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MAPK Implications in the Biological Rhythms of Suprachiasmatic Nuclei and Peripheral Oscillators

Roles des MAPK dans les Rythmes Biologiques des Noyaux Suprachiasmatiques et des Oscillateurs Peripheriques

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ABSTRACT

Most living organisms have developed endogenous biological clocks to adapt to and anticipate daily and seasonal changes in the environment. In mammals, a main circadian clock is located within the suprachiasmatic nuclei (SCN) of the hypothalamus, and multiple oscillators exist in the periphery. Autoregulatory feedback loops, where clock proteins regulate their own rhythmic expression by inhibiting the transcription of their cognate genes, constitute the circadian molecular machinery. At the level of the SCN, an afferent system conveys the light information to the SCN to entrain the clock to 24 h, while efferent systems, including the synthesis of the pineal melatonin hormone, distribute the time information to the whole organism. Transcriptional and post-translational regulations are critical to define the 24-h period of the molecular clockwork, and to regulate the arylalkylamine *N*-acetyltransferase, a key enzyme for the melatonin synthesis. Recent studies have suggest that two subfamilies of mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase 1/2 and $p38^{MAPK}$, are involved in these transcriptional and post-translational regulations by phosphorylating target proteins.

Little is known on the role of a third subfamily of MAPK, JNK, in the circadian system. The aim of this thesis was to investigate the potential regulatory functions of JNK isoforms in both the molecular clockwork and the melatonin synthesis.

Our results demonstrate that JNK isoforms display distinct activation patterns in rat-1 fibroblasts: p46 kDa and p54 kDa isoforms are both acutely phosphorylated while only p46 kDa phosphorylation level is rhythmic over the circadian cycle. We showed that the activation level of JNK is critical to properly define the period of the clock in rat-1 cells. Also, we found that JNK activation determines the pace of the oscillations in the mouse SCN and some but not all peripheral tissues. In the rat pineal gland, we established that the transient cAMP-induced activation of JNK isoforms is also necessary for the cAMP-induced transcription of several genes including *Aa-Nat*. Besides, both JNK and $p38^{MAPK}$ activation contribute to the expression of *Mkp-1*, a MAPK phosphatase.

Altogether, our results support a role for JNK isoforms at two levels of the circadian system: the molecular clockwork by regulating the period of the clock, and the efferent system by driving the transcription of enzymes that participate to the melatonin synthesis.

RESUME

1. Introduction.

Les horloges biologiques endogènes ont été développées par la plupart des organismes au cours de l'évolution pour s'adapter aux fluctuations journalières et saisonnières des conditions environnementales. Elles permettent aux organismes d'anticiper les changements dans leur milieu et d'adapter leur biologie en conséquence, leur offrant ainsi un avantage déterminant face aux contraintes écologiques (Dunlap, 1999). Ces horloges génèrent un ensemble de rythmes physiologiques et comportementaux, tels que le cycle veille/sommeil, l'activité locomotrice, la température, la libération de corticostérone ou la libération de mélatonine, qui persistent en l'absence de tout signal environnemental. Les oscillations endogènes ont une période proche mais rarement égale à 24 heures et présentent un phénomène de compensation thermique. Elles sont dites circadiennes (*circa* : environ ; *dies* : jour). Divers signaux environnementaux tels que l'alternance cyclique du jour et de la nuit ou la disponibilité et variabilité de la nourriture participent à l'ajustement de leur période à 24 heures exactement (Challet et al., 2003; Schibler et al., 2003).

Chez les Mammifères, de multiples rythmes sont originaires des noyaux suprachiasmatiques de l'hypothalamus (NSC), petite structure constituée de deux noyaux denses localisés immédiatement au-dessus du chiasma optique, de part et d'autre du troisième ventricule (Moore & Eichler, 1972; Stephan & Zucker, 1972) et qui constitue l'horloge principale. Diverses études récentes indiquent que d'autres structures centrales et périphériques, telles que les poumons, les reins, le foie, la *pars tuberalis* ou le noyau du lit de la strie terminale contiennent des oscillateurs périphériques (Amir et al., 2004; Yoo et al., 2004; Nishide et al., 2006; Wagner et al., 2007). A la différence de l'horloge des NSC, les rythmes des oscillateurs, à l'échelle du tissu, s'amortissent et disparaissent en quelques jours *in vitro* (Yamazaki et al., 2000; Abe et al., 2002; Yoo et al., 2004), et *in vivo* en l'absence des NSC (Terazono et al., 2003; Amir et al., 2004; Lamont et al., 2005). Des analyses à l'échelle cellulaire ont montré que la synchronisation intercellulaire des rythmes est nécessaire au maintient des oscillations à l'échelle du tissu (Welsh et al., 2004; Yoo et al., 2004) et l'hypothèse la plus récente suggère que les NSC seraient nécessaires à la synchronisation des rythmes des cellules à la périphérie.

Les oscillations qui naissent au sein de ces structures reposent sur un certain nombre de protéines dites 'horloges' dont l'expression cyclique est contrôlée par l'inhibition de la transcription de leurs propres gènes. La boucle de rétrocontrôle principale est constituée des facteurs de transcriptions BMAL1 et CLOCK qui s'hétérodimérisent pour activer l'expression des protéines inhibitrices PERIOD1/2/3 et CRYPTOCHROME1/2. Deux boucles de régulation supplémentaires stabilisent la boucle principale. Elles impliquent d'une part les protéines DEC1/2 qui s'hétérodimérisent et inhibent l'activité transcriptionnelle du complexe CLOCK-BMAL1 ; et d'autre part des récepteurs orphelins REV-ERBα, controlés par les hétérodimères CLOCK-BMAL1, et RORα qui régulent tous deux la transcription de BMAL1. (Reppert & Weaver, 2001; Ko & Takahashi, 2006). En plus de la régulation transcriptionnelle de ces gènes 'horloges', les modifications post-traductionnelles des protéines 'horloges', telles que leur phosphorylation, sont critiques dans le contrôle de la rythmicité des protéines et la détermination de la période des rythmes (Lowrey et al., 2000; Iwahana et al., 2004; Xu et al., 2005). L'état de phosphorylation des protéines 'horloges' en particulier influence leur stabilité et leur localisation subcellulaire (Dey et al., 2005; Vanselow et al., 2006), et les protéines kinases « casein kinase Iε/δ » (CKIε/δ) sont en partie responsables de ces modulations. Ainsi, le syndrome familial d'avance de phase du sommeil résulte d'une mutation sur la protéine 'horloge' PERIOD2 d'un site de phosphorylation par la CKIε, ce qui empêche la rétention nucléaire de la protéine (Vanselow et al., 2006). Le rythme s'en trouve accéléré, entraînant ainsi l'avance de phase du sommeil et le raccourcissement de la période des rythmes de température et de mélatonine. De plus, chez le hamster Syrien, la mutation *tau*, localisée sur le gène codant pour la CKIε, entraîne aussi un raccourcissement de la période des oscillations de l'horloge : l'activité de la CKIε mutée étant réduite, la phosphorylation des protéines PERIOD est altérée, ce qui entraîne leur évacuation précoce du noyau et leur dégradation rapide par le protéasome cytoplasmique (Lowrey et al., 2000; Dey et al., 2005).

Les régulations transcriptionnelles et post-traductionnelles apparaissent aussi essentielles à un autre aspect du système biologique circadien : la régulation de la synthèse de mélatonine. La mélatonine constitue l'une des efférences majeures de l'horloge des NSC en distribuant une partie de l'information temporelle issue des NSC à l'ensemble de l'organisme *via* la circulation générale. Sa synthèse est contrôlée par la libération nocturne de noradrénaline sur la glande pinéale et implique plusieurs enzymes, dont l'« arylalkylamine *N*-acetyltransferase » (AA-NAT) et l'« hydroxyindole *O*-

methyltransferase » (HIOMT) (Simonneaux & Ribelayga, 2003). Des études ont révélé que la phosphorylation par la « protein kinase A » (PKA) de l'AA-NAT (Ganguly et al., 2001; Obsil et al., 2001) et du facteur de transcription « cAMP-response element binding protein » (CREB) (Roseboom & Klein, 1995), qui participe à la transcription de l'*Aa-Nat*, est essentielle à leur activation.

Plus récemment, il a été montré que d'autres protéines kinases, les « mitogen-activated protein kinases » (MAPK), membres de la superfamille des sérine/thréonine kinases, sont impliquées dans la machinerie moléculaire de l'horloge des mammifères. Les MAPK sont des enzymes activées en réponse à des stimuli externes tels que les stimulations mécaniques, les facteurs de croissance, les hormones ou les cytokines. Elles jouent un rôle essentiel dans la régulation de nombreux processus cellulaires tels que la croissance, la survie, la différentiation ou la mort cellulaire (Barr & Bogoyevitch, 2001; Pearson et al., 2001). Des études récentes ont ainsi démontré que les « extracellular-signal regulated kinases 1/2 » (ERK1/2), dont l'activation montre une rythmicité circadienne dans les noyaux suprachiasmatiques (Obrietan et al., 1998; Pizzio et al., 2003), participent aux processus d'entraînement photique de l'horloge (Butcher et al., 2002; Dziema et al., 2003; Cheng et al., 2006). De plus, des associations entre les ERK1/2 et les protéines 'horloges' CRYPTOCHROME1/2 et BMAL1 ont également été mises en évidence et suggèrent une implication active des ERK1/2 dans la régulation des mécanismes moléculaires de l'horloge des mammifères (Sanada et al., 2002; Sanada et al., 2004). D'autres MAPK, les $p38^{MAPK}$, semblent participer à la détermination de la période des oscillations endogènes, car leur inhibition allonge significativement la période de l'horloge contenue dans la glande pinéale de poulet (Hayashi et al., 2003). Enfin, il a aussi été suggéré que ERK1/2 et $p38^{MAPK}$ régulent l'une des efférences majeures de l'horloge des NSC, en modulant l'activité de l'AA-NAT au niveau de la glande pinéale (Ho et al., 2006).

Toutefois, très peu d'informations ont été recueillies sur les rôles potentiels d'une troisième sous-famille de MAPK, les « c-JUN N-terminal kinases » (JNK), dans les mécanismes de régulation du système circadien. Compte tenu des rôles multiples des ERK1/2 et $p38^{MAPK}$ dans la régulation de l'horloge biologique, il est envisageable que les JNK soient également impliquées à différents niveaux de l'organisation circadienne. L'objectif de cette thèse a été d'évaluer les éventuelles fonctions régulatrices des

isoformes des JNK sur les mécanismes moléculaires de l'horloge et les mécanismes de la synthèse de la mélatonine.

2. Rôles des isoformes des JNK dans la régulation des mécanismes moléculaires du système circadien.

Plusieurs études récentes indiquent que les fibroblastes peuvent être utilisés comme modèle d'étude des mécanismes de l'horloge car ils contiennent une horloge fonctionnelle similaire à l'horloge des NSC (Balsalobre et al., 1998; Yagita et al., 2001). Nous avons donc utilisé les fibroblastes pour évaluer le rôle des isoformes des JNK dans les mécanismes de l'horloge. D'abord, nous avons montré par Western blot que **les isoformes p46 kDa et p54 kDa sont rapidement et transitoirement activées** suite à la synchronisation des rythmes de fibroblastes en culture. Toutefois, **seules les isoformes p46 kDa montrent une rythmicité dans leur niveaux de phosphorylation sur les 24 h qui suivent la synchronisation**. Nous avons alors décidé d'évaluer le rôle potentiel de ces phosphorylations en empêchant l'activation des JNK *via* un traitement par l'inhibiteur SP600125. Pour cette étude, nous avons travaillé sur des cellules (fibroblastes) ou tissus (NSC, glande pinéale, poumons, reins) transformés génétiquement par inclusion du gène rapporteur *luciférase* lié au promoteur du gène codant pour la protéine 'horloge' PERIOD1 (fibroblastes et souris *Period1-luciferase*) (Izumo et al., 2003; Herzog et al., 2004) ou par production d'une protéine de fusion PERIOD2::LUCIFERASE (souris $Period2^{Luciferase}$) (Yoo et al., 2004). Nous avons alors enregistré en continu l'émission de bioluminescence, née du clivage de la luciférine par l'enzyme Luciférase grâce à un système de mesure de la bioluminescence en temps réel. Nos résultats montrent que **l'inhibition de la phosphorylation des isoformes des JNK entraîne un allongement important, et dose-dépendant, de la période des oscillations de l'intensité de la bioluminescence** dans notre modèle de fibroblastes. Une concentration en inhibiteur de 30µM allonge notamment la période des oscillations de plus de 7 heures. De plus, nous avons obtenu des **effets similaires** de l'inhibiteur des JNK sur des **cultures de coupes de NSC et des cultures organotypiques de glande pinéale et de poumon** provenant de souris transgéniques pour les constructions *Period1-luciferase* ou $Period2^{Luciferase}$. Néanmoins, l'inhibition de la phosphorylation des JNK dans des cultures organotypiques de **rein** induit des **effets différents** : cela altère les oscillations dirigées par le promoteur du gène *Period1* chez les animaux *Period1-*

luciferase tandis que cela n'a pas d'effet sur la rythmicité induite par le promoteur du gène *Period2* chez des souris *Period2*^{Luciferase}. Nos résultats suggèrent donc que l'activation des isoformes des JNK est nécessaire au contrôle de la période des rythmes endogènes de certaines structures contenant une horloge ou un oscillateur. Nous avons renforcé cette conclusion en montrant que **l'acide valproique**, un anticonvulsant utilisé pour traiter les symptômes du syndrome bipolaire, et connu pour ses effets sur la période des rythmes comportementaux (Rietveld & van Schravendijk, 1987; Klemfuss & Kripke, 1995; Dokucu et al., 2005), **active les JNK et réduit simultanément la période des oscillations** de l'intensité de la bioluminescence mesurée dans les fibroblastes *Period1-luciferase*. Ces travaux originaux suggèrent un rôle important du niveau de phosphorylation des isoformes des JNK dans la définition de la période des oscillations endogènes de divers horloge et oscillateurs.

En utilisant notre modèle de fibroblastes *Period1-luciferase*, nous avons aussi démontré que **l'inhibition des p38^{MAPK} par l'inhibiteur SB203580 allonge de manière remarquable la période des oscillations de bioluminescence** dirigées par le promoteur du gène *Period1*. En confirmant des données préalables obtenues dans la glande pinéale de poulet (Hayashi et al., 2003), nous validons non seulement notre modèle d'étude, mais démontrons aussi que les deux sous-familles de MAPK, JNK et p38^{MAPK}, qui constituent les « stress-activated protein kinases » (SAPK), possèdent des fonctions régulatrices semblables du rythme des oscillations circadiennes. A l'inverse, ERK1/2 ne participent pas à la détermination de la période des oscillations. Dans notre modèle, leur inhibition semble plutôt empêcher la synchronisation intercellulaire des rythmes des fibroblastes *Period1-luciferase* en culture, ce qui confirme des résultats antérieurs menés sur des fibroblastes de souris (Akashi & Nishida, 2000).

3. Rôles des isoformes des JNK dans la modulation de la synthèse de mélatonine dans la glande pinéale.

La sécrétion rythmique de mélatonine par la glande pinéale est reconnue comme une sortie majeure de l'horloge des NSC. Cette hormone est impliquée dans la synchronisation des rythmes circadiens (Pevet, 2003), et joue surtout un rôle crucial dans la synchronisation de phénomènes saisonniers, en particulier la reproduction (Malpaux et al., 1999). Plusieurs études ont montré que les ERK1/2 (Ho et al., 1999), ainsi que les p38^{MAPK} (Chik et al., 2004), sont activées la nuit dans la glande pinéale, par des

mécanismes impliquant la libération de noradrénaline – qui accroît les niveaux intracellulaires d'AMPc et de GMPc – et le recrutement des récepteurs α_1-et β_1-adrénergiques. Nos analyses par Western blot montrent que **les isoformes des JNK, comme les p38^{MAPK}, sont également phosphorylées par une stimulation à l'AMPc dans la glande pinéale de rat**. Toutefois, les dynamiques d'activation diffèrent, les JNK étant rapidement et transitoirement activées (pic d'activation observé 20 min après stimulation), tandis que l'activation des p38^{MAPK} est lente et maximale 4 h après la stimulation (Chik et al., 2004). Ces différences dans la cinétique de l'activation des SAPK suggèrent que les JNK et les p38^{MAPK} possèdent des rôles distincts dans la glande pinéale.

En plus de l'activation des SAPK, la stimulation nocturne de noradrénaline conduit la transcription d'un certain nombre de gènes impliqués dans la synthèse de la mélatonine, tels que l'*Aa-Nat*. La stimulation adrénergique induit aussi l'expression de *Mkp-1*, qui code pour une MAPK phosphatase capable de désactiver les trois principales sous-familles de MAPK, et du gène 'horloge' *Period1*. Afin d'étudier le rôle de l'activation des SAPK dans la stimulation de la transcription de ces gènes, nous avons utilisé les inhibiteurs SP600125 (JNK) et SB203580 (p38^{MAPK}). Nos résultats indiquent que **suite à une stimulation à l'AMPc, l'activation des JNK est nécessaire à la transcription des gènes *Aa-Nat* et *Period1*. De plus, seule l'expression de *Mkp-1* requiert la double activation des JNK et des p38^{MAPK}**. Ces données rejoignent une étude antérieure qui a montré que les p38^{MAPK} ne participe pas à la régulation de la transcription de l'*Aa-Nat* (Man et al., 2004).

4. Identification des mécanismes mis en jeu – Perspectives.

L'ensemble des résultats obtenus suggère une implication importante des SAPK dans la régulation de divers aspects de l'horloge. Nos données indiquent d'abord que les JNK, au même titre que les p38^{MAPK} (Hayashi et al., 2003), sont susceptibles de jouer un rôle critique dans la régulation des mécanismes moléculaires de l'horloge et notamment dans la détermination de la période des oscillations endogènes. De multiples régulations aident à définir la période de 24 h. Il s'agit notamment d'une régulation transcriptionnelle des gènes 'horloges' et de modifications post-traductionnelles des protéines 'horloges'. Il est ainsi envisageable que l'activation des JNK affecte l'une ou l'autre de ces voies de régulations.

Les mécanismes de régulation transcriptionnelle pourraient mettre en jeu les facteurs de transcription « activator protein-1 » (AP-1), dont l'activité est stimulée par les isoformes des JNK, et qui sont susceptibles d'induire l'expression de gènes 'horloges'. Diverses approches pourraient être envisagées pour évaluer la validité de cette hypothèse.

- Si les complexes AP-1 sont impliqués, leur activité de liaison à l'ADN devrait être élevée par l'activation des JNK. A l'inverse, le traitement par l'inhibiteur SP600125 devrait réduire cette activité de liaison. Cette analyse pourrait être effectuée par « electrophoretic mobility shift assay » (EMSA).
- De plus, le facteur de transcription c-JUN forme avec d'autres facteurs apparentés, tels que c-FOS ou FRA-2, les facteurs AP-1. La surexpression de c-JUN dans notre modèle de fibroblastes *Period1-luciférase* est ainsi susceptible d'élever l'activité des complexes AP-1, promouvoir la transcription des gènes 'horloges' cibles et ainsi accélérer les oscillations de bioluminescence dirigées par le promoteur du gène *Period1*. La transfection dans les fibroblastes de c-JUN recombinant insérés dans un vecteur d'expression permettrait ainsi de valider notre hypothèse.
- L'induction de la transcription *via* les complexes AP-1 s'effectue par la liaison des facteurs à des sites de liaison spécifiques au niveau du promoteur des gènes. La construction de gènes rapporteurs *luciférase,* placés sous le contrôle de ces promoteurs mutés pourrait permettre d'identifier l'implication des complexes transcriptionnels AP-1.

D'autre part, les isoformes des JNK pourraient moduler la phosphorylation d'une ou plusieurs protéine(s) 'horloge(s)' cible(s) comme cela a été démontré pour les ERK1/2 qui interagissent et phosphorylent les protéines BMAL1 (Sanada et al., 2004) et CRYPTOCHROME1/2 (Sanada et al., 2002).

- L'identification de ces protéines 'horloges' cibles pourrait être effectuée par immunoprécipitation des isoformes phosphorylées des JNK et détection par Western blot des protéines 'horloges' co-immunoprécipitées.
- Après identification de la protéine candidate, il serait possible de déterminer si la liaison des JNK avec la protéine cible entraîne sa phosphorylation. Une étude de l'activité kinase des JNK sur la protéine candidate pourrait être réalisée.

La phosphorylation de la protéine cible pourrait influencer la période des oscillations *via* la modification de la stabilité (Eide et al., 2005) ou de la localisation subcellulaire de la protéine candidate (Vanselow et al., 2006), comme cela a été démontré pour la CKIε sur les protéines PERIOD.

- L'inhibition des JNK induisant un allongement de la période des oscillations, il est envisageable que la phosphorylation de cette protéine la stabilise et la protège de la dégradation par le protéasome cytoplasmique. Cette hypothèse pourrait être testée *in vitro* en surexprimant la protéine candidate avec des isoformes des JNK en présence ou non d'un inhibiteur du protéasome.
- D'autre part, la phosphorylation de la protéine candidate pourrait promouvoir sa nucléarisation. Une surexpression de la protéine cible et des JNK dans des fibroblastes, couplée à l'identification par immunohistochimie de la localisation cellulaire de la protéine, en présence ou non de l'inhibiteur SP600125, permettrait de vérifier cette hypothèse.

Les effets rapportés sur l'activation des JNK d'une part et la participation des JNK et des $p38^{MAPK}$ dans la synthèse de certains gènes, tels que l'*Aa-Nat*, d'autre part, suggèrent un rôle jusqu'à présent méconnu des SAPK dans la synthèse et la libération rythmique de la mélatonine.

Bien que les mécanismes impliqués ne soient pas encore élucidés, l'activation des JNK par l'AMPc suggère que de manière similaire à l'activation des $p38^{MAPK}$, la libération nocturne de noradrénaline régule l'activation des JNK dans la glande pinéale.

- Il serait intéressant d'identifier la voie de signalisation menant à l'activation des JNK. Cette étude ferait appel à des techniques d'immunodétection sur des échantillons provenant de glandes pinéales de rat traitées par divers stimulants et inhibiteurs.
- S'il s'avère que les isoformes des JNK sont activées par la noradrénaline, il est probable que la phosphorylation des JNK sur 24 h présente un rythme avec un pic de phosphorylation nocturne, du fait de la libération nocturne de la noradrénaline. Le prélèvement sur 24 h de glandes pinéales de rat et l'analyse par Western blot des niveaux des JNK phosphorylées permettrait de tester notre hypothèse.

Divers mécanismes sont susceptibles d'être utilisés par les SAPK pour réguler l'expression de l'*Aa-Nat*, de *Mkp-1* et de *Period1* par l'AMPc. D'une part, il est peu probable que l'*Aa-Nat* et *Period1* soient régulés par des facteurs de transcriptions néosynthétisés dans la mesure où l'inhibition de la synthèse protéique antérieure à une stimulation par la noradrénaline n'inhibe pas les niveaux d'expression des deux gènes suite à la stimulation (Fukuhara et al., 2002). Néanmoins, une régulation de *Mkp-1* par ces facteurs de transcription ne peut pas être exclue. L'activation des complexes AP-1 survenant après l'initiation de la transcription de *Mkp-1* (Guillaumond et al., 2000; Price et al., 2004b), il est peu probable que les facteurs AP-1 contribuent à l'expression de *Mkp-1*. Toutefois, il est envisageable que les JNK phosphorylent d'autres complexes transcriptionnels, comme les facteurs SP1, pour promouvoir leur activité transcriptionnelle au niveau du promoteur de *Mkp-1*. Cette hypothèse pourrait être validée en bloquant d'abord la traduction de SP1 dans des pinéalocytes de rat *via* la transfection d'ARNsi, et en analysant ensuite les niveaux d'expression de *Mkp-1* par « real-time quantitative polymerase chain reaction » suite à une stimulation à l'AMPc.

L'activation du facteur de transcription CREB par la PKA sur le résidu Ser-133 est essentielle à la transcription de l'*Aa-Nat* (Roseboom & Klein, 1995). Gau et al. (2002) ont montré que la phosphorylation de CREB sur plusieurs résidus est nécessaire à la médiation des décalages de phases induits par la lumière chez la souris. Il est ainsi envisageable que l'activation de la transcription des gènes *Aa-Nat*, *Period1* et *Mkp-1* dépende de la phosphorylation de CREB sur plusieurs sites, PKA phosphorylant le résidu Ser-133 et JNK phosphorylant d'autres résidus.

- En utilisant un assortiment d'anticorps générés contre les sites connus de phosphorylation de CREB (Kornhauser et al., 2002), il serait possible d'identifier le ou les résidus phosphorylés par les isoformes de JNK après stimulation à l'AMPc en présence ou non de l'inhibiteur SP600125.
- La surexpression de CREB muté au niveau des sites de phosphorylation identifiés, permettrait d'évaluer l'importance de la phosphorylation de ces résidus dans la transcription des gènes *Aa-Nat*, *Period1* et *Mkp-1* par l'AMPc. Cette étude serait menée par « real-time quantitative polymerase chain reaction ».

La démonstration que l'expression de *Mkp-1* seule, et non pas celle de l'*Aa-Nat* et de *Period1*, est activée par $p38^{MAPK}$ suggère que les mécanismes mis en jeu par ces kinases

sont différents de la voie de signalisation initiée par les isoformes des JNK. Ces mécanismes pourraient impliquer d'autres facteurs de transcription. Plusieurs expériences permettraient de les identifier.

- La comparaison des niveaux d'expression du gène rapporteur *luciférase,* placé sous le contrôle de différents segments du promoteur de *Mkp-1,* et stimulé à l'AMPc en présence ou non de l'inhibiteur SB203580, permettrait d'identifier une zone restreinte du promoteur responsable de l'activation de la transcription du gène par les $p38^{MAPK}$.
- Pour identifier spécifiquement le facteur de transcription impliqué, les pinealocytes de rat pourraient être stimulés à l'AMPc après transfection avec des plasmides rapporteurs *luciférase* construits avec la zone restreinte du promoteur *Mkp-1*, préalablement mutée au niveau de sites spécifiques de liaison à l'ADN de facteurs de transcription.

5. Conclusion.

L'organisation circadienne est composée de trois entités essentielles : un système afférent qui participe à l'entraînement des rythmes à 24 h, l'horloge moléculaire en elle-même qui génère les rythmes physiologiques et comportementaux, et un système efférent qui distribue l'information issue de l'horloge moléculaire à l'ensemble de l'organisme. **Nos données, associées aux travaux préalablement publiés démontrent que les MAPK sont impliquées dans la régulation du système circadien à tous les niveaux**. Ainsi, les ERK1/2 contrôlent l'entraînement de l'horloge des NSC par la lumière et participent à la synchronisation intercellulaire des rythmes à la périphérie. De plus, les $p38^{MAPK}$ et les isoformes des JNK contribuent au contrôle de la période de l'horloge moléculaire. Enfin, les trois sous-familles de MAPK sont impliquées dans la modulation de la synthèse de la mélatonine, qui constitue une efférence majeure des NSC. Ainsi, $p38^{MAPK}$ et JNK sont susceptibles de contrôler la transcription nocturne d'un certain nombre de gènes, dont l'*Aa-Nat*, *Mkp-1* et *Period1*, tandis que ERK1/2 et $p38^{MAPK}$ régulent l'activité de l'AA-NAT

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RECURRENT ABBREVIATIONS

5-HT, 5-hydroxytryptamine, serotonin
AA-NAT, arylalkylamine *N*-acetyltransferase
AC, adenylyl cyclases
AP-1, activator protein-1
ASK, apoptosis signal-regulated kinase
ATF, activating transcription factor
ATP, adenosine triphosphate
AVP, arginine vasopressine
bHLH, basic Helix-Loop-Helix
BMAL1, brain and muscle ARN-t like protein 1
BNSTov, oval nucleus of the bed nucleus of the stria terminalis
CBP, CREB-binding protein
CDK, cyclin-dependent kinase
CEA, central nucleus of the amygdale
CKI, casein kinase I
CLOCK, circadian locomotor output cycles kaput
CRE, cAMP-response element
CREB, CRE binding protein
CREM, CRE modulator
CT, circadian time
DBP, D-element binding protein
DD, constant darkness
DIO2, type 2 iodothyronine deionidase
DMEM, dulbecco's eagle modified medium
DSP, dual-specific protein phosphatases
ERK, extracellular signal-regulated kinases
GABA, γ-amino butyric acid
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GC, guanylyl cyclases
GEF, guanine nucleotide exchange factors
GLU, glutamate
GPCR, G protein-coupled receptors
GRP, gastrin-releasing peptide
GSK3, glycogen synthase kinase 3
HAT, histone acetyltransferase
HDAC, histone deacetylase
HIOMT, hydroxyindole *O*-methyltransferase
ICER, inducible cAMP early repressor
IEG, immediate early gene
IGL, intergeniculate leaflets
IL, interleukin
IML, intermediolateral nucleus
ir, immunoreactivity
IRAK, IL-1 receptor associated kinase
JNK, c-JUN N-terminal kinases
KO, knockout
LD, light/dark
LL, constant light
MAP-2, microtubule-associated protein 2
MAPK, mitogen-activated protein kinases
MAPKAPK, MAPK-activated protein kinase
MAPKK, **MKK**, **MEK**, MAPK kinases
MAPKKK, **MKKK**, **MEKK**, MAPKK kinases
MEL, melatonin
MKB, MAPK binding motif
MKP, MAPK phosphatases
MOP, members of the PAS superfamily of proteins
MP-1, MEK-partner 1
MSK, mitogen- and stress-activated kinase
MT, melatonin receptor
NE, norepinephrine
NO, nitric oxide
NPY, neuropeptide Y
OB, olfactory bulbs
PACAP, pituitary adenylate cyclase-activating peptide
PAS, Per-ARN-t-Sim
PBS, phosphate buffer saline
PCR, polymerase chain reaction
PER, period
PG, pineal gland
PK2, prokineticin 2
PKG, cGMP-related protein kinase
PMT, photomultiplier tube
PP, protein phosphases
PRC, phase response curve
PT, pars tuberalis
PTP, tyrosine-specific protein phosphatases
PVN, paraventricular nucleus of the hypothalamus
PVT, paraventricular thalamic nucleus
Q-PCR, real-time quantitative reverse transcriptase polymerase chain reaction
RGC, retinal ganglion cell
RHT, retinohypothalamic tract
ROR, retinoic acid related orphan receptor
RORE, ROR response elements
RSK, p90 ribosomal S6 kinase
RTK, tyrosine kinase receptors
RyR, ryanodine receptors
SAPK, stress-activated protein kinases
SCG, superior cervical ganglions
SCN, suprachiasmatic nuclei
STEP, striatal enriched phosphatases
SUMO, small ubiquitin-related modifier protein
TAB, TAK1 binding protein
TAK, transforming growth factor-β-activated protein kinase

TIEG, transforming growth factor β-inducible early gene
TNF, tumor necrosis factor
TNF-R, TNF receptor
TPOH, tryptophane hydroxylase
TRAF, TNF receptor-associated factor
TRE, tetradecanoyl phorbol acetate response element
VIP, vasoactive intestinal peptide
ZT, Zeitgeber time
α1-AR, α_1-adrenergic receptor
β1-AR, β_1-adrenergic receptor

INTRODUCTION

A. INTRODUCTION

The animal behavior is characterized by a succession of redundant events that occur with a defined frequency, like the onset of activity, mealtime, awakening, sleep… Remarkably, these events are adequately phased with the ecosystem and their timing is correlated to the geophysical constraints met on Earth. On a daily basis, the Earth rotates on its axis, which generates a highly reproducible light/dark cycle (LD). Many animals have phased their behavior with this LD cycle, some being active during the day, others being active at night. Similarly, the tilted rotation of the Earth around the sun generates major environmental changes that define seasons, and many animals exhibit behaviors in phase with seasons. This is the case of mating, which precisely occurs at the same season every year to allow the offspring to be born at the mildest season. In species with a short gestation period like rodents, animals mate during spring while species with a longer gestation period, like sheeps, breed during fall. The whole physiology of organisms displays oscillations that are correlated to the cyclic behavioral changes.

Over the years, it has become evident that most living organisms have developed an **endogenous internal timing system** that not only allows them to **measure time**, but also permits an **anticipation** of the major events that arise from the highly reproducible geophysical cyclic changes. They are thus able to cyclically modify their physiology to adapt to changes that are about to happen in their environment. This endogenous system, referred to as the **circadian system** (*circa*: about, *dies*: day), cycles independently from external cues but can be reset by various environmental signals to keep the system in phase with the environment.

The circadian system orchestrates a wide range of circadian rhythms, which can be defined as cyclic endogenous events with a **period close to but not equal to 24 h**. Remarkably, **circadian rhythms persist in constant conditions** and are **temperature compensated**. Besides, circadian rhythms can be **reset** by various environmental cues, such as light, temperature or food availability. The lighting regimen, the most reproducible event in the environment, has been characterized as the strongest synchronizer (*Zeitgeber*: time giver) of circadian rhythms. In constant conditions, there

are no synchronizing cues and the circadian system free-runs at its own endogenous pace. A remarkable model of study of the circadian system consists in monitoring the locomotor activity rhythms of rodents housed in cages equipped with a running-wheel (Figure 1). Under daily cycle of 12 h of light and 12 h of darkness (12:12 LD), animals are synchronized to the lighting regimen and their locomotor activity period equals exactly 24 h. When animals are transferred from the LD schedule to a constant darkness environment (DD), they start to free-run and display a locomotor activity period that reflects their endogenous pace. However, daily oscillations in activity and rest phases persist and nocturnal animals keep running during the subjective night and rest during the subjective day. The endogenous period can be subdivided in 24 circadian hours. One circadian hour thus equals 1/24 of the circadian endogenous period and **circadian time** (CT) 12 is defined as the onset of locomotor activity or as subjective dusk in nocturnal animals. As opposed to circadian time, ***Zeitgeber* time** (ZT) refers to 1 hour exactly and ZT0 is defined as the onset of light.

In this introduction, I will describe the cellular and molecular mechanisms of the central clock located in the **suprachiasmatic nuclei** (SCN), introduce the **circadian oscillators** located in the periphery and describe the regulation of the **melatonin** (MEL), a main output of the circadian system. But first, as this work mainly focused on the implication of **mitogen-activated protein kinases** (MAPK) in the regulation of several aspects of the circadian system, I will present the MAPK family and exemplify their activation mechanisms.

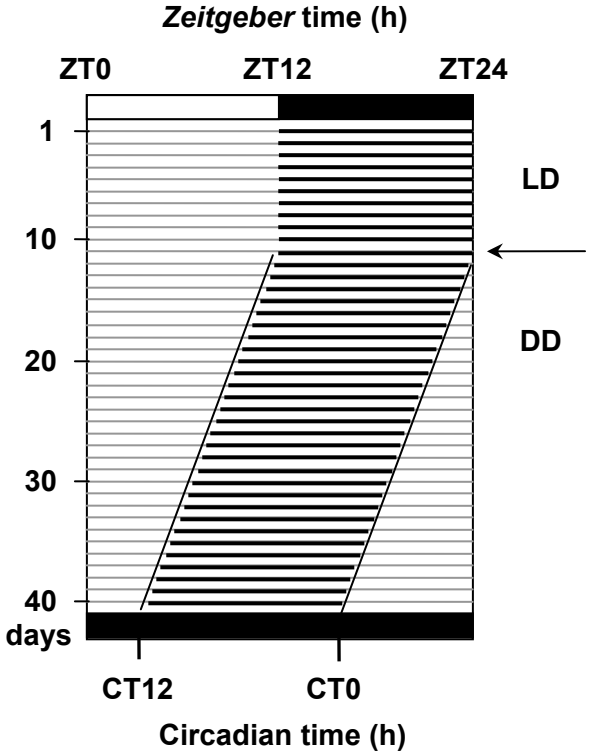

Figure 1. Locomotor activity of a nocturnal rodent.

Under a light/dark cycle (LD), locomotor activity (dark lines) is restricted to night time and entrained to dusk. On day 11 (arrow), animals are placed in constant darkness (DD). They stay active during the subjective night but start to free-run at their endogenous period. These animals have an endogenous circadian period shorter than 24 h. Each day, they become active earlier than the previous day. Dark boxes represent periods of obscurity.

1. THE MAPK FAMILY

1.1. MAPK: A CRITICAL PROTEIN KINASE FAMILY WHICH MEDIATES SIGNAL TRANSDUCTION

In all organisms, cells must respond to a large variety of extracellular stimuli, which include **mitogens**, **growth factors**, **cytokines**, **pheromones**, **hormones**, **UV radiation**, **osmotic stress**, **oxidative stress**, **mechanical forces** and **neurotransmitters**. Highly interactive networks allow these signals to be transduced across the cell membrane and through the cytoplasm to the nucleus. Within these signal transduction networks, the **MAPK** play a key role. The MAPK family (Pearson et al., 2001) belongs to the large superfamily of eukaryotic protein kinases, and MAPK have been classified within the C-M-G-C group of proteins, which comprises cyclin-dependent kinases, MAPK, glycogen synthase kinases 3 (GSK3) and cell-division cycle-dependent (cdc)-like kinases. By describing a serine/threonine kinase which phosphorylated the microtubule-associated protein (MAP)-2 upon insulin stimulation in mouse adipocytes, Ray & Sturgill identified the first member of the MAPK family (Ray & Sturgill, 1987). Since then, more than a hundred evolutionary well-conserved MAPK, which all share a common sequence located on loop 12 of the three-dimensional structure (Figure 2), have been reported. A phylogenetic study allowed the distinction of nine subfamilies of MAPK classified in three subgroups (Kultz, 1998): the **stress-activated protein kinases** (SAPK), the **extracellular signal-regulated kinases** (ERK) and the **MAPK3** subgroups. Among these nine subfamilies, the animal SAPK1 subfamily (also named **c-JUN N-terminal kinases**, JNK), the animal SAPK2 subfamily (also named **p38^{MAPK}**) and the animal ERK1 subfamily (also named **ERK**) are the major and the most widely studied groups (Table 1, Figure 3). While JNK and p38^{MAPK} are mainly activated by various **stress stimuli** including cytokines (Dunn et al., 2002), osmotic shock (Yamagishi et al., 2001), nitric oxide (NO) (Ghatan et al., 2000; Cheng et al., 2001) and UV radiation (Derijard et al., 1994), ERK are mostly stimulated by **extracellular signals** such as growth factors (Miyasaka et al., 1990; Boulton et al., 1991), neurotrophins (Cavanaugh et al., 2001), NO (Kanterewicz et al., 1998) or neurotransmitters like norepinephrine (NE) (Ho & Chik, 2000).

