presenilin-2*. We demonstrated that *presenilin-2* is controlled by the mammalian biological clock in the mouse liver. However, we may ask why *presenilin-2* circadian oscillation would be important. Considering the functional polyvalence of presenilin proteins, circadian modulation could have a high impact on the functions associated with presenilins. For example, presenilins are responsible for regulated intramembraneous proteolysis (RIP) (Urban and Freeman, 2002; Koo and Kopan, 2004). The function of RIP consists on the regulation of intracellular signalling. Here, we can easily evaluate the consequences that circadian modulation of presenilins could have on RIP. It would imply that the signalling pathways targeted by the presenilin-dependant RIP would be rhythmically regulated. Among the molecules regulated by presenilins, we find

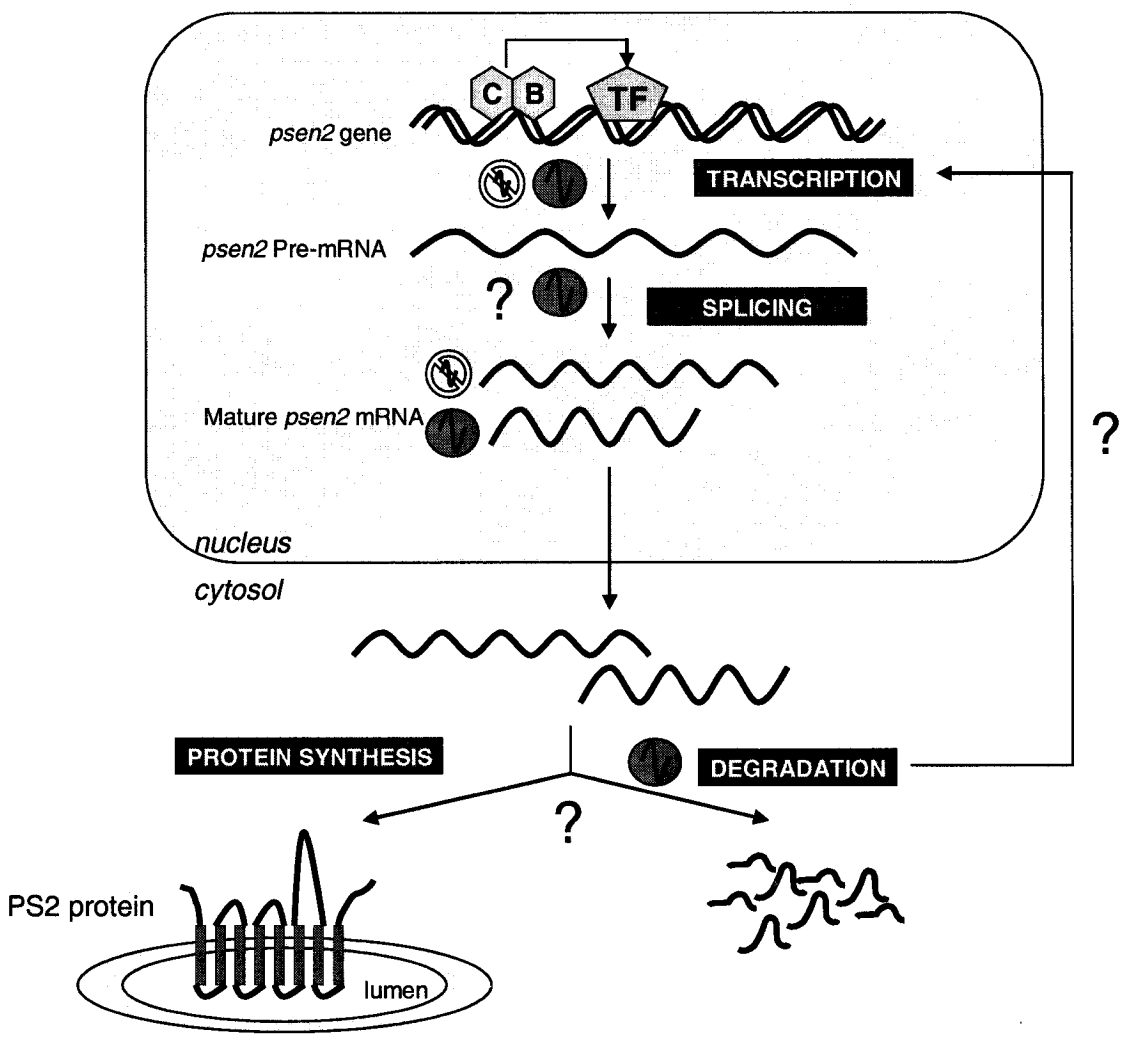

FIG. 13: Speculative model of the mechanism of circadian modulation of *presenilin-2* gene in the mouse liver. As demonstrated in the current research project, the circadian oscillation of *presenilin-2* in the mouse liver is regulated at multiple levels: direct transcriptional modulation by CLOCK/BMAL1 transcription factors, indirect transcriptional modulation by another transcription factor controlled by CLOCK/BMAL1 and posttranscriptional modulation either via alternative splicing and regulated destabilization of the mRNA molecules. C (CLOCK); B (BMAL1); TF (Transcription factors).

transcriptional factors, transcriptional factor activators, molecules involved in cell adhesion and junctions, etc. (Urban and Freeman, 2002; Koo and Kopan, 2004). One could investigate the impact of presenilins circadian regulation by measuring the abundance of intracellular domain (ICD) released