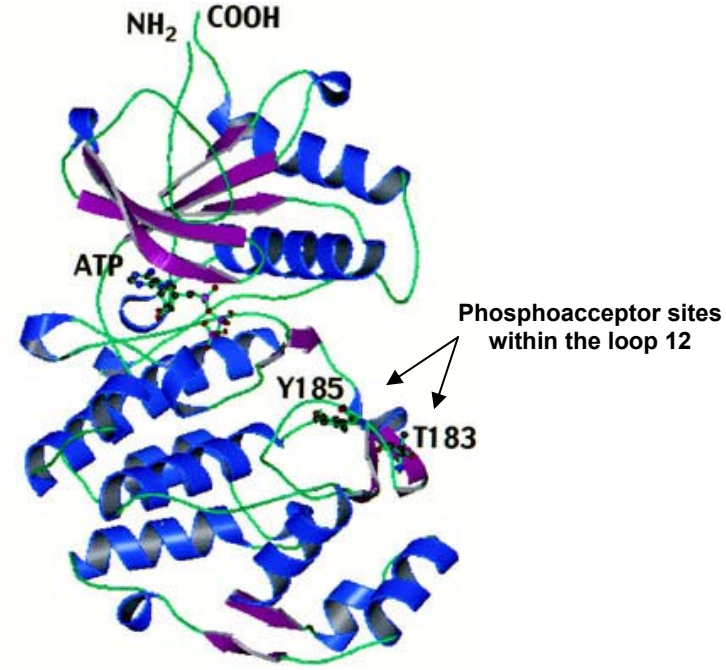

Figure 2. Unphosphorylated 3D structure of ERK2.

MAPK activity is controlled by phosphorylation of the tyrosine and threonine residues within the activation loop (loop 12). ATP binds in the interior of the active site.
(Adapted from Paerson et al., 2001)

MAPK	Molecular weight	Phosphorylation motif	Expression
ERK1	42 kDa	TEY	Ubiquitous
ERK2	44 kDa	TEY	Ubiquitous
ERK3	63/95-100 kDa	SEG	Ubiquitous
ERK4	45 kDa	TEY	
ERK5	98 kDa	TEY	Widely expressed except in the liver
ERK6	42-45 kDa	TGY	Widely expressed, at various levels
ERK7	61 kDa	TEY	Widely expressed, at various levels
ERK8	60 kDa	TEY	Widely expressed with a maximum in lungs and kidneys
p38α	38 kDa	TGY	Widely expressed with a maximum in the skeletal muscle
p38β	38 kDa	TGY	Widely expressed with a maximum in the heart and skeletal muscle
p38γ	42-45 kDa	TGY	Skeletal muscle
p38δ	38-42 kDa	TGY	Lungs, kidneys and testis
JNK1	46/54-55 kDa	TPY	Ubiquitous
JNK2	46/54-55 kDa	TPY	Ubiquitous
JNK3	46/54-55 kDa	TPY	Brain, heart and testis

Table 1. Mammalian MAPK.

MAPK are part of signal transduction modules. These modules whose structure is identical among distinct subgroups are characterized by a **cascade of phosphorylation of several protein kinases** (Figure 3). Typically, MAPK are activated through phosphorylation on both **threonine and tyrosine** residues of the **TXY** motif, conserved within the SAPK and ERK subgroups. This phosphorylation is achieved by the **dually-specific protein kinases MAPK kinases** (MAPKK, also named MKK or MEK) (Matsuda et al., 1992; Wu et al., 1993), located immediately upstream of MAPK in the signaling cascade. MAPKK present narrow substrate specificity, with each kinase phosphorylating only one or few MAPK. They are themselves activated by upstream kinases, the **MAPKK kinases** (MAPKKK, also named MKKK or MEKK), which phosphorylate either the two serine or the two threonine residues of the MAPKK activation loop (Kosako et al., 1992). Additional kinases may be required to activate this cascade.

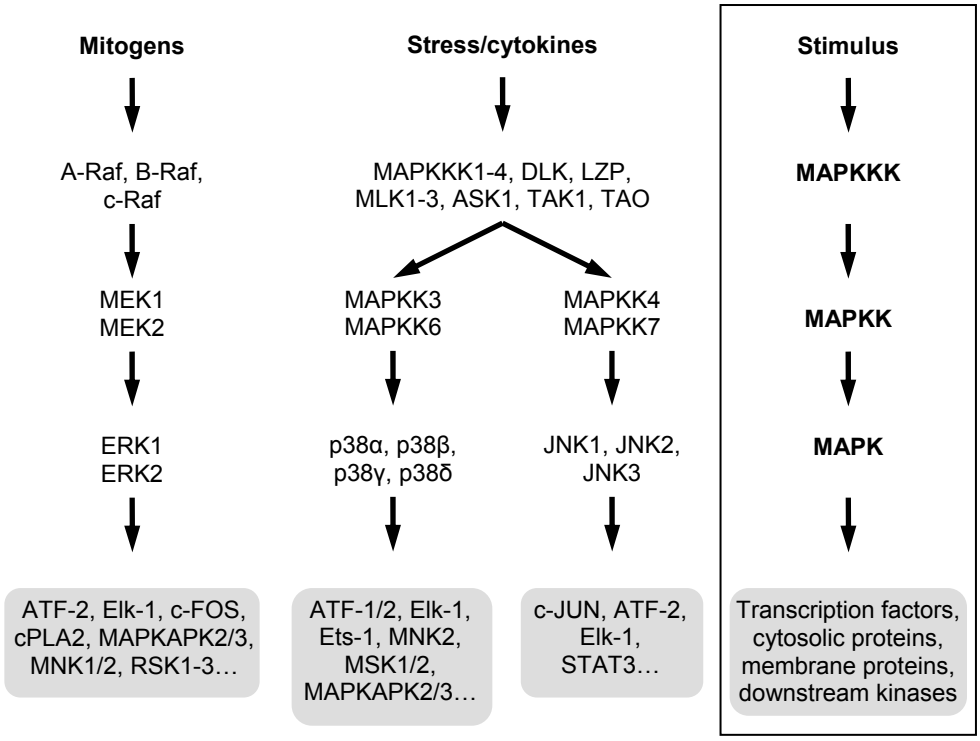

Figure 3. Signaling transduction modules of MAPK.

Mitogens and stress/cytokines stimuli lead to activation of many intracellular protein targets through a cascade of phosphorylation of MAPKKK/MAPKK/MAPK organized in three-kinase modules. See text for more details and abbreviations used.

The **hierarchical organization** of these **three-kinase modules** provides several characteristics that are essential for the transduction of the signal:

- The skeleton of the transduction modules, which include intermediate mediators, permit **additional controls** for the definition of the cell response. In this system, intermediate kinases can receive inputs from other signaling pathways (Frost et al., 1997; Schönwasser et al., 1998; Mackova et al., 2000) to either enhance or suppress the signal to the MAPK and eventually refine the cell response.
- Another important ability of the three-kinase modules is **signal amplification**. This will happen if each successive kinase in the cascade is more abundant than its regulator. This could hypothetically occur at any step of the module. However, Ferrell demonstrated that in the case of the ERK pathway, the amplification occurs at the MAPKKK – MAPKK step, since this latter one is much more abundant than its regulator (Ferrell, 1996). On the contrary, amplification is unlikely to happen at the MAPKK – MAPK step since amounts of regulator kinases and target kinases are approximately similar.
- Several studies indicated that the dynamic of the phosphorylation of the TXY motif of MAPK by dually-specific MAPKK follows a **distributive model** (Robbins & Cobb, 1992; Ferrell & Bhatt, 1997). In this mechanism, MAPKK firstly phosphorylate the tyrosine residue of the MAPK targets. The inactive monophosphorylated MAPK accumulate before being activated through phosphorylation on the threonine residue. The major advantage of this two-collision mechanism is the establishment of a **threshold**: the inactive tyrosine-phosphorylated MAPK must accumulate before being converted to the active state by phosphorylation on threonine, which allows additional modulation by other signaling pathways.

It has also been described that the three-kinase modules are part of complexes where additional proteins including **scaffold proteins** (Morrison & Davis, 2003) and **protein phosphatases** (Farooq & Zhou, 2004) interact to control the activation of MAPK cascades (*sections A1.5 and A1.6*). This tight regulation of MAPK leads to the phosphorylation of targets which all contain a **serine/threonine-proline** activation motif (Roux & Blenis, 2004).

1.2. THE EXTRACELLULAR SIGNAL-REGULATED KINASE SUBGROUP

1.2.1. THE ERK SIGNALING MODULE

1.2.1.1. ERK subfamily is constituted of many isoforms

Using the initial work of Ray and Sturgill (1987), Boulton and colleagues made a major contribution to the discovery of MAPK families (Boulton et al., 1990). By using degenerate primers based on short peptide sequences of an insulin-stimulated protein kinase that phosphorylates MAP-2, they succeeded in amplifying and cloning a novel protein kinase, which they named **ERK1**. They suggested several related kinases may exist and indeed identified a new component of the recently-established ERK subfamily, **ERK2** (Boulton & Cobb, 1991; Boulton et al., 1991). ERK1 and ERK2 proteins, 42 and 44 kDa respectively (Table 1), are highly similar with 83% sequence identity. A greater identity is even found in the activation loop and the core regions involved in binding substrates (Boulton et al., 1991). In both kinases, a glutamate (GLU) residue separates the common phosphoacceptor sites threonine and tyrosine to constitute the **TEY** motif (Payne et al., 1991). ERK1 and ERK2 have been the most widely studied so far in the ERK subfamily, in part because of their ubiquitous expression among tissues and cell lines (Boulton et al., 1991) and their wide range of extracellular stimulants, which include **mitogens** and numerous **growth factors**.

Additional studies allowed the identification of other related ERK subfamily members, namely **ERK3, ERK4, ERK5, ERK6, ERK7 and ERK8** (Bogoyevitch & Court, 2004). They exhibit distinct expression profiles and their molecular weight ranges from 42 kDa to 98 kDa (Table 1). To date, only ERK1 and ERK2 have been implicated in circadian biology and I will therefore describe in the following sections the known mechanisms of ERK1/2 signaling cascade.

1.2.1.2. Nature of the ERK signaling cascade

ERK1/2 isoforms are involved in many aspects of the cellular physiology including the regulation of the **cell cycle** and the **circadian rhythms**. To illustrate the mechanisms of

ERK1/2 activation, I will further depict the well-described stimulation of the ERK1/2 pathway by activation of **tyrosine kinase receptors** (RTK) (Figure 4) and **G protein-coupled receptors** (GPCR).

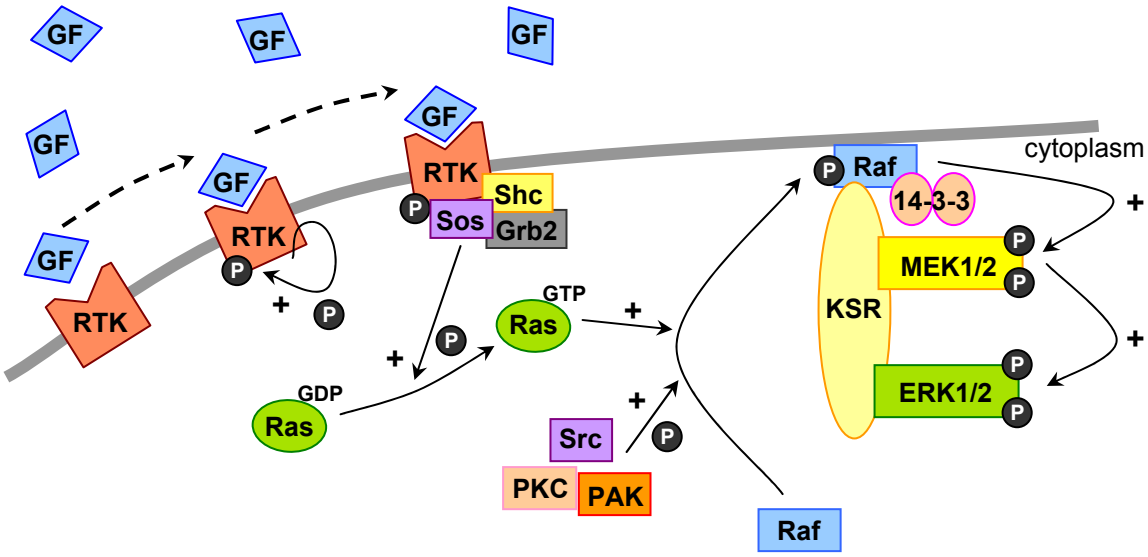

Figure 4. Simplified mechanism of ERK1/2 activation: example of growth factor-induced activation by recruitment of tyrosine kinase receptors.

Upon binding of growth factors (GF), tyrosine kinase receptors (RTK) activate themselves by autophosphorylation. This allows the recruitment by RTK of adaptor proteins (Grb2, Shc, Sos) and the formation of mutliprotein complexes. These complexes promote the GTP/GDP exchange on Ras. Raf is further recruited by the active GTP-Ras and targeted to the membrane where it is phosphorylated by Src, protein kinase C (PKC) or p21-activated kinase (PAK). Activated Raf binds 14-3-3 protein dimers, which stabilize it. The scaffold protein kinase suppressor of Ras (KSR) interacts with the kinase components of the cascade and facilitates the activation cascade that leads to MEK1/2 and ERK1/2 phosphorylation. GF, growth factor; P, phosphate. "+" represents facilitating steps. Dotted arrows indicate successive events occurring on the same RTK.

Activation of RTK by growth factors and activation of GPCR by specific ligands are indeed the main signaling pathways leading to ERK1/2 phosphorylation (Hunter, 1995; Pawson & Scott, 1997). Upon attachment of the ligand, the **RTK** are activated through autophosphorylation. This allows the **recruitment of adaptor proteins** such as Shc and Grb2 as well as son of sevenless (Sos) and other **Ras-activating guanine nucleotide exchange factors** (GEF), which contribute to the formation of multiprotein complexes.

By exchanging GDP for GTP, these GEF stimulate GTP-binding proteins of the Ras family, which is composed of several related proteins: **H-Ras, K-Ras, N-Ras** and **Rap-1**, all expressed ubiquitously in mammals. Activated Ras-GTP can then activate one of the MAPKKK of the **Raf** family, involved in ERK1/2 activation, as described below.

Alternatively, **GPCR** can also trigger the ERK1/2 activation through the enhancement of Raf isoforms activity. However, the mechanisms involved are not fully understood yet. Several models have been proposed. In one model, $\mathbf{G_S\alpha}$ or $\mathbf{G_I\alpha}$ can directly interact with the protein kinase **Src** following stimulation of the GPCR to promote the phosphorylation of **Raf** enzymes (Ma et al., 2000). Another model proposed that free **Gβγ** subunits, resulting from the activation of $G_I\alpha$, interact with Shc and Grb2 **adapter proteins** to enhance **Src** autoactivation (Luttrell et al., 1996), a phenomenon that would also require the phosphatidylinositol-3-kinase (PI-3K) (Lopez-Ilasaca et al., 1997). Over-activated Src then interacts with RTK (Linseman et al., 1995) to support the activation of Raf kinases *via* the Ras proteins. The cascade of events is here similar to the one described earlier for RTK-originated signaling pathway.

Three isoforms of Raf enzymes have been described: **A-Raf, B-Raf** and **Raf-1** (also termed c-Raf-1) (Hagemann & Rapp, 1999). These three cytosolic MAPKKK have a respective molecular weight of 68 kDa, 95 kDa and 74 kDa. Although Raf enzymes possess great variable regions, they also share three highly conserved domains – CR1, CR2 and CR3. The first two regions are located within the N-terminal tail of the proteins and seem to regulate the catalytic domain since their deletion leads to a constitutively active Raf-1 (Bruder et al., 1992). On the contrary, CR3 region is embedded in the C-terminal tail of the proteins and contains the kinase domain. Raf isoforms are differentially distributed. While Raf-1 is widely expressed in the body, B-Raf expression is mainly localized within neuronal tissues and testis, and A-Raf expression is restricted to the urogenital tissue (Hagemann & Rapp, 1999). Similarly, A-Raf, B-Raf and Raf-1 isoforms exhibit distinct modes of regulation. However, two major events are redundant: the **binding of activated GTP-Ras** to the Raf-RBD (Ras binding domain) located within the CR1 conserved region, and the **phosphorylation of one or multiple phosphoacceptor sites**. In the case of Raf-1, on which most studies focused, mostly K-Ras – but also N-Ras and H-Ras – recruits the MAPKKK at the cell membrane and activates it through phosphorylation mediated by Src protein kinase (Fabian et al., 1993; Diaz et al., 1997), p21-activated kinase (Chaudhary et al., 2000) or protein kinase C

(Kolch et al., 1993) among others. Raf-1 is part of a protein complex which is constituted of the heat shock protein 90 and p50 proteins, important for the Raf signaling, as well as the 14-3-3 proteins, which stabilize active and inactive Raf proteins and may function as a protein adapter (Hagemann & Rapp, 1999).

Once activated, Raf enzymes phosphorylate **MEK1** and **MEK2** (Dent et al., 1992; Kyriakis et al., 1992) at the two serine or the two threonine residues of the MAPKK activation loop (Alessi et al., 1994; Zheng & Guan, 1994a). Phosphorylation on each of the two residues partially enhances the activity of MEK1/2 (Alessi et al., 1994). This latter is increased over 7000-fold when both residues are phosphorylated (Alessi et al., 1994). In addition to the kinase domain, MEK1/2 contain three regions that are essential for the regulation of their functions. An ERK1/2 binding domain, also known as a docking domain or D domain, is located within the N-terminal tail of the enzymes and acts on the MEK1/2 – ERK1/2 interactions (Tanoue et al., 2000). A proline-rich domain located within the C-terminal half of the catalytic domain of MEK1/2 contain sites that will allow the association of MEK1/2 with linker proteins. This is the case of the **MEK-Partner 1** (MP-1), a protein essential for promoting the interaction of MEK1 and ERK1 (Schaeffer et al., 1998) (*section A1.5*). A very active nuclear export sequence lies in the N-terminal region of MEK1/2 and is mostly responsible for the cytoplasmic subcellular localization of MEK1/2 (Zheng & Guan, 1994b).

Following activation through phosphorylation of the two phosphoacceptors, MEK1/2 transduce the signal and activate **ERK1/2** enzymes (Crews et al., 1992). This phosphorylation step may involve the formation of a complex with MEK1/2 and MP-1 (Schaeffer et al., 1998). This complex facilitates the interaction between MEK1/2 and ERK1/2 *via* the D domain located on both sets of enzymes (Tanoue et al., 2000). The phosphorylation occurs at the **tyrosine and threonine residues** of the TEY motif located in the loop 12 of the target proteins. As previously described (*section A1.1*), ERK1/2 phosphorylation occurs primarily on tyrosine before threonine (Robbins & Cobb, 1992; Ferrell & Bhatt, 1997). Once fully activated by phosphorylation of both residues, ERK1/2 promotes the activation of numerous targets through interaction *via* various docking domains and phosphorylation of serine and threonine residues followed by a proline residue. These targets range from nuclear proteins – such as transcription factors

– to cytoplasmic proteins – such as cytoskeletal proteins and downstream kinases – and membrane proteins.

1.2.2. FUNCTIONS OF ERK, EXAMPLE OF ERK1/2

The functions of the MAPK are defined by the nature of their substrates. In the case of the ERK family, many different targets are known, which all contain serine/threonine-proline activation motifs (Pearson et al., 2001; Roux & Blenis, 2004). By phosphorylating nuclear transcription factors directly in the nucleus, ERK1/2 have a well-described role in **transcriptional regulation**. c-JUN, c-FOS and activating transcription factor (ATF)-2, which belong to the **activator protein-1** (AP-1) family of transcription factors, are phosphorylated by ERK1/2. Elk-1, a ternary complex factor is also activated through phosphorylation by ERK1/2 to enhance the formation of complexes with promoters of numerous genes and eventually facilitate transcription (Janknecht et al., 1993). ERK1/2 role as a transcriptional regulator is also mediated *via* phosphorylation of several **downstream kinases**, like p90 ribosomal S6 kinase-1, -2 and -3 (RSK1-3) (Smith et al., 1999), mitogen- and stress-activated kinases (MSK) 1/2, and MAPK-activated protein kinase (MAPKAPK) 2/3 (Stokoe et al., 1992). These enzymes are known to activate and regulate a wide range of transcription factors such as transcription intermediary factor (TIF) 1A, **cAMP-response element** (CRE) **binding protein** (CREB), **CREB-binding protein** (CBP), ATF-1, or signal transducer and activator of transcription-3 (STAT3).

Moreover, ERK1/2 exert a **regulation on the cell cycle** and as a consequence on the cell proliferation through the RSK family of kinases, which are known to act on the cyclin-dependent kinase (CDK) inhibitor p27kip1 or the protein kinase Myt1, an inhibitory kinase of CDC2 (Roux & Blenis, 2004).

ERK1/2 are also involved in **promoting cell survival** through actions mediated by RSK. It has been described that RSK2 phosphorylates the pro-apoptotic protein Bad to repress its death-promoting activity. Similarly, RSK2 activates the transcription factor CREB, which in turns increases the transcription of survival-promoting genes (Roux & Blenis, 2004). Recent studies suggested that RSK1 may promote survival as well by phosphorylating IκBα, a NFκB inhibitor. This phosphorylation promotes IκBα

degradation and subsequent NFκB nuclearization that can activate the transcription of survival-promoting genes (Ghoda et al., 1997; Schouten et al., 1997).

More recently, ERK1/2 have been suggested to play a role in the mechanisms that regulate **circadian rhythms**. This will be thoroughly detailed in sections below (*sections A2.2.2.3, A2.2.3.2 and A2.4.3.3*).

Moreover, because ERK1/2 signaling pathway is involved in the regulation of essential functions within cells, such as cell survival, cell proliferation and transcription regulation as described above, they have been associated to many diseases. One of the most studied is cancer and it has been strikingly reported that 25% of all cancers display an activating mutation of the Ras protein (Bos, 1989).

1.3. THE p38^{MAPK} SUBGROUP

1.3.1. THE p38^{MAPK} SIGNALING MODULE

1.3.1.1. The p38^{MAPK} subfamily is constituted of several isoforms

Similarly to the ERK subfamily of MAPK, several members of the **p38^{MAPK}** have been identified. In 1994, three independent studies described the same first member of the family: **p38α** (Table 1). It was either characterized as a new kinase tyrosine-phosphorylated upon inflammatory cytokines treatment like endotoxin lipopolysaccharides (Han et al., 1994), as a target kinase of pyridinyl imidazole drugs, which blocks tumor necrosis factor (TNF) α production and as a consequence is named cytokine-supressive anti-inflammatory drug-binding protein (CSBP) (Lee et al., 1994), or as a MAPKAPK2 reactivating kinase (Rouse et al., 1994). The 38 kDa p38α enzyme is widely expressed within tissues with a maximum in the skeletal muscle. Its amino-acid sequence is 50% analogous to ERK2 and like other MAPK, p38α is a **dually-phosphorylated kinase** that becomes activated upon phosphorylation of a threonine and a tyrosine residues. Contrary to kinases of the ERK subfamily, p38α possesses a **TGY** motif within its activation loop. Besides, it has been shown that the phosphorylation lip sequence is **six residues shorter** than the one of ERK2 (Wilson et al., 1996; Wang et al.,

1997). These differences with ERK subfamily are thought to contribute to p38α substrate specificity.

Soon after the identification of p38α, a new isoform of the $p38^{MAPK}$ family was identified: **p38β** (also termed p38-2, Table 1). The 38 kDa protein sequence is only 47% similar to ERK2 but 73-74% identical to p38α. It is similarly shown to be a dually-phosphorylated kinase with a **TGY** activation motif lying within a phosphorylation lip **six residues shorter** than ERK2 (Jiang et al., 1996; Stein et al., 1997). These two latter characteristics were further recognized as a **hallmark** of the $p38^{MAPK}$ family of protein kinases. Like p38α, p38β is ubiquitously expressed, although levels of expression vary among tissues. The highest level of expression is found in heart tissue and skeletal muscle (Stein et al., 1997). Stress-inducing stimuli as well as proinflammatory cytokines activate p38β, and like p38α, the kinase activity is blocked by pyridinyl imidazole drugs (Stein et al., 1997).

A third member of the $p38^{MAPK}$ family was identified in 1996 by three independent studies. This new kinase was either termed **p38γ** (Li et al., 1996), **SAPK3** (Mertens et al., 1996) or **ERK6** (Lechner et al., 1996) (Table 1) since investigators were unable to clearly classified this 42-45 kDa enzyme, which possesses qualities of both $p38^{MAPK}$ and ERK subfamilies. Even though the kinase sequence is 33-41% identical to ERK1-3 sequences, the intrinsic structure of the protein is closer to the $p38^{MAPK}$ family of kinases. p38γ sequence is indeed 63% and 62% identical to p38α and p38β respectively. Moreover, both the activation motif **TGY** and the overall phosphorylation lip are similar to the two previously identified members of the $p38^{MAPK}$ family. However, unlike p38α and p38β kinases, p38γ expression is limited to the skeletal muscle (Lechner et al., 1996; Li et al., 1996) and its activation is not abrogated by pyridinyl imidazole drugs (Goedert et al., 1997; Kumar et al., 1997).

The fourth kinase and last identified to date has been described by two separate groups. It was either termed **p38δ** (Jiang et al., 1997) or **SAPK4** (Kumar et al., 1997). Although p38δ and SAPK4 molecular weight were found different, 38 kDa and 42 kDa respectively (Table 1), both groups showed that this new kinase is highly identical to the already-described members of the $p38^{MAPK}$ family. p38δ sequence is indeed 61%, 59% and 65% identical to p38α, p38β and p38γ kinases respectively while identity with ERK1

is much lower, 41% (Jiang et al., 1997). Like the three other $p38^{MAPK}$, p38δ activation loop contains the **TGY** motif and the phosphorylation lip is similar to the previously-identified kinases of the subfamily. p38δ can be activated by cytokines and environmental stress signals in a variety of tissues different from related enzymes. The expression of p38δ is indeed restricted to mostly lungs, kidneys and testis (Hu et al., 1999). Similarly to p38γ with which it shares 67% amino-acid residues, p38δ does not seem to be sensible to pyridinyl imidazole drugs (Goedert et al., 1997; Kumar et al., 1997).

1.3.1.2. Nature of the $p38^{MAPK}$ signaling cascade

Like other kinases of the MAPK family, the $p38^{MAPK}$ signaling cascade is structured in a **three-kinase module**. Many different components participate to these cascades (Kyriakis & Avruch, 2001). The nature of each cascade is on the one hand dependent on the cellular system and on the other hand dependent of the extracellular stimulus which initiates the phosphorylation cascade. To exemplify the structure and components of $p38^{MAPK}$ signaling cascades, I will depict the well-described **interleukin** (IL)**-1**-induced $p38^{MAPK}$ activation (Figure 5).

Although many various extracellular stimuli are able to activate $p38^{MAPK}$, ranging from heat shock and irradiation to cytokines and osmotic stress, the cytokine IL-1 is probably the best characterized stimulant for the $p38^{MAPK}$ pathway. It has been shown that in a situation of inflammation, the binding of IL-1 ligand to its membrane receptor **IL-1 receptor type 1** (IL-1R1) (Sims et al., 1988) entails the formation of a complex with the **IL-1 receptor accessory protein** (IL-1RAcP) (Greenfeder et al., 1995; Wesche et al., 1997). This heterocomplex then leads to the recruitment of the protein adaptor **MyD88** (Wesche et al., 1997; Burns et al., 1998) through a Toll domain in the C-terminal tail. The pre-formed **IL-1 receptor associated kinase** (IRAK) – **Toll-interacting protein** (Tollip) complex binds to this protein aggregate, which entails the autophosphorylation of IRAK through interaction between the death domains. Upon phosphorylation, IRAK is eventually released (Burns et al., 2000). It further interacts with and phosphorylates the **TNF receptor-associated factor** (TRAF) **6** (Cao et al., 1996). This latter phosphorylation allows the downstream activation of the **transforming**

growth factor-β-activated protein kinase (TAK) **1**, a MAPKKK upstream of the $p38^{MAPK}$.

Heterotrimeric G proteins associated with **GPCR** are also known upstream activators of the $p38^{MAPK}$ cascade. G proteins recruit **Rho GEFs**, which are activated through an exchange of GDP to GTP. Upon activation, GEFs stimulate the GTP-binding proteins **Rac** and **Cdc42**, which belong to the Rho family of GTPases, which in turn activate the **p21-activated kinase** *via* interaction on the Cdc42/Rac GTPase-binding (CRIB) domains (Kyriakis & Avruch, 2001). Rac and Cdc42 are notably recognized to be necessary to the IL-1-induced activation of the $p38^{MAPK}$ cascade (Bagrodia et al., 1995; Zhang et al., 1995). Although the role of p21-activated kinase remains partly unknown, the kinase is believed to support the activation of downstream MAPKKK.

Numerous proteins have been identified as MAPKKK of the $p38^{MAPK}$ cascade. They include MAPKKK1-4, TAK1, apoptosis signal-regulated kinase (ASK) 1, and thousand and one amino-acid protein kinases although MAPKKK4, TAK1, ASK1 and thousand and one amino-acid protein kinases have been shown the most effective (Kyriakis & Avruch, 2001). **TAK1** (also termed MAPKKK7) was identified by Yamaguchi and colleagues as a new serine/threonine protein kinase of the MAPKKK family (Yamaguchi et al., 1995). This 64 kDa protein is expressed in many tissues. Although it has been demonstrated that TAK1 and **TRAF6** interact (Ninomiya-Tsuji et al., 1999), a more recent study established that the activation of TAK1 requires the involvement of an additional partner (Sakurai et al., 2000). This widely expressed protein, identified as the **TAK1 binding protein** (TAB) **1** (Shibuya et al., 1996), binds to TAK1 *via* the C3 fragment lying within the TAB1 C-terminal domain. Upon interaction, C3 fragment, coupled with C1 and C2 fragments rich in serine and threonine residues, promotes a full autophosphorylation at two threonine residues in the activation loop of TAK1 (Sakurai et al., 2000). The related **TAB2** and **TAB3** identified more recently are known to participate in the TRAF6 – TAK1 interaction (Takaesu et al., 2000; Ishitani et al., 2003).

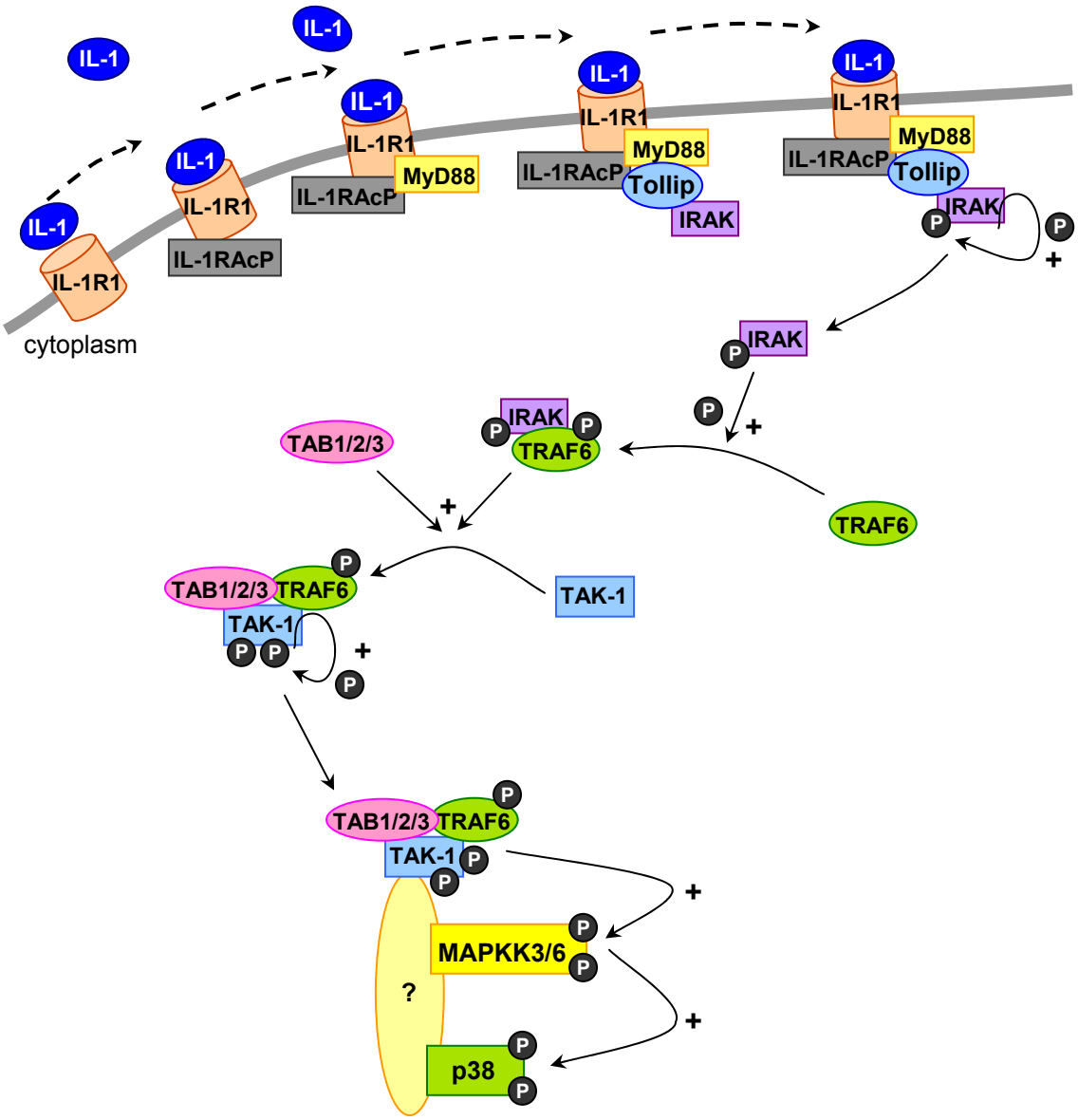

Figure 5. Simplified mechanism of p38^{MAPK} activation: example of interleukin-1-induced activation.

Upon binding of interleukin-1 (IL-1), interleukin-1 receptor type 1 (IL-1R1) successively recruits the IL-1 receptor accessory protein (IL-1RAcP), the adapter protein MyD88 and the pre-existing IL-1 receptor associated kinase (IRAK) – Toll-interacting protein (Tollip) multiprotein complex. This leads to the autophosphorylation of IRAK and its subsequent release from the multiprotein complex. Phosphorylated IRAK further interacts with and phosphorylates tumor necrosis factor receptor-associated factor 6 (TRAF6). The transforming growth factor-β-activated protein kinase 1 (TAK1)-binding protein 1/2/3 (TAB1/2/3) promote an interaction between TRAF6 and TAK1 which leads to autophosphorylation of TAK1. Activated TAK1 induces the phosphorylation of MAPKK3/6 and p38^{MAPK} (p38) after binding to a scaffold protein (?) that could either be JNK interacting protein 2 (JIP-2) or JIP-4. Note that this is a simplified schema which does not show additional components like adapter proteins that interact with scaffold proteins and define spatial regulation of p38^{MAPK} activation. P, phosphate. "+" represents facilitating steps. Dotted arrows indicate successive events occurring on the same IL-1R1.

In the $p38^{MAPK}$ cascade, four MAPKK have been described as potential activators of $p38^{MAPK}$ isoforms: MAPKK3, MAPKK4, MAPKK6 and MAPKK7. Even though MAPKK4 (Derijard et al., 1995) and MAPKK7 (Hu et al., 1999) are capable of activating $p38^{MAPK}$ isoforms *in vitro*, MAPKK3 and MAPKK6 are the major kinases responsible for their activation in cells. The 39 kDa kinase **MAPKK3** was first identified by Derijard and colleagues (1995), as a kinase expressed in many tissues with a maximum level of expression within the skeletal muscle. Later on, **MAPKK6** was identified by four independent studies (Han et al., 1996; Moriguchi et al., 1996; Raingeaud et al., 1996; Stein et al., 1996). It is a 37 kDa kinase ~80% identical to the previously-characterized MAPKK3 amino-acid sequence, but only 37% and 34% similar to the MEK1 and MEK2 enzymes of the ERK1/2 signaling pathway (*section A1.2.1.2*). Contrary to MAPKK3, expression of this novel kinase is restricted to the skeletal muscle and to a lesser extent to the thymus, small intestine and pancreas. Both kinases possess a serine and a threonine residue within their activation loop, which must be both phosphorylated to lead to the activation of the enzymes. Strikingly, the MAPKKK TAK1, activated following IL-1 stimulation, is able to phosphorylate both MAPKK3 and MAPKK6, as initially established *in vitro* (Moriguchi et al., 1996). This participates to the transduction of the signal towards the $p38^{MAPK}$ isoforms.

Following their activation, MAPKK3 and MAPKK6 direct the further phosphorylation of the $p38^{MAPK}$ isoforms. It has been documented that MAPKK3 targets p38α, p38γ and p38δ isoforms, while MAPKK6 potently triggers the phosphorylation of all four isoforms of $p38^{MAPK}$ (Jiang et al., 1997; Enslen et al., 1998). As described in a previous section (*section A1.3.1.1*), the phosphorylation occurs at the **TGY** motif. Like other MAPK proteins, $p38^{MAPK}$ phosphorylate their targets on **serine/threonine-proline motifs**. Various targets of the activated $p38^{MAPK}$ isoforms are identified. They include cytoskeletal and cytosolic proteins, nuclear substrates like transcription factors, and downstream protein kinases.

1.3.2. FUNCTIONS OF $p38^{MAPK}$

Due to the elevated number of $p38^{MAPK}$ substrates, many various functions have been described (Kyriakis & Avruch, 2001; Roux & Blenis, 2004). The most widely

recognized is an implication in the **transduction of inflammatory signals** that eventually lead to cytokine biosynthesis. It has been notably shown that $p38^{MAPK}$ can participate in the maturation of naïve T cells into T_H1 cells by eliciting IL-12 gene transcription and synthesis by antigen-presenting cells (Lu et al., 1999). Besides, $p38^{MAPK}$ seem to enhance the mRNA stability of mRNA encoding proinflammatory cytokines, as demonstrated by Winzen and colleagues who showed that activation of MAPKAPK2 by p38α and p38β leads to a stabilization of mRNA through a AU-rich sequences-targeted mechanism (Winzen et al., 1999).

Several other roles of $p38^{MAPK}$ have been described. They include a role in the **development**, as it has been seen that p38α is critical for normal angiogenesis within the placenta (Mudgett et al., 2000). Morevover, in addition to a function in **differentiation**, as exemplified previously with the T_H1 cells, $p38^{MAPK}$ isoforms are involved in **cell proliferation** and **cell survival** *via* interaction with a plethora of transcription factors and protein kinases notably, and in regulating **cell cycle progression**. Interactions of $p38^{MAPK}$ isoforms with **transcription factors** have either been depicted as direct, in the case of ATF-1/2, myocyte enhancer factor 2C and myocyte enhancer factor 2A, serum response factor-associated protein (Sap)-1, Elk-1, Ets-1 and the heat shock transcription factor-1, or through downstream intermediate kinases. Among these kinases, MAPKAPK2/3 are activated by $p38^{MAPK}$ isoforms and further phosphorylate various substrates as CREB, ATF-1, serum response factor and heat shock protein 27. MAPK-interacting kinases-1 phosphorylate eukaryotic initiation factor 4E (eiF-4E), which participates to mRNA translation, while MSK-1 activate both CREB and ATF-1.

Similarly to ERK1/2 kinases, $p38^{MAPK}$ have been recently implicated in the regulation of **circadian rhythms**. This will be the object of a later section (*section A2.4.3.3*).

1.4. THE JNK SUBGROUP

1.4.1. THE JNK SIGNALING MODULE

1.4.1.1. The JNK subfamily is constituted of many isoforms

In 1990, shortly after the discovery of the first MAPK of the ERK subfamily, a group identified a new MAPK different from ERK. The protein inhibitor cycloheximide was found to enhance the activation of a novel kinase, which phosphorylated the MAP-2 in rats (Kyriakis & Avruch, 1990). This **dually-phosphorylated kinase**, initially termed pp54 MAP-2 kinase, was activated through phosphorylation of both a **threonine and a tyrosine residue** (Kyriakis et al., 1991). In parallel, another group identified new serine/threonine kinases of 46 kDa and 54/55 kDa participating in the transforming oncogenes- and UV radiation-induced activation of c-JUN. They were named **c-JUN N-terminal kinases**. The activation occurred by binding on the N-terminal trans-activating domain of c-JUN and phosphorylation of two serine residues localized in its N-terminal extension (Hibi et al., 1993). The later cloning and characterization of these enzymes in 1994 in rat and human showed that those two kinases were actually identical (Derijard et al., 1994; Kyriakis et al., 1994).

JNK isoforms are coded from three genes, ***Jnk1*** (also termed *SAPK-γ*), ***Jnk2*** (also termed *SAPK-α*) and ***Jnk3*** (also termed *SAPK-β*), which are alternatively spliced to lead to a minimum of **10 isoforms** (Gupta et al., 1996). Splicing yields proteins of either 46 kDa or 54/55 kDa and occurs at two levels (Table 1): the C-terminal extension for all genes, and an exon of the protein kinase domain for *Jnk1* and *Jnk2* specifically (Barr & Bogoyevitch, 2001). *Jnk* genes sequences were 40-41% identical to ERK2 sequences but 87-88% identical among each others. These similarities included the **TPY** activation motif located within the activation loop (Gupta et al., 1996). Although no study has shown the expression pattern of each of the 10 isoforms, it has been described that *Jnk1* and *Jnk2* are ubiquitously expressed while *Jnk3* expression is mostly restricted to the brain and to a lesser extent to the heart and testis (Martin et al., 1996).

1.4.1.2. Nature of the JNK signaling cascade

Alike $p38^{MAPK}$, JNK signaling cascade may be activated by numerous stimuli among which **cytokines** are believed to be the most potent stimulants. The well-described induction of JNK pathway activation by the multi-functional **TNFα cytokine** (Wajant et al., 2003) will be further detailed (Figure 6).

Upon activation by the immune system, TNFα is produced and acts as a ligand on two receptors of the **TNF receptor** (TNF-R) family, the 55 kDa TNF-R1 and the 75 kDa TNF-R2 (Brockhaus et al., 1990). Although TNF-R1 seems to be constitutively expressed, TNF-R2 expression is mostly restricted to the cells of the immune system. Apart from their cellular distribution, these two receptor types also differ in their intracellular signaling domain: contrary to TNF-R2, **TNF-R1** possesses a protein-protein interaction domain called death domain, which allows association with partners that also contain a death domain (Tartaglia et al., 1993). Upon TNFα stimulation, the **silencer of death domain** (SODD) protein, which inhibits the self-association of receptors through their death domains in the quiescent cell, dissociates from TNF-R1 (Jiang et al., 1999). This dissociation allows TNF-R1 to recruit the adaptor proteins **TNF receptor-associated death domain proteins** to the death domain (Hsu et al., 1995). The TNF-R1-bound TNF receptor-associated death domain proteins further interact with **TRAF2** – *via* their TRAF interaction domain (Hsu et al., 1996) –, an adaptor protein that most likely allows the autoactivation of the **MAPKKK1** (Xia et al., 2000). This latter step involves serine/threonine kinases of the **germinal center kinases**, group 1, which are known to interact with TRAF2 to promote MAPKKK1 autoactivation (Yuasa et al., 1998; Chadee et al., 2002). In parallel to this TNF-R1-related pathway, TNF-R2 is capable of directly interacting with the TRAF2 adaptor protein to further promote MAPKKK1 activation (Rothe et al., 1994).

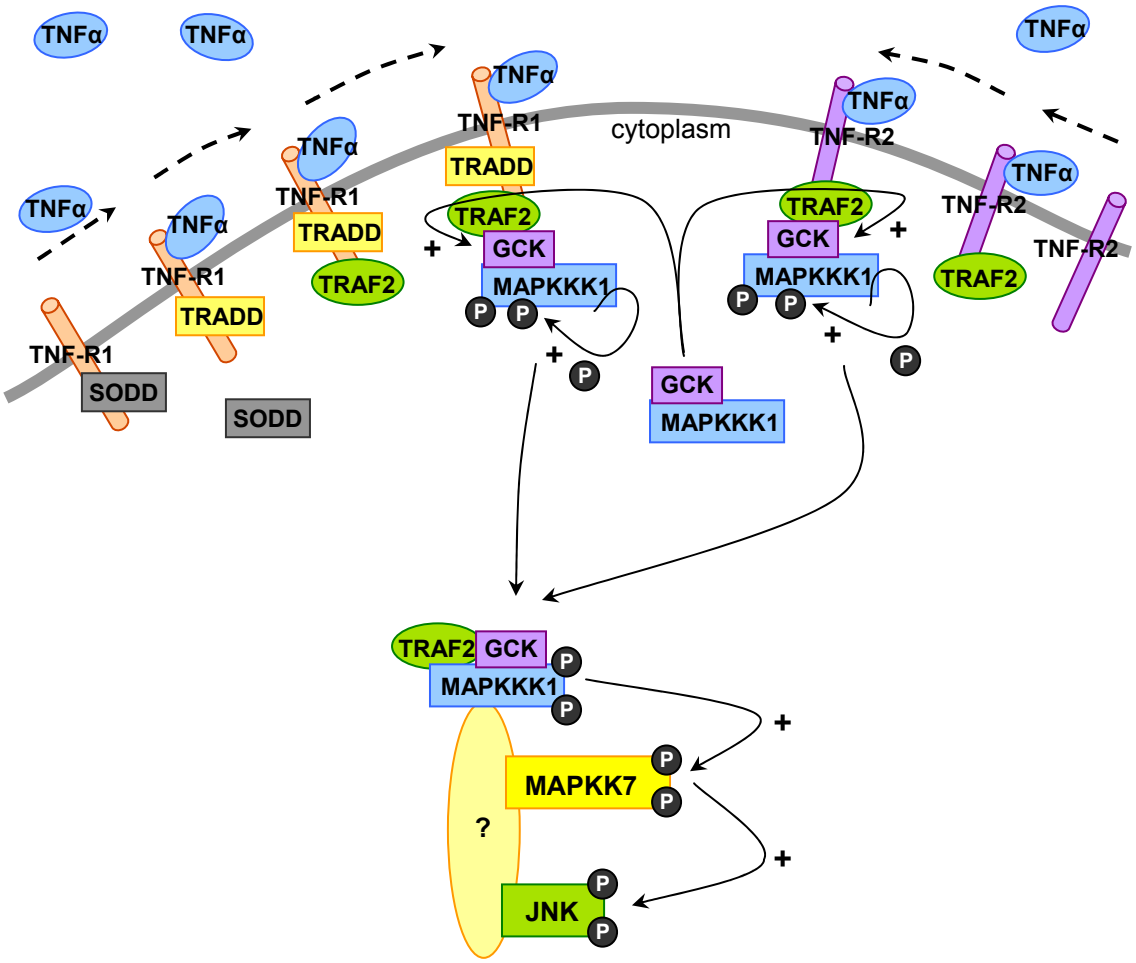

Figure 6. Simplified mechanism of JNK activation: example of TNFα-induced activation.

Upon binding of tumor necrosis factor α (TNFα) on TNFα-receptor 1 (TNF-R1), the silencer of death domain protein (SODD) dissociates from the receptor which allows the recruitment of the adaptor protein TNF receptor-associated death domain protein (TRADD). The TNF receptor-associated factor 2 (TRAF2) then interacts with TNF-R1 and promotes the binding of the pre-existing germinal center kinases (GCK) – MAPKKK1 complex. Upon attachment, TRAF2 facilitates the activation of GCK and the autophosphorylation of MAPKKK1 on 2 phosphoacceptor sites. TRAF2 can directly bind TNF-R2 once stimulated. This also leads to GCK – MAPKKK1 binding, GCK activation and MAPKKK1 autophosphorylation. Activated MAPKKK1 interacts with a scaffold protein (?) that could either be filamin or JNK-interacting protein (JIP) which facilitates MAPKK7 and JNK activation by interacting with the components of the signaling module. Note that this is a simplified schema. Additional components are likely involved, especially adapter protein interacting with scaffold proteins and defining spatial regulation of JNK activation. P, phosphate. "+" represents facilitating steps. Dotted arrows indicate successive events occurring on the same TNF-R.

Many MAPKKK have been reported to activate the JNK signaling pathway. They include members of the MAPKKK group (MAPKKK1-4), the mixed-lineage protein kinases group (mixed-lineage protein kinases 1-3, dual-leucine zipper kinases (DLK), leucine zipper-bearing kinases (LZP)), and the ASK and TAK groups (Davis, 2000). In the TNFα-induced activation of JNK pathway, **MAPKKK1** plays a major role. In mouse and rat, it was described as a 195 kDa kinase expressed in various tissues (Lange-Carter et al., 1993; Xu et al., 1996). Similarly to Raf in the ERK1/2 activation pathway, MAPKKK1 presents catalytic domains within the C-terminal extension and regulatory domains within the N-terminal tail. Activation of the kinase occurs through interaction and phosphorylation with group 1 **germinal center kinases** (Diener et al., 1997).

Two ubiquitous MAPKK were shown to participate in JNK activation: MAPKK4 (Derijard et al., 1995) and MAPKK7 (Tournier et al., 1997), which are both widely expressed in mammalian tissues (Tournier et al., 1997). Although these 42 kDa and 47 kDa dually-specific kinases are able to phosphorylate both threonine and tyrosine phosphoacceptor sites, it has been suggested that they possess some preferences: **MAPKK7** preferentially phosphorylates **threonine** residues while **MAPKK4** mainly activates **tyrosine** residues. This implies a complementary action of both sets of kinases to mediate the downstream JNK activation (Lawler et al., 1998), which allows integration of additional extracellular signals. This is confirmed with the TNFα-mediated activation of JNK, where MAPKKK1 interacts with and activates MAPKK7 (Moriguchi et al., 1997) whilst TNFα does not promote MAPKK4 activation. This suggests the involvement of other signaling pathways initiated by various extracellular signals to regulate MAPKK4 phosphorylation. Moreover, MAPKK7 can associate with **ASK1** (Zama et al., 2002), which is itself turned on through interaction with the C-terminal region of TRAF2 and through activation by its RING domain (Nishitoh et al., 1998). This suggests that ASK1 may participate as well in the TNFα-induced regulation of JNK pathway.

Once activated, MAPKK4 and MAPKK7 phosphorylate the downstream **JNK isoforms**. As mentioned earlier, at least 10 isoforms of JNK exist (*section A1.4.1.1*). They are dually-phosphorylated kinases that become activated through phosphorylation on both threonine and tyrosine residues of the characteristic TPY motif embedded in the

phosphorylation lip of the proteins. Numerous targets are known. They include cytoplasmic proteins and nuclear substrates like transcription factors.

1.4.2. FUNCTIONS OF JNK

Like kinases from the two previously-described MAPK subfamilies, JNK are involved in many cellular aspects. They are notably known to phosphorylate numerous **transcription factors**, as various as c-JUN, ATF-2 – which participate to the formation of AP-1 transcriptional complexes – (Barr & Bogoyevitch, 2001), Elk-1, heat shock transcription factor-1 or signal transducer and activator of transcription-3 among others. Membrane targets also exist, like the insulin receptor substrate (IRS)-1 which activation by insulin is blocked *via* phosphorylation by JNK (Chen et al., 2001).

Various studies described JNK as a mediator of both **apoptosis** and **cell survival** in cells. For many years, it was unclear how JNK could lead those two contradictory actions. However, the numerous studies that tried to explain these actions revealed that JNK signal interpretation by cells most likely depends on several parameters. They include the **cellular context** (normal *versus* tumoral cell), the **duration of the JNK activation** (transient *versus* sustained), which is linked to the **nature of the initial stimuli**, and the **various other signaling pathways activated simultaneously** within the cell (Davis, 2000; Liu & Lin, 2005).

1.5. PHYSIOLOGICAL RELEVANCE OF SCAFFOLD PROTEINS IN DETERMINING LOCALIZATION, EFFICIENCY AND SPECIFICITY OF SIGNALING CASCADES

As mentioned earlier (*section A1.1*), a body of evidence suggests that additional proteins, including **scaffold proteins**, that are part of or interact with kinases of the three-kinase modules are needed to regulate the cascades of phosphorylation (Morrison & Davis, 2003; Dard & Peter, 2006). The first of the scaffold proteins identified in MAPK signaling was the yeast protein Ste5p involved in the signaling cascade controlling the mating. It was shown to associate with the three main components of the cascade: the MAPKKK, the MAPKK and the MAPK (Choi et al., 1994). Because of their central position within modules, scaffold proteins are believed to play a central role in the regulation of the signaling cascade.

This has been confirmed later on through the discovery of new scaffold proteins and the description of their inherent functions. First, **scaffold proteins increase the efficiency of signaling cascades** by locally concentrating proteins and positioning kinases closer to their substrates. Hence, they promote kinase/substrate interactions. This is the case of the **kinase suppressor of Ras** (KSR) which is part of the ERK module, and which subsequently to Ras activation targets MEK1/2 to the plasma membrane, where their activator Raf-1 and their effectors ERK1/2 locate (Therrien et al., 1996; Roy et al., 2002).

Through their interactions with transmembrane or membrane-associated adaptor proteins, **scaffold proteins additionally restrict the activation of MAPK in time and space**. This has been demonstrated with several proteins including **MP-1**. This scaffold protein promotes the formation of complexes between MEK1 and ERK1 at endosomes through its interaction with the p14 adaptor protein, highly localized at the surface of endosomal compartments (Wunderlich et al., 2001). Because down-regulation of p14 expression leads to both a magnitude-reduced and a time-restricted ERK activation, it is believed that MP-1 and p14 impact both the spatial and temporal control of the ERK cascade (Teis et al., 2002).

A third identified characteristic of scaffold proteins is the ability to form complexes that will not only promote the activation of kinases but also **regulate its kinetics and termination**. This was suggested by the observation that scaffold proteins bind **phosphatases** in addition to kinases. Indeed, the **JNK interacting protein-1** known to bind members of the mixed-lineage protein kinases, as well as MAPKK7 and JNK, associates with the **dually-specific MAPK phosphatase** (MKP)**-7**. This association reduces JNK and c-JUN phosphorylations (Willoughby et al., 2003). Although the physiological functions of this interaction remain unclear, activation of JNK might require the previous inhibition or dissociation of MKP-7. Alternatively, the recruitment of MKP-7 by JNK interacting protein-1 might participate in the timing of JNK activation, by determining its termination.

Scaffold proteins thus appear to have a critical role in MAPK signaling, not only by interacting with some or all of the three-kinase module components, but also by **modulating efficiency, specificity and the kinetics of MAPK signaling**.

1.6. MAPK SIGNALING INACTIVATION THROUGH MAPK PHOSPHATASES

As seen previously, scaffold proteins are essentials to ensure the selective activation of a specific MAPK pathway. However, activation of MAPK is not limited to a switch from an OFF to an ON state and present variability in both **magnitude** and **duration**. Both characteristics appear critical to determine the physiological outcome in cells (Marshall, 1995). Accordingly, the magnitude and duration of MAPK activation reflect a balance between the upstream activating and inactivating signals. As MAPK are activated through phosphorylation of both phosphoacceptor sites within the TXY motif of the phosphorylation lip, dephosphorylation of either is sufficient for inactivation. Three kinds of **MKP** have been reported: the **serine/threonine-specific protein phosphatases**, and the **tyrosine-specific protein phosphatases** (PTP), which also comprise the **dually-specific protein phosphatases** (DSP) that are capable of dephosphorylating both threonine and tyrosine residues.

While DSP have been the most studied, little is known on serine/threonine-specific protein phosphatases and not-DSP PTP physiological relevance in determining MAPK signaling termination. Two serine/threonine-specific protein phosphatases, the protein phosphatase (PP) 2A and to a lesser extent the PP2C are known to promote the inactivation of the ERK1 pathway (Alessi et al., 1995b). Similarly, three not-DSP PTP have been described as interacting with and inactivating ERK. Indeed, striatal enriched phosphatases (STEP) and PTP-STEP-like phosphatases interact with ERK1/2 through a kinase interacting motif (KIM), which induces the phosphorylation of STEP and PTP-STEP-like phosphatases N-termini by ERK1/2 and the subsequent dephosphorylation of the tyrosine residue of ERK1/2 TEY motif (Pulido et al., 1998). Likewise, the haematopoietic PTP (HePTP) associate to both ERK and $p38^{MAPK}$ through their N-terminal tail and allow the inactivation of these kinases (Saxena et al., 1999).

Moreover, **DSP** are currently extensively studied. Thirteen have been identified to date that act on MAPK (Farooq & Zhou, 2004). Although they present some variability in their overall sequence, DSP all share a highly conserved signature motif with particularly 3 crucial amino-acid residues for MAPK dephosphorylation: a cysteine and arginine residue within the activation loop and a highly conserved aspartate residue. Four groups have been identified on the basis of the structural and functional characteristics of each DSP. Structurally, type 1 DSP contain only a DSP domain involved in the threonine and tyrosine dephosphorylation of active MAPK. Type 2 DSP

include an N-terminal MAPK binding motif (MKB) in addition to the DSP domain. Type 3 DSP contain an N-terminal domain of unknown function in addition to the MKB and DSP domains, while type 4 DSP contain both MBK and DSP domain, as well as an additional domain rich in prolines, glutamates, serines and threonines (PEST) which is thought to regulate the half-life of the phosphatases (Farooq & Zhou, 2004). It is suggested that the binding of MAPK on the MKB domain alters its interaction with the DSP domain. This association between MKB and the MAPK triggers the active residues cysteine, arginine and aspartate of the signature sequence to allosterically change the conformation of the DSP so that dephosphorylation of the active MAPK becomes possible. Importantly, it has been demonstrated that DSP MKP gene transcription can be induced by transcription factors themselves phosphorylated by MAPK. This system thus provides a **negative feedback mechanism** that can participate in the control of the MAPK activity.

The interactions of the three-kinase modules with regulatory proteins such as scaffold proteins and MKP thus contribute to the formation of **protein complexes that tightly regulate the localization, efficiency, specificity, magnitude and duration of the MAPK activation** in order to build appropriate answers to specific stimuli.

2. THE BIOLOGICAL CLOCK

The system developed by most organisms to anticipate and adapt to daily changes in environmental conditions, exemplified by the changes in lighting conditions, is constituted of three major components: **1)** a **self-sustained circadian clock** which oscillates with an **approximate 24-h period**, **2)** an **afferent system** that allows the **entrainment** of the clock to an exact period of 24 h and **3)** an **efferent system** which **distributes** the circadian information to the whole organism to adjust the physiology accordingly. The structural and mechanical characteristics of this clock will be successively described in the present section.

2.1. THE BRAIN HOSTS THE BIOLOGICAL CLOCK

2.1.1. LOCALIZATION WITHIN THE SUPRACHIASMATIC NUCLEUS OF THE HYPOTHALAMUS

In 1972, two groups identified the **SCN** as the site of the biological clock in mammals (Moore & Eichler, 1972; Stephan & Zucker, 1972). By performing bilateral lesions of the SCN in rats, they indeed observed a loss in the circadian rhythmicity of corticosterone rhythm as well as drinking and locomotor activity behavior. Strikingly, Lehman and colleagues managed to restore part of the circadian behavior, including the free-running locomotor activity in animals placed in DD, by **transplanting** fetal SCN tissue in the third ventricle (Lehman et al., 1987). Additionally, the transplantation of fetal SCN tissue coming from homozygous ***tau*** **mutant hamsters**, which present a locomotor activity period of 20 h (Ralph & Menaker, 1988), restored a rhythmicity in the activity of wild-type SCN-lesioned hamsters. The restored period of the activity was characteristically of 20 h, comparable to the graft donor. The converse experiment showed similar results. Likewise, a recent study demonstrated that arrhythmic mice (Vitaterna et al., 1994; van der Horst et al., 1999), where the clock genes *Clock* or *Cryptochrome1* and *Cryptochrome2* (*section A2.2.1*) are disabled, could recover a behavioral circadian rhythm following transplantation of fetal SCN tissue from wild-type mice (Sujino et al., 2003). The crucial role of SCN neurons in leading circadian rhythmic

behavior was also strengthened by a study which revealed that a suspension of dissociated SCN neurons transplanted in the brain of SCN-lesioned hamsters can restore a rhythmic pattern of activity (Silver et al., 1990).

2.1.2. ANATOMY OF THE SCN – NEUROPEPTIDES AND NEUROTRANSMITTERS CONTENT

The SCN, initially described in the rat (van den Pol, 1991), is a small paired nucleus which lies in the anterior hypothalamus. It is localized immediately on top of the optic chiasm and adjacent to the third ventricle (Figure 7). Each of the two nuclei contains about 10500 neurons in mice and Abrahamson and colleagues described each nucleus as a 300 µm wide, 350 µm high and 600 µm long "pear-like" shaped structure (Abrahamson & Moore, 2001).

Based mostly on phenotypical studies, the SCN can be subdivided into a **ventrolateral part** (vlSCN, also named **core**) that is encapsulated into a **dorsomedian part** (dmSCN, also called **shell**) (Leak et al., 1999) (Figure 8). Commonly to most mammals, the shell contains neurons that mostly express the neuropeptide **arginine vasopressin** (AVP) (Abrahamson & Moore, 2001) (Figure 8). In rats exposed to LD cycles, AVP mRNA and protein content oscillate in the shell neurons, peaking in the late day and the early light phase respectively. These rhythmic patterns persist in the absence of light inputs (Tominaga et al., 1992; Cagampang et al., 1994). Besides, neurons expressing AVP in rat were shown to project to the core region of the SCN (Romijn et al., 1997).

Furthermore, most neurons located in the core region do not express AVP. Instead, several studies revealed the expression of multiple neuropeptides. In rat and mice, the most ventral neurons rhythmically express the **vasoactive intestinal peptide** (VIP) (Abrahamson & Moore, 2001; Dardente et al., 2002) (Figure 8). Although high levels of VIP proteins are present late at night in rats housed under LD cycles, the rhythmicity is abrogated in DD (Takahashi et al., 1989; Shinohara et al., 1993), which suggests that the VIP protein oscillation is driven by the lighting schedule.

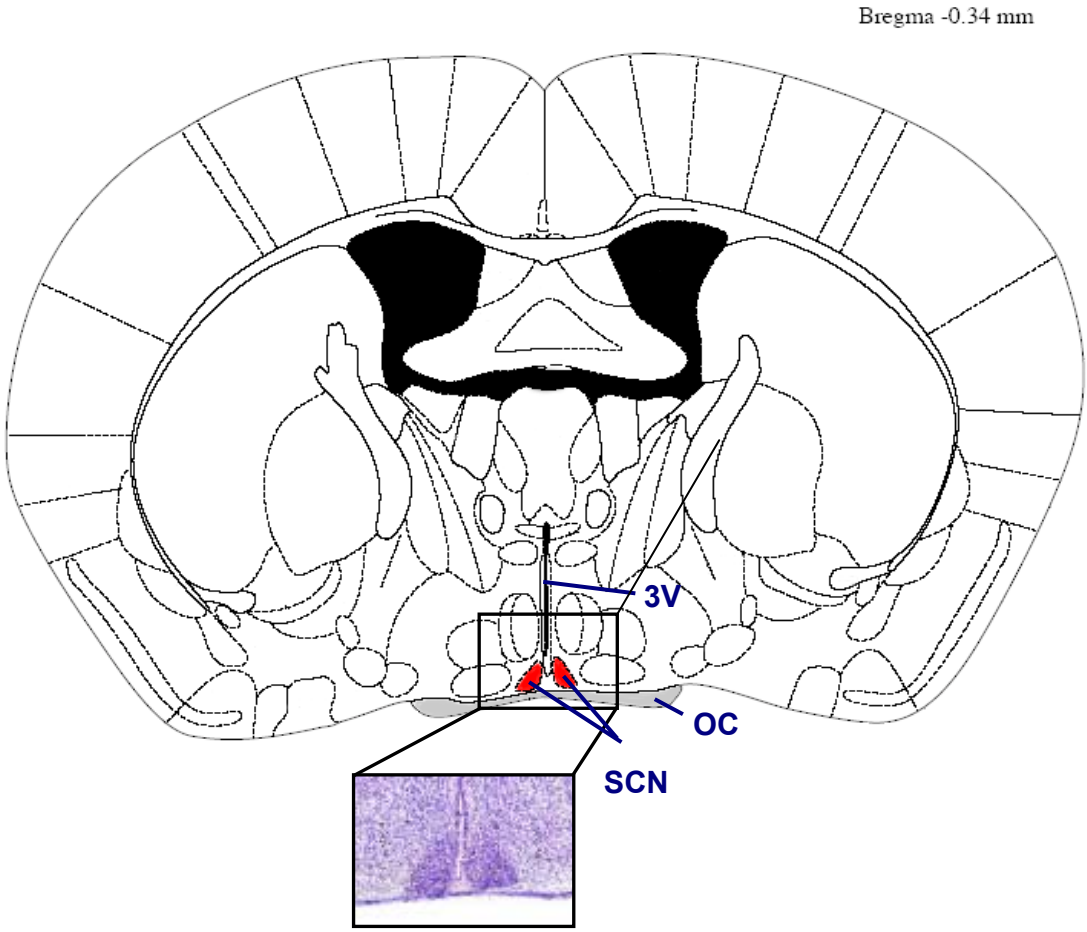

Figure 7. Suprachiasmatic nuclei localization in the mouse.

Coronal section of a mouse brain showing the position of the suprachiasmatic nucleus (SCN; red) on top of the optic chiasm (OC; gray). Enlarged panel shows the SCN stained with cresyl violet. 3V, third ventricle.
(Adapted from (Paxinos & Franklin, 2001)

Similarly located in the core, the **gastrin-releasing peptide** (GRP)-expressing neurons are mostly localized in the central core, although a colocalization with VIP neurons has been described in rats and mice (Mikkelsen et al., 1991; Abrahamson & Moore, 2001) (Figure 8). GRP protein levels were shown to oscillate on a daily basis in rats kept under LD cycles, peaking at the transition between day and night (Shinohara et al., 1993; Okamura & Ibata, 1994). Like VIP, the rhythmic expression of GRP disappears in DD conditions.

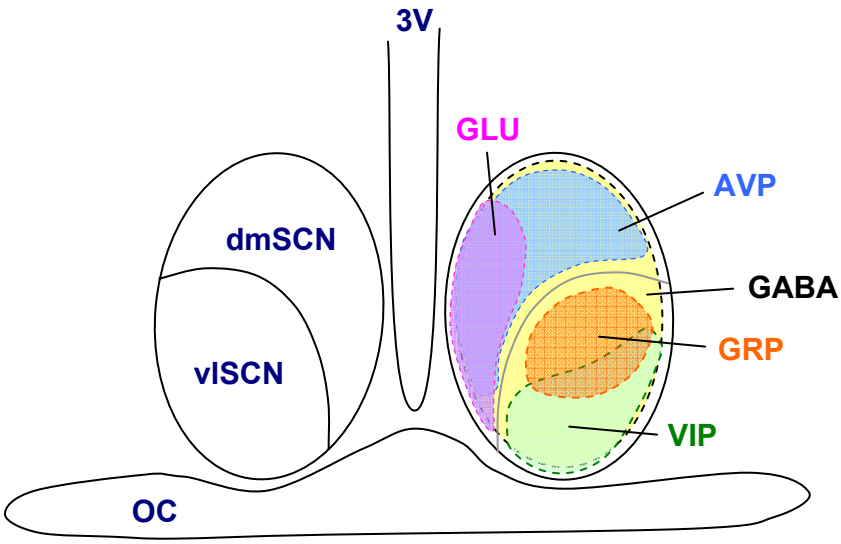

Figure 8. Subdivision and simplified representation of the neuropeptides and neurotransmitters content of the mouse SCN.

Subdivisions of the SCN are represented on the left nucleus and distribution of the main neuropeptides and neurotransmitters on the right nucleus. 3V, third ventricle; AVP, arginine vasopressin (blue); dmSCN, dorsomedian SCN; GABA; γ-amino-butyric acid (yellow); GLU, glutamate (purple); GRP, gastrin-releasing peptide (orange); OC, optic chiasm; VIP, vasoactive intestinal peptide (green); vlSCN, ventrolateral SCN.

In addition to the neuropeptides mentioned above and several others not cited here (Abrahamson & Moore, 2001), the neurotransmitter **γ-amino butyric acid** (GABA) is widely expressed in both the shell and core regions of the rat SCN (Moore & Speh, 1993) (Figure 8). The content of GABA presents a circadian rhythmicity in the SCN, with levels oscillating in both LD and DD conditions (Aguilar-Roblero et al., 1993). Similarly, the activity of the glutamic acid decarboxylase (GAD), which converts GLU into GABA, shows a diurnal oscillation. The release of GABA during the light phase is believed to drive a strong inhibitory signal toward the rat SCN targets (Kalsbeek et al., 1999; Kalsbeek et al., 2000).

Neurons expressing **GLU** are also found in the rat SCN, mostly in the shell (Csaki et al., 2000) (Figure 8). They are believed to drive an excitatory signal at night, which stimulates downstream targets (Perreau-Lenz et al., 2004).

2.1.3. THE COUPLING OF SINGLE CELL ACTIVITY LEADS TO A FUNCTIONAL CLOCK

One of the earliest observation concerning the activity of the SCN neurons was the discovery by Inouye and Kawamura that neurons exhibit a circadian rhythm of **electrical discharge**, with higher firing rate in the daytime (Inouye & Kawamura, 1979). Besides, they noticed that after *in situ* surgical isolation of the SCN, the rhythmic discharge of SCN neurons persists, while the rhythmic firing of neurons of other brain regions disappears. This initial study thus demonstrated that contrary to other brain regions, **circadian firing rates within the SCN are autonomous**. This was confirmed *in vitro* on culture of SCN slices (Green & Gillette, 1982; Shibata et al., 1982; Herzog et al., 1997). Since then, many studies attempted to decipher the mechanisms of the circadian oscillator that lead to the rhythmic electrical pattern.

A major advance in our understanding of the clock cellular machinery was achieved by the use of an array of 61 electrodes, which allowed the simultaneous record of multiple SCN neurons on cultures of SCN dispersed cells (Welsh et al., 1995). This study revealed that **individual neurons exhibit robust circadian rhythms**. But, these rhythms are not synchronized and show various phases. This led to the hypothesis that the SCN oscillator is constituted of **many single cell oscillators**. In the intact SCN, 76.1% of neurons show a circadian electrical activity, a larger proportion being localized in the dorsal compartment (86.8%) as compared to the ventral part (62.1%) (Nakamura et al., 2001).

Moreover, additional studies of the electrical activity of single SCN neurons revealed that neurons not only present a great variability in phase (Welsh et al., 1995) but they also show a large range of periods, from 20.0 to 28.3 h in rat (Honma et al., 1998a) (Figure 9). This is in contrast with the period of behavioral locomotor activity of these rats, which only moderately varies from 24.0 to 24.8 h among individuals. Remarkably, the average of the single neuronal activities is highly similar to the average locomotor activity period. This result led to the hypothesis that SCN neurons may **synchronize and integrate** the various circadian rhythms to constitute a **multiple oscillator system** that drives many physiological and behavioral outputs including the locomotor activity.

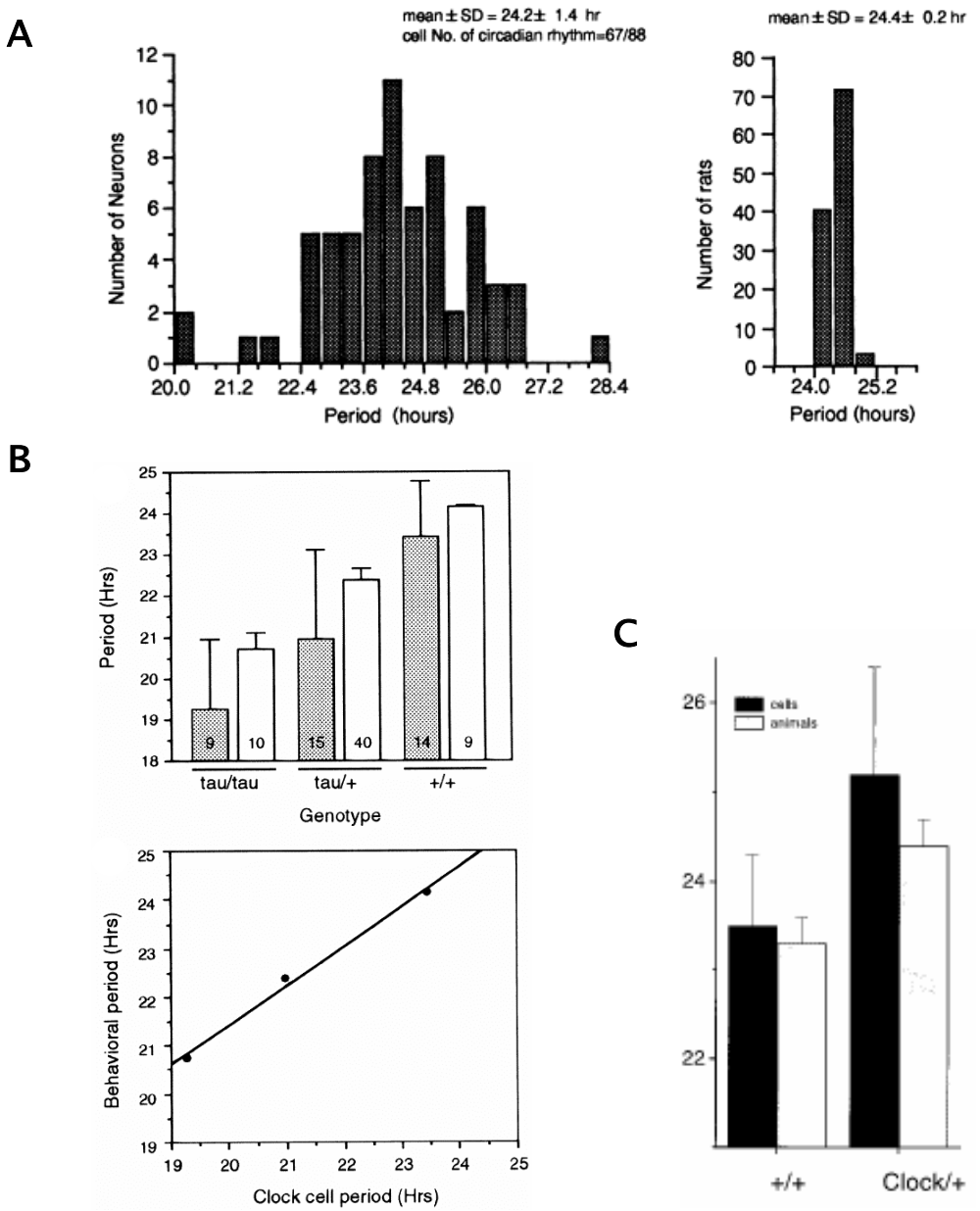

Figure 9. Correlation between the average firing rate period of individual SCN neurons and the average period of locomotor activity in rodents.

A. Distribution of circadian period of neuronal activity rhythm in individual rat SCN neurons (left panel), and of the locomotor activity rhythm (right panel). (From Honma et al., 1998a)
B. The upper panel represents the mean ± standard deviation of clock cell periods (shaded vertical bars) and behavior periods (open bars) for wild-type (+/+), heterozygous *tau* mutant (*tau*/+) and homozygous *tau* mutant (*tau*/*tau*) Syrian hamsters. The regression analysis of these data is shown on the lower panel. The regression line ($r^2 = 0.99$) is described by the formula $y = 0.81x + 5.20$, where y = behavioral period and x = clock cell period (in hours). (From Liu et al., 1997a)
C. The mean ± standard deviation circadian periods of *Clock* mutant mice SCN neurons (black bars) and wheel-running behavior (gray bars) were similarly lengthened by the mutation (Student's t-test, $p > 0.08$). (From Herzog et al., 1998)

This hypothesis was later confirmed by two independent studies (Figure 9). First, Liu and colleagues cultured dissociated SCN neurons from wild-type, heterozygous or homozygous *tau* mutant hamsters and monitored electrical activity (Liu et al., 1997a). The two mutant strains are known to have a period of locomotor activity close to 22 h and 20 h respectively (Ralph & Menaker, 1988). Although they recorded a great variability in the period of SCN neurons within a same strain, the authors noticed that the average of the periods of neuronal electrical activity for each strain was closely correlated to the mean period of locomotor activity of the same strain. *Clock* mutant mice (Vitaterna et al., 1994), which present a mutation in the clock gene *Clock* (*section A2.2.1.1*) that lengthens the behavioral locomotor activity in DD, were similarly used (Herzog et al., 1998). It has been observed that the average of the scattered electrical activity period, recorded in single SCN neurons in dispersed culture, equals the period of the locomotor activity: the average electrical discharge period of heterozygous *Clock* mutant cells is longer than the wild-type neurons.

This model in which the SCN clock is constituted of autonomous single cell clocks thus suggests that **synchronization** among cells must occur. It has been proposed that synchronization might involve **electrical synapses** between neurons. Indeed, synchronized firing rates observed between two SCN neurons (Honma et al., 2000; Long et al., 2005) are lost when tetrodotoxin, a Na^+-channel blocker is used (Honma et al., 2000). This was confirmed recently by monitoring the *Period1* (*Per1*) clock gene promoter activity in organotypic slice cultures following treatment with tetrodotoxin (Yamaguchi et al., 2003).

Another synchronization mechanism might involve **GAP junctions** among neurons. GAP junctions are intercellular channels composed of 12 connexin proteins subunits that notably allow the passage of small ions contributing to electrical coupling. Although an initial study reported that GAP junctions do not participate in the SCN neuronal coupling *in vitro* (Welsh & Reppert, 1996), a recent study reveals these pores are most likely involved in the coupling, both *in vitro* and *in vivo* (Long et al., 2005). Indeed, the coupling of SCN neuronal activity observed on paired neurons in wild-type mice is absent in cultures of connexin36 knockout mice neurons. In addition, the locomotor activity of these knockout mice show a diminishment in the overall circadian amplitude as compared to wild-type mice (Long et al., 2005).

VPAC2-deficient mice fail to exhibit circadian rhythms in firing activity, and in locomotor activity in LD and DD (Cutler et al., 2003) and a recent study established that the activity of each cell is likely desynchronized from other cells in those mutant mice (Maywood et al., 2006). It is thus likely that the neuropeptide **VIP** and its receptor **VPAC2** participate to the coupling of the SCN neurons.

The neurotransmitter **GABA** might also synchronize SCN neurons activity. It has been recently demonstrated that GABA can synchronize rhythms among dispersed SCN neurons showing opposite phases (Liu & Reppert, 2000).

Last but not least, **astrocytes** are believed to play a determinant role in synchronizing SCN neurons. Indeed, in SCN slice culture, the number and organization of astrocytes are more important than in dissociated cell culture and accordingly firing activity among neurons is more synchronized in SCN slice culture (Welsh & Reppert, 1996).