by RIP in rhythmic cell culture (rhythmicity induced by a serum shock, see (Balsalobre et al., 1998)) by immunoblotting. This could also answer the question whether the shorter *presenilin-2* transcripts result in a functional protein or not. Indeed, we don't know yet for sure whether *presenilin-2* is alternatively spliced and whether this gives rise to a functional protein. Generally, alternative splicing results in different mRNA molecules from a common pre-mRNA precursor molecule in order to generate different forms of the final protein, either with an enhanced, abolished or novel function (Reviewed in (Stamm et al., 2005). From the current working hypothesis, splicing of the exons 6-7 or 6-7-8 would greatly modify the structure of the protein as three transmembrane domains of the full-length protein would be excluded of the final protein (Fig. 14). Also, as Presenilin-2 is a transmembrane protein, this could seriously impair its integration and/or its orientation into the membrane. Intracellular localisation by immunofluorescence microscopy using antibodies directed toward epitopes in specific regions of the protein could allow us to determine whether the protein is spanning the membrane and the orientation of its C- and N-terminal extremities (Dewji and Singer, 1997). That point raises many questions: What is the function of the new protein? Why is it only expressed in the liver?

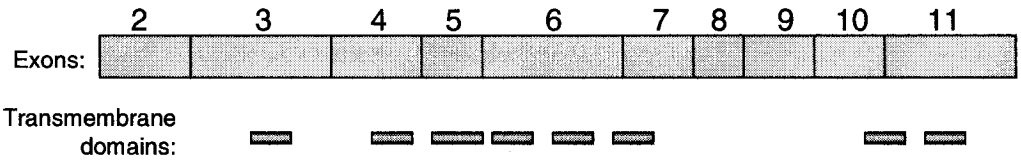

FIG. 14: Schematic view of Presenilin-2 protein. Here are shown the transmembrane domains and their exonic equivalent. According to our hypothesis, exons 6 and 7 would be skipped together or not at all. Taken at the protein level, this would mean that three transmembrane domains would be excluded from the mature protein.

5.3 *Presenilin-2*: from a clock-controlled gene to a clock gene

Up to now, we have demonstrated that *presenilin-2* is controlled in a circadian fashion. But, what if *presenilins* were not only clock-controlled genes but were clock genes themselves? Indeed, among all the reported functions of presenilin proteins, one is particularly interesting for molecular chronobiology. Presenilins have been shown to have a scaffolding function in the stepwise phosphorylation of β-catenin by bringing together the target protein (β-catenin) and the kinases (GSK-3, PKA) (Kang et al., 1999; Kang et al., 2002). Indeed, by bringing close to each other the substrate and all the necessary kinases, presenilins facilitate the stepwise phosphorylation of the target protein, resulting in a very efficient mechanism. In that particular case, phosphorylation of β-catenin tags the protein for degradation by the ubiquitin pathway, thus presenilins are also involved in the turnover of that protein. This immediately reminded us the importance of phosphorylation and protein turnover in the molecular clockwork (Fig. 2). It was shown in many organisms, from Drosophila to humans, that proper equilibrium of phosphorylation-degradation of clock proteins is necessary to maintain a 24-hour

circadian period (Lowrey et al., 2000; Toh et al., 2001; Harms et al., 2004). In mammals, interaction between CKIε and PER (1 and 2) is critical to maintain the period (Camacho et al., 2001; Lee et al., 2004; Nawathean and Rosbash, 2004; Eide et al., 2005a; Eide et al., 2005b). Even more interestingly, it was recently demonstrated that GSK-3β (glycogen synthase kinase-3β) phosphorylate PER2 protein in the SCN and in the liver (Iitaka et al., 2005). Moreover, we already know that presenilins and GSK-3β directly interact together (Kang et al., 2002). From these pieces of evidence, a speculative model emerges for a possible role of presenilins in the mammalian clockwork (Fig. 15). In this model, clock-controlled Presenilin-2 would serve as a scaffolding protein, bringing together PER proteins, GSK-3β (Glycogen synthase kinase 3β) and CKIδ/ε (Casein kinase I δ/ε). It would facilitate successive phosphorylation of the PER proteins and participate to clock proteins turnover, which is critical for the well functioning of the molecular clockwork as well as for the maintenance of the circadian period. The first step in testing this model would be to perform co-immunoprecipitation and immunoblotting in transfected cells. This would identify the proteins present in the «phosphorylation complex». Also, two-hybrid experiments could confirm the protein-protein interactions between presenilins, PERs, GSK-3β and CKI δ/ε .