2.1.4. *INPUT AND OUTPUT PATHWAYS OF THE SCN*

2.1.4.1. Input pathways

Most of the input pathways of the SCN connect the ventrolateral part of the nucleus, suggesting the core acts as a relay station which integrates the various SCN inputs. Three major inputs have been identified: the **retinohypothalamic tract** (RHT), the **geniculohypothalamic tract** (GHT) and the **serotoninergic input** from the raphe nucleus (Figure 10).

a. *The retinohypothalamic tract*

The **RHT**, which is involved in the photic entrainment of the circadian system (*section A2.2.3*), has been widely studied (Moore & Lenn, 1972). It originates from a small distinct subset of **retinal ganglions cells** (RGC) widely distributed over the retina, identified using both retrograde and anterograde tracers (Moore et al., 1995). The RGC which connect to the SCN represent only 0.1% of the total optic nerve fibers (Provencio et al., 1998) and convey only **circadian information**. Indeed, a lesion of the RHT only alters photic entrainment, without affecting the vision (Johnson et al., 1988). Contrary to other RGC, these particular SCN-connected RGC are sensitive to light and express the

photopigment **melanopsin**, which plays a critical role in entraining the circadian clock (Gooley et al., 2001; Hattar et al., 2002) (*section A2.2.3.1*).

Multiple studies demonstrated that these RGC project to the ventrolateral area of the SCN, at the level of the VIP neurons (Ibata et al., 1989; Tanaka et al., 1993). Neurotransmitters of the RHT notably include the excitatory neurotransmitter GLU (Castel et al., 1993) and the neuropeptide pituitary adenylate cyclase-activating peptide (PACAP) (Hannibal et al., 1997).

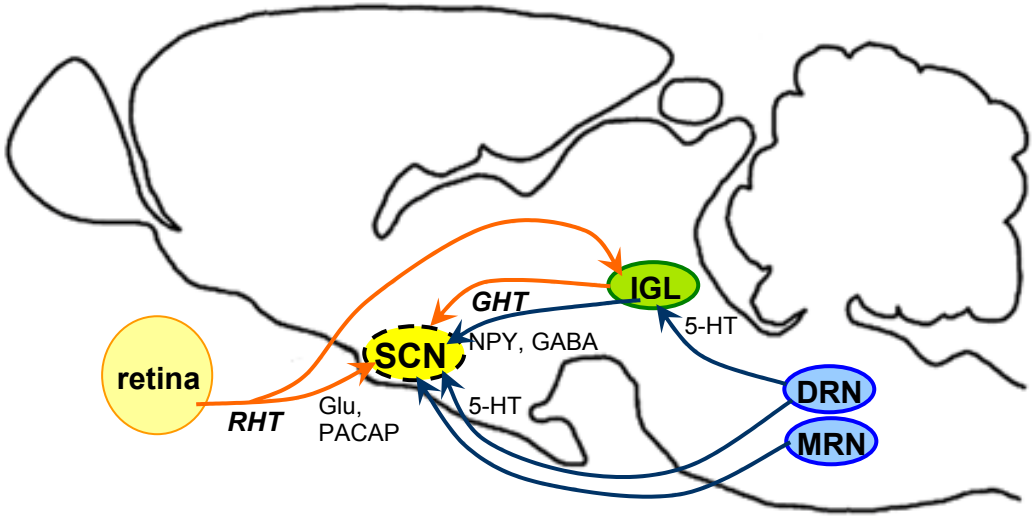

Figure 10. Main SCN afferent pathways in the rat.

Orange and dark blue arrows represent photic and non-photic inputs respectively. 5-HT, serotonin; DRN, dorsal raphe nucleus; IGL, intergeniculate leaflets; GABA; γ-amino-butyric acid; GHT, geniculohypothalamic tract; Glu, glutamate; MRN, median raphe nucleus; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase-activating peptide; RHT, retinohypothalamic tract; SCN, suprachiasmatic nucleus.

The light information conveyed by the RHT entrains the endogenous circadian oscillations in physiology and behavior to 24 h exactly by resetting the phase and the period of the clock daily. Strikingly, the effect of light on the phase and period of the clock depends upon the time of the daily cycle at which the light information is perceived. This can thus be translated into a **phase response curve** (PRC) (Figure 11). It has been early documented that a light pulse is capable of shifting the phase of the clock only when it is administered during the **night phase** (Aschoff, 1969; Daan & Pittendrigh, 1976). Furthermore, a light pulse will not induce the same patterns of phase-shift when it

is given in the **early subjective night** or in the **late subjective night**. While the former induces a **phase-delay** in the locomotor activity onset of nocturnal rodents on the following day, the latter entails a **phase-advance** in the activity onset on the day after (Daan & Pittendrigh, 1976). Animals showing an endogenous period shorter than 24 h are thus phase-delayed by the light perceived at the subjective day/subjective night transition to reset a 24 h period, while animals with an endogenous period longer than 24 h perceive the light at the subjective night/subjective day transition as a phase-advancing cue that retunes the period to 24 h exactly.

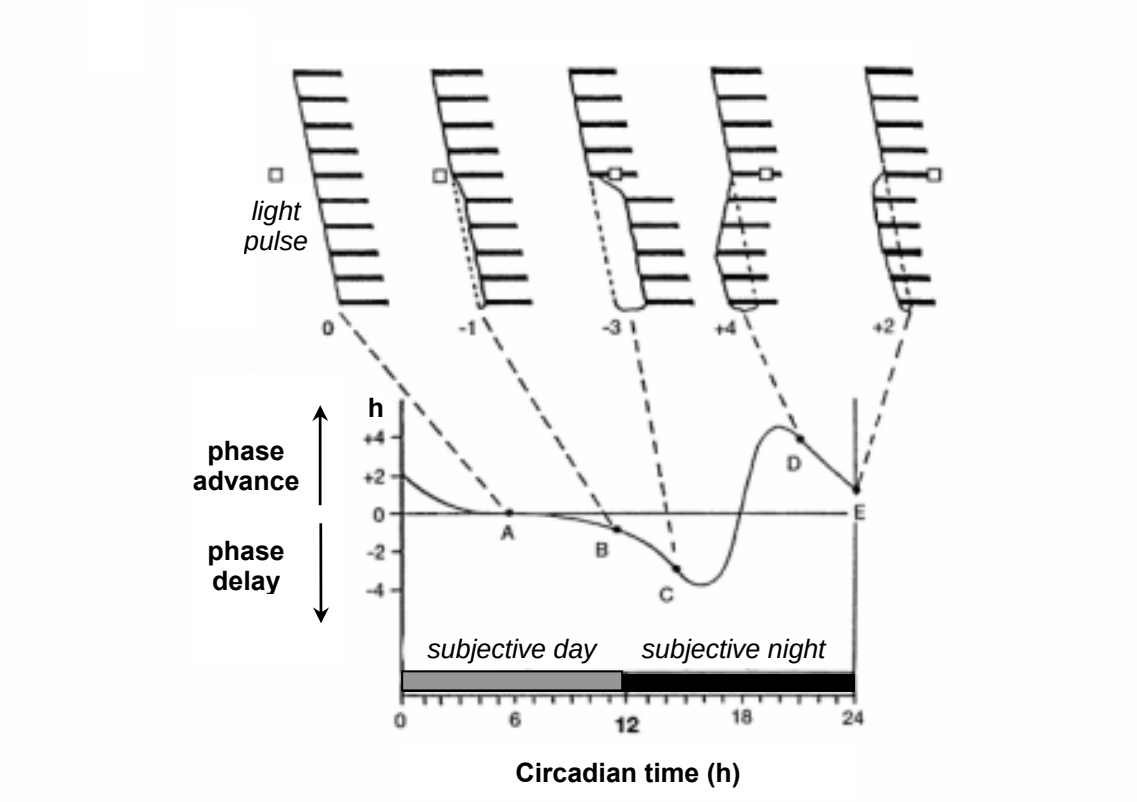

Figure 11. Photic-induced phase-shifts and phase-response curve of the hamster locomotor activity.

A light pulse exposure (open square) during the day (A) has no effect on the locomotor activity (thin solid bars, which represent periods of activity). On the contrary, light pulse stimulations at the beginning (B, C) and the end (D) of the night induce a phase-delay and a phase-advance of the locomotor activity respectively. Plotting the magnitude of the phase-advances or phase-delays as a function of the time of light pulse exposure allows the drawing of a phase-response curve.
(Adapted from (Serviere & Lavialle, 1996)

b. *The geniculohypothalamic tract*

The second major SCN afferent pathway originates in the **intergeniculate leaflets** (IGL) of the thalamus, located between the dorsal and ventral parts of the lateral geniculate nucleus. The IGL receive a dense bilateral input from the retina (Hickey & Spear, 1976) and each RGC that innervates the core region of the SCN also connects the IGL (Pickard, 1985; Moore & Card, 1994). The neuropeptide Y (NPY) and the neurotransmitter GABA are the major neurotransmitters of the IGL (Harrington et al., 1985; Moore & Speh, 1993; Moore & Card, 1994) and NPY-immunoreactive cells were found to overlap with IGL retinal afferents (Harrington et al., 1985). Several studies further demonstrated that NPY-immunoreactive cells of the IGL project to the core of the SCN (Harrington et al., 1987; Card & Moore, 1989; Morin et al., 1992) where NPY-immunoreactive fibers form a dense plexus (Harrington et al., 1985). NPY-immunoreactive axon terminals make synaptic contact on neurons that contain VIP in the core SCN (Hisano et al., 1988). Similarly, GABA fibers, which colocalize in the IGL with the NPY fibers that project to the SCN (Moore & Speh, 1993; Moore & Card, 1994), innervate the VIP neurons of the core SCN (Francois-Bellan & Bosler, 1992).

Considering first that IGL neurons that project to the SCN receive information from the same RGC than those which directly connect to the SCN, and second that IGL receive additional important afferent inputs from structures like the dorsal raphe nucleus (Meyer-Bernstein & Morin, 1996), it appears that the **IGL** constitute the integration point of a complex circuit that allow a **tight regulation of photic and non-photic** (related to behavior) **entrainment** of the circadian clock.

c. *The serotoninergic tract*

The third main SCN input pathway is a serotoninergic input originating in the **median raphe nuclus** in hamsters (Meyer-Bernstein & Morin, 1996; Leander et al., 1998), and in both **the median raphe nuclus and the dorsal raphe nucleus** in rats (Moga & Moore, 1997). Early studies reported that serotoninergic nerve fibers colocalize with the retinal afferents within the core region of the SCN (van den Pol & Tsujimoto, 1985). Additional investigations showed that serotoninergic fibers terminate on VIP-immunoreactive cells

of the ventrolateral SCN (Kiss et al., 1984; Bosler & Beaudet, 1985; Francois-Bellan & Bosler, 1992).

The serotoninergic tract, which terminates at the level of the retinal afferents in the SCN, participates in a **non-photic regulation of the entrainment** of the circadian clock.

2.1.4.2. Output pathways

The circadian rhythm generated within the SCN network is further **distributed** to various structures to drive rhythmic behavioral and physiological functions, like locomotor activity, water and food intake, body temperature or hormone synthesis. Two different pathways are now suggested to allow the transmission of the SCN information: **neuronal pathways** (Figure 12) and pathways involving a **diffusible signal**.

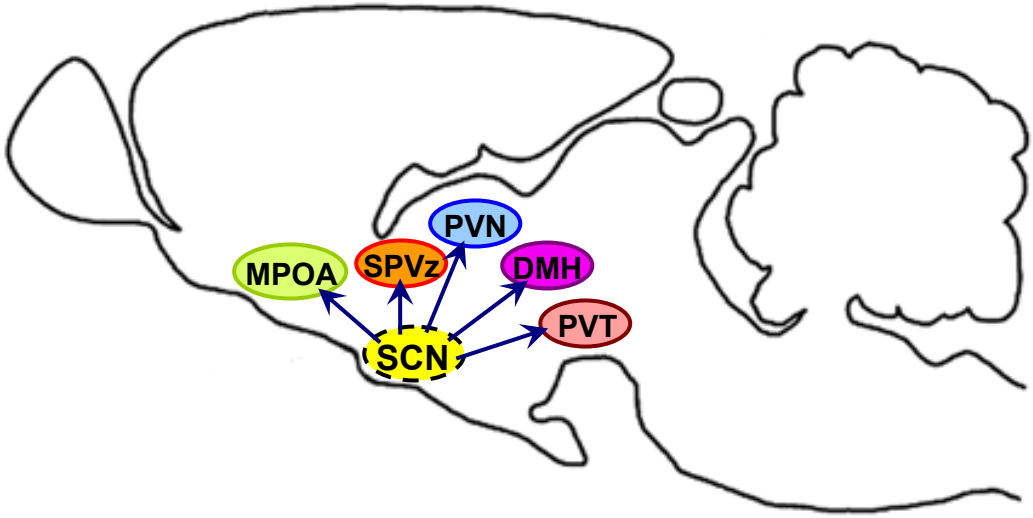

Figure 12. Main SCN efferent pathways in the rat.

DMH, dorsomedian hypothalamus; MPOA, median preoptic area; PVN, paraventricular nucleus of the hypothalamus; PVT, paraventricular thalamic nucleus; SCN, suprachiasmatic nuclei; SPVz, subparaventricular zone.

a. *Neuronal pathways*

Several brain structures mostly located within the thalamus and hypothalamus are innervated by the SCN. Using retrograde and anterograde tracers, Watts et al. and other groups reported that the nature of the target structures may depend on the SCN subdivision. The **median preoptic area** (MPOA) indeed specifically receives projections from the shell of the SCN, while the **subparaventricular zone** (SPVz) is targeted by the core SCN. Besides, both core and shell parts of the SCN project to the **dorsomedian hypothalamus**, the **paraventricular nucleus of the hypothalamus** (PVN) and the **paraventricular thalamic nucleus** (Watts & Swanson, 1987; Watts et al., 1987; Leak & Moore, 2001).

The role of the AVP release on the PVN in the regulation of the rhythmic corticosterone secretion is notably well-described (Buijs et al., 1998). Besides, the axis that leads to the rhythmic **melatonin** hormone secretion has been the most studied and the best-characterized (Simonneaux & Ribelayga, 2003). It is initiated by SCN projections to the PVN and further implicates the intermediolateral nucleus (IML) of the spinal cord and the superior cervical ganglions (SCG), which eventually connect to the pineal gland (PG), as thoroughly described in a section below (*section A2.4.3*).

b. *Diffusible factors*

In 1987, when Lehman and colleagues performed a SCN graft on SCN-lesioned hamsters placed in DD, they noticed the circadian locomotor behavior could be restored but not the photoperiodic response (Lehman et al., 1987). Later, additional groups showed that oestrous cycle, luteinizing hormone (LH) rhythm, corticosterone rhythm and MEL rhythm could not be recovered as well following a SCN transplant (Meyer-Bernstein et al., 1999). Thus, even though SCN afferent and efferent pathways are not functionally restored, circadian locomotor activity behavior can be recovered, suggesting the existence of a **diffusible factor**. This was confirmed by another transplantation experiment: a SCN enveloped in a semipermeable polymeric capsule that prevents neural outgrowth but not hormone diffusion could restore the circadian locomotor activity of hamsters (Silver et al., 1996). Additionally, the surgical isolation of the SCN *in vivo*

disrupted the photoperiodic response but not the circadian rhythms of locomotor activity, strengthening the hypothesis of a diffusible signal (Hakim et al., 1991).

As far as the locomotor activity is concerned, two candidates have been identified. First, **transforming growth factor α** is expressed rhythmically in the SCN and inhibits the locomotor activity when injected in the third ventricle. It is believed to induce the masking phenomenon (suppression of behavioral activity by light) through binding on epidermal growth factor receptors localized in the SPVz. Indeed, a mutation in the epidermal growth factor receptor increases the locomotor activity during the daytime and prevents the inhibition of the activity by light at night in mice (Kramer et al., 2001). Secondly, the **prokineticin 2** (*Pk2*) mRNA is rhythmically expressed in the SCN and PK2 level peaks during the daytime. A night-time delivery of PK2 in the third ventricle inhibits the locomotor activity of mice through binding on its PK2 receptor, which is expressed in several structures like the PVN and the dorsomedian hypothalamus and at high levels in the paraventricular thalamic nucleus (Cheng et al., 2002).

2.2. THE MOLECULAR MACHINERY OF THE CIRCADIAN CLOCK

2.2.1. THE MOLECULAR CLOCKWORK

Studies on the fruit fly *Drosophila melanogaster* allowed the identification of genes that appeared crucial to sustain circadian oscillations (Konopka & Benzer, 1971). This prompted scientists to search for cognate genes in mammals. The genetic essence of the circadian clock was notably supported by studies using protein and RNA synthesis inhibitors, which revealed alterations of the circadian clock (Takahashi & Turek, 1987; Raju et al., 1991). Today, 12 mammalian genes have been identified as relevant to the circadian clock molecular mechanism and named accordingly "**clock genes**". A clock gene is defined by one or several of the following characteristics:

- the loss-of-function mutant should lead to arrhythmicity or at least affect the period of the rhythm;
- the dynamic of the gene or protein expression should follow a circadian rhythm;
- the rhythm of gene/protein expression should be entrained and possibly shifted by entrainment input signals;

- the rhythmic pattern of expression should be altered when modifying cellular amounts of the given gene.

None of the currently identified mammalian clock genes followed all of the four rules. As described below, these genes assemble in interlocked multiple feedback loops that constitute the molecular clockwork (Figure 13).

2.2.1.1. The primary feedback loop

Constituted of the *Clock*, *Bmal1*, *Per1*, *Per2*, *Per3*, *Cry1* and *Cry2* genes in mammals, this loop is considered as the **core loop of the clock**. It is the basis of the oscillatory movement and appears critical in initiating behavioral and physiological circadian oscillations.

a. Clock *and* Bmal1*: positive elements of the loop*

Clock, which stands for circadian locomotor output cycles kaput, has been the first mammalian clock gene identified (Vitaterna et al., 1994). This has been achieved by randomly inducing mutations using *N*-ethyl-*N*-nitrosourea in mice and further screening for circadian clock alterations by monitoring the wheel-running circadian activity. The *Clock* mutation identified by this strategy is a semidominant autosomal mutation, which **lengthens the period of heterozygous animals** by about 1 h. In homozygous animals, the mutation dramatically lengthens the period by about 4 h and eventually leads to a **complete arrhythmicity after 2 weeks in constant conditions**. A two-step approach combining positional cloning and functional rescue identified the locus of the mutation and allowed the cloning of the full *Clock* gene (Antoch et al., 1997; King et al., 1997). The gene was found to code for a **bHLH-PAS** protein since the CLOCK protein harbors two characteristic domains: a **basic Helix-Loop-Helix** (bHLH) domain that allows interactions with DNA, and a **Per-Arnt-Sim** (PAS) domain, which facilitates protein-protein interactions with other proteins that harbor a **PAS** domain (Antoch et al., 1997; King et al., 1997). Proteins from the **bHLH-PAS** family are transcription factors known to bind DNA and modulate gene transcription following dimerization (Hogenesch et al., 1997). The *Clock* mutant phenotype is caused by a defect in the gene transcription induced by the heterodimer made of CLOCK and an unidentified bHLH-PAS protein,

since the point mutation occurs in the glutamine-rich region of CLOCK which is necessary to promote transcription (King et al., 1997). Interestingly, *Clock* mRNA (Sun et al., 1997; Tei et al., 1997; Oishi et al., 1998) and CLOCK protein (Maywood et al., 2003) are mostly present in the SCN where they are both constitutively expressed under LD and DD conditions in rat and mice.

Several studies further attempted to identify the functional partner of CLOCK. In 1998, Hogenesch and colleagues showed that CLOCK can bind several members of the **members of the PAS** superfamily of proteins (MOP) *in vitro* (Hogenesch et al., 1998). These MOP include **BMAL1** (Brain and muscle Arnt like protein 1, also called MOP3) and **NPAS2** (also named MOP4). **NPAS2** is highly homologous to CLOCK and as such is considered as a functional analog of CLOCK, especially in the forebrain where it binds BMAL1 (Reick et al., 2001). *Bmal1* mRNA levels oscillate in the rat SCN. In LD, mRNA levels peak at night, at ZT14-18, and present a trough at midday (ZT2-6). A similar pattern of expression exists in DD with a maximum mRNA level found at CT14-18 and a minimum level at CT2-6 (Honma et al., 1998b). Similarly, BMAL1 protein levels oscillate in the rat SCN under both LD and DD conditions, peaking at midnight (ZT/CT18-22) and dipping at midday (ZT/CT6) (Tamaru et al., 2000).

The functional relevance of BMAL1 in the molecular clockwork was initially suggested by Hogenesch and colleagues who showed that the **CLOCK-BMAL1 heterodimer** is functionally active to **promote gene transcription** (Hogenesch et al., 1998). This is achieved by the binding of the heterodimer on a consensus **CACGTG** sequence that constitute an **E-box** element and is located in the promoter of multiple genes (Hogenesch et al., 1997; Hogenesch et al., 1998).

The importance of BMAL1 in the circadian molecular machinery was further confirmed by the generation of BMAL1/MOP3 mutant mice in which the bHLH domain is impaired (Bunger et al., 2000). Heterozygous **BMAL1/MOP3 mutants mice** present a normal behavioral phenotype in both LD and DD. On the contrary, homozygous mutants are less active in LD and become immediately **arrhythmic when placed in a DD** environment. Besides, wild-type animals are capable of phase-shifting when a light pulse is administered at ZT14 or CT15 (Tamaru et al., 2000). This ability is lost in BMAL1/MOP3 mutant mice that receive a light pulse at CT16 (Bunger et al., 2000).

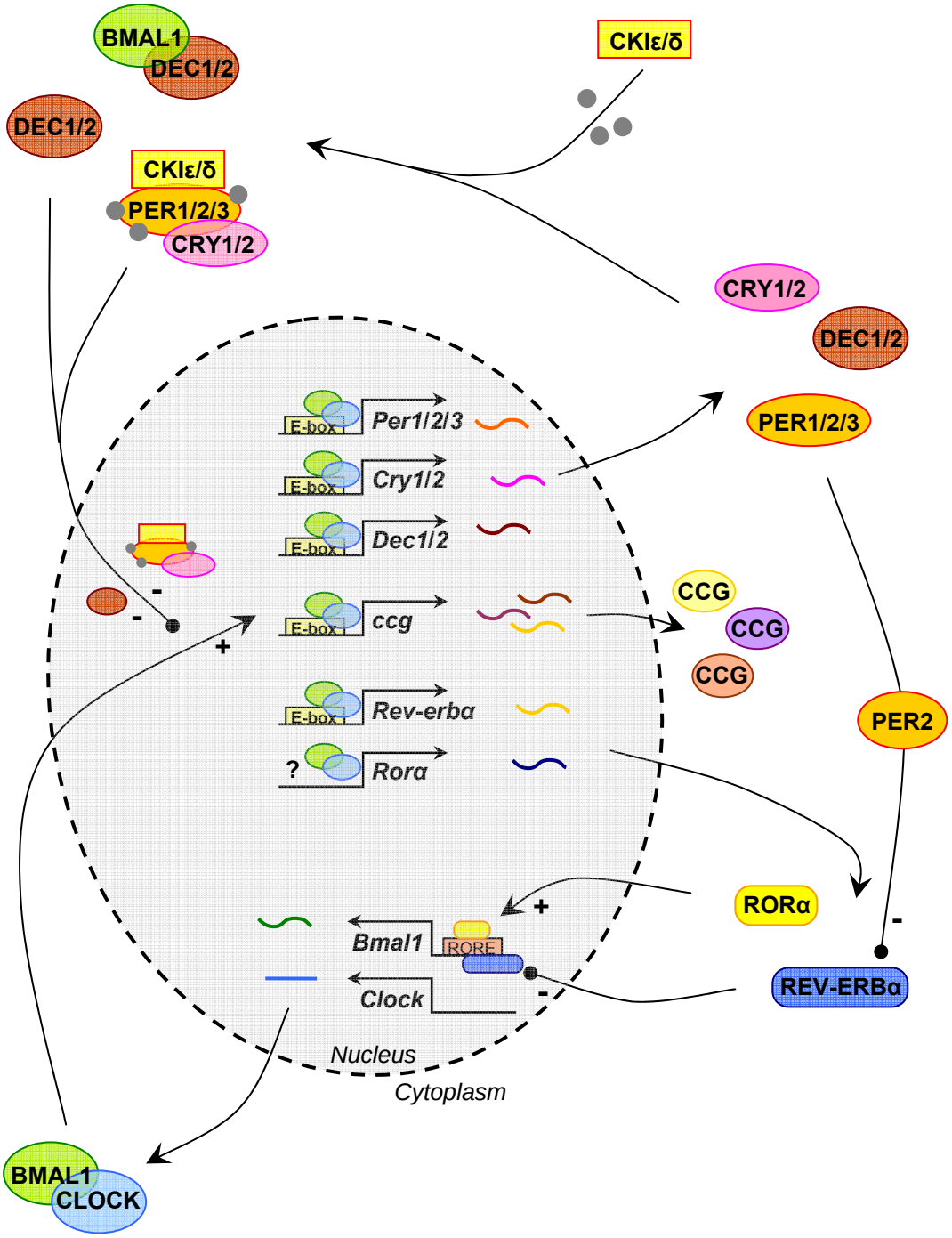

Figure 13. Simplified model of the molecular machinery of the circadian clock in mammals.

"+" and "-" represent stimulatory and inhibitory steps respectively. ● represents phosphate. See text for explanations. This representation is simplified and it should be noted that additional elements, notably ERK and GSK3, are involved (*section A2.2.2.3*). ccg, clock-controlled gene.

BMAL1 and the CLOCK-BMAL1 heterodimer thus appear to be critical for the generation and maintenance of circadian rhythms, for normal entrainment of behavior and for normal locomotor activity of animals.

However, it should be noted that a recent study by Debruyne and colleagues challenges the role of CLOCK in the circadian clock function by demonstrating that a null mutation of *Clock* does not prevent the rhythmic locomotor behavior of mice in DD (Debruyne et al., 2006). They suggest that NPAS2 could substitute CLOCK in the *Clock* knockout (KO) mice (Debruyne et al., 2006). Another group came to a similar conclusion after demonstrating that *Clock* + MEL mutant mice exhibit a rhythmic release of MEL in DD (Kennaway et al., 2003) and that *Per2* and *Pk2* gene expressions oscillate in the SCN (Kennaway et al., 2006). This suggests that these mice retain central rhythmicity despite the *Clock* mutation and that NPAS2 may substitute CLOCK.

b. Per *and* Cry*: negative elements of the loop*

In search for new clock genes, ***Per1***, which stands for *Period1* (initially named *Rigui*), has been identified in mice (Sun et al., 1997; Tei et al., 1997). The gene, recognized as an ortholog of the *Drosophila melanogaster Period* gene, codes for a protein that encompasses a PAS domain but no bHLH domain. *Per1* is expressed widely, although at a stronger rate in the SCN. Here, *Per1* mRNA levels oscillate throughout the day, peaking in the middle of the day/subjective day (ZT/CT6) and reaching a trough at midnight/midsubjective night (ZT/CT18) in mice (Sun et al., 1997; Tei et al., 1997; Maywood et al., 2003), rat (Yan et al., 1999) and hamsters (Maywood et al., 1999). Likewise, PER1 protein levels in the rodent SCN fluctuate over 24 h. The maximum content in PER1 is delayed by 4-6 h as compared to the mRNA levels and occurs at the day/night (ZT12) or subjective day/subjective night transitions (CT12) in LD and DD respectively. The least amount of PER1 is accordingly found at the night/day (ZT20-24) and subjective night/subjective day transitions (CT20-24) (Hastings et al., 1999; Maywood et al., 1999; Field et al., 2000; Maywood et al., 2003). Remarkably, *Per1* expression can be acutely **induced by a light pulse** administered in early subjective night (CT14) (Shearman et al., 1997) or at midnight (ZT18) (Field et al., 2000).

Although the generation of mutant mice for the *Per1* gene by several groups provided evidence for a role of *Per1* in the molecular clockwork, it led to various

phenotypes. Zheng and Cermakian groups showed that *Per1* mutant mice are still **rhythmic in constant conditions** (DD), although the period of locomotor activity is shorter by less than 1 h and less stable than wild-type animals (Cermakian et al., 2001; Zheng et al., 2001). In contrast, Bae and colleagues found that *Per1* mutant mice become eventually **arrhythmic** approximately 2 weeks following transfer in DD (Bae et al., 2001).

Per2 has been later identified as a homologous gene of *Per1*. Similar to *Per1*, *Per2* is widely expressed in mice with a maximum within the SCN (Shearman et al., 1997; Takumi et al., 1998). The PER2 protein, which shares ~45% amino acid residues with PER1, contains the protein-protein interaction PAS domain but no bHLH DNA binding domain (Shearman et al., 1997; Takumi et al., 1998). *Per2* gene expression oscillates over 24 h in the SCN. In mice (Takumi et al., 1998; Field et al., 2000), rats (Yan et al., 1999) and hamsters (Maywood et al., 1999) housed in LD, the peak of mRNA content is found late during the day (ZT8-12) and the minimum at the end of the night (ZT20-24). In mice kept in DD, the mRNA levels peak at midsubjective day (CT8) and reach a trough late at subjective night (CT20) (Shearman et al., 1997; Takumi et al., 1998; Field et al., 2000). In rats, *Per2* mRNA maximum levels are found late during the subjective day (CT8-12) and minimum levels at subjective dawn (CT20-24) (Yan et al., 1999). Moreover, mouse SCN content in PER2 proteins varies throughout 24 h, peaking at ZT12-16/CT12 and dipping at ZT/CT0-4 in LD/DD (Field et al., 2000). Similarly to *Per1*, *Per2* expression can be induced by subjecting animals to a **light pulse** in early subjective night (CT14) (Shearman et al., 1997) or at midnight (ZT18) (Field et al., 2000).

Disruption of the *Per2* gene, notably by altering the PAS domain, in *Per2* mutant mice leads to major impairment of the circadian behavioral rhythm (Zheng et al., 1999; Bae et al., 2001). Mutant mice behave normally in LD but free-run at a **shorter period** when housed in DD. Eventually, after more or less 2 weeks in constant conditions, mice become **arrhythmic** (Zheng et al., 1999; Bae et al., 2001). Mice mutant for both *Per1* and *Per2* gene behave normally in LD but immediately become arrhythmic when they are transferred in DD (Zheng et al., 2001).

The cloning of a third homologue gene was achieved in 1998 (Zylka et al., 1998). ***Per3*** gene codes for a protein 36% and 37% identical to PER1 and PER2 respectively.

The homology notably includes a well-conserved PAS domain. Similar to *Per1* and *Per2* genes, *Per3* is ubiquitously found and highly expressed in the SCN where it shows circadian oscillations in its expression pace. In mice housed in DD, the *Per3* mRNA content peaks at midsubjective day (CT6-9) and reaches a minimum at the end of the subjective night (CT21) (Zylka et al., 1998). Contrary to its two homologues (Shearman et al., 1997), *Per3* expression is **not responsive to light pulses** (Zylka et al., 1998).

Besides, *Per3* KO mice do not present dramatic changes in behavior despite a shortening of the period of activity by ~0.5 h when animals are placed in DD (Shearman et al., 2000a). This result has challenged the role of *Per3* in the molecular clockwork. Generation of double mutant mice for *Per1*/*Per3* and for *Per2*/*Per3* supports a minor role of *Per3* in the circadian clock machinery since they respectively behave like *Per1* and *Per2* mutant mice (Bae et al., 2001).

Cry1 and ***Cry2*** genes, which stand for *Cryptochrome 1* and *2*, have been identified as two critical components of the primary feedback loop. In plants, these flavoproteins constitute blue-light photoreceptors which resemble DNA photolyases (DNA repairing enzymes) that are deprived of repairing activities (Cashmore et al., 1999). In the circadian clock system, they are notably known to participate to the entrainment of circadian rhythms in *Drosophila melanogaster* (Stanewsky et al., 1998), which has led to suspect a role for *Cry* genes in mammals as well. In mice, *Cry1* and *Cry2* are expressed widely, although at a stronger level in the SCN (Miyamoto & Sancar, 1998). In the SCN, *Cry1* is rhythmically expressed throughout 24 h, and *Cry1* mRNA levels reach a peak at CT8-12 and a trough at CT20-24 in DD (Miyamoto & Sancar, 1998; Kume et al., 1999; Okamura et al., 1999). On the contrary, *Cry2* studies are not conclusive as *Cry2* mRNA levels are either found not rhythmic (Kume et al., 1999) or rhythmic (Okamura et al., 1999). This latter study showed a circadian rhythm of *Cry2* mRNA content characterized by a peak at subjective dusk (CT12) and a trough at subjective dawn (CT0). In rats, both *Cry1* and *Cry2* expressions oscillate in the SCN in LD, with a maximum in mRNA levels reached at ZT12 and a minimum at ZT0 (Park & Kang, 2004). Moreover, CRY1 and CRY2 protein levels oscillate in mice SCN peaking at subjective dusk (CT12-14) and reaching a trough at subjective dawn (CT2) (Kume et al., 1999). Interestingly, in contrast to *Per1* and *Per2*, *Cry1* and *Cry2* expression **do not seem to be responsive to a light**

pulse stimulus given at night (ZT14) or in the middle of the subjective night (CT18) (Miyamoto & Sancar, 1999; Field et al., 2000).

KO mice for each of the *Cry* genes as well as double KO mice were generated to pursue their involvement in the molecular machinery of the clock. *Cry1* KO and *Cry2* KO mice both behave normally in LD but present alterations in the free-running period of locomotor activity when animals are transferred to DD: the locomotor activity period is indeed **shorter in *Cry1* KO** than wild-type animals (van der Horst et al., 1999), while the period of ***Cry2* KO mice is longer** (Thresher et al., 1998; van der Horst et al., 1999). Despite these opposing effects on period length, both *Cry1* KO and *Cry2* KO animals **maintain rhythmicity in DD**. This is in contrast with the double *Cry1*/*Cry2* KO mice in which locomotor activity rhythms are **immediately disrupted upon transfer to DD** (van der Horst et al., 1999). These KO mice thus provide strong evidence that *Cry1* and *Cry2* are both crucial to maintain circadian rhythmicity and that they play, at least partly, redundant functions.

The rhythmic transcription of the three *Per* genes as well as the two *Cry* genes is directly dependent upon the binding of the **CLOCK-BMAL1 heterodimer** (Gekakis et al., 1998) (Figure 13). The *Per* and *Cry* genes all encompasses within their promoter **E-box** elements consensus sequences **CACGTG** or **E-box-like** elements on which CLOCK-BMAL1 heterodimers bind and initiate gene transcription. This has been demonstrated *in vitro* on *Per1* transcription (Gekakis et al., 1998). Moreover, *Per* and *Cry* genes expression are blunted or eliminated respectively in *Clock* mutant mice (Jin et al., 1999; Kume et al., 1999) and *Per1* and *Per2* expression lack rhythmicity in BMAL1/MOP3 null mutant mice (Bunger et al., 2000). This highlights the role of CLOCK-BMAL1 heterodimer as a **positive regulator** in the molecular clockwork. Strikingly, the rhythmic pattern of *AVP* gene expression in the SCN is also dependent upon the CLOCK-BMAL1 binding on the E-box of the *AVP* promoter, as rhythmic AVP mRNA content is absent in *Clock* mutant mice (Jin et al., 1999).

To complete a full cycle in *Per1*/*2*/*3* and *Cry1*/*2* mRNA and protein content over 24 h, **PER1/2/3 and CRY1/2 proteins inhibit the CLOCK-BMAL1-induced transcription of *Per1*/*2*/*3* and *Cry1*/*2***. *In vitro*, PER1/2/3 proteins interact among each other to form **heterodimers** (Kume et al., 1999). These interactions entail the nuclearization of heterodimers, which inhibit the CLOCK-BMAL1-induced transcription of *AVP* (Jin et al., 1999; Kume et al., 1999). CRY1 and CRY2 proteins also strongly

inhibit the CLOCK-BMAL1-mediated transcription of *Per1* and *AVP* following binding with PER1/2/3 proteins and nuclearization (Kume et al., 1999). This is confirmed in *Cry1*/*Cry2* KO mice for which the magnitude of *Per1*/*2*/*3* genes constitutive transcription is higher than in wild-type animals (Okamura et al., 1999). PER2-CRY1 heterodimer does not imply a PAS-mediated interaction, and inhibition of CLOCK-BMAL1-induced transcription does not involve binding with an E-box (Shearman et al., 2000b). Since CRY1/2 can bind BMAL1, it is thus likely that inhibition of CLOCK-BMAL1-induced transcription is mediated *via* a direct contact of PER-CRY heterodimers with BMAL1 (Griffin et al., 1999). This is confirmed by a recent study, which revealed that the CRY-mediated repression of the transcriptional activity of the CLOCK-BMAL1 heterodimer, required for maintenance of circadian rhythmicity (Sato et al., 2006), occurs through binding of CRY on the C-terminal extension of BMAL1 (Kiyohara et al., 2006).

Several *in vivo* studies support this **feedback loop model**. First, PER1/2/3 and CRY1/2 proteins are all capable of binding each other *in vivo* (Field et al., 2000). In addition, the rhythmic pattern of clock proteins as compared to cognate mRNA is consistent with a role of the PER and CRY proteins in inhibiting the transcription of their own gene: the decline of mRNA levels coincides with the high protein levels within the SCN (Hastings et al., 1999; Maywood et al., 1999), and *Bmal1* mRNA and protein levels are in phase opposition with that of *Per1*/*2*/*3* and *Cry1*/*2*. Besides, *Per1* and *Per2* mRNA levels in SCN of *Cry1*/*Cry2* KO mice lack rhythmicity (Okamura et al., 1999).

2.2.1.2. The secondary loops

a. Dec1 *and* Dec2*: the interlocked loop*

Dec1 and ***Dec2*** genes have been identified by Honma and colleagues (2002). Expressed in the SCN, they code for bHLH transcription factors which are both rhythmically expressed in the SCN. *Dec1* and *Dec2* mRNA levels peak at early day/midday or early subjective day/midsubjective day and reach a trough at midnight/late night or midsubjective night/late subjective night in LD and DD respectively (Honma et al., 2002). As suggested in *in vitro* studies (Hamaguchi et al., 2004; Kawamoto et al., 2004), transcription of *Dec* genes is directly **activated by CLOCK-BMAL1 heterodimers** interacting with the three and two E-box elements of *Dec1* (Kawamoto et al., 2004) and

Dec2 (Hamaguchi et al., 2004) promoters respectively. Accordingly, *Dec1* and *Dec2* SCN expression in *Clock* mutant mice are diminished (Butler et al., 2004).