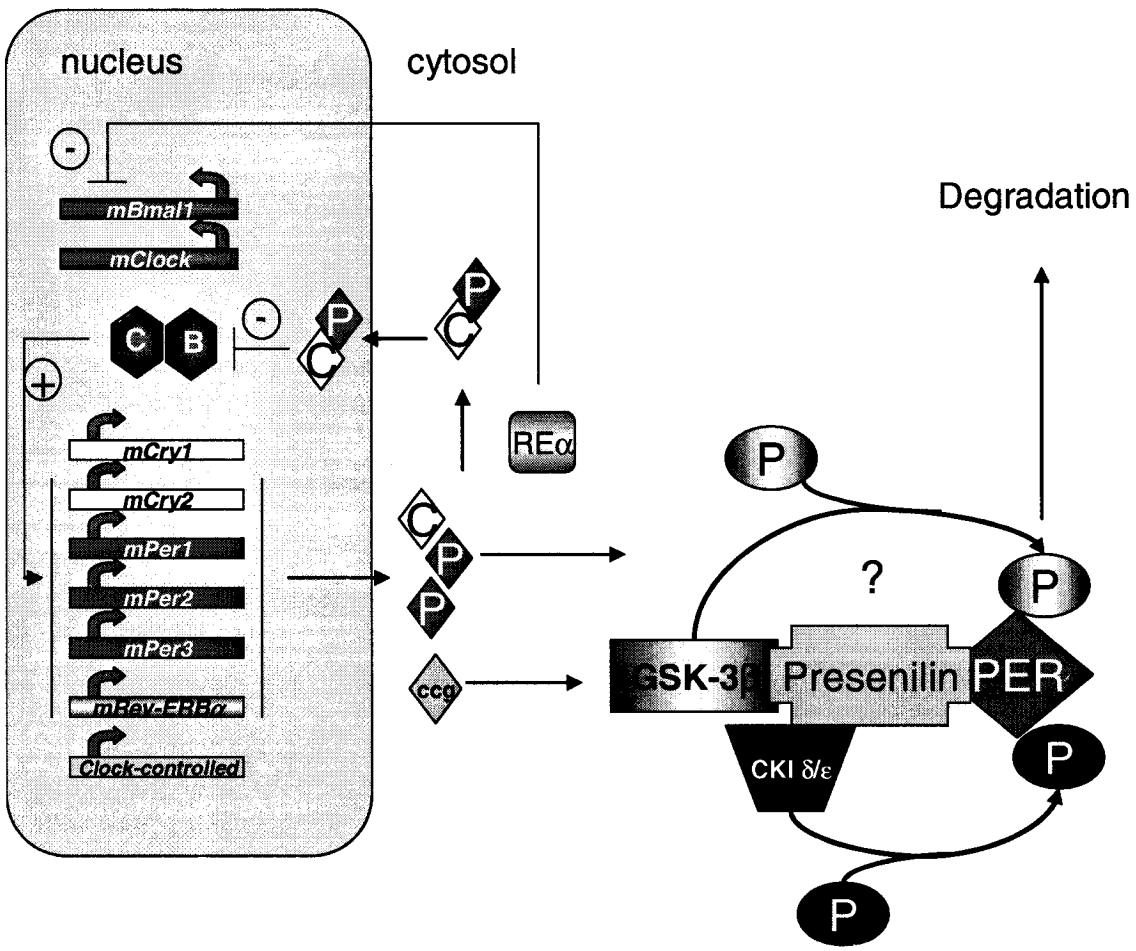

FIG. 15 Speculative model of the potential function of Presenilin-2 in the molecular clockwork of the mouse hepatocytes. Presenilin-2 could act as a scaffolding protein in the molecular clockwork of the mouse hepatocyte by coupling the PERIOD proteins and the kinases (GSK-3β, CKIε) to facilitate its phosphorylation.

6. CONCLUSION

The current research project studied the mechanism of expression of the *presenilin-2* gene. We determined that CLOCK and BMAL1, the transcriptional regulators of the mammalian circadian clock, modulate its expression. However, our results suggested multiple levels of circadian modulation of the *presenilin-2* gene (combination of direct and indirect mechanisms of regulation). We demonstrated that *presenilin-2* mRNA spliced variants oscillate in the mouse liver but not in other tissues (muscle, spleen, thymus, heart and kidney). This study represents the first demonstration of the differential circadian regulation of one mRNA molecule. Indeed, we showed that the *presenilin-2* mRNA molecule does not oscillate in its full-length form but shorter transcripts oscillate in a circadian fashion. We propose a model involving multiple levels of circadian modulation of the *presenilin-2* gene and speculate that presenilins could even have a role in the molecular clockwork in mammals. The circadian modulation of Presenilin-2 could have a strong impact in intracellular signalling via regulated intramembraneous proteolysis. Moreover, it could also have an impact on Alzheimer's Disease as APP (amyloid precursor protein) cleavage is regulated by presenilins (APP cleavage by mutant presenilin proteins lead to accumulation of neurotoxic metabolite in the brain tissue). On the other hand, if *presenilins* are also clock genes, this could be the missing link between Alzheimer's Disease and sleep disturbance in AD patients. Overall, this study brought many new ideas in the fields of chronobiology as well as in intracellular signalling and Alzheimer's Disease.

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8. APPENDIX

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Circadian regulation of presenilin-2 gene: how is the molecular clockwork involved?

First, I'D like to thank the examiners who revised my thesis: Dr. Nicolas Cermakian and Dr Fiona Bedford. They both provided me an excellent feedback on my work as well as good commentaries and useful corrections to make. Here is a summary of the corrections I did on my thesis, in light of the examiners suggestions.