A function for *Dec1* and *Dec2* in the circadian clockwork has been revealed by the observation that *Dec* genes are capable of **inhibiting the CLOCK-BMAL1-mediated transcription** of *Per1*, *Dec1* and *Dec2 in vitro* (Honma et al., 2002; Hamaguchi et al., 2004; Kawamoto et al., 2004). Inhibition of *Per1* by *Dec1/2* is comparable to the *Cry1/2*-induced inhibition of *Per1* (Honma et al., 2002). Inhibition of *Dec1* and *Dec2* transcription is notably possible through a direct **binding** of DEC1 and DEC2 on *Dec1* and *Dec2* E-box elements, thus competing with CLOCK-BMAL1 heterodimer (Hamaguchi et al., 2004; Kawamoto et al., 2004). However, an inhibition mediated through interaction with BMAL1 cannot be excluded since DEC1/2 can bind BMAL1 (Honma et al., 2002). Interestingly, *Per1/2* and *Cry1/2* can also inhibit *Dec1/2* transcription *in vitro* (Hamaguchi et al., 2004; Kawamoto et al., 2004).

The *Dec1/2* loop thus highly resembles the *Per1/2/3* and *Cry1/2* loop. Components of both loops interact among each other, constituting **tightly interlocked feedback loops**, which role is to **stabilize** the molecular clockwork.

b. Rev-erbα *and* Rorα*: the stabilizing loop*

In the mouse SCN, the cycling ***Bmal1*** gene possesses **RORE** (retinoic acid related orphan receptor (ROR) response elements) in its promoter (Preitner et al., 2002; Ueda et al., 2002). This has suggested the involvement of **REV-ERBα** and **RORα** in the circadian regulation of *Bmal1* since both orphan receptors can bind RORE. *Rev-erbα* expression in the mouse SCN is rhythmic and follows a pattern similar to CLOCK-BMAL1-controlled genes and in phase opposition to *Bmal1* rhythm: the maximum in *Rev-erbα* mRNA content is reached at midday/midsubjective day and the minimum is found at midnight/midsubjective night in LD/DD (Onishi et al., 2002; Panda et al., 2002b; Preitner et al., 2002; Ueda et al., 2002). Similarly, *Rorα* expression in the mouse SCN is rhythmic with a peak at midday and a trough at midnight (Sato et al., 2004). It is likely that *Rorα* transcription is controlled by CLOCK-BMAL1 since the peak in *Rorα* expression is delayed in *Clock* mutant mice in a similar fashion to *Per2* expression (Sato et al., 2004). However, this has not been confirmed yet.

REV-ERBα and RORα **compete** *in vitro* for the binding to RORE encompassed in the *Bmal1* promoter: **REV-ERBα represses** *Bmal1* expression while **RORα induces** it (Ueda et al., 2002; Sato et al., 2004; Guillaumond et al., 2005). *Bmal1* rhythmic expression is completely abolished in *Rev-erbα* KO mice (Preitner et al., 2002) and strongly reduced in *Staggerer* mice (loss-of-function mutation of *Rorα*) (Sato et al., 2004), which confirms the *in vivo* implication of REV-ERBα and RORα in *Bmal1* regulation.

By constituting a link between the negative and the positive elements of the primary loop, REV-ERBα **stabilizes** the circadian molecular machinery: CLOCK-BMAL1 heterodimers induce *Rev-erbα* expression, and consequently the increased amounts of REV-ERBα inhibit *Bmal1* transcription. Meanwhile, PER-CRY heterodimers levels increase and eventually inhibit the REV-ERBα-mediated inhibition of *Bmal1* to start a new cycle and allow *Bmal1* expression. This is consistent with the demonstration that PER2 positively regulates *Bmal1* expression (Shearman et al., 2000b; Bae et al., 2001). It is suggested that PER2 inhibits REV-ERBα and authorizes the *de novo* synthesis of *Bmal1*.

2.2.1.3. CKIε and CKIδ: timing of the loops

We have depicted above the critical role of the primary loop in generating the oscillatory movement and of the secondary loops in stabilizing this oscillation. However, transcription, translation, as well as nuclear translocation are not sufficient to explain how the approximate 24-h period of the clock is determined. Additional **post-translational regulations** of clock proteins that modulate their stability are required to precisely time the molecular oscillations of clock genes expression and clock proteins synthesis. Several **protein kinases**, like the **casein kinase I (CKI) ε and its highly homologous CKIδ**, contribute to these modifications.

Inhibition of CKIε/δ kinase activity occurs through autophosphorylation of 8 phosphoacceptor sites situated in the C-terminal tail of the enzymes (Graves & Roach, 1995; Cegielska et al., 1998; Gietzen & Virshup, 1999). Conversely, dephosphorylation of these amino acid residues by PP1, PP2A, PP2B (also known as calcineurin) and PP5 increases CKIε/δ kinase activiy, *in vitro* (Cegielska et al., 1998; Lowrey et al., 2000; Partch et al., 2006).

CKIε/δ are expressed constitutively in the mammalian SCN (Lowrey et al., 2000; Takano et al., 2000; Ishida et al., 2001; Lee et al., 2001). They both physically **interact with and phosphorylate PER1/2 proteins** in mice (Keesler et al., 2000; Camacho et al., 2001) and human (Vielhaber et al., 2000; Akashi et al., 2002; Eide et al., 2002) (Figures 13 and 14). Recent studies suggested that PER phosphorylation leads to a **bimodal regulation** of PER functions (Partch et al., 2006; Vanselow et al., 2006). Vanselow and colleagues proposed a model based on PER2 phosphorylation. They suggested that **1)** at the beginning of the circadian cycle, newly synthesized PER2 proteins shuttle between nucleus and cytoplasm, where they are rapidly **phosphorylated by the CKIε/δ**. This phosphorylation occurs on specific sites that target PER2 to the **proteasomal degradation pathway** (Vanselow et al., 2006). Previously, several studies indicated that phosphorylation of PER proteins by CKIε/δ promote their ubiquitylation and subsequent degradation by the proteasome (Keesler et al., 2000; Camacho et al., 2001; Akashi et al., 2002; Miyazaki et al., 2004; Eide et al., 2005). **2)** The neo-synthesized CRYs then bind on PERs to form **ternary complexes PERs-CKIε/δ-CRYs**. The interaction with CRY proteins (Eide et al., 2002) as well as the phosphorylation of Ser-661 and Ser-663 of PER1 by CKIε (Takano et al., 2004) promote the **nuclearization** of PERs-CKIε/δ-CRYs complexes. This translocation of the multiprotein aggregates is associated with a **stabilization** of PER proteins. CRY1 notably binds the C-terminal tail of PER2 in mice, preventing it from being ubiquitylated (Yagita et al., 2002). Reciprocally, PER2 also prevents ubiquitylation of CRY1 (Lee et al., 2001; Yagita et al., 2002). In search for a mechanism responsible for the stabilization, Vanselow et al. proposed that **upon complex formation with CRY proteins, additional kinases are activated or recruited** to the PER2-CKIε/δ-CRYs complexes. These yet-unidentified kinases phosphorylate PER2 at Ser-659 and Ser-662 in mice (Ser-662 and Ser-665 in human). These initial phosphorylations prime the phosphorylation by CKIε/δ of the serine residues downstream from Ser-659 and Ser-662. This prevents an **early nuclear clearance** and thereby **stabilizes** PER2 proteins by protecting them from the cytosolic proteasomal degradation (Vanselow et al., 2006). **CKIε/δ thus promote both cytoplasmic proteasomal degradation and nuclear retention by phosphorylating PER on different residues**. This complex bimodal regulation creates a **4-6 h delay** between the accumulation of neo-synthesized PERs and CRYs, which is slowed down by the CKIε/δ-directed proteasomal degradation, and the nuclear retention of the PERs-CKIε/δ-CRYs

multiprotein complexes. This delay contributes to the **definition of the approximate 24-h circadian period**.

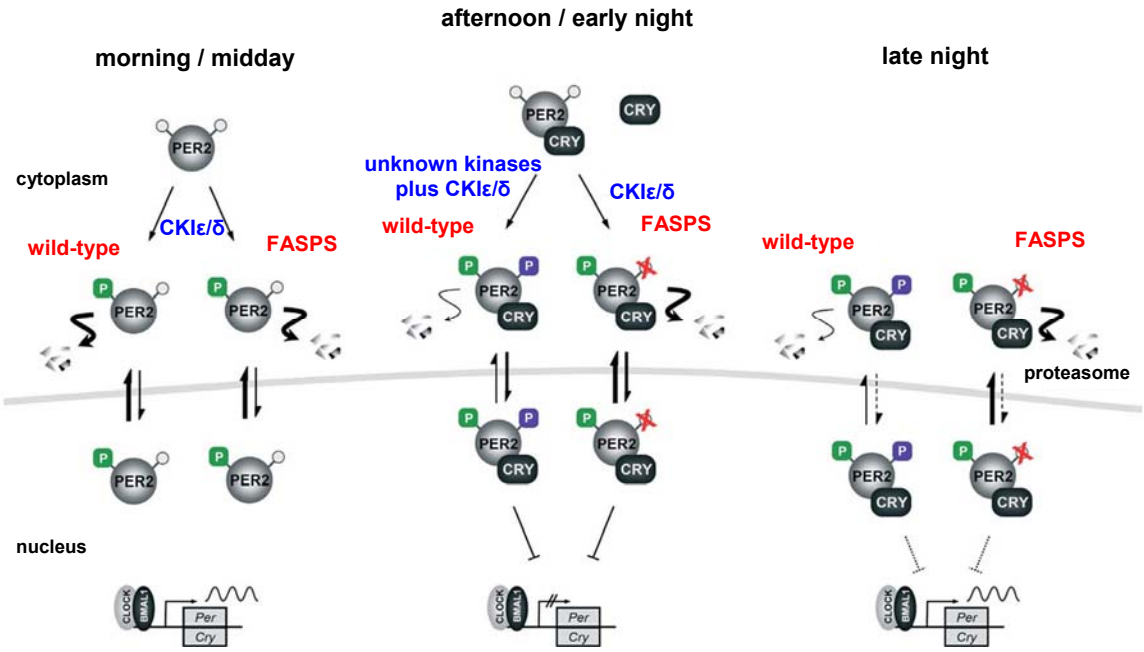

Figure 14. Model of CKIε/δ implication in the regulation of the murine molecular clockwork in a wild-type or family advanced sleep phase syndrome genotype.

PER2 contains two functionally different sets of phosphorylation sites (P) – one primarily mediating proteasomal degradation (green), the other inducing nuclear retention (purple). In the family advanced sleep phase syndrome (FASPS) genotype (right side of the panels), the latter cannot be phosphorylated because Ser-659 is mutated (red crosses). At the beginning of the circadian cycle (morning/ midday), newly synthesized PER2 proteins shuttle between nucleus and cytoplasm, where they are phosphorylated by CKIε/δ at sites that target it for rapid proteasomal degradation in the cytoplasm. Later (afternoon/early night), complex formation with CRY proteins enhances the nuclear localization of the PER2–CRYs complexes and likely activates or recruits additional kinases to the PER2–CRYs complexes. These yet-unknown kinases phosphorylate PER2 at the Ser-659 site, which serves as a priming site for CKIε/δ phosphorylation at downstream serine residues. Together, this leads to nuclear accumulation of the PER2–CRYs complexes and thereby to transcriptional repression of CLOCK–BMAL1 transactivation. At the end of the circadian cycle (late night), the PER2–CRYs repression is released because the PER2–CRY complexes are degraded in the cytoplasm after nuclear export. In FASPS, however, the region responsible for nuclear retention on PER2 cannot be phosphorylated (red crosses), leading to premature nuclear export of the PER2–CRYs complexes, and thus to an earlier cytosolic degradation and to a faster circadian cycle.
(From Vanselow et al., 2006)

It should be noted however that another study showed that PP5, which activates CKIε, is down-regulated *via* its interaction with CRY1/2 proteins (Partch et al., 2006). Consequently, CKIε is less active and PER phosphorylation levels decrease, which accelerates the nuclear clearance and reduces PER stability.

Altogether, these studies suggest that CKIε/δ ensure a tight regulation of the timing of transcriptional repression. This is supported by *in vivo* data. First, the ***tau* mutation** discovered in 1988 in hamsters (Ralph & Menaker, 1988) is attributed to a point mutation in the *CKIε* gene that reduces the enzymatic activity *in vitro* (Lowrey et al., 2000). Initially, it was believed that this mutation leads to a hypophosphorylation of PER proteins *in vivo* and consequently to an impairment in CKIε-mediated proteasomal degradation. Hence, the nuclearization of the inhibitory PERs-CKIε/δ-CRYs complexes would occur sooner. This model was consistent with the shorter behavioral period observed in *tau* mutant hamsters (Ralph & Menaker, 1988). However, recent findings indicate that the nuclear entry of PERs-CKIε/δ-CRYs complexes is not advanced in *tau* mutant hamsters (Dey et al., 2005). Instead, it appears that the nuclear clearance of PER proteins occurs sooner in mutants. Considering the new model of CKIε/δ regulation by Vanselow and colleagues, it is likely that altered CKIε in *tau* mutants is unable to promote the phosphorylation of the serine residues that assure nuclear retention, while it can still promote the cytosolic proteolysis (Vanselow et al., 2006). It should be noted however that a recent study predicted a gain-of-function *tau* mutation that would lead to hyperphosphorylation and increased degradation of PER1/2 (Gallego et al., 2006).

An additional study showed that the **family advanced sleep phase syndrome** is associated with a point mutation at Ser-662 in the CKIε binding site of the human PER2 protein (Toh et al., 2001). This nucleotide substitution prevents normal phosphorylation by CKIε of PER2 on serine residues immediately downstream of Ser-662. As a consequence, PER2 is cleared sooner from the nucleus (Vanselow et al., 2006) (Figure 14). This correlates with the advanced sleep onset and shorter period of temperature and melatonin rhythms (Toh et al., 2001). In addition, an *in vitro* study revealed that the mutation of Ser-714 of PER1 leads to a significant phase advance of the clock, which suggests that Ser-714 phosphorylation by CKIε might be critical to properly regulate the phase of the clock (Takano & Nagai, 2006). A point mutation in the CKIδ gene that reduces the kinase activity also entails a defect in PER1/2/3 phosphorylation *in vitro* (Xu et al., 2005). *In vivo*, this mutation leads to a shortening of the activity period in both mice and humans (Xu et al., 2005).

Another study similarly suggests that a mutation in the CKIε phosphorylation motif of the human PER3 protein may be responsible for the **delayed sleep phase syndrome** (DSPS), which symptoms are a sleep-onset insomnia and an inability to wake up in the morning (Ebisawa et al., 2001).

2.2.2. MULTIPLE REGULATIONS OF THE MOLECULAR CLOCKWORK

Although the model of transcriptional/translational interconnected feedback loops is now well-accepted, many studies suggest that it alone cannot justify the dynamic and the timing of the oscillations. The CKIε/δ-mediated phosphorylation of clock proteins depicted above (*section A2.2.1.3*) illustrates the requirement for additional modulation. In the mammalian molecular clockwork, these supplementary levels of regulation involve **transcriptional regulation** through several regulatory elements lying within clock genes promoters, **post-transcriptional regulation** of the transcripts, and **post-translational regulation** of clock proteins, such as their phosphorylation.

2.2.2.1. Transcriptional regulation

In addition to the above-mentioned E-box, clock genes expression requires several other regulatory elements and physical reorganization of the genomic DNA.

a. *E-box element*

As mentioned earlier (*section A2.2.1*), the E-box element is an enhancer consensus sequence that lies within the promoters of many genes. In the circadian clock system, several clock genes and many clock-controlled genes (Panda et al., 2002b) encompass one or more E-Box elements, which are implicated in the rhythmic oscillations of clock gene expression. This is the case for the negative elements of the primary feedback loop. Five E-box elements have been identified in the mouse *Per1* promoters (Hida et al., 2000; Yamaguchi et al., 2000). *Per2* promoter also contains E-box elements, although data are less conclusive. A group identified 7 E-box-like elements in the mouse *Per2* promoter (Travnickova-Bendova et al., 2002) while two other groups described a single functional E-box-like element (Yoo et al., 2005; Akashi et al., 2006). However, Yoo and

colleagues described 3 additional E-box-like elements and 1 E-box that do not seem to be implicated in driving *Per2* rhythmic expression (Yoo et al., 2005). Similarly, not all 5 E-box elements (Yamamoto et al., 2004) or 5 E-box elements and 3 E-box-like elements described in the *Rev-erbα* promoters are involved in the rhythmic expression of the gene (Triqueneaux et al., 2004). Thus, the presence of E-box elements in clock genes promoters appears **necessary** to drive circadian rhythmicity but **not all E-box elements are critical** for cyclic gene expression.

b. *cAMP response element*

In mouse, *Per1* (Hida et al., 2000) and *Per2* (Travnickova-Bendova et al., 2002) promoters possess at least one **CRE TGACGTCA** as an additional transcriptional regulatory sequence. *In vitro*, it has been demonstrated that CRE is essential in promoting signaling-mediated *Per1*/*2* transcription (Travnickova-Bendova et al., 2002). This study also established that CRE-mediated transcription links the light-stimulated ERK pathway to the light-stimulated *Per1* expression through the **CREB** (Obrietan et al., 1999; Travnickova-Bendova et al., 2002) (*section A2.2.3.1*). The mechanism involves the phosphorylation and activation of CREB by ERK1/2, which further binds to the CRE of the *Per1* promoter. This binding requires the interplay of two coactivators: **CBP** and **p300**, which physically interact with CREB to promote its transcriptional activity (Kwok et al., 1994; Lee et al., 1996; De Cesare & Sassone-Corsi, 2000). Strikingly, using a CRE-β-galactosidase transgenic reporter strain, Obrietan and colleagues showed that the **CRE-mediated transcription in the mouse SCN is rhythmic in DD**, peaking at early to midsubjective day (CT2-6) and reaching a trough at midsubjective night (CT18) (Obrietan et al., 1999). Consistently, CREB shows a circadian rhythm of phosphorylation (p-CREB) in the SCN, with the zenith reached at mid- to late subjective night (CT18-22) and the nadir at CT10 (Obrietan et al., 1999). This CRE-mediated and CLOCK-BMAL1-induced regulations of *Per1*/*2* gene expression seem independent since CLOCK-BMAL1 activation of *Per1*/*2* promoters occurs regardless of an intact CRE (Travnickova-Bendova et al., 2002).

c. *Rev-erbα/Rorα element*

Several clock genes including *Bmal1* (Preitner et al., 2002; Yin & Lazar, 2005), *Cry1* (Etchegaray et al., 2003) and *Rev-erbα* (Yamamoto et al., 2004) contain RORE in their promoter. As suggested earlier (*section A2.2.1.2*), *Bmal1* regulation by REV-ERBα and RORα through RORE play a critical role in regulating the molecular clockwork and the 2 RORE of the *Bmal1* promoter seem to be required for REV-ERBα-mediated *Bmal1* gene expression (Yin & Lazar, 2005).

d. *Structural modifications of chromatin*

In addition to enhancer and inhibitory regulatory elements embedded in promoters, gene transcription is also dependent upon changes in the **chromatin structure**. The chromatin is organized in a succession of basic building units constituted of 8 histones among H_2A, H_2B, H3 and H4 and nearly 200 bp of DNA (Kornberg, 1974). Various **post-translational modifications** of the N-terminal tail of histones allow changes in chromatin structure. These modifications include **acetylation**, **phosphorylation**, **methylation**, **ubiquitylation** or **ADP-ribosylation** (Strahl & Allis, 2000). Many studies demonstrated that these modifications **authorize** or reversely **prevent** gene transcription mostly by modulating the **compaction level** of chromatin and by facilitating the recruitment of transcriptional factors and coactivators (Workman & Kingston, 1998; Cheung et al., 2000a; Strahl & Allis, 2000). Recent studies revealed that, like any other gene, clock gene transcription is modulated by chromatin structure modifications. Acetylation, phosphorylation and methylation of histones in clock genes promoter have been identified (Figure 15).

In 2003, a group showed a circadian rhythm in **H3 acetylation** within the promoters of mouse *Per1*/*2* in liver in phase with the rhythm of *Per1*/*2* mRNA (Etchegaray et al., 2003). Similar rhythms were found in the vascular tissues (Curtis et al., 2004) and fibroblasts (Naruse et al., 2004). Besides, the induction of *Per1*/*2* gene transcription by a **light pulse** in early subjective night (CT16) is associated with an **increased acetylation of H3 and H4** in *Per1*/*2* promoters (Naruse et al., 2004). Together, these data thus suggest that acetylation of histones may participate in the induction of *Per1*/*2* genes transcription by the CLOCK-BMAL1 heterodimer. **CBP** and **p300 coactivators**, which

have a **histone acetyltransferase** (HAT) activity, rhythmically interact with CLOCK and BMAL1 proteins. They associate the most at time of maximal *Per1*/*2* transcription (Takahata et al., 2000; Etchegaray et al., 2003; Curtis et al., 2004) and mediate H3 and H4 acetylation. Additionally, **CLOCK** itself has been recently identified as a HAT (Doi et al., 2006). Upon binding with BMAL1, its HAT activity is potentiated and it mediates the acetylation of H3 and H4 on *Per1* promoters that lead to *Per1* rhythmic transcription (Doi et al., 2006).

Consistently, gene **transcription repression** is associated with **deacetylation** of histones. First, *in vitro* and *in vivo*, **CRY1**-mediated repression of *Per1*/*2* transcription is associated with the rhythmic recruitment of the co-regulator **mSin3** and the **histone deacetylases** (HDAC) **1/2** (Naruse et al., 2004). Likewise, **CRY2** disrupts the association of the HAT p300 with CLOCK (Curtis et al., 2004). It thus participates in the deacetylation of histones in *Per1*/*2* promoters that eventually leads to transcriptional repression (Curtis et al., 2004). Secondly, the rhythmic repression of *Bmal1* expression through REV-ERBα binding on RORE of the *Bmal1* promoter has recently been associated with the recruitment of a **HDAC3** (Yin & Lazar, 2005). In mouse liver cells, HDAC3 decreases H3 and H4 acetylation, which leads to *Bmal1* repression (Yin & Lazar, 2005).

Methylation of histones also follows circadian oscillations, albeit in antiphase with the rhythm of acetylation This has been demonstrated in mouse on the promoter of the clock-controlled gene *D-element binding protein* (*Dbp*) (Ripperger & Schibler, 2006) and on the *Per1* promoter (Etchegaray et al., 2006). **Histone methyltransferase WDR5** interacts with *Per1*/*2* promoters and decreases CLOCK-BMAL1-induced transcription of *Per1*/*2* through methylation of histones (Brown et al., 2005). Likewise, another histone methyltransferase, **EZH2**, rhythmically forms a complex *in vivo* with the CLOCK-BMAL1 heterodimers and CRY1 (peak at CT18) to enhance CRY1-mediated repression of *Per1*/*2* transcription (Etchegaray et al., 2006).

The acetylation of H3 in *Per1*/*2* promoters in the SCN after a light pulse (Naruse et al., 2004) is followed by the **phosphorylation** of the same H3 (Cheung et al., 2000b; Crosio et al., 2000). Strikingly, inhibiting the light signaling at early (CT15) or late (CT21) subjective night, *via* a systemic treatment with the $GABA_B$ receptor agonist baclofen, prevents the light-induced H3 phosphorylation in parallel to the *c-fos* and *Per1*

transcription (Crosio et al., 2000). This suggests the involvement of H3 phosphorylation in the transcriptional response to light in the SCN (*section A2.2.3*).

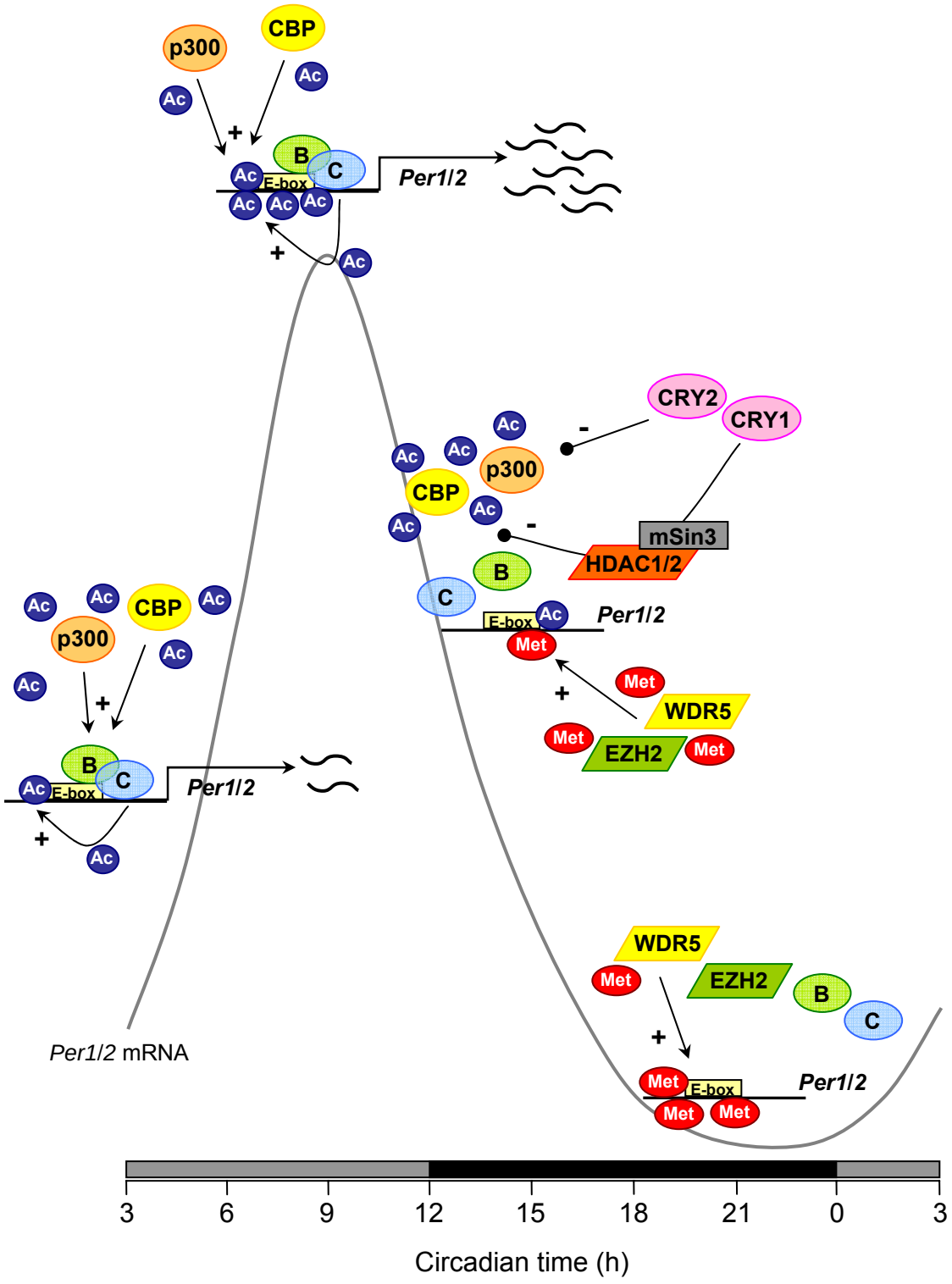

Figure 15. Circadian chromatin modifications participate to the regulation of *Per1/2* genes transcription.

The gray solid line represents the circadian rhythm in *Per1/2* mRNA levels in mouse liver. The gray and dark boxes of the time scale represent the subjective day and subjective night respectively. See text for explanations. Ac, acetyl; B, BMAL1; C, CLOCK; Met; methyl. "+" and "-" represent activating and inhibitory events respectively.

2.2.2.2. Post-transcriptional regulation

As we have seen, the oscillations of clock genes mRNAs levels are mostly considered to be under transcriptional regulation. Nevertheless, rhythmic synthesis also requires the **half-lives** of neo-synthesized mRNAs to be short since long half-lives would lead to an accumulation of mRNA and as a consequence to a dampening of the amplitude of the oscillations. Multiple mechanisms are known to **post-transcriptionally** regulate mRNAs. These processes include **capping**, **splicing**, and **polyadenylation** of the 3' end of the mRNA. Few mechanisms of post-transcriptional regulation are currently known in the circadian system.

The **3'-UTR** of mRNAs play a crucial role in determining stability, localization and translational efficiency of its transcripts (Grzybowska et al., 2001). It has been recently demonstrated that a short sequence of 196nt localized within the 3'-UTR region of the mouse *Per1* promoter contributes to the post-transcriptional repression of its transcripts (Kojima et al., 2003). Although the mechanism remains unknown, Kojima and colleagues found that the AU-rich element – usually implicated in mRNA degradation – localized within this 196nt segment of *Per1* is not involved.

The regulation of the **poly(A) tail** of mRNA is critical in regulating mRNA stability (Wilusz et al., 2001). In mammals, the decapping of the 5' end of the mRNA that initiates mRNA degradation by exonucleases is triggered by the shortening of the poly(A) tail (Couttet et al., 1997). The clock-controlled gene ***AVP*** is the sole clock-related gene identified to date which exhibits a diurnal rhythm in the length of the poly(A) tail of its mRNA (Robinson et al., 1988). Strikingly, this rhythmic oscillation peaks at day and reaches a nadir at night, thus paralleling the rhythmic variation of AVP within the cerebrospinal fluid (Schwartz & Reppert, 1985; Gillette & Reppert, 1987). Therefore, the **rhythmic deadenylation** could participate to the definition of the rhythmic pattern of AVP release. A single candidate for regulating the length of the poly(A) tail of clock gene mRNA is described to date. **NOCTURNIN** is a poly(A)-specific 3' exonuclease first identified in *Xenopus laevis* (Green & Besharse, 1996) and later cloned in mammals (Dupressoir et al., 1999). In both *Xenopus* and mammals, *Nocturnin* expression is rhythmic and found in cell-types and tissues known to contain a circadian oscillator. Indeed, *Nocturnin* mRNA and protein levels oscillate in the cones

and rods of the photoreceptor layer of the *Xenopus* retina (Baggs & Green, 2003). Likewise, mouse *Nocturnin* mRNA levels are rhythmic in the SCN as well as several oscillators including liver, heart and kidney (*section A2.3*) under both LD and DD conditions (Wang et al., 2001). Besides, circadian expression of *Nocturnin* is dampened but not abolished in *Clock* mutant mice (Vitaterna et al., 1994) whilst continuously high *Nocturnin* levels have been reported in *Cry1/2* double KO mice (van der Horst et al., 1999). This suggests a partial control of *Nocturnin* rhythmic expression by the heterodimer CLOCK-BMAL1 (Oishi et al., 2003).

2.2.2.3. Post-translational regulation

The initial demonstration that **post-translational regulation** of clock genes plays a critical role in regulating the pace of the circadian clock came with the identification of the *tau* mutant hamsters (Ralph & Menaker, 1988). As suggested previously, the mutation, which leads to the characteristic 20 h locomotor activity period of homozygous mutants, is a point mutation located within the CKIε gene (Lowrey et al., 2000). This mutation entails a change in PER phosphorylation level that accelerates its nuclear clearance and the overall cycle (Lowrey et al., 2000; Takano et al., 2000; Miyazaki et al., 2004; Vanselow et al., 2006).

a. *Phosphorylation*

The identification of rhythms in the phosphorylation levels of several clock proteins (PER1, PER2, CLOCK, BMAL1) in mouse liver prompted scientists to elucidate the mechanisms and roles of these phosphorylations (Lee et al., 2001).

- CKIϵ and CKIδ

The involvement of **CKIε** and **CKIδ** in the post-translational regulation of clock protein has been the subject of a previous section (*section A2.2.1.3*).

- MAPK

As we have seen in the first part of this manuscript, **MAPK** are involved in a wide range of cellular events. Recent studies have suggested the involvement of these protein kinases in the modulation of the molecular clockwork (Figure 16). The first evidence came from the demonstration that phosphorylated **ERK** are capable **of binding and phosphorylating BMAL1** *in vitro* on 3 phosphoacceptor sites (Ser-527, Thr-534 and Ser-599) (Sanada et al., 2002). This BMAL1 phosphorylation leads to a substantial **decrease in the CLOCK-BMAL1-mediated transcription** in HEK-293 cells. In the molecular clockwork, PER and CRY protein levels decrease from mid-subjective night to late subjective night, which would release the inhibition of the BMAL1-CLOCK-mediated transcription. However, in mice, the BMAL1-CLOCK-induced transcription is suppressed until late subjective night when PER and CRY protein levels are at their nadir (Reppert & Weaver, 2001). ERK activation oscillates over 24 h in various structures of vertebrates with a zenith during the mid-subjective night and a nadir during the subjective day in peripheral structures [bullfrog retina (Harada et al., 2000) and chick pineal gland (Sanada et al., 2000)] and in the central part of the mouse SCN (Nakaya et al., 2003). Sanada and colleagues (2002) thus proposed that the phosphorylated **ERK-mediated inhibition of BMAL1 that occurs at night keeps the BMAL1-CLOCK-mediated transcription suppressed until late during the night**. Suppression of BMAL1-CLOCK-mediated transcription by ERK may contribute to the establishment of a time-lag participating in the definition of the 24-h period.

More recently, **CRY1/2** proteins were also identified as a potential substrate of ERK in mice (Sanada et al., 2004). Upon phosphorylation of **CRY1** on Ser-247 and **CRY2** on Ser-265, the CRY1/2 activity in inhibiting the CLOCK-BMAL1-induced transcription is partially decreased (Sanada et al., 2004). While CRY1/2 proteins levels decline at night, **ERK phosphorylation of CRY1/2 proteins could permit a progressive release of the inhibition on the transcriptional heterodimer to permit the start of the next cycle**.

ERK may thus contribute to the regulation of the timing of the CLOCK-BMAL1-induced transcription from mid- to late subjective night. Phosphorylation of CRY1/2 proteins promotes gene transcription while phosphorylation of BMAL1 suppresses CLOCK-BMAL1-mediated transcription to create a time-lag.

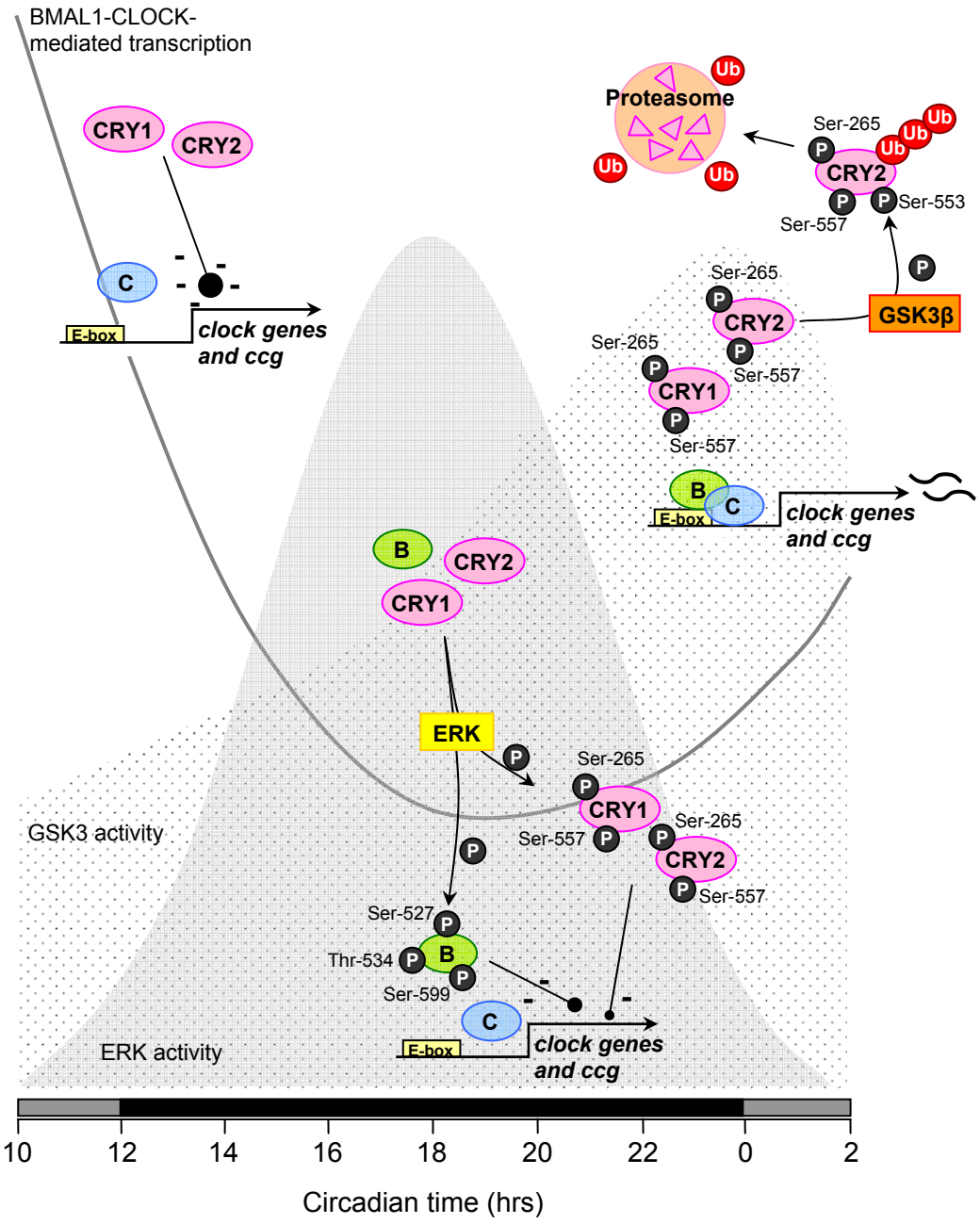

Figure 16. Model of ERK and GSK3 regulation of the BMAL1-CLOCK-induced transcription in the SCN.

At the beginning of the subjective night, CRYs strongly inhibit the BMAL1-CLOCK-induced transcription. Later in the night, ERK phosphorylates neosynthesized BMAL1 and declining levels of CRYs on multiple phosphoacceptors sites. Phosphorylated BMAL1 prevents the BMAL1-CLOCK-mediated transcription while phosphorylation of CRYs gradually releases the CRYs inhibition. At the end of the night and following CRY2 phosphorylation on Ser-557, GSK3 phosphorylates CRY2 on Ser-553 which induces CRY2 ubiquitylation and degradation by the proteasome. BMAL1-CLOCK-induced transcription then initiates a new cycle. The gray line represents the BMAL1-CLOCK induced transcription. The shaded and stippled areas represent ERK and GSK3 rhythmic activities respectively. "-" represent inhibitory steps. B, BMAL1; C, CLOCK; ccg, clock-controlled genes; P, phosphate; Ub, ubiquitin.

- GSK3α/β

GSK3 were initially identified as protein kinases that phosphorylate glycogen synthase, the rate-limiting enzyme of glycogen deposition. Two isoforms coded from two separate genes have been identified, **GSK3α** and **GSK3β** that share 85% homology (Woodgett, 1990). GSK3α/β are expressed in many tissues and involved in multiple cellular processes ranging from cell proliferation and differentiation to regulation of the microtubule dynamics (Woodgett, 1990; Frame & Cohen, 2001). Contrary to many other protein kinases, GSK3α/β are **inactivated upon phosphorylation** by various signaling pathways including the ERK-MAPKAPK1 (also named RSK2) pathway (Sutherland et al., 1993; Welsh & Proud, 1993; Sutherland & Cohen, 1994; Eldar-Finkelman et al., 1995), whilst they are **activated through dephosphorylation** by PP2A (Sutherland et al., 1993; Sutherland & Cohen, 1994).

The first suggestion of a role of the GSK3 in the circadian system came from a study by Iwahana and colleagues (2004). Phosphorylated levels of GSK3α (p-GSK3α) are found rhythmic in the SCN of mice housed in DD, rising to a maximum at the subjective day/subjective night transition and declining to a minimum at the subjective night/subjective day transition (Iwahana et al., 2004). The treatment of mice with **lithium**, which directly **inhibits GSK3 activity** (Klein & Melton, 1996; Stambolic et al., 1996), decreases the active levels of GSK3 and increases the levels of inactive p-GSK3 in the SCN (Iwahana et al., 2004). This effect on GSK activity is correlated with a **lengthening of the period of locomotor activity** of mice kept in DD (Iwahana et al., 2004), which supports the effect of lithium on the period of the firing rate of individual mouse SCN neurons (Abe et al., 2000). It has thus been suggested that GSK3 might affect the molecular clockwork through a change in the phosphorylation level of its substrate(s).

A recent study, designed to identify such a substrate, revealed that **CRY2** might be the target of GSK3 (Harada et al., 2005) (Figure 16). In addition to its phosphorylation by ERK1/2 on Ser-265 (Sanada et al., 2004), CRY2 is rhythmically phosphorylated at Ser-557 in the liver of mice kept in DD, reaching a zenith during the late subjective night (CT22) and a nadir during the late subjective day (CT10). The kinase implicated has not been identified yet but ERK1/2 are likely candidates since they are capable of

phosphorylating Ser-557 *in vitro* (Sanada et al., 2004). Following phosphorylation of CRY2 on Ser-557, GSK3β has been shown to specifically phosphorylate the closely-located Ser-553 residue of CRY2 to promote its **degradation** by the 26S proteasome (Harada et al., 2005). In the molecular clockwork, **it is thus likely that GSK3β contributes to the clearance of the CRY2 proteins at the end of the subjective night** at a time when GSK3β activity is the highest. Interestingly, ERK1/2 activation levels in the mouse liver overlap with the phosphorylation levels of GSK3β, which supports a role for ERK1/2 in the control of the GSK3-mediated CRY2 destabilization (Harada et al., 2005).

Another study identified **PER2** as an additional potential substrate of GSK3β in the mouse liver (Iitaka et al., 2005). Indeed, **GSK3β interacts with and phospohrylates PER2** *in vitro* and *in vivo*, which promotes PER2 **nuclearization** (Iitaka et al., 2005). As GSK3β activity peaks when PER2 protein levels are high in the liver (CT22) (Harada et al., 2005; Iitaka et al., 2005), it is likely that GSK3β activity promotes nuclear entry of the PERs-CRYs complexes.

b. *Degradation*

• Role of the proteasome

The rhythmic patterns of clock proteins such as PERs and CRYs implicate not only the rhythmic translation of the transcripts but also their **tightly timed degradation**. A strong evidence about the critical role of protein degradation in maintaining normal oscillations comes from a recent study which showed that inhibition of the 26S proteasome lengthens the period of circadian oscillations in rat-1 fibroblasts (Eide et al., 2005). As already mentioned previously, CRY2 degradation by the 26S proteasome is mediated through phosphorylation by GSK3β (*section A2.2.2.3*) (Harada et al., 2005) (Figure 16). Moreover, CKIε/δ promote the phosphorylation and degradation of PER1/2 proteins (*section A2.2.1.3*) (Keesler et al., 2000; Camacho et al., 2001; Akashi et al., 2002; Miyazaki et al., 2004; Gallego et al., 2006). In an attempt to decipher the mechanisms implicated in this latter CKIε/δ-mediated degradation, a recent study demonstrated that CKIε-mediated phosphorylation of PER2 promotes the binding of the E3 ubiquitin ligase β-Transducin repeat containing protein (β-TrCP). Consequently, β-TrCP initiates the

ubiquitylation of PER2 that eventually leads to its degradation by the 26S proteasome (Eide et al., 2005).

- SUMOylation

SUMOylation is a reversible post-translational modification recently discovered and controlled by an enzymatic pathway analogous to the ubiquitin pathway. It consists of a covalent linking of **small ubiquitin-related modifier protein** (SUMO) on a consensus sequence ΨKxE/D where Ψ is a hydrophobic residue and x may be any amino acid. SUMOylation has been shown to participate to the regulation of several mechanisms including post-transcriptional regulation and modulation of **protein stability**. A recent study showed that **BMAL1** is SUMOylated *in vitro* and *in vivo* on the highly conserved Lys-259 (Cardone et al., 2005). This specific residue is embedded in the ΨKxE/D consensus sequence located between the two PAS domains involved in the interaction with CLOCK. SUMOylation of BMAL1 is CLOCK-dependent and exhibits a daily oscillation in parallel to BMAL1 rhythm in mouse liver extracts with a peak at ZT9 and a trough at ZT21 (Cardone et al., 2005). The relevance of the SUMOylation of BMAL1 in the molecular clockwork is confirmed in mouse fibroblasts transfected with a mutated BMAL1 on Lys-259. In those cells, BMAL1 cannot be SUMOylated and no rhythmicity in BMAL1 levels are found in synchronized fibroblasts (Cardone et al., 2005). Although the mechanisms involved remain unknown, it is likely that SUMOylation of BMAL1 leads to its degradation since an inhibition of the SUMO proteases (*N*-ethylmaleimide) stabilizes SUMOylated BMAL1 (Cardone et al., 2005).

2.2.3. CELLULAR AND MOLECULAR REGULATION OF THE PHOTIC ENTRAINMENT OF THE SCN

The SCN generate an endogenous rhythmicity with a period close to 24 h that needs to be in phase with the environment. Two types of external cues are able to accurately synchronize daily oscillations: **photic factors** and **non-photic factors**. Non-photic synchronizing cues include **induced physical activity**, **restricted feeding** or treatment with **chronobiotic** drugs (Challet & Pevet, 2003). In the following sections, I will only describe the cellular and molecular mechanisms of the photic entrainment.

2.2.3.1. The light-triggered signaling cascade

As mentioned in a previous section, the SCN receives the light information mostly *via* the **RHT** constituted of the RGC of the retina which connect to the core SCN (*section A2.1.4.1*). **IGL**, another target of the RGC, may participate as well in the photoentrainment by modulating the signal conveyed by the RHT to the SCN, but this implication will not be described here. The **melanopsin photopigment** is present in a small subpopulation of RGC that are sensitive to light (Gooley et al., 2001) and project to the SCN (Provencio et al., 1998). **Melanopsin participates in the entrainment** processes of mice since knockout mice for melanopsin show a reduced magnitude of photic responses (Ruby et al., 2002). However, these mutant mice are still entrained to the LD cycle, which suggests that the visual photoreceptors rods and cones might be involved as well in the circadian photoentrainment (Panda et al., 2002a; Ruby et al., 2002). Triple knockout mice for melanopsin, rods and cones lose their ability to entrain to the LD cycle, which supports **a role for each of the three photopigments in the photoentrainment** (Hattar et al., 2003). Following light stimulation at night, **GLU** and **PACAP** are released from the RHT terminals in the ventrolateral part of the SCN. Although GLU is able to induce light-like phase-shifts of the SCN neurons firing rate *in vitro* when administered at both CT14 and CT19, PACAP is mainly associated to light-induced phase-shifts in the late night (Kawaguchi et al., 2003).

Upon release, GLU recruits **NMDA receptors** (Ding et al., 1994). This recruitment appears essential since the NMDA receptor antagonist 2-amino-5-phosphonovaleric (APV) abolishes the GLU-induced phase-shifts (Ding et al., 1994). Conversely, microinjections of NMDA in the third ventricle at CT13.5 and CT19 induce phase-delays and phase-advances respectively in the locomotor activity of hamsters (Mintz & Albers, 1997). Following NMDA receptor recruitment, calcium is free to enter cells. The subsequent calcium gradient most likely activates a **calcium/calmodulin kinase**, which phosphorylates the **NO synthase** (Agostino et al., 2004) (Figure 17). **NO** increases are known to induce phase-delays and phase-advances at early (CT14) and late (CT20) subjective night respectively, *in vitro* and *in vivo* (Ding et al., 1994).

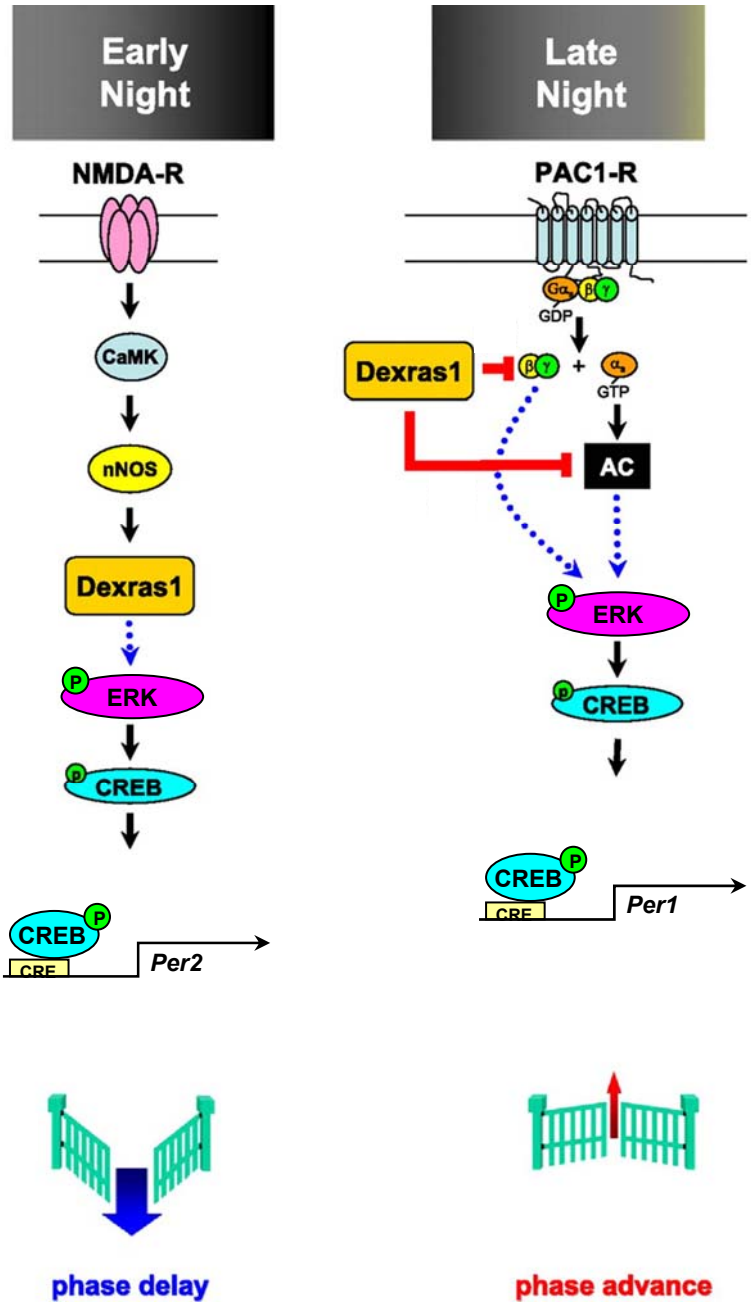

Figure 17. Simplified model of the signaling mechanisms of photic entrainment.

Early at night, Glutamate release following light exposure activates *Per2* clock gene expression through a mechanism that involves the activation of NMDA receptors (NMDA-R), calcium/calmodulin kinases (CaMK), nitric oxide synthases (nNOS), Dexras1, extracellular-signal regulated kinases (ERK) and cAMP response element (CRE) binding proteins (CREB). Alternatively, PACAP, released late at night after light exposure, binds to PAC1 receptors (PAC1-R), which activates *Per1* expression through ERK and CREB activations. Note that Dexras1 limits the phase-advance by inhibiting $G_S\alpha$ and $G\beta\gamma$ subunits. See text for full explanations. Dotted arrows indicate the likely involvement of undetermined intermediates. P, phosphate.
(Adapted from Cheng et al., 2006)

The mechanisms elicited by the increase in NO levels seem to differ according to the time at which the light pulse is given. Late at night (CT19), the inhibition of the **cGMP-related protein kinase** (**PKG**) prevents the phase-advancing effect of light and GLU *in vivo* (Weber et al., 1995; Mathur et al., 1996) and *in vitro* respectively (Ding et al., 1998). It thus appears that the elevation in NO levels subsequent to the light pulse stimulates guanylyl cyclases (GC), which increase the content of cGMP that activates PKG. In contrast, early at night, PKG inhibition does not prevent the GLU-induced phase-delay, and the NO-GC-cGMP-PKG pathway is thus not implicated (Weber et al., 1995; Mathur et al., 1996; Ding et al., 1998). Instead, NO activates the **Ras-like G protein Dexras1** (Fang et al., 2000), which participates to the photic induced phase-delay by mediating the ERK activation (see below) (Cheng et al., 2004; Cheng et al., 2006). Dexras1-deficient mice notably fail to exhibit large phase-delays upon light exposure early at night (Cheng et al., 2004). **Ryanodine receptors** (**RyR**) – which are integral membrane proteins associated with cellular organelles that sequester calcium, such as endoplasmic reticulum (McPherson et al., 1991) – are also likely to be involved. Indeed, inhibition of RyR prevents GLU-induced phase-delays early during the subjective night (CT14) *in vitro* and *in vivo* while direct activation of RyR by caffeine at the same time (CT14) induces GLU-like phase-delays (Ding et al., 1998).

In 1998, Obrietan and colleagues demonstrated that light pulses given at either early (CT15) or late (CT22.5) subjective night promotes **ERK activation** in the SCN of mice (Obrietan et al., 1998; Butcher et al., 2002; Dziema et al., 2003). Similarly, GLU is able to enhance the levels of phosphorylated ERK (p-ERK) in the mouse SCN (Obrietan et al., 1998). Inhibition of ERK activation prevents the light-induced phase-shifts *in vivo*, which suggests that **ERK couple light to clock entrainment** (Butcher et al., 2002). In addition to an increase in ERK activation, light and GLU stimuli lead to an elevation in **CRE-mediated transcription** within the SCN through CREB phosphorylation at Ser-133 and Ser-142 (Ginty et al., 1993; Obrietan et al., 1998; Obrietan et al., 1999; Gau et al., 2002; Tischkau et al., 2003). A recent study showed that the light-, GLU- and PACAP-induced **MSK-1** phosphorylation in the SCN at both early (CT15) and late (CT22) subjective night is prevented when ERK activation is inhibited (Butcher et al., 2005). It thus indicates that MSK-1, known regulators of CREB phosphorylation, couple light stimuli to CREB phosphorylation, and may thus couple light stimuli to clock entrainment.

Late at night, the photic-induced release of PACAP recruits the **PAC1 receptors** since their genetic ablation in mice strongly attenuate the light-induce phase-advances (Kawaguchi et al., 2003). Upon activation, PAC1 receptors, which belong to superfamily of the GPCR, induce the GTP-dependent dissociation of **$G_S\alpha$** and **$G\beta\gamma$** subunits that independently activate downstream effectors, including **ERK** and **adenylyl cyclases** (AC**)**. Importantly, Cheng et al. recently demonstrated that **Dexras1**, which potentiates light-induced phase-delays early at night, also participates to the mediation of PACAP-mediated phase-advances late at night (Cheng et al., 2006) (Figure 17). In contrast with its role at the beginning of the night, they found that **Dexras1 reduces the magnitude of the PACAP-induced phase-advances** by inhibiting the activation of ERK by $G_S\alpha$ and $G\beta\gamma$ subunits (Cheng et al., 2006).

2.2.3.2. Photic-induced changes in gene expression

Consistent with an increase in H3 acetylation and phosphorylation (Crosio et al., 2000), and CRE-mediated transcription found after a light pulse at night, several genes are overexpressed in response to light. First, several **immediate early genes** (IEG) quickly respond to a **light stimulation** as assessed in the SCN of mice. This has been notably well described for ***c-fos*** whose mRNA and protein levels are increased in the SCN by light (Aronin et al., 1990; Rusak et al., 1990) through the binding of **p-CREB** on the **CRE** cassette contained in the promoter of *c-fos* (Ginty et al., 1993). The promptness in the light-induced expression of *c-fos* associated with the demonstration that oligodeoxynuclotides of *c-fos* prevents light-induced phase-delays at CT15 (Wollnik et al., 1995) had suggested that IEG might participate to the photic entrainment. However, null mutant mice for *c-fos* are still entrained by the LD cycle (Honrado et al., 1996). They exhibit some capacity to phase-shift and show a PRC to light more or less similar to wild-type mice (Honrado et al., 1996).

Similar to IEG, ***Per1*** (Shigeyoshi et al., 1997), ***Per2*** (Shearman et al., 1997) and ***Dec1*** (Honma et al., 2002) are induced by a light pulse only at subjective night, at a time when behavioral phase-shifting can occur. *Per1* gene expression have been correlated to behavioral phase-shift since its inhibition by injection of oligodeoxynucleotide reduces the light- and GLU-induced phase-shifts at early (CT16) and late subjective night (CT18-

20), *in vivo* and *in vitro* (Akiyama et al., 1999; Tischkau et al., 2003). This transient *Per1* induction that follows illumination is most likely dependent upon a CRE-controlled transcription since *Per1* and *Per2* contain CRE domains in their promoters (Travnickova-Bendova et al., 2002) (Figure 17).

Strikingly, ***Per1*** and ***Per2*** are differently regulated by light during the night phase. Several studies showed that following a light pulse early in the subjective night, *Per1* and *Per2* expression are enhanced and both mRNA and protein levels are increased within the SCN (Shigeyoshi et al., 1997; Yan et al., 1999; Field et al., 2000; Yan & Okamura, 2002). On the contrary, only *Per1* mRNA are increased following a light pulse given in the late subjective night (Albrecht et al., 1997; Field et al., 2000; Yan & Silver, 2002). This has led to the hypothesis that ***Per1*** **may be implicated in the mechanism that phase-advances the clock** and ***Per2*** **in the mechanism that phase-delays** it. This hypothesis is supported by the study of ***Per1*** mutant mice and ***Per2*** mutant mice. While ***Per1*** mutant mice fail to advance the clock after a light pulse given late in the subjective night (CT22), ***Per2*** mutant mice are unable to delay the clock after a light pulse administered early in the subjective night (CT14) (Albrecht et al., 2001). However, it should be noted that additional studies challenge the role of ***Per1*** and ***Per2*** in mediating phase-shifts by showing that ***Per1*** mutant mice and ***Per2*** mutant mice are still capable of phase-advances and phase-delays respectively (Cermakian et al., 2001; Bae & Weaver, 2003). Nevertheless, the apparent differential role of ***Per1*** and ***Per2*** is further supported by a differential localization of ***Per1***/***2*** expression within the SCN. Early in the subjective night, ***Per1*** large expression that follows light stimulation is restricted to the core SCN and more specifically the GRP-expressing cells, while ***Per2*** expression occurs in both core and shell, both GRP- and AVP-immunoreactive cells (Dardente et al., 2002; Yan & Silver, 2002). Later during the subjective night, *Per1* expression is increased initially in the core SCN and later in the shell (Yan & Silver, 2002).

Few additional studies focused on other clock genes. Although *Dec1* is induced by light (Honma et al., 2002), its possible role in the light-induced phase-shifts has not been elucidated yet. On the contrary, ***Bmal1***, in addition to *Per1*/*2* genes, could also be implicated in the light entrainment resetting since BMAL1 protein levels are **reduced** by a light pulse exposure at early (CT15) or late (CT21) subjective night (Tamaru et al., 2000). This degradation by light can be mimicked by application of GLU and NMDA to SCN slices (Tamaru et al., 2000). However, this was not confirmed by another group (von Gall et al., 2003) and the participation of *Bmal1* in the light-induced phase-shifting

mechanisms remains uncertain. Besides, ***Cry1/2*** genes are unlikely to participate to photic resetting since their expression is unaffected by a light exposure at the late subjective night (Okamura et al., 1999).

2.3. OSCILLATIONS IN THE PERIPHERY

Soon after the discovery of clock genes and the identification of their roles in the mechanisms of the biological clock, many studies revealed that clock genes expression is not restricted to the SCN in mammals. In addition to the retina, where oscillations have first been described (Tosini & Menaker, 1996; Green & Besharse, 2004), a pioneer study led by Schibler's group demonstrated that **clock genes** are rhythmically expressed in **rat-1 fibroblasts** (Balsalobre et al., 1998) (Figure 18). Additional micro-array analyses revealed that clock genes are actually widely expressed in many mammalian tissues such as **liver** (Akhtar et al., 2002; Panda et al., 2002b; Storch et al., 2002; Ueda et al., 2002), **heart** (Storch et al., 2002) and **adrenal glands** (Oster et al., 2006a) among others. Strikingly, nearly 2% and 10% of total analyzed genes in fibroblasts (Grundschober et al., 2001; Duffield et al., 2002) and liver (Panda et al., 2002b) present oscillatory levels of expression. This has led to the hypothesis that besides the SCN, some **neuronal and non-neuronal tissues may host functional clocks** that would regulate local physiology.

A terminological distinction between the hypothalamic clock and peripheral entities – "clock", "pacemaker", "oscillator" – has been made after the demonstration that oscillations of clock genes in some structures outside the SCN are disrupted in SCN-lesioned animals suggesting some dependence towards the SCN (Sakamoto et al., 1998; Akhtar et al., 2002). However, the use of "clock", "pacemaker" and "oscillator" is not always consistent in the literature. Besides, the **discovery of clock entities similar to the hypothalamic clock in the periphery** has made the distinction of each clock entity more complex. In the following sections, "**clock**" will refer to any tissue or cell that rhythmically expresses clock genes with an ~24-h period, independently from any external cue. Hence, the SCN, defined in the previous sections (*sections A2.1 and A2.2*), contain a clock. An "**oscillator**" is a tissue that also rhythmically expresses clock genes over 24-h. However, as opposed to a clock, these oscillations require external cues. I will refrain from using "pacemaker" since it is often used to describe both clocks and oscillators in the literature.

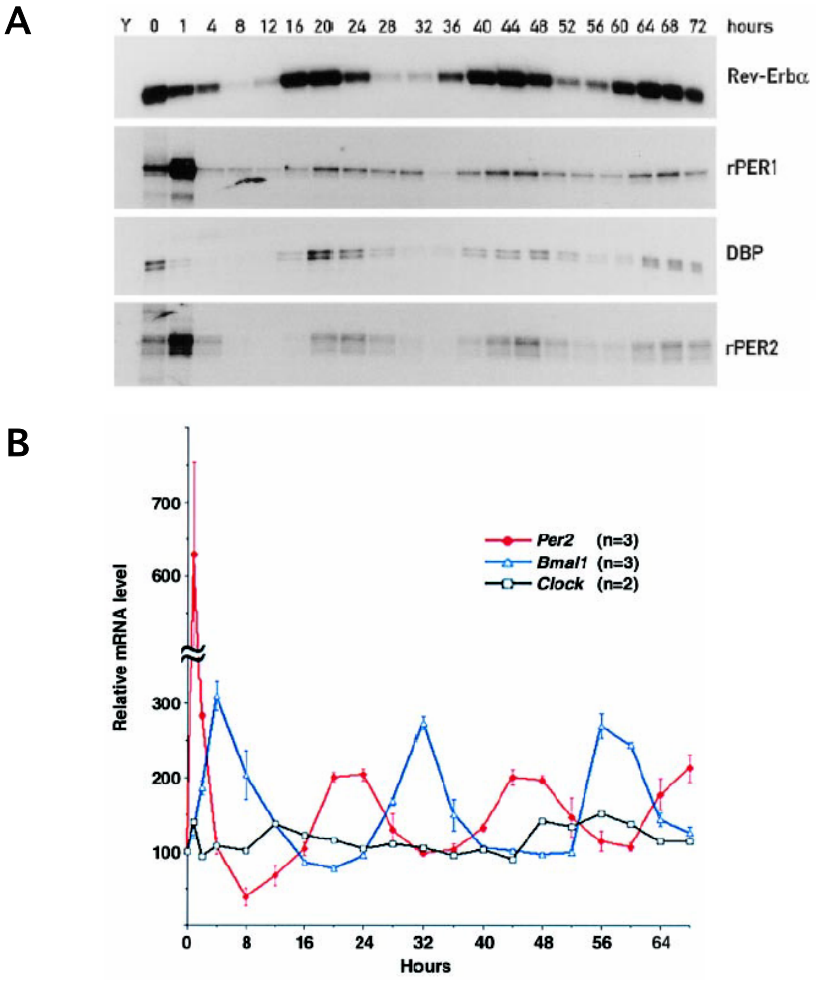

Figure 18. Clock genes oscillations in rat-1 fibroblasts.

A. Rhythmic circadian accumulation of various clock genes (*Rev-erbα*, *Per1* and *Per2*) and a clock-controlled gene (*Dbp*) mRNAs in confluent rat-1 cells serum-shocked for 2hrs. (Adapted from Balsalobre et al., 1998)
B. Temporal expression profiles of clock genes (*Per2*, *Bmal1*, *Clock*) mRNA in rat-1 cells after endothelin-1 treatment. Note that the circadian profile of *Bmal1* expression is in anti-phase with *Per2*, like in the SCN, which suggests that a functional clock resides in fibroblasts. (From Yagita et al., 2001)

Since SCN integrity is essential to oscillators' function, it has been suggested that **SCN communicate with peripheral oscillators to synchronize the oscillations in the periphery**. SCN are believed to use both **neuronal** (Inouye & Kawamura, 1979) and **diffusible** (humoral) **signals** (Silver et al., 1996; Pevet et al., 2006), and recent studies have shown that the regulation of oscillators by the SCN greatly varies. Although the relationship between SCN and periphery is not fully understood yet, I will describe in the

following sections our current knowledge about the cells and tissues that received most attention in the literature.

2.3.1. CLOCKS IN THE PERIPHERY

2.3.1.1. Clocks in cells.

In 1998, Balsalobre and colleagues demonstrated that a 2-h **serum shock** – treatment for 2 h with a high (50%) concentration of serum – induces the **circadian expression** of *Per1*/*2*, *Rev-erbα* and the clock-controlled gene *Dbp* in a culture of **rat-1 fibroblasts** for at least 3 days (Balsalobre et al., 1998). (Figure 18) Later on, the same group showed that **dexamethasone**, a glucocorticoid receptor agonist, also initiates rhythmic circadian transcription of *Per1*/*2*/*3*, *Cry1*, *Rev-erbα* and *Dbp* in the same cells (Balsalobre et al., 2000). Other cell lines, like NIH3T3 fibroblasts (Akashi & Nishida, 2000) and C6 glioma cells (Fujioka et al., 2006), display the same properties. A recent study demonstrated that primary astrocytes, removed and cultured from mice carrying either the *Period1-luciferase* or *Period2*^{Luciferase} reporter constructs, display circadian oscillations in the transcription of those constructs (Prolo et al., 2005). These discoveries suggest that many cell types may contain a clock. Importantly, the expression patterns of clock genes in rat-1 fibroblasts were similar to those of the SCN, with *Bmal1* cycling in anti-phase to the *Per1*/*2*/*3* genes and PER1/2 proteins rhythms following *Per1*/*2* mRNA cycles by 4-8 h (Yagita et al., 2001). Moreover, **temperature compensation**, a typical property of circadian clocks, was demonstrated in rat-1 fibroblasts (Izumo et al., 2003). Altogether, these results suggest that **the circadian machinery is functional**. The physiological relevance of a circadian clock in fibroblasts is still unclear. However, several genes participating in transcription regulation, protein turnover, cell signaling and cell motility oscillate throughout 24 h, which suggests that the clock may regulate the timing of these particular cellular functions (Duffield et al., 2002).

a. *Induction of clock genes oscillations*

A wide variety of signals, including dexamethasone, endothelin-1, forskolin, glucose, serum and prostaglandin E2 (Balsalobre et al., 1998; Akashi & Nishida, 2000; Balsalobre

et al., 2000; Yagita & Okamura, 2000; Yagita et al., 2001; Hirota et al., 2002; Tsuchiya et al., 2005; Fujioka et al., 2006), are able to induce the rhythmic expression of clock genes in isolated cells. Among them, the mechanisms used by **glucose** have been well described.

A serum shock induces a **quick and transient increase** in *Per1*/*2*, *Rev-erbα* and *Dbp* genes expression that eventually leads to the cyclic expression of each gene (Balsalobre et al., 1998). In contrast, glucose treatment leads to **a rapid and transient decrease** in *Per1*/*2* mRNA levels followed by a rhythmic expression of both genes (Hirota et al., 2002). Two candidates for the mediation of the glucose effect on *Per1*/*2* expression are transiently up-regulated by glucose stimulation. First, the **transforming growth factor β-inducible early gene** (TIEG) **1**, which is known to mediate the transforming growth factor β signaling (Cook & Urrutia, 2000), possesses 3 zinc finger DNA binding motifs within its C-terminal tail that allow binding on GC-rich sequences identified as **SP1** motifs. Upon binding, TIEG1 **represses** gene transcription. As human *Per1* (Hida et al., 2000) and *Bmal1* (Yu et al., 2002) promoters both contain 3 well-conserved **SP1** motifs, it is likely that TIEG1 contributes to the glucose-mediated down-regulation of *Per1*/*2* genes (Hirota et al., 2002). Besides TIEG1, the **vitamin D3 up-regulated protein-1** negatively regulates thioredoxin. Thioredoxin activates the helix-loop-helix-PAS transcription factors **hypoxia-inducible factor-1α** (HIF-1α**)** and **hypoxia-inducible factor-1α-like factor** (HLF), and promotes their interaction with the co-activators **CBP/p300** (Ema et al., 1999). These latter intermediates themselves facilitate CLOCK-BMAL1-regulated transcription. By negatively regulating thioredoxin, up-regulated vitamin D_3 up-regulated protein-1 thus **down-regulates** CLOCK-BMAL1-induced transcription of clock genes (Hirota et al., 2002). Since glucose plasma levels oscillate in rodents under the control of the SCN (Yamamoto et al., 1987), glucose may constitute one hand of the SCN in timing peripheral oscillators *in vivo*.

b. *Damping of clock genes oscillations*

Although oscillations of clock gene expression could be initiated in isolated cells, the cycle of expression appeared to **dampen** after a few days (Balsalobre et al., 1998; Nagoshi et al., 2004). Balsalobre and colleagues proposed two hypotheses to explain this damping: peripheral oscillations could either **1)** be **enslaved by the SCN** that would

induce oscillations through a specific undetermined signaling, or **2)** SCN function could be restricted to **coupling and synchronization of pre-existing oscillations** in the periphery (Figure 19).

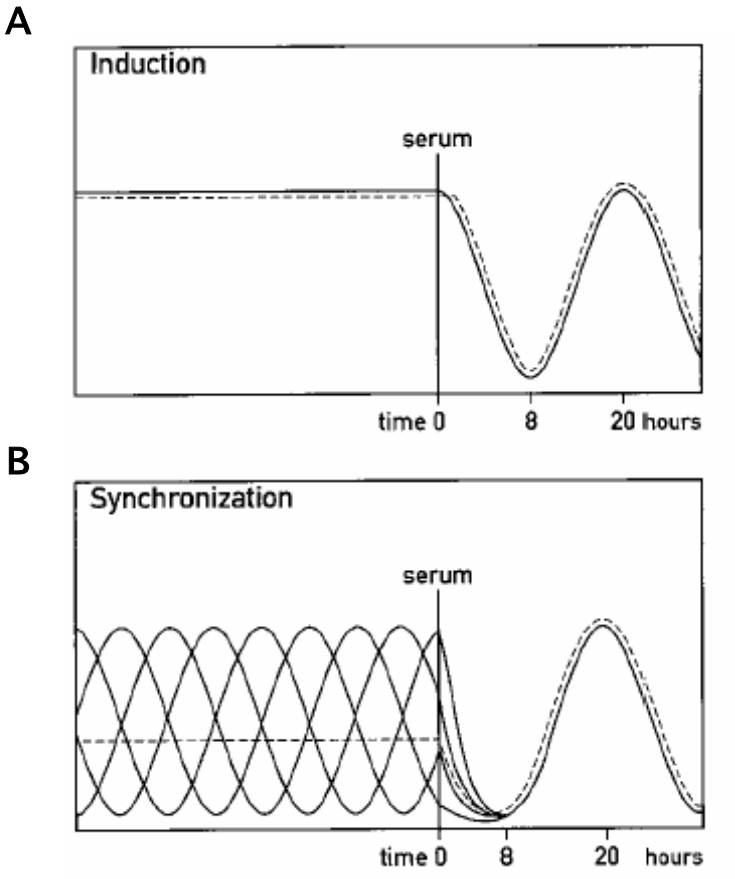

Figure 19. Hypothetical models for serum-induced circadian gene expression.

As exemplified here in serum-shocked rat-1 fibroblasts, circadian gene expression in the periphery could arise either from induction or synchronization mechanism.
A. In the induction model, serum factors induce rhythmicity in otherwise arrhythmic cells.
B. In the synchronization model, serum factors synchronize pre-existing circadian oscillations among desynchronized cells.
The dotted lines represent for each model the expected mRNA accumulation curves determined by biochemical approaches before and after serum induction.
(From Balsalobre et al., 1998)

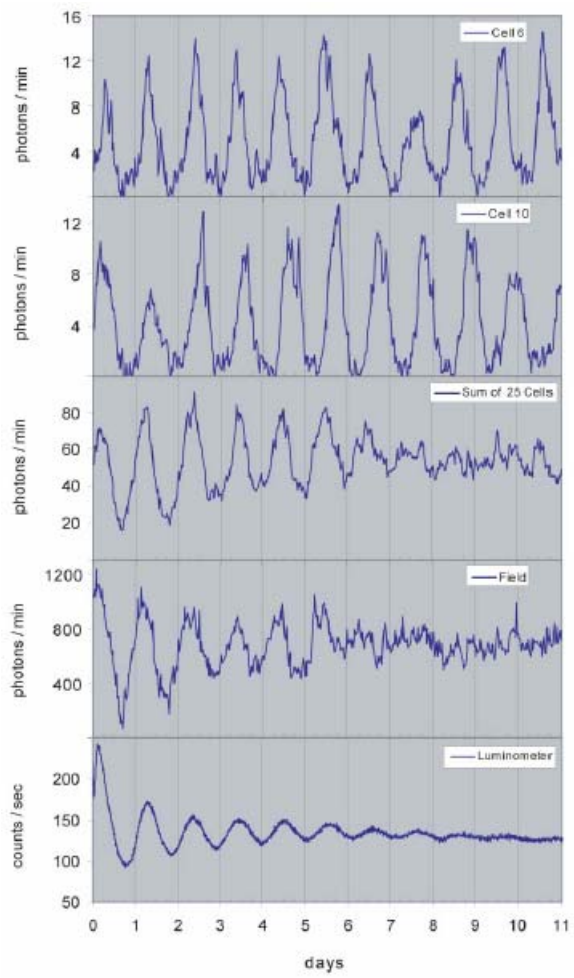

Figure 20. The damping of peripheral oscillations reflects the desynchrony of single-cell clocks.

Plots of bioluminescence records from *Period2*^{Luciferase} knock-in mice primary fibroblasts are represented. Plots are of two representative fibroblasts (6 and 10), the sum of all 25 cells monitored, the entire microscope field and of the same culture dish in the luminometer. Individual cells begin with similar phases but drift completely out of phase by the end, due to differences in period. As cells become desynchronized, they begin to cancel out one another's rhythms, and this is reflected in the rapidly damped oscillations seen in the arithmetic sum of luminescence from all 25 cells monitored, as well as in the luminescence from the entire microscope field, and finally also in the luminometer recording luminescence from the entire dish.
(From Welsh et al., 2004)

The use of rat-1 fibroblasts and NIH3T3 fibroblasts stably transfected with a *Bmal1-luciferase* reporter construct allowed to monitor oscillations in **individual cells** (Nagoshi et al., 2004; Welsh et al., 2004) (Figure 20). Strikingly, **each single cell exhibits oscillations of *Bmal1*-driven transcription with its own period and phase without damping** (Nagoshi et al., 2004; Welsh et al., 2004). In *Bmal1-luciferease* rat-1

fibroblasts, period remarkably ranges from 19.1 to 30.3 h. When synchronized, most of the bioluminescence oscillations originated from the promoter activity peak at roughly the same time, which allows investigators to monitor overt circadian oscillations at the level of the culture dish. However, because of the period variability, **cells gradually lose phase** when an appropriate synchronizer is missing. The drifting of the phases of the single-cell oscillations leads to an **apparent damping** at the level of the dish (Welsh et al., 2004). A mathematical analysis of the damping of the oscillations supports the hypothesis that the damping reflects the dephasing of detuned single-cell oscillators (Nagoshi et al., 2004). Besides, the rhythmicity of dampened *Bmal1-luciferase* oscillations at the level of the dish can be restored by a medium change, which is likely to resynchronize rhythms among cells (Welsh et al., 2004). The study of primary fibroblasts from *Period2*^{Luciferase} knock-in mice (Yoo et al., 2004) shows very similar results (Welsh et al., 2004).

These recent results suggest that **cell lines and primary cells outside the SCN constitute self-sustained clocks** highly similar to SCN neurons (Welsh et al., 1995; Liu et al., 1997a; Herzog et al., 1998). However, **in a network or tissue, they require a synchronizing stimulus** – from the SCN *in vivo* – to be in phase, and the network or tissue is considered as an **oscillator**.

2.3.1.2. Olfactory bulbs

The use of a *Period1-luciferase* reporter construct in rats initially demonstrated that **OB** express a circadian rhythm in gene transcription *in vitro* (Abe et al., 2002). Strikingly, **the oscillations found in the mitral neurons of the OB persist *in vitro*** when OB are removed from SCN-lesioned rats (Granados-Fuentes et al., 2004a). Moreover, OB cultured from rats housed in LL maintain circadian oscillations of *Period1-luciferase* transcription *in vitro*. This is in sharp contrast with the *Period1*-driven oscillations in the SCN and the locomotor activity of the same rats that lose rhythmicity (Granados-Fuentes et al., 2004a). These results suggest that the clock genes expression within the OB oscillate independently from SCN signaling. The definite demonstration of this hypothesis comes from an elegant study by Herzog's group. They developed a method that allows to record *Period1-luciferase* transcription levels in the OB ***in vivo*** and nicely demonstrated that **oscillations persist in SCN-lesioned rats** (Abraham et al., 2005)

(Figure 21). This clearly established that SCN are not necessary to synchronize rhythms among individual mitral cells. It thus appears that OB differ from peripheral oscillators and resembles a true clock. The possibility to **entrain** OB oscillations by temperature cycle *in vitro* as well as the demonstration that the period of *Period1*-driven transcription is **temperature compensated** *in vitro* (Granados-Fuentes et al., 2004b) strongly supports that **OB is an independent clock similar to the hypothalamic clock of the SCN**. Besides, it should be noted that the phase between SCN and OB oscillations is lost in explants removed and cultured from rats housed in LL *in vitro* and in SCN-lesioned rats *in vivo* (Granados-Fuentes et al., 2004a; Abraham et al., 2005). This suggests that an SCN-originated cue determines the phase of the OB oscillations.