1. **In figure 6, it would be useful to mention the presence of the binding complex formed by an endogenous COS-7 protein and in addition to annotate this band by an arrow. Also, it would be interesting to discuss about this particular band before the discussion.**

CORRECTION: I added an open arrow head indicating the complex in the figure and added the following to the figure legend: «*The open arrow head represents a secondary complex formed by COS-7 endogenously expressed proteins and the presenilin-2 E-box probe.* ». Also, I added the following details on this complex to the results section: « *Another proteins-DNA complex was formed in the gel shift experiment (see Fig. 6, open arrow head). Indeed, we used COS-7 whole-cell protein extracts and found that endogenous COS-7 cell proteins specifically bind to presenilin-2 E-box as this complex was competed the same way as the specific complex by the cold probe, the Per1 probe and the mutated E-box probe.* ».

2. **In figure 7, as mentioned above. It should be noted that the background COS-7 complex is not supershifted. This makes a positive, more convincing data and this should be mentioned.**

CORRECTION: I added to the results section: « *It is noteworthy to mention that the other complex formed by the endogenous COS-7 proteins was not displaced by the specific antibodies (Fig. 7, open arrow head). This further confirms our initial thoughts about the specific CLOCK/BMAL1-DNA complex.* ». Also, I added to the figure legend: « *The other complex formed of endogenous COS-7 proteins with presenilin-2 E-box DNA sequence (open arrow head) was not supershifted by the specific antibodies (α-CLOCK and α-BMAL1).* », in addition to modifying the figure in order to identify the background COS-7 complex by adding an open arrow head as in figure 6.

3. **In figure 8 and 9, there is an apparent discrepancy in the activation of the luciferase by different upstream regions of the presenilin-2 gene. It would be important to add some discussion to explain this discrepancy.**

CORRECTION: There was no discrepancy in the activation level by the different upstream regions used in the luciferase assays. The differences in the folds of induction mostly reflect normal experimental variations. However, I added the following speculations on the «discrepancy»: « *The difference in the level of induction observed in the two luciferase assay experiments (Fig. 8 compared to Fig. 9) can be explain by two reasons: the technical reason is the variability between experiments or between DNA preparations and the biological reason is that an inhibitory or a repressive element in the promoter region may have been removed between the 0.8 kb and the 0.25 kb promoters. However, we did not search for such element in the presenilin-2 promoter region.* ».

4. **In figure 9, mutation of the 2 E-box sites does not completely abolish the enhancement on the minimal promoter. Can a possible control be suggested that could be used to the conclusion that is presented that enhancement actually occurs?**

CORRECTIONS: I did not ad anything about this because it was already discussed in the text: «*When both canonical and non-canonical E-boxes were disrupted, approximately half of the activation was lost, but a 7-fold induction still remained. Total abolition of induction of gene expression by CLOCK/BMAL1 was expected, as no DNA response elements for CLOCK/BMAL1 were present anymore. The remaining induction may be due to another transcription factor that is itself induced by the CLOCK/BMAL1 complex in the COS-7 cells. Since, CLOCK and BMAL1 were overexpressed in this assay, the proposed secondary mechanism may have overridden the effect of CLOCK/BMAL1; alternatively, this could indicate cooperativity in the gene expression mechanisms.* ». Moreover, it is mentioned in the figure legend: « * *All activations were statistically significant according to the repeated measures ANOVA test:* $F_{(1,6)} = 185.5$, $p = 0.00001$. ** *Post-Hoc Tukey HSD test also revealed a significant difference between the activation with 2 E-boxes as compared to the activation with no E-box:* $p = 0.0005$. ».

5. **In p. 39, the description of the mutations could have been summarized in a table.**

CORRECTION: I added the reference to Table 2 in the text and deleted the long explanation on the mutagenesis. «*By overlapping PCR, the non-canonical E-box was mutated (see Table 2) and mutation was confirmed by sequencing. Following this modification, a slight decrease in the activation by CLOCK/BMAL1 was observed (Fig. 9). The canonical E-box was then also mutated by the same strategy (see Table 2).* ».