Similarly to the clock of the SCN, the OB thus meet all the parameters of a functional circadian clock: the period of the clock genes transcription oscillations approximates **24 h** in the absence of any external cue, these oscillations can be **synchronized** to daily cycles and are **temperature compensated**. A link between the SCN and the OB exists since an OB stimulation enhances the light-induced phase-shifts of the locomotor activity, and c-FOS expression within the SCN (Amir et al., 1999b). Besides, an olfactory bulbectomy shortens the free-running period of locomotor activity and alters the resetting time after a 6-h phase shift in mice (Granados-Fuentes et al., 2006). It should be noted however that a previous study by the same group failed to report any effect of OB on photic entrainment and period regulation of the locomotor activity of rats (Granados-Fuentes et al., 2004b).

The mitral neurons of the OB send olfactory information to other brain centers like the **piriform complex** and the **hypothalamus**. In 1999, Amir et al. reported day/night differences in the odor-induced responses in multiple brain areas associated with olfaction (Amir et al., 1999a). It has thus been suggested that the OB clock might regulate these daily fluctuations in olfactory responsiveness. Recently, Granados-Fuentes and colleagues reported that the circadian oscillations in odor-evoked c-FOS-ir in the OB and piriform complex are maintained in SCN-lesioned mice (Granados-Fuentes et al., 2006). Moreover, removal of both OB induces the loss of c-FOS-ir rhythmicity within the piriform complex (Granados-Fuentes et al., 2006). Altogether, these data support a role for the OB circadian clock in the regulation of the daily response to olfactory stimulations.

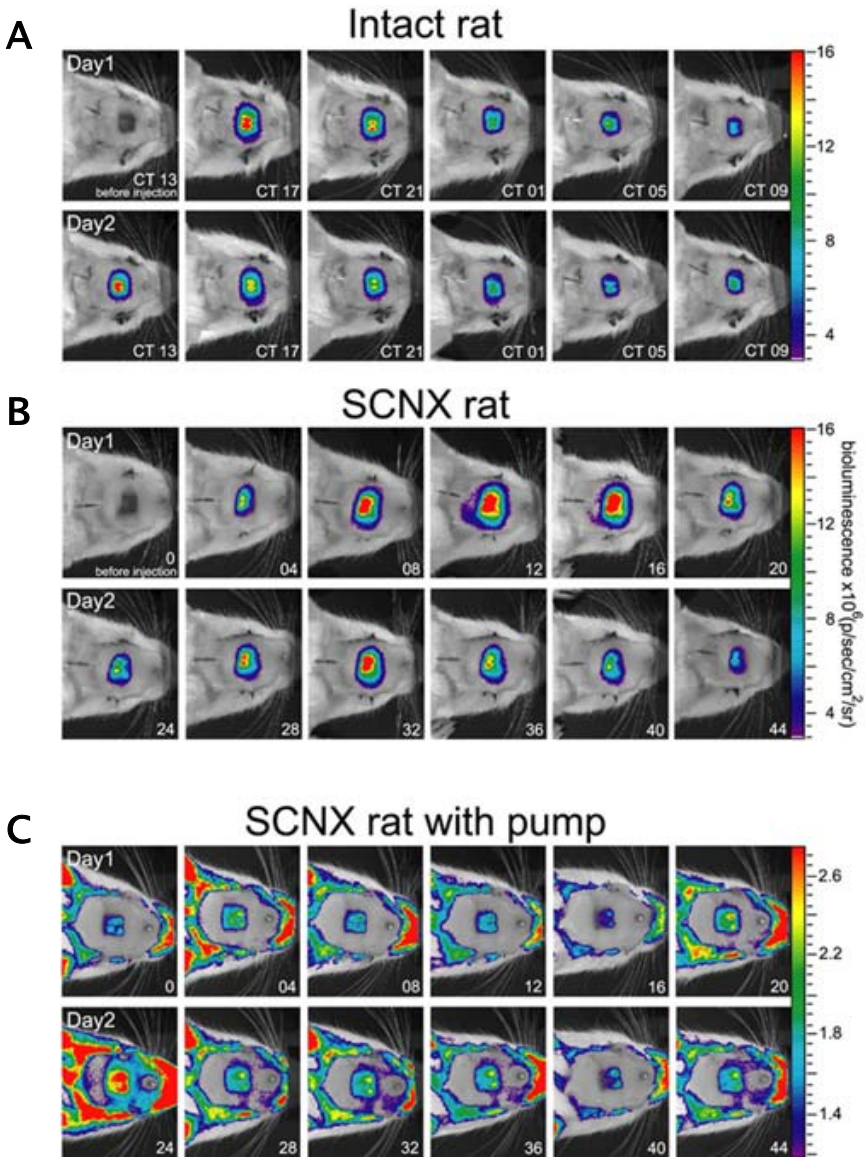

Figure 21. Representative *in vivo Period1-luciferase* bioluminescence images from the olfactory bulbs of three transgenic rats.

Light from the bilateral olfactory bulbs (OB) is imaged for 1 min at 4-h intervals through a glass window in the skull of an intact (**A**) and SCN-lesioned (SCNX) (**B**) rat, 4 h after luciferin was delivered to the OB through a cannula. Light emission peaks daily around CT13–17 in the intact rat and around recording time 8–12 (15:00 h – 18:00 h) in the SCN-lesioned rat. Similarly, a circadian pattern of *Per1*-driven activity is seen in a SCN-lesioned rat carrying an osmotic pump releasing luciferin (30 mg/mL) at a rate of 10µL/h (**C**). The pump was implanted in the intraperitoneal cavity 2 days before the start of imaging. Bioluminescence above background is emitted from the OB and exposed skin. The intact rat was enucleated 2 days before the creation of the OB window, so all times are reported relative to the LD cycle to which the animal was entrained previously. CT0 denotes the daily onset of the previous light phase (subjective dawn), and CT12 refers to subjective dusk. For SCN-lesioned rats, which were enucleated and lesioned 1 month before the start of the imaging, recording started at 7:00 h (time 0). Pseudocolored images were superimposed over a photograph of the anesthetized rats taken immediately before the bioluminescence integration. (From Abraham et al., 2005)

2.3.2. OSCILLATORS IN THE PERIPHERY

Over the years, many oscillators have been identified outside the SCN (Hastings et al., 2003). The nature of these oscillators is of many kinds since **non-neuronal tissues**, **endocrine glands** and **neuronal structures** display clock genes oscillations similar to those found in the SCN. Understanding how time-keeping of those unique peripheral oscillators are controlled by the SCN, and their function in peripheral tissues is one of the current challenges in the field of circadian neurobiology. In the following sections, I will detail our present knowledge on several oscillators that received most attention.

2.3.2.1. Oval nucleus of the bed nucleus of the stria terminalis and central nucleus of the amygdala

Located in the limbic forebrain, the oval nucleus of the stria terminalis (BNSTov) and the central nucleus of the amygdala (CEA) are two anatomically and functionally related structures involved in the control of **emotional and motivational states**. BNSTov and SCN present a synchronous neuronal activity correlated with a daily fluctuation in PER2-immunoreactivity (-ir) that peaks at the day/night or subjective day/subjective night transition in both structures (Amir et al., 2004). Similarly, PER2-ir in the CEA is synchronized with the SCN PER2-ir rhythm with a zenith reached at ZT13 (Lamont et al., 2005). In both BNSTov and CEA, **a lesion of the SCN disrupts the rhythmicity of PER2-ir** and this latter remains constitutive, at intermediary levels. This suggests that the SCN participate in the rhythmic expression of PER2, but not in its basal expression (Amir et al., 2004; Lamont et al., 2005). Importantly, **adrenalectomy**, which abrogates the rhythmic SCN-controlled circadian release of **glucocorticoids** from adrenal glands (Szafarczyk et al., 1983), **interrupts the rhythmic expression of PER2** in both BNSTov and CEA where PER2 levels remain constant and intermediary (Amir et al., 2004; Lamont et al., 2005). The drinking behavior is a rhythmic behavior controlled by the SCN. As a consequence, the daily administration of glucocorticoids in the dinking water provides an artificial daily rhythm that restores PER2-ir rhythms in both structures of adrenolectomized rats (Segall et al., 2006).

Altogether, these results indicate that the **synchronization of rhythms among single cells** in BNSTov and CEA oscillators is likely mediated through the SCN-driven rhythmic release of glucocorticoids from the adrenal gland. The chronic exposure to stressors, which would affect the glucocorticoid rhythm, could directly alter the circadian expression of clock genes in the BNSTov and CEA. Since those two structures have important roles in the control of emotional and motivational states, such persistent disruptions of local circadian functioning could lead to the development of abnormal autonomic, endocrine and behavioral responses to motivational and emotional stimuli.

2.3.2.2. Liver

In 2004, Yoo and colleagues attempted to characterize the role of the SCN in the regulation of the **liver oscillator** by culturing liver explants removed from *Period2*^{Luciferase} knock-in mice that have been previously SCN-lesioned. They demonstrated that **oscillations persist *in vitro* for more that 20 cycles** (Yoo et al., 2004). Since SCN lesions do not abolish the rhythmic expression in liver explants *in vitro*, SCN may not be required to synchronize rhythmicity within liver cells. Additional results indicate that *Bmal1-luciferase* oscillations persist for more than 3 weeks *in vitro* (Nishide et al., 2006). Considering the recent results obtained on individual fibroblasts (Nagoshi et al., 2004; Welsh et al., 2004), it is likely that **coupling among single-cell clocks in liver is maintained in isolated organ culture**. Besides, Yoo et al. showed that the phase of each peripheral tissue including liver is changed in cultures from SCN-lesioned animals as compared to controls. From these experiments, it is thus likely that **the SCN serve to coordinate the phase among peripheral tissues within individuals** (Yoo et al., 2004).

Nevertheless, *in vivo* experiments revealed that SCN lesions abolish the rhythmic clock genes expression in the liver (Terazono et al., 2003; Guo et al., 2005). This strongly suggests that **SCN are necessary to synchronize rhythms among single liver cells *in vivo***. Several attempts to identify the signal used by the SCN to synchronized liver rhythms *in vivo* have not been conclusive yet. The SCN connect to the liver through a **polysynaptic autonomic pathway** that encompasses the PVN, IML and dorsal vagal nucleus (la Fleur et al., 2000; Kalsbeek et al., 2004). It has been initially proposed that the SCN recruit the sympathetic branch of this pathway to **synchronize liver rhythms**

through adrenergic signaling. The elegant work by Terazono et al. (2003) supports this hypothesis. They managed to monitor *in vivo* levels of *Period1*-driven bioluminescence in the liver of *Period1-luciferase* transgenic mice. A stimulation of the sympathetic nerves increases the *Period1*-driven bioluminescence in liver. Moreover, they found that daily injections of adrenaline can restore a rhythm in *Per2* and *Bmal1* expression in SCN-lesioned mice *in vivo* (Terazono et al., 2003).

In contrast, a recent study proposed a different model (Cailotto et al., 2005). In this work the liver sympathectomy did not affect clock gene expression suggesting that the sympathetic branch is not responsible for the rhythmicity observed in the liver. However, in this study, the SCN are not lesioned and are still capable of sending **non-neuronal diffusible signals** to the liver. This hypothesis was further supported by the outstanding work by Guo and colleagues (2005). The experiment paradigm consisted in a parabiotic linkage between an intact mouse and a SCN-lesioned mouse. Although the liver of SCN-lesioned mice lost rhythmicity in *Per1*/*2* and *Bmal1* expression, the parabiotic linkage with an intact mouse restored these rhythms (Guo et al., 2005). Interestingly, the same results were obtained with kidney but not with heart, muscle and spleen. This strongly suggests that **blood-borne cues or behavioral cues** – since the mice are attached together – are likely involved in the liver cells synchronization. Although it cannot be excluded that neuronal inputs and blood-borne/behavioral cues are somewhat redundant, adrenergic stimulation of liver cells is not critical to liver oscillations. Another recent study by Guo et al. showed that liver rhythms of SCN-lesioned *tau* mutant hamsters transplanted with a wild-type SCN are restored but with a phase that resembles the phase of wild-type animals (Guo et al., 2006). The neuronal efferent connections from the SCN graft are likely altered and it is thus likely that the new phase is determined by non-neuronal diffusible signals.

Glucocorticoids, another SCN-controlled signal, regulate the liver oscillator (Balsalobre et al., 2000). In mice, **dexamethasone** injections phase-delay the liver *Per1* mRNA oscillations when performed at early (ZT14) and late night (ZT21) (Figure 22). Conversely, injections of the glucocorticoids agonist given in early morning (ZT1) phase-advance the *Per1* rhythm. Strikingly, the PRC that can be obtained from these data showed that phase-shifts mediated by dexamethasone happen at any time of the day (Balsalobre et al., 2000). This is in strict opposition with the PRC of light in the SCN, which shows that phase-shifts can only occur during night-time.

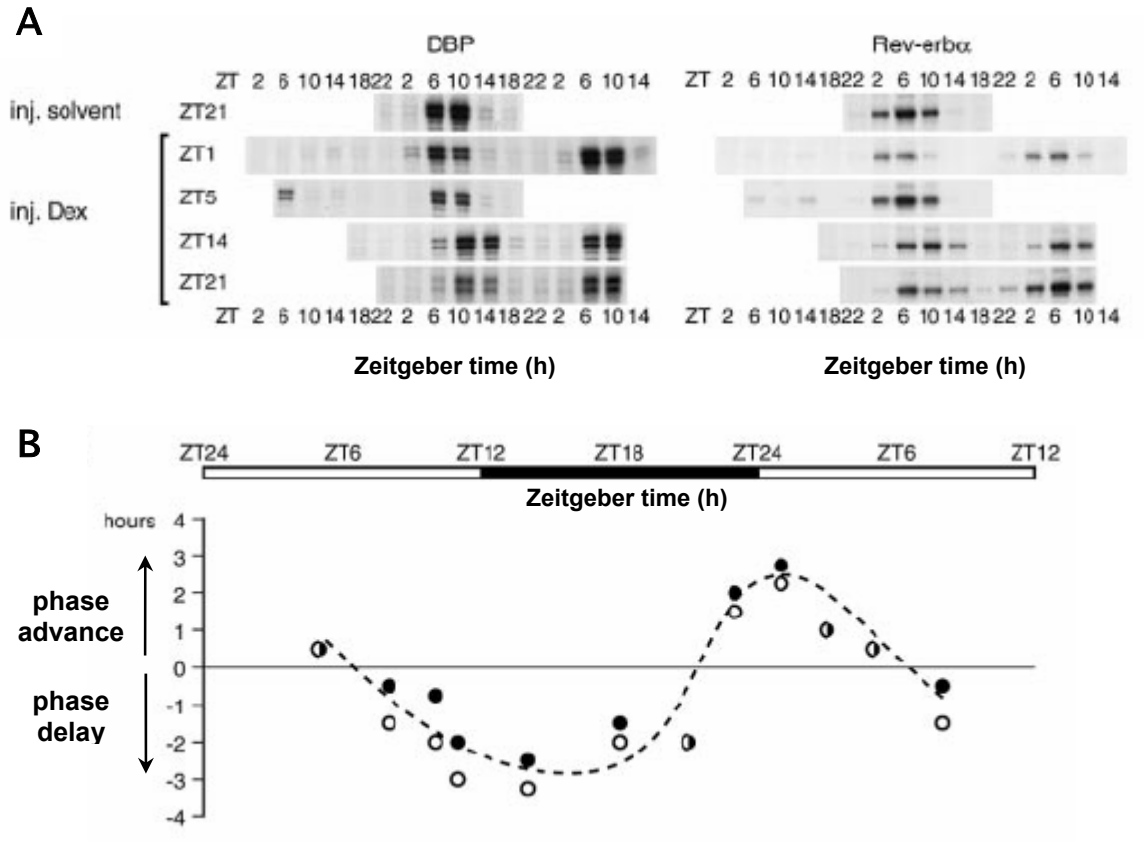

Figure 22. Dexamethasone induces phase shifts in circadian gene expression in the periphery (mouse liver).

A. *Rev-erbα* and *Dbp* mRNA levels are determined by ribonuclease protection experiments following injections of solvent or the glucocorticoids receptor agonist dexamethasone (Dex) at ZT1, ZT5, ZT14 and ZT21.
B. Dexamethasone-induced phase-shifts in the expression of *Dbp* and *Rev-erbα* transcripts were recorded after injections at ZT1, ZT3, ZT5, ZT8, ZT10, ZT11, ZT14, ZT18, ZT21, and ZT23. Open circles, *Dbp* mRNA; filled circles, *Rev-erbα* mRNA. Note that dexamethasone induces phase-shifts at any time of the circadian cycle which is in opposition with photic-induced phases-shifts that occur in the SCN.
(Adapted from Balsalobre et al., 2002)

A critical role of the liver oscillator has been recently elucidated (Matsuo et al., 2003). Although the **cell cycle** of liver cells in a normal mouse is stopped in G_0, a partial hepatectomy will make hepatocytes enter the **mitotic cycle**. Matsuo and colleagues showed that the time necessary for the cells to reach and complete the S phase is about 36 h, and is independent from the time at which partial hepatectomy is performed. On the contrary, the G_2/M transition always occurs at the same time of the day, which suggests that **the G_2/M transition is controlled by the circadian clock**. Matsuo et al. revealed that the gene expression of the kinase **WEE1** oscillates throughout 24 h and *Wee1* is

likely to be a clock-controlled gene. They proposed that G_2/M transition occurs when WEE1 levels are low. At that time, the protein phosphatase CDC25 removes a tyrosine phosphate from the CDC2-cyclin B complex, which becomes active and allows G_2/M transition. Strikingly, hepatocyte proliferation is impaired in *Cry1*/*2*-deficient mice through the disruption of the expression profiles of many genes involved in the cell cycle progression. Remarkably, *Wee1* expression is more elevated than in control mice, which blocks the G_2/M transition. This elegant work thus provides compelling evidence that **the liver oscillator gates the entry of liver cells in mitosis to certain time of the day**.

2.3.2.3. Adrenal glands

In a recent report, Oster and colleagues confirmed the existence of a functional circadian oscillator in the **adrenal gland** (Oster et al., 2006b). They cultured adrenal glands from wild-type and *Per2*/*Cry1* mutant mice (Oster et al., 2002) and stimulated them with adrenocorticotropic hormone at the peak and trough time of the glucocorticoids rhythm in wild-type animals. Although the adrenocorticotropic hormone moderately increases glucocorticoids production at the trough time in both wild-type and mutant adrenals, the increase at the peak time is higher in wild-type adrenals (Oster et al., 2006b). This strongly suggests that **the glucocorticoids response to adrenocorticotropic hormone stimulation is gated by the adrenal oscillator** that defines a time window of higher efficiency. Furthermore, Oster et al. transplanted wild-type adrenals in *Per2*/*Cry1* mutant hosts. Strikingly, they found that the LD cycle is able to evoke a rhythmic corticosterone production, even though the SCN are not functional. This suggests that the light may directly entrains the adrenal oscillator. Besides, in DD, there is no rhythm in glucocorticoids production and the SCN are thus likely needed to entrain the adrenal clock (Oster et al., 2006b). Altogether, these well-designed experiments demonstrate that **the adrenal glands oscillator is entrained by the SCN, but it can also be directly entrained by light**. The light-induced entrainment of the adrenal glands is likely to use the **polysynaptic sympathetic pathway** that connects the SCN to the PVN, the IML and eventually the adrenal glands (Buijs et al., 1999). The requirement for this sympathetic branch has been confirmed by Ishida and colleagues who demonstrated that a light exposure successively induces the *Period1*-driven expression in *Period1-luciferase* mice *in vivo* and the corticosterone release through a mechanism requiring **adrenergic**

signaling. The denervation of adrenal glands notably eliminates the light-induced *Period1-luciferase* expression (Ishida et al., 2005).

It is of particular importance that light may directly entrain the adrenal oscillator *via* the autonomic nervous system and regulate the corticosterone rhythm since this hormone is believed to time other peripheral oscillators to the environmental LD cycle. This is notably the case for BNSTov and CEA as we have described previously (*this section*). A recent study also revealed that about a hundred of liver genes lose rhythmicity when mice are adrenalectomized (Oishi et al., 2005). Some of these genes code for key enzymes of the metabolism like 3-hydroxy-3-methylglutaryl-Coenzyme A, a key enzyme of the cholesterol biosynthetic pathway. This suggests that **glucocorticoids play a critical role in mediating circadian rhythmic expression in peripheral oscillators**.

2.3.2.4. Pars tuberalis

The **PT** located at the anterior lobe of the pituitary is an **endocrine structure** involved in the **seasonal secretion of prolactin** (Morgan & Williams, 1996). Several clock genes, including *Per1*/*2*, *Cry1*, *Bmal1* and *Rev-erbα* are rhythmically expressed in the PT throughout a day (Hazlerigg et al., 2004; Johnston et al., 2006; Wagner et al., 2007). For several years, the rhythmic clock gene expression was thought to be dependent upon the daily MEL rhythm *via* a mechanism implicating the MEL receptors MT1. Several studies supported this model. First, *Cry1* expression rises at the night onset, in response to the nocturnal MEL input (Dardente et al., 2003; Hazlerigg et al., 2004). Besides, *Per1* expression is inhibited by MEL and is initiated at dawn when the MEL levels start to decrease (von Gall et al., 2002). The mechanisms of *Per1* regulation are well-described: at night, MEL inhibits the cAMP-mediated *Per1* expression and sensitizes the adenosine receptors A_{2b}. At dawn, MEL levels decrease and its inhibitory effect is gradually cleared up. Circulating or locally released adenosine subsequently acutely induces *Per1* expression through AC activation and subsequent increase in cAMP and p-CREB levels (von Gall et al., 2002). This model suggested that the PT was less than an oscillator since no clock gene expression would occur in the absence of MEL (von Gall et al., 2002).

However, Hazlerigg and colleagues recently demonstrated that the **rhythmic expression** of *Per1* and *Rev-erbα* **persists** in the PT of Siberian hamsters maintained in

constant light (LL) – a lighting condition which strongly inhibits the synthesis and release of MEL (Wagner et al., 2007). Besides, *Per1*/*2*, *Bmal1* and *Rev-erbα* rhythmic expressions also continue in sheep exposed to a LL regimen (Johnston et al., 2006). To complement their study, they showed that infusions of MEL at any time of the day potently increase *Cry1* expression while it phase-dependently inhibits *Per1*/*2*, *Bmal1* and *Rev-erbα* when their endogenous expression levels are at their zenith (Johnston et al., 2006). These new important data support the existence of an **entrainable circadian oscillator in the PT**. The arrhythmicity observed in previous experiments in pinealectomized or MT1-deficient mice (von Gall et al., 2002) most likely derives from the progressive damping of the circadian oscillator in the PT due to the desynchronization among individual cells that occurs in the absence of MEL, the synchronizing input.

The fact that the MEL signal, which is correlated to the duration of the night, is capable to regulate the transcription of *Cry1* and *Per1* at the onset and offset of the night is of particular importance. It allows a photoperiodic control of the circadian oscillator of the PT, which is thus likely involved in the decoding of the MEL photoperiodic signal that leads to the photoperiodic release of prolactin (Hazlerigg & Wagner, 2006).

2.3.2.5. Pineal gland

The **PG** is an **endocrine structure** of importance, which mediates the timing information from the SCN through the **rhythmic synthesis and release of MEL** (*section A2.4*). Remarkably, several clock genes were shown to oscillate *in vivo* in the PG, both in LD and DD. In mice, *Per1*/*2*/*3* and *Cry1*/*2* oscillations peak at early to midnight under both lighting regimen with *Bmal1* in anti-phase (Karolczak et al., 2004). *Clock* expression oscillates in LD, but not in DD. Apart from *Per3* and *Cry1* in DD (Takekida et al., 2000; Simonneaux et al., 2004), similar oscillatory patterns were reported for the rat PG: *Per1*/*2*/*3* and *Cry1*/*2* expression are rhythmic in both lighting regimen with a maximum expression level at night/subjective night (Fukuhara et al., 2000; Takekida et al., 2000; von Gall et al., 2001; Simonneaux et al., 2004) while *Bmal1* oscillates in anti-phase (Namihira et al., 1999). Contrary to regular peripheral oscillators (*sections A2.3.2.1-4*), a unique mechanism of regulation of clock genes expression in the PG has

been proposed. A NE treatment *in vitro* is able to induce *Per1* expression (von Gall et al., 2001; Fukuhara et al., 2002) and *in vivo* injections of a β-adrenergic receptor (β-AR) agonist isoproterenol during the daytime are able to induce both *Per1* and *Cry2* expression in the rat PG (Takekida et al., 2000; Simonneaux et al., 2004). Besides, *Per1* is not induced at night in rats from which SCG have been removed (Takekida et al., 2000). Altogether, these results strongly support a role for the SCN-driven NE release in the control of the clock genes expressions in the PG. Although these findings are compatible with the oscillator model, the observation that *Per1* and *Cry2* expression are immediately abolished by injections of the β-AR antagonist propranolol or by light exposure at night does not corroborate this model (Fukuhara et al., 2000; Takekida et al., 2000; Simonneaux et al., 2004). The clock genes expressions within the PG may thus be directly **dependent upon the SCN through the NE release**, and in that case the PG should not be considered as a peripheral oscillator. Nevertheless, a recent report using the *Period1-luciferase* reporter construct in mice managed to monitor *Per1* transcription oscillation for few cycles *in vitro* (Fukuhara et al., 2005). This supports a similar result obtained from *Period1-luciferase* transgenic rats (Abe et al., 2002) and suggests that the PG might behave like an oscillator in which pinealocytes require a SCN-originated cue to keep synchronized rhythms. Additional studies involving *in vivo* imaging in SCN-lesioned animals may provide a definite answer for the nature of the PG entity.

2.3.3. CONCLUSION

Recent studies have contributed to improve our understanding of the physiology of peripheral oscillators. To date, it appears that both resetting mechanisms and physiological functions of peripheral oscillators are unique. Resetting involves several synchronizing cues, like glucocorticoids, MEL and NE, which can be used by the SCN. The study by Guo et al. (2005) suggests that unidentified blood-borne or behavioral signals might add to the complexity of the communication between SCN and peripheral oscillators. The study of the locally regulated genes in the periphery will permit the understanding of the functions of each of the peripheral oscillators.

The identification of a new clock located within the OB suggests that additional SCN-like clocks are still likely to be discovered.

2.4. THE MELATONIN OF THE PINEAL GLAND, AN ENDOCRINE OUTPUT SIGNAL OF THE SCN CLOCK

The **PG** constitutes a major target of the central clock in that it contributes to the diffusion of the SCN-elaborated timing information to the whole organism through the synthesis and release of the **MEL** hormone. In this section, I will successively depict the MEL **physiological functions**, its **sympathetic pathway** and its **regulation** (Simonneaux & Ribelayga, 2003).

2.4.1. PHYSIOLOGICAL ROLES OF MELATONIN

The MEL synthesis is characteristically restricted to the **night phase** and the duration of its synthesis is positively correlated to the length of the night that fluctuates with seasons (Figure 23). This provides organisms with a time information and MEL is thus mostly implicated in the **regulation of seasonal physiological functions**. Besides, a role in the **regulation of circadian rhythms** has been proposed.

2.4.1.1. Regulation of seasonal rhythms

The most critical role played by MEL in mammals is the regulation of **seasonal physiological functions** (Pevet, 1988; Goldman, 2001). Remarkably, the photoperiodic variations of MEL appear to regulate the **reproduction** in seasonal breeders. Depending on species, several hypothalamic structures expressing MEL binding sites have been implicated in this regulation (Morgan & Mercer, 1994). In ewes, MEL microimplants within the **premammillary area of the hypothalamus** in ovariectomized, oestradiol-treated animals stimulate the luteinizing hormone secretion (Malpaux et al., 1998). In Syrian hamsters, the destruction of the **dorsomedian hypothalamus** prevents the gonadal regression of pinealectomized animals to short day-like infusions of MEL (Maywood et al., 1996). Besides, seasonal changes in secretion of prolactin from lactotropic cells of the *pars distalis* of the pituitary depend upon the MEL-controlled secretion of the tuberalin – an unknown factor – by cells of the **PT** (Morgan & Williams, 1996).

In the past few years, several groups have made a major contribution in the understanding of the regulation of the reproduction by MEL. They discovered that **the expression of a set of genes changes under various photoperiods *via* photoperiodic fluctuations in the MEL input**. Indeed, the level of **retinoid X receptor γ** (RXR/RAR) and **retinoid binding protein** (CRBP1) expressions vary in the **dorsal tuberomammilary nucleus** of Siberian hamsters under various photoperiods and these variations are associated with body weight changes (Ross et al., 2004; Barrett et al., 2006). Similarly, the expression of ***type 2 iodothyronine deionidase*** (*Dio2*), an enzyme that converts the prohormone T_4 in the bioactive T_3, depends upon the photoperiod: under a long photoperiod, *Dio2* expression is increased in the mediobasal hypothalamus (Watanabe et al., 2004; Revel et al., 2006a). Besides, T_3 injections mimic the photoperiodically-induced testicular growth in Siberian hamsters (Watanabe et al., 2004) while MEL injections block the *Dio2* expression in Syrian hamsters (Revel et al., 2006a).

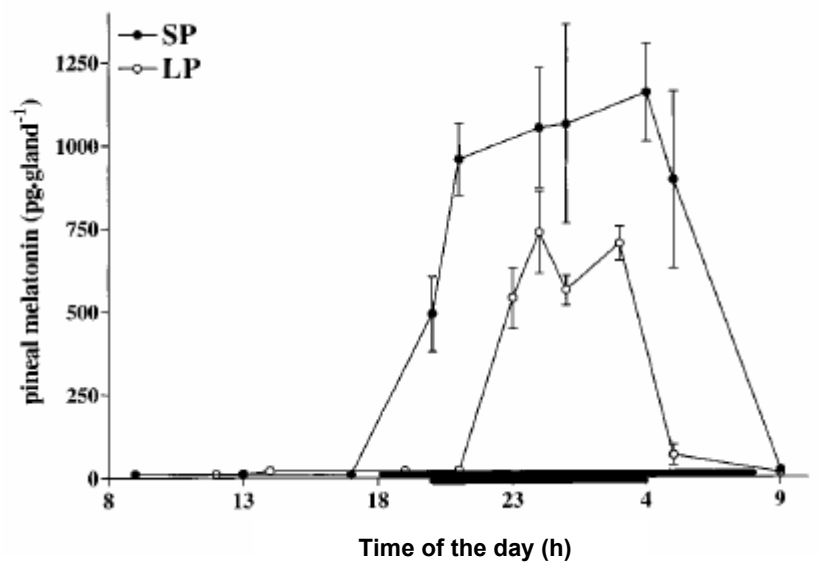

Figure 23. Duration of the melatonin synthesis is positively correlated to the length of the night.

Daily patterns of pineal melatonin content in Siberian hamsters kept under short (SP; LD 10:14) or long photoperiod (LP; LD 16:8) are shown. Black bars indicate dark periods. The duration of the MEL synthesis at night is longer in SP than LP. Note that in this species amplitude of the MEL synthesis is also higher in SP than LP.
(From (Ribelayga et al., 2000)

KISS-1 that codes for **kisspeptin**, the ligand of the **GPR54** receptor is also differently expressed under a short and long photoperiod. In the arcuate nucleus of Syrian hamsters, *KISS-1* expression is low under a short photoperiod and it appears regulated by MEL since pinealectomy prevents the down-regulation of *KISS-1* under a short photoperiod. Besides, a chronic administration of kisspeptin is able to restore testicular growth, even under a short photoperiod (Revel et al., 2006b). Those recent findings indicate that the MEL control of seasonal functions is likely mediated through a seasonal regulation of specific genes.

2.4.1.2. Regulation of circadian rhythms

In addition to its seasonal functions, MEL may have a **chronobiotic effect on the central clock of the SCN** (Pevet, 2003). This means that **MEL is able to influence the phase and/or the period of the circadian clock**. *In vivo* investigations first showed that MEL and/or its agonist S20098, administration entrains circadian rhythms of either temperature or locomotor activity in rats and Syrian hamsters housed in constant conditions (DD) (Redman et al., 1983; Pitrosky et al., 1999; Schuhler et al., 2002). Strikingly, entrainment only occurs when MEL administration coincides with the onset of activity at the subjective day/subjective night transition, at a time when endogenous levels of MEL are low in the plasma (Redman et al., 1983; Pitrosky et al., 1999; Schuhler et al., 2002). Outside of this narrow sensitive time window, MEL is unable to entrain the clock. Additional *in vitro* studies on SCN slices confirmed this chronobiotic effect. Firing rate activity of SCN neurons is **phase-advanced** when MEL is administered for 1 h at the subjective day/subjective night transition (McArthur et al., 1991). Besides, MEL **inhibits** the SCN neurons firing rate when slices are treated in a 6-h period straddling the subjective day/subjective night transition, but is much less effective outside of this time window (Stehle et al., 1989). MEL injections in rats carried out immediately before the onset of darkness also induce a phase-advance in the rise of the arylalkylamine *N*-acetyltransferase (AA-NAT) activity, the rhythm-generating enzyme of the MEL synthesis (*section A2.4.3*) (Humlova & Illnerova, 1990). This suggests that **exogenous MEL is capable of synchronizing the circadian rhythms of locomotor activity, temperature and MEL synthesis**.

The mechanisms of action of MEL on the circadian system are still unclear. In an attempt to clarify those mechanisms, Poirel and colleagues stimulated rats with MEL at the subjective day/subjective night transition and evaluated whether the clock genes expression was affected in the SCN. Surprisingly, they found that none of the *Per1*/*2*/*3*, *Cry1* and *Bmal1* genes expressions are immediately affected by the MEL treatment, although some were phase-shifted the second night after injections (Poirel et al., 2003). This suggests that none of these clock genes participate to the immediate phase-shift induced by MEL. On the contrary, ***Rev-erbα* rhythmic expression is phase-advanced by MEL** when administered at the subjective dusk (Agez et al., 2007). Strikingly the magnitude of the phase-advance is correlated to the magnitude of the phase-advance in *Bmal1* expression, which suggests a role of the orphan receptor in the MEL-induced phase-shifts (Agez et al., 2007). The mechanisms linking the exogenous MEL stimulation to the subsequent regulation of *Rev-erbα* are not elucidated yet but MEL is likely to mediate its effects through the recruitment of the high-affinity receptors **MT1** and **MT2** (*section A2.4.2*). In the SCN, MT1 are indeed believed to mediate MEL-induced inhibition of neurons firing activity, while MT2 may participate to MEL-induced phase-shifts (Liu et al., 1997b).

2.4.2. MELATONIN RECEPTORS

The use of the radioiodinated MEL ligand ^{125}I-MEL allowed the identification of several MEL binding sites (Vanecek et al., 1987). So far, two subtypes of membrane receptors have been identified: **high- and low-affinity receptors** (Dubocovich, 1995). Three high-affinity MEL-binding sites have been characterized: **MT1**, **MT2**, and **Mel1c,** which is only expressed in non-mammalian vertebrates (Reppert et al., 1996). They are characterized by a Kd < 300 pM and their expression pattern is highly variable depending on species. However, the **PT** and the **SCN** to a lesser extent express MEL-binding sites in many mammalian species (Morgan & Mercer, 1994).

Both MT1 (Reppert et al., 1994) and MT2 (Reppert et al., 1995) are G protein-coupled receptors negatively associated to AC. Upon activation, MT1 notably inhibits the forskolin-induced cAMP accumulation in the PT of Siberian hamsters (Carlson et al., 1989). This inhibition of AC is likely mediated through recruitment of a pertussis toxin

G protein-sensitive G_i or G_o since pertussis toxin blocks the ability of MEL to inhibit forskolin-induced cAMP accumulation (Carlson et al., 1989; Reppert et al., 1994).

MT1 is mostly expressed in the brain, with the highest density in the PT and the SCN (Reppert et al., 1994). MT2 is differently expressed since it presents high levels in the retina and low levels in the brain and SCN (Reppert et al., 1995). However, both receptors are likely involved in the chronobiotic effect of MEL (*section A2.4.1.2*), MT1 mediating the inhibitory effect on electrical activity and MT2 mediating phase-shifts (Liu et al., 1997b). As suggested in a previous section (*section A2.4.1.1*), MEL receptors are found in many **hypothalamic structures** like the dorsal tuberomammilary nucleus and the mediobasal hypothalamus, and their recruitment by MEL is believed to mediate the photoperiodic control of seasonal functions like the reproduction (Ross et al., 2004; Watanabe et al., 2004; Barrett et al., 2006; Revel et al., 2006b; Revel et al., 2006a).

2.4.3. MELATONIN SYNTHESIS PATHWAY AND REGULATION

The structures and mechanisms accountable for the tight temporal regulation of the **MEL** secretion at night show significant species differences, as thoroughly reviewed recently (Simonneaux & Ribelayga, 2003). Here, I will mainly describe MEL regulation in rat.

2.4.3.1. The hypothalamo-pineal axis

The **PG** is an evagination of the diencephalic roof in rodents. The axis that connects the SCN of the hypothalamus to the PG, by which they send photoperiodic information, is constituted of several nuclei and implicates various neurotransmitters (Figure 24).

The **PVN** of the hypothalamus is the primary target of the SCN in the hypothalamo-pineal axis. Indeed, bilateral lesions of the PVN abolish the rhythm of MEL in the pineal gland (Klein et al., 1983). Viral transneuronal tracing technique using a pseudorabies virus showed that neurons from the shell of the SCN project to the dorsal parvocellular subdivision of the PVN and form synaptic contact with neurons descending to the **IML** of the upper thoracic spinal cord (Larsen, 1999). The inhibitory neurotransmitter **GABA** is likely involved in the transmission of SCN signal to the PVN since daytime infusion of a GABA antagonist in the PVN increases daytime levels of MEL (Kalsbeek et al., 2000) while infusion of GABA agonist during the night inhibits the night-time MEL secretion

(Kalsbeek et al., 1996; Kalsbeek et al., 1999). Besides, **GLU** antagonist infusions within the SCN or PVN at night decrease the night-time MEL secretion, which suggests that GLU release is a positive SCN signal during the night (Perreau-Lenz et al., 2004).

The neurons of the PVN that project to the IML cells are likely to transmit the SCN information through the release of **oxytocin** and **AVP** whose infusion in the IML inhibits the neuronal electrical activity locally (Gilbey et al., 1982). The IML neurons targeted by the PVN neurons innervate the **SCG** (Strack et al., 1988; Larsen, 1999) and **acetylcholine** is the main neurotransmitter released (Kasa et al., 1991). Eventually, a small percentage of SCG neurons constitute the **sympathetic innervation** of the PG and release **NE** (Moller, 1999).