6. **In figure 10, Mrs Bélanger concludes that there is no circadian rhythm detected in the tissues analyzed. However, in the figure, there is no example (control) that the experiment could detect such a delicate rhythm. It would be nice to see a control circadian regulated mRNA in this figure. [...] Also, nothing is mentioned in the legend about β-actin.**

CORRECTION: I agree that some details were missing in this figure. In addition to improving the figure itself, I added the following information to the figure legend: «*β-actin gene was used as a negative control (non-oscillating transcript) and to normalize mRNA quantity at each timepoints in each tissue. Only one representative β-actin RPA is shown here. Bmal1 gene was used as a positive control (oscillating transcript).* ». Also, I modified figure 11 as the following: I added a «non-oscillating» curve among the oscillating ones in order for the reader to draw his own conclusion about «oscillating» vs. «non-oscillating» transcripts.

7. **In figure 10, 11 and 12, how many times were these experiments performed?**

CORRECTION: The following modification has been done in the discussion section: «*All RPA experiments were performed at least two times. The fact that we pooled RNA extracts from a common tissue of all mice killed at the same timepoint (n=6 per timepoints) did not allow us to do statistical analysis on the data presented in Fig. 10. However, we believe that these results are relevant to draw conclusions on the circadian rhythm of presenilin-2 mRNA.* ».

8. **The order of section 5.2 needs to be changed or at least the emphasis altered. [...]**

CORRECTION: I also agreed on the commentaries made for this section. I decided to change the order of the subsections in order to present a stronger discussion.

Previous order	New order
5.2.1 *Presenilin-2* expression in the SCN	5.2.2 *Presenilin-2* expression in the peripheral tissues
5.2.2 *Presenilin-2* expression in the peripheral tissues	5.2.3 What is happening in the liver?
5.2.3 What is happening in the liver?	5.2.1 *Presenilin-2* expression in the SCN
5.2.4 Why would *presenilin-2* oscillates?	5.2.4 Why would *presenilin-2* oscillates?

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October 14, 2003

The McGill University Animal Care Committee certifies that
Valerie Belanger has successfully completed a
Mouse Methodology Workshop on ***October 2, 2003***.

The training included the following procedures:

- ✔ Handling and restraint
- ✔ Injections: subcutaneous, intramuscular, intraperitoneal, intravenous *
- ✔ Gavage (tube feeding)
- ✔ Blood collection: saphenous and cardiac puncture
- ✔ Determination of anaesthetic depth
- ✔ Euthanasia by cervical dislocation

** Intravenous injection has only been demonstrated, for certification of this procedure, a special session is needed*

Certification is valid for 5 years, starting on the date of the workshop.

Suzanne Smith
Research Ethics Officer for Animal Studies
animalcare@mcgill.ca

(Confirmation of training can be obtained by request to the above email address)

Note: Trainee must keep this certificate as other institutions may request it as evidence of training

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11. Introduction aux rayonnements et structure de la matière
12. Unités de rayonnement.....
13. Rayonnement et radioactivité.....
14. Détection et mesure du rayonnement.....
15. Effets biologiques du rayonnement.....
16. Effets des rayonnements sur le fœtus.....
17. Exigences réglementaires et limites d'exposition.....
18. Contrôle de la radioexposition.....
19. Procédures d'exploitation et d'urgence.....
20. Exigences en matière de transport.....

Certificat émis le 24 octobre 2003

CONTEX ENVIRONNEMENT

certifie que **Valérie Bélanger**

a réussi avec succès une session de formation
d'une durée de 6:00 heures

PRINCIPES DE RADIOPROTECTION

Laboratory Safety procedures : Douglas Hospital
03-10-06, 3 hours

1. Regulations and standards.....
2. Essential Health and Safety Procedures in a Research Facility
3. Biohazards containment levels and safety rules
4. Emergency Response

Certificate issued on October 7, 2003

CONTEX ENVIRONNEMENT

Confirms that ***bélanger Valérie***

Has participated in a formal training which was given at the
Douglas Hospital on

Laboratory Safety Procedures