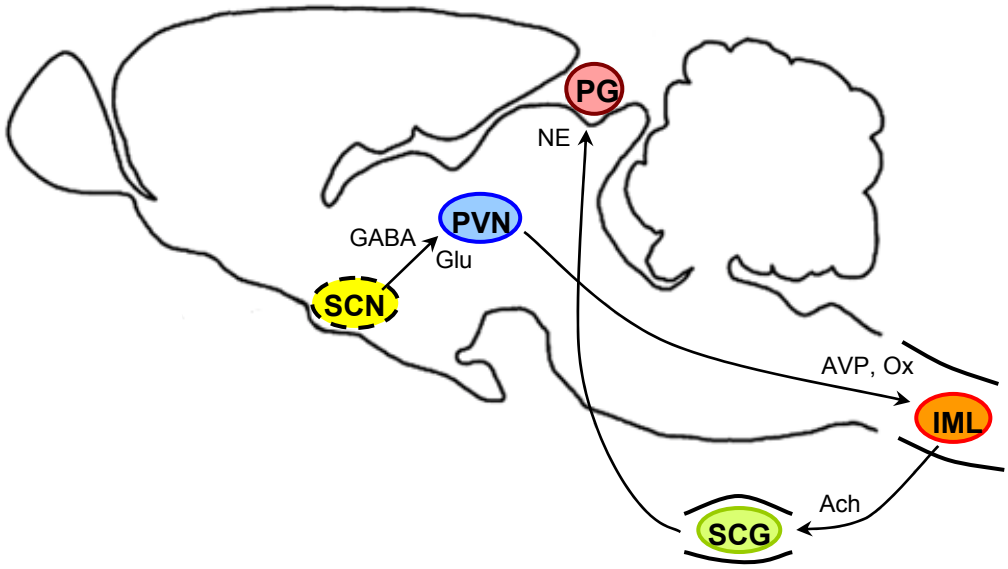

Figure 24. The hypothalamo-pineal axis.

Major neurotransmitters involved are indicated. Note that Glutamate (Glu) is likely involved in the night-time stimulatory signaling on the PVN, while γ-amino-butyric acid (GABA) is implicated in the daytime inhibitory signaling. Ach, acetylcholine; AVP, arginine vasopressin; IML, intermediolateral nucleus of the spinal cord; NE, norepinephrine; Ox, oxytocin; PG, pineal gland; PVN, paraventricular nucleus of the hypothalamus; SCG, superior cervical ganglions; SCN, suprachiasmatic nuclei.

2.4.3.2. Melatonin synthesis pathway

The **N-acetyl-5-methoxytryptamine** named **MEL** is an **indoleamine**. Its synthesis requires the recruitment of four enzymes (Figure 25). The essential amino-acid **tryptophan** is the MEL synthetic precursor. Upon uptake from the bloodstream, tryptophan is metabolized to **5-hydroxytryptophan** by the pineal **tryptophan hydroxylase** (TPOH). *Tpoh* gene expression barely change on the course of the day in the rat PG – 20% increase at night (Besancon et al., 1996) – while TPOH activity, already high during the day, is elevated by 100% during the night phase mostly by post-translational modifications (Shibuya et al., 1977; Ehret et al., 1991). The **aromatic amino acid decarboxylase**, present in large amounts in the pinealocytes, then converts 5-hydroxytryptophan into **5-hydroxytryptamine** (5-HT, serotonin). The rhythmic use of 5-HT for the nocturnal MEL biosynthesis (Snyder et al., 1965) as well as the release of 5-HT from the PG (Sun et al., 2002) leads to a rhythmic content of 5-HT in rat pinealocytes with accordingly low nocturnal levels.

The **AA-NAT** has been identified as the enzyme catalyzing *N*-acetylation within the rat PG (Voisin et al., 1984). The 23 kDa cytosolic enzyme converts 5-HT into *N*-acetylserotonine. The **high instability** of the AA-NAT – which presents a half-life of 3-5 min – as well as its tight daily regulation by the SCN (*section A2.4.3.3*) has given AA-NAT a major role in regulating the rhythm of MEL synthesis. This has also led scientists to refer to the AA-NAT as the **MEL rhythm-generating enzyme**. Both *Aa-Nat* expression and AA-NAT activity display high amplitude circadian oscillations in the rat PG where nocturnal mRNA levels are ~150-fold increased, and activity is ~100 times more elevated at night (Borjigin et al., 1995; Roseboom et al., 1996; Garidou et al., 2001).

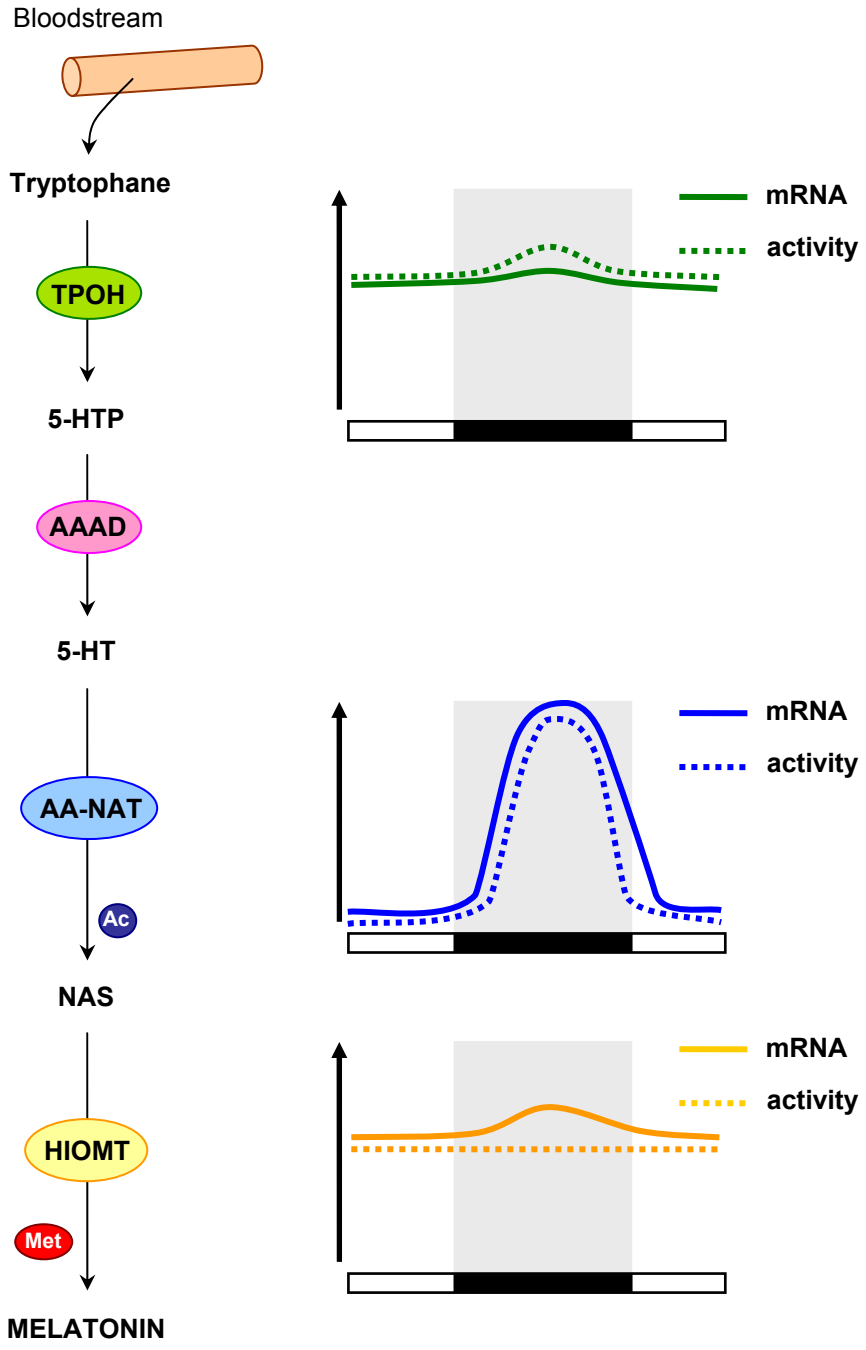

Figure 25. The melatonin synthesis pathway.

Tryptophane taken from the bloodstream is metabolized to 5-hydroxytryptophane (5-HTP) by the tryptophane hydroxylase (TPOH). 5-HTP is further decarboxylated by the aromatic amino acid decarboxylase (AAAD) into 5-hydroxytryptamine (5-HT, serotonine). The arylalkylamine *N*-acetyltransferase (AA-NAT) acetylates 5-HT into *N*-acetylserotonine (NAS) which is then methylated by the hydroxyindole *O*-methyltransferase (HIOMT) to form *N*-acetyl-5-methoxytryptamine (Melatonin). The panels on the right represent daily pattern of TPOH, AA-NAT and HIOMT mRNA and activity. Black boxes and shaded areas indicate night-time. Ac, acetyl; Met, methyl.

The final step of the MEL synthesis is performed by the **hydroxyindole-O-methyltransferase** (HIOMT, 38 kDa), which transfers a methyl group from the cofactor *S*-adenosylmethionine to *N*-acetylserotonine (Baldessarini & Kopin, 1966; Sugden et al., 1987). *Hiomt* expression exhibits a circadian fluctuation with higher levels at night. However, levels are already elevated during the day and the amplitude of the rhythm is low with a night-time increase limited to 100% (Gauer & Craft, 1996; Ribelayga et al., 1997; Ribelayga et al., 1999a). Contrary to AA-NAT, **HIOMT is greatly stable** and characterized by a half-life superior to 24 h (Sugden et al., 1987). Consistently, HIOMT accumulates and HIOMT activity levels does not display daily changes (Ribelayga et al., 1999a).

2.4.3.3. Circadian regulation of the MEL synthesis by noradrenergic input

MEL synthesis is regulated by several mechanisms, which include a **noradrenergic** control, a **peptidergic** modulation and a **non-adrenergic, non peptidergic** regulation as thoroughly described in a recent review (Simonneaux & Ribelayga, 2003). In the present section, I will focus on the noradrenergic control of the MEL synthesis in rodents, as it is believed to be the main regulatory mechanism responsible for the rhythmic release of MEL (Figure 26).

a. *Initiation of the MEL synthesis*

In mammals, **NE** is considered as the major neurotransmitter involved in the control of the MEL synthesis in the PG. *Aa-Nat* rhythmic expression is notably lost in superior cervical ganglionectomized rats while NE injections can restore *Aa-Nat* expression. Similarly, AA-NAT activity drops after a light pulse exposure at night (Roseboom et al., 1996; Garidou et al., 2001). Besides, NE participates to the daily regulation of *Hiomt* expression (Gauer & Craft, 1996; Ribelayga et al., 1999a) and to the long-term modulation of the HIOMT activity (Ribelayga et al., 1997; Ribelayga et al., 1999a).

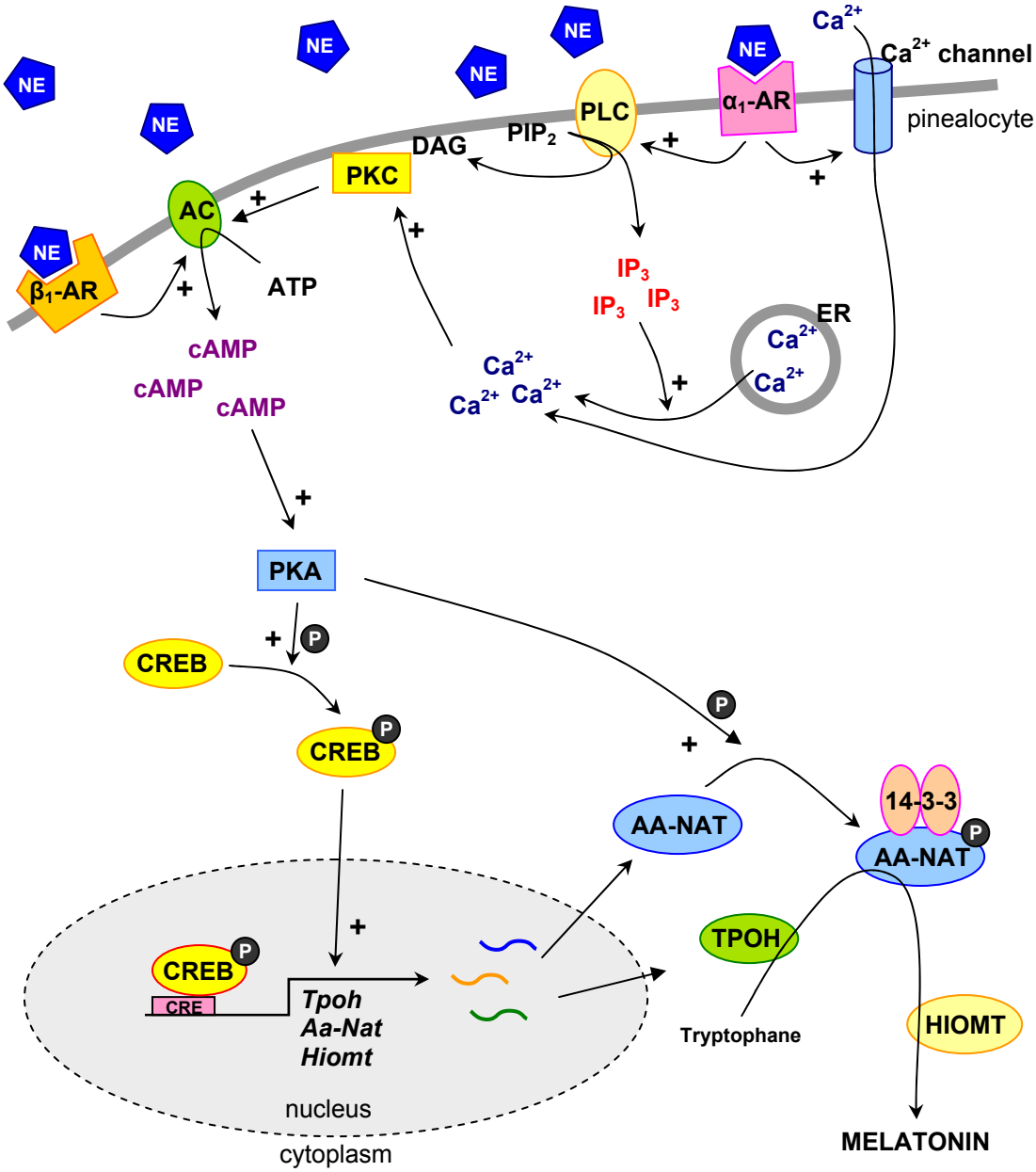

Figure 26. Noradrenergic induction of melatonin synthesis.

Norepinephrine (NE) release at night increases intracellular levels of cAMP upon binding on β_1-adrenergic receptors (β_1-AR) coupled to membrane adenylyl cyclases (AC). Binding of NE on α_1-adrenergic receptors (α_1-AR) potentiates the β_1-AR-induced increase in cAMP levels. Mechanisms implicated involve activation of a phospholipase C (PLC), release of calcium (Ca^{2+}) from calcium stores and through Ca^{2+} channels, activation of the protein kinase C (PKC) which eventually activates the β_1-AR-coupled AC. Increasing levels of cAMP promote the protein kinase A (PKA) activation which phosphorylates the cAMP response element-binding protein (CREB), permitting *tryptophane hydroxylase* (*Tpoh*), *arylalkylamine* N-*acetyltransferase* (*Aa-Nat*) and *hydroxyindole* O-*methyltransferase* (*Hiomt*) transcription. AA-NAT activity is increased and the protein stabilized through phosphorylation by PKA and binding with a dimer of 14-3-3 proteins. Melatonin synthesis can be initiated. "+" indicate promoting steps. CRE, cAMP response element; DAG, diacylglycerol; ER, endoplasmic reticulum; IP_3, inositol triphosphate; P, phosphate; PIP_2, phosphatydilinositol biphosphate.

The amount of NE released from the SCG sympathetic terminals on the PG is under the control of the SCN (Drijfhout et al., 1996a) and exhibits a circadian oscillation with NE levels ~100-fold higher during the night than during the day (Drijfhout et al., 1996b). Two subtypes of adrenergic receptors expressed in the PG are involved: **α1-adrenergic receptor** (α_1-AR) coupled to the **phospholipase C** transduction system (Sugden & Klein, 1984) and **β1-AR** coupled to the membrane **AC** (Strada et al., 1972; Zatz et al., 1976). In rat, *in vivo* injections of β_1-AR agonist isoproterenol stimulate AA-NAT activity during the day (Deguchi & Axelrod, 1972a) while β_1-AR antagonist propranolol injections inhibit the AA-NAT activity increase at night (Deguchi & Axelrod, 1972b). Upon stimulation, the AC-coupled β_1-AR increases the intracellular **cAMP** levels (Strada et al., 1972). α_1-AR activation further **potentiates** the β_1-AR-induced increase in cAMP (Vanecek et al., 1985) by activating the phospholipase C transduction system. This leads to an **increase in intracellular levels of Ca^{2+}** through the initial depletion of the Ca^{2+} stores and the subsequent opening of membrane Ca^{2+} channels. Intracellular Ca^{2+} further activates protein kinase C (Ho et al., 1988), which is likely to enhance AC activity (Sugden & Klein, 1988).

The transregulator element **CREB** constitutively expressed in the rat PG is phosphorylated *in vivo* and *in vitro* following NE treatment and isoproterenol injections respectively (Roseboom & Klein, 1995). After activation of β_1-AR, the cAMP-dependent **PKA phosphorylates CREB** (Roseboom & Klein, 1995). Once activated, p-CREB induces the expression of ***Aa-Nat*** (Baler et al., 1997; Burke et al., 1999) and ***Hiomt*** (Rodriguez et al., 1994), which are endowed with putative **CRE** motifs in their promoter. This is supported by distinct studies, which demonstrated that *de novo* protein synthesis is not required to promote both *Aa-Nat* (Roseboom et al., 1996) and *Hiomt* (Ribelayga et al., 1999a) expression in the rat PG, suggesting the requirement of a constitutive element. Besides, PKA directly phosphorylates AA-NAT, allowing the formation of a complex with the **dimer of 14-3-3 chaperones proteins**, which activates AA-NAT (Ganguly et al., 2001; Obsil et al., 2001). A recent study revealed that **proteasomal proteolysis** is required for NE-induced *Aa-Nat* transcription (Terriff et al., 2005). This suggests that a repressor protein that prevents *Aa-Nat* mRNA and protein levels accumulation must be degraded to allow MEL synthesis at night.

b. *Termination of the MEL synthesis*

The **termination** of the MEL synthesis at the end of the night is achieved by several mechanisms. First, early at night, NE-stimulated p-CREB induces the gene expression of *CRE modulator* (*Crem*). A splice variant of *Crem* codes for the transcriptional repressor **inducible cAMP early repressor** (ICER), which accordingly exhibits a circadian rhythm with a high level of expression at night (Stehle et al., 1993). ICER-ir peaks at the night/day transition and it is thus likely that ICER participates in the inhibition of the cAMP-induced transcription at the end of the night (Maronde et al., 1999). In support of this hypothesis, silencing *Icer* in rat pinealocytes potentiates *Aa-Nat* expression and MEL synthesis (Maronde et al., 1999). It should be noted however that a recent study challenged the role of the level of expression of ICER in the regulation of *Aa-Nat* expression (Ho et al., 2007). This group shows that ICER down-regulation by siRNA does not affect NE-induced *Aa-Nat* expression while overexpression of ICER before the NE stimulation potently inhibits the NE-induced *Aa-Nat* transcription. They proposed that not only the ICER protein levels but also the temporal relationship between the expression of the repressor and the transcriptional activity of the *Aa-Nat* are critical to determine the effectiveness of the repressor.

Secondly, at the end of the dark phase, the **NE** release stops and the intracellular cAMP levels return to daytime values. This eventually leads to a decrease in *Aa-Nat* mRNA levels. However, the decline of MEL levels in rat occurs prior to the decrease in *Aa-Nat* mRNA levels (Roseboom et al., 1996; Ribelayga et al., 1999b), which suggests that **cessation of the expression of *Aa-Nat* is not involved in MEL decline**. Instead, MEL synthesis arrest is associated with the **post-translational inhibition of the AA-NAT enzyme**. After cAMP levels returned to daytime levels, PKA activity decreases and as a consequence, AA-NAT is dephosphorylated. 14-3-3 proteins subsequently dissociate from AA-NAT, which is then unprotected and quickly degraded by **proteasomal proteolysis** (Gastel et al., 1998; Ganguly et al., 2001; Klein et al., 2002). The degradation of AA-NAT is immediately followed by the decrease in MEL synthesis and release.

Third, the sensibility of β_1-AR in the PG is 10 times lower at the end of the dark period than during the light period (Romero & Axelrod, 1974). Subsensitivity of

receptors is a frequent phenomenon that results from a repetitive administration of drugs. In the PG, supersensitivity of β_1-AR results from a decreased release of NE during the daytime while subsentivity results from the increased release at night. Although it is not believed to be the main mechanism of MEL synthesis termination, subsensitivity of the receptors at the end of the night might participate in the decreased *Aa-Nat* expression and AA-NAT activity that prelude the decline in MEL levels.

c. *Potential modulation of the MEL synthesis by MAPK*

The nocturnal **NE** release on the PG leads to a **rhythmic phosphorylation of ERK1/2** with high levels during the night (Ho et al., 2003a). Similarly, the activity of both ERK1/2 upstream regulators MEK1/2 and downstream target p90RSK peaks at night (Ho et al., 2003b; Ho et al., 2003a). The night-time activation of ERK1/2 requires the recruitment of both **α_1- and β_1-AR** and the activation of the **cGMP pathway**. cGMP intracellular levels increase following the stimulation of α_1- and β_1-AR. This leads to the recruitment of **GC** and the subsequent activation of the cGMP-dependent **PKG** (Ho et al., 1999; Ho & Chik, 2000). As opposed to PKG, PKA activation seems to down-regulate ERK1/2 phosphorylation at night, which suggests that ERK1/2 activation results from integration of the PKA and PKG opposed signaling mechanisms (Ho & Chik, 2000). Alike ERK1/2, **$p38^{MAPK}$ activation is rhythmic** in LD with a maximal activation level at midnight. *In vitro*, $p38^{MAPK}$ are dose-dependently phosphorylated by NE treatment and both **α_1- and β_1-AR** are involved. A closer analysis identified **Ca^{2+}**, **cAMP** and **PKA** as elements mediating NE-induced $p38^{MAPK}$ activation (Chik et al., 2004).

In the PG, inhibition of ERK1/2 decreases the NE-induced AA-NAT activity (Ho et al., 2003b) while $p38^{MAPK}$ inhibition causes a sustained increase in AA-NAT activity and protein levels (Man et al., 2004). It thus seems **that MAPK participate in the modulation of the MEL synthesis, ERK1/2 promoting the induction of AA-NAT activity and $p38^{MAPK}$ limiting the AA-NAT maximal response to NE stimulation**. The opposing effects of ERK1/2 and $p38^{MAPK}$ are consistent with the $p38^{MAPK}$-induced inhibition of ERK1/2 activation observed in rat pinealocytes (Mackova et al., 2000; Ho et al., 2003b). It is remarkable that both NE-induced *Aa-Nat* transcription and AA-NAT activity are regulated by duet of proteins showing opposite effects despite a common NE

activation: ICER and CREB for transcriptional regulation and ERK1/2 and $p38^{MAPK}$ for the control of AA-NAT activity.

d. *Species differences*

Importantly, major differences in the regulation of the MEL synthesis exist within rodent species and between rodents and non-rodent species. These variations mostly affect the mechanisms downstream of the NE-α_1/β_1-AR-cAMP-PKA in the signaling cascade that leads to AA-NAT activation and MEL synthesis (Simonneaux & Ribelayga, 2003; Schomerus & Korf, 2005).

In **rodents**, one striking difference noticed when comparing the Syrian hamster or mouse and the rat regulation processes of MEL is the inability for NE and isoproterenol to induce *Aa-Nat* expression or MEL synthesis during the daytime in the Syrian hamster (Reiter et al., 1987; Garidou et al., 2003) and the mouse (Fukuhara et al., 2005). Theses results suggest that in **Syrian hamsters and mice, MEL synthesis is gated to night-time**. Mechanisms of the gating remain unclear. However, few studies dedicated to the understanding of the gating phenomenon showed that as opposed to the rat, the nocturnal increase in *Aa-Nat* transcription in the Syrian hamster is strongly inhibited by a protein synthesis inhibitor (Garidou et al., 2003). This suggests that inducible stimulatory transcription factors are required during the early night. A recent study suggests that c-FOS and c-JUN, which are transiently expressed early in the night, may participate in this transcriptional regulation of *Aa-Nat* expression (Sinitskaya et al., 2006).

MEL regulation in **ungulates** has been well-described. *Aa-Nat* expression is high during the day and increase only by 50% at night (Coon et al., 1995; Craft et al., 1999). In contrast, AA-NAT protein levels, enzyme activity and MEL content are low during the day and increase 10 times during the night, suggesting that **AA-NAT changes are primarily regulated at the protein level** (Klein et al., 1997). During the day, in the absence of NE-α_1/β_1-AR-cAMP-PKA activation, AA-NAT protein is constitutively translated but immediately degraded by proteasomal proteolysis. In contrast, at night, the PKA activation that follows NE release on the PG phosphorylates AA-NAT. This phosphorylation promotes the recruitment of the 14-3-3 protein dimers, which prevent

AA-NAT from being degraded (Schomerus et al., 2000; Ganguly et al., 2001). In addition to its protection toward the proteasome, 14-3-3 proteins increase AA-NAT affinity to 5-HT (Ganguly et al., 2005). As a consequence of these regulations, AA-NAT proteins accumulate, the enzyme activity increases and MEL synthesis is initiated. At the end of the night, NE release stops and AA-NAT protection is cancelled. This leads to the degradation of AA-NAT and the arrest of MEL synthesis (Gastel et al., 1998; Schomerus et al., 2000).

AIMS OF THE STUDY

B. AIMS OF THE STUDY

The mammalian circadian system, which drives behavioral and physiological oscillations, is constituted of a set of clock genes that are organized in autoregulatory feedback loops (*section A2.2.1*). A primary feedback loop controls the cyclic expression of clock genes and multiple clock-controlled genes, which drive the timing information to the rest of the organism. Two additional loops interlocked with the primary loop participate in the reinforcement and the stabilization of the cyclic clock genes expression. In addition to the transcriptional regulation of clock genes, **post-translational regulation of clock proteins** are critical to precisely determine the 24-h period of the oscillations (*section A2.2.2.3*). Remarkably, phosphorylation of clock proteins affects their stability and eventually adjusts the pace of the molecular oscillations.

Post-translational modifications also appear critical in another aspect of the circadian biology: the regulation of the MEL synthesis (*section A2.4.3.3*). MEL is one major output of the SCN clock and is known to distribute a part of the SCN timing information to the whole organism through the bloodstream. The synthesis pathway of MEL requires post-translational phosphorylation of the transcription factor CREB and of AA-NAT, the MEL rhythm-generating enzyme. In both cases, phosphorylation is performed by PKA and promotes the activity of the target proteins.

It has been recently highlighted that additional kinases, the **MAPK**, seem to be implicated in the circadian biology. Among the three main subfamilies of MAPK, ERK subfamily has been the most studied and its role is mainly associated with the mediation of the photic entrainment (*section A2.2.3.1*). Moreover, both ERK and $p38^{MAPK}$ subfamilies seem to modulate the melatonin synthesis in the PG (*section A2.4.3.3*).

On the contrary, **JNK isoforms** have been less studied, despite their rhythmic activation in the hamster SCN (Pizzio et al., 2003), and their roles in the circadian system remain elusive. **The aim of this study was thus to expand our knowledge on the functions of JNK isoforms by determining whether they are involved in the regulation of the circadian system**. Considering that the related ERK and $p38^{MAPK}$ subfamilies have been implicated in the regulation of both the molecular clockwork and the MEL synthesis, we planned to evaluate JNK involvement in both mechanisms.

In the first part of this work, **the implication of JNK in the mechanisms that regulate the circadian clockwork was studied**. Several data in the literature indicate that the phosphorylation of PER proteins by the CKIε/δ is essential to properly determine the 24-h period of the clock (Vanselow et al., 2006). Besides, the $p38^{MAPK}$ has been suggested to set the pace of the chick PG clock (Hayashi et al., 2003). We thus hypothesized that **JNK activation might tune the pace of the circadian rhythms in mammals**. Our study, based on the chemical modulation of JNK activation, focused on both SCN and peripheral oscillators to establish whether JNK functions might be a feature common to several cell types.

In the second part of this work, **the potential roles that JNK might play in the regulation of the melatonin synthesis were investigated**. The melatonin synthesis pathway and its regulation are well described in the literature (Simonneaux & Ribelayga, 2003). At night, the NE-cAMP signaling initiates the transcription of several genes in the PG, including the key MEL regulatory enzyme *Aa-nat*, and *Per1* genes (Humphries et al., 2002; Fukuhara et al., 2003). Interestingly, recent studies revealed that ERK1/2 and $p38^{MAPK}$, which are activated by NE at night, likely modulate the melatonin synthesis by controlling the activation level of AA-NAT (Ho et al., 2006). However, little is known about the roles of JNK in the regulation of the genes expression or of AA-NAT. Based on the previous studies, we hypothesized that **JNK isoforms might be activated by NE in the PG and participate to the genes expression**. The involvement of $p38^{MAPK}$ in the signaling cascades of melatonin synthesis was further investigated since $p38^{MAPK}$ and JNK share common upstream regulatory pathways (*sections A1.3 and A1.4*).

MATERIALS AND METHODS

C. MATERIALS AND METHODS

1. ANIMALS, TISSUES AND CELLS

1.1. ANIMALS AND TISSUES

1.1.1. ANIMALS

All animals used were housed under a 12:12 LD (lights on, 07:00 h – 19:00 h) in a temperature-controlled environment and had access to food and water *ad libitum*. During the dark phase, they were exposed to a constant red dim light with an intensity inferior to 1 lux. Wistar rats were purchased from Charles River Laboratories (Frederick, MD, USA). *Period1-luciferase* transgenic mice and *Period2*^{Luciferase} knock-in mice (both detailed below) were generously provided by Dr Hajime Tei (Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan), and Dr Joseph S. Takahashi (Northwestern University, Evanston, IL, USA) respectively.

Transgenic mice carrying a *Period1-luciferase* transgene were constructed as follows (Yamazaki et al., 2000; Herzog et al., 2004): a mouse *Per1* genomic fragment of 6.7 kb was ligated directly to the second codon of the firefly *luciferase* cDNA flanked by the SV40 late polyadenylation signal. The mouse *Per1* (*mPer1*) fragment includes five functional **E-box** regions, a transcription initiation site, the first and second exons, which are split by the first intron, and a translational start codon in the second exon (Figure 27). The linearized reporter fragment was microinjected into fertilized eggs of Wistar rats. Transgenic rats were further identified by polymerase chain reaction (PCR), and the copy number of the transgene was determined by Southern analysis. A transgenic line with approximately 12 copies per genome of the transgene showed circadian oscillations of luciferase activity in the SCN. The period of the wheel-running activity rhythm in constant darkness was 24.43 ± 0.02 h (n = 20) for *Period1-luciferase* male and 24.33 ± 0.01 h (n = 26) for wild-type males controls. This indicates that the transgene does not disrupt molecular circadian time keeping.

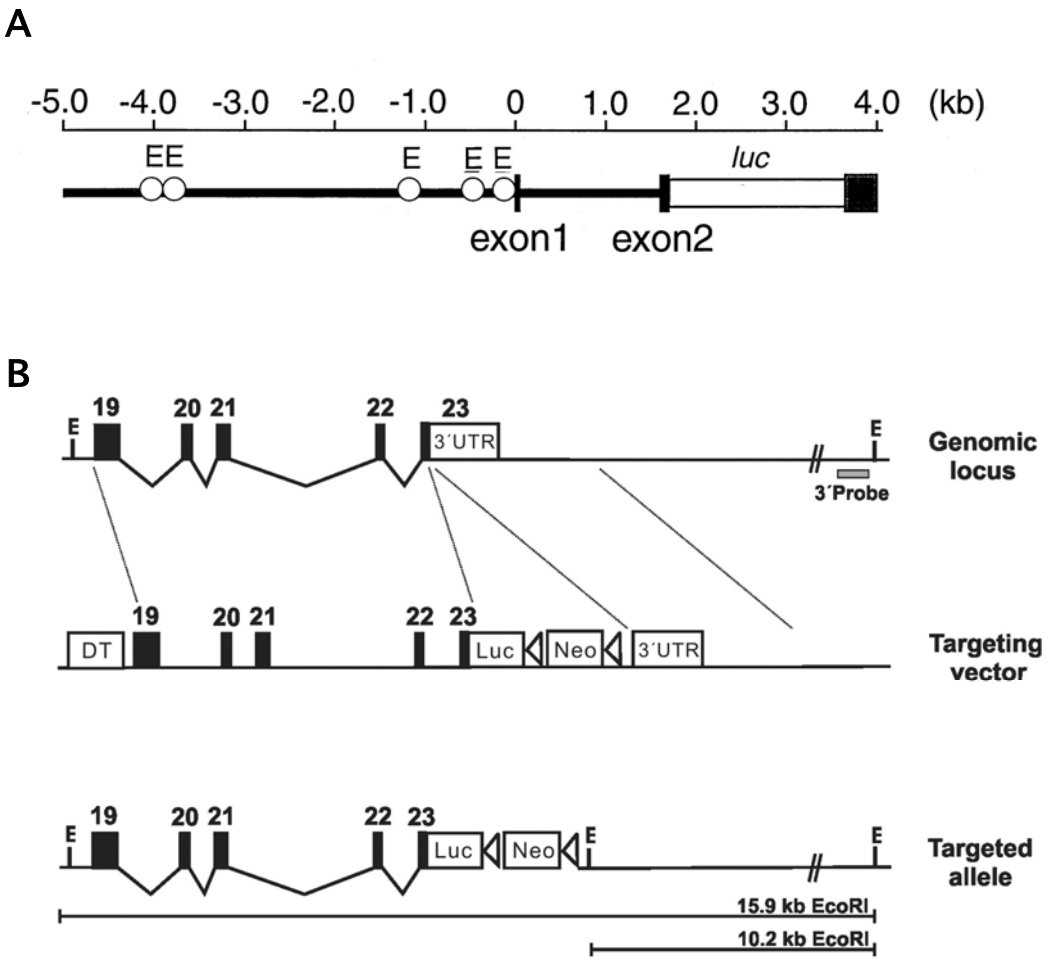

Figure 27. Generation of *Period1-luciferase* and *Period2^{Luciferase}* mice.

A. Diagram of the mouse *Period1-luciferase* transgene. Heavy line, *mPer1* fragment; open bar, *luciferase* (*luc*) fragment; shaded box, polyadenylation fragment; circles represent E-box elements (E).
(From Yamazaki et al., 2000)
B. Diagram of the *mPer2* locus, targeting vector, and targeted knockin allele. Filled blocks with numbers, exons; E, *Eco*RI; DT, diphtheria toxin A chain; *Neo*, neomycin resistance gene; triangle, loxP site.
(From Yoo et al., 2004)

Period2^{Luciferase} knock-in mice with a *luciferase* insert under the control of the *Per2* promoter were constructed as follows (Yoo et al., 2004): a mouse bacterial artificial chromosome (BAC) library generated from 129Sv embryonic stem cells was screened with a full-length mouse *Per2* (*mPer2*) cDNA probe. A 15.9-kb *Eco*RI fragment was isolated from a hybridizing BAC clone and was partially digested with *XmaI* to yield a 6.4-kb fragment, which was subsequently ligated in-frame to a 1.7-kb PCR-amplified

luciferase gene. The resulting 8.1- and 3-kb fragments from the 3' UTR of the *mPer2* gene were used as the long and short arms of the targeting construct, respectively, in the pKO Scrambler 916 vector (Lexicon, The Woodlands, TX, USA) (Figure 27). The targeting construct (40 µg) was inserted into W4 embryonic stem cells by electroporation and homologous recombinants were detected by Southern analysis after G418 selection (200 µg/mL). Targeted embryonic stem cell clones were injected into C57BL/6J mice blastocysts and transferred to pseudopregnant C57BL/6J female recipients. Resulting male chimeras were bred with C57BL/6J females. Germ-line transmission was confirmed by agouti coat color and Southern analysis using a 3' *mPer2* probe.

1.1.2. TISSUE COLLECTION AND DISSECTION

Animals were sacrificed by decapitation following an overdose of halothane anesthesia. If tissues were to be cultured for Western blotting or immuno-histochemistry (*sections C3 and C4*), they were maintained in 0.01 M phosphate buffer saline (PBS) pH 7.4 until the beginning of the culture. If tissues were to be cultured in the real-time bioluminescence monitoring system (*section C6*), they were placed in ice-cold Hank's Balanced Salt Solution until dissection.

Tissues removed at ZT11 for the bioluminescence assay were dissected as follows. Brain coronal sections of 300-µm thickness were prepared using a vibratome. The brain sections containing the SCN were further dissected under a binocular loop and SCN were isolated as 1 x 1 mm square. Under a binocular loop, lungs and kidneys were dissected into cubes of ~1 mm^3 in volume.

1.2. CELLS

Rat-1 fibroblasts stably transfected with a *Period1-luciferase* reporter construct (Izumo et al., 2003) were obtained from Dr Carl H Johnson (Vanderbilt University, Nashville, TN, USA). The cytomegalovirus promoter of pcDNA3.1/Hygro(+) (Invitrogen, Carlsbad, CA, USA) was replaced by the 3.0-kb region that is immediately upstream of the *mPer1* transcription start site. The *mPer1* promoter was fused to firefly luciferase cDNA (from pGL3-Basic, Promega, Madison, WI, USA). This expression vector is

hereafter called *Period1-luciferase*. Rat-1 fibroblasts were transfected with 5 µg of linearized *Period1-luciferase* by LipoFectin (GIBCO, Carlsbad, CA, USA). Forty-eight hours after transfection, hygromycin (200 µg/mL) was added to select stable lines. In stable cell lines, bioluminescence respond to a 50% horse serum shock and oscillate for at least two cycles, as shown for *mPer1* mRNA expression by Balsalobre et al. (1998). The clone that showed the highest amplitude and the most robust circadian rhythm of luciferase activity was selected.

2. TISSUE AND CELL CULTURE

2.1.1. TISSUE CULTURE

PG for Western blot or immuno-histochemistry analyses were cultured on Millicell culture membranes (Millipore, Billerica, MA, USA) inserted in a well of a 6-well plate. The tissue was incubated in BGJb medium (Invitrogen) supplemented with antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin). Cultures were maintained at 36°C under a 95% O_2 / 5% CO_2 atmosphere throughout the experiments. All experiments were performed on the third day of culture.

Tissues removed for the bioluminescence assay were cultured in assay medium constituted of serum-free Dulbecco's Eagle Modified Medium (DMEM, Cellgro, Herndon, VA, USA), 10 mM HEPES, 350 mg/mL sodium bicarbonate, 2% B27 Supplement (Gibco), antibiotics (25 units/mL penicillin and 25 µg/mL streptomycin) and 0.1 mM luciferin (Promega). All pieces of tissues were cultured individually in 35 mm Petri dishes. SCN slices and PG were placed on Millicell culture membranes (Millipore). Each Petri dish was sealed with silicon grease and a circular glass coverslip and cultured in a 36°C air incubator.

2.1.2. CELL CULTURE

Rat-1 fibroblasts (10^6 cells) were seeded in 35 mm Petri dishes and cultured for 3 days at 36°C under 5% CO2 / 95% air atmosphere until cells reach confluence. Medium used was constituted of DMEM (Gibco) supplemented with 5% fetal bovine serum and

antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin). On the fourth day of culture, circadian rhythms were synchronized among the cells by replacing the culture medium with 2 mL of assay medium (same as in *section C2.1.1*) as described by Hirota et al. (2002). Petri dishes were sealed with silicon grease and circular glass coverslips and cultured in a 36°C air incubator.

3. WESTERN BLOT

PG, and cells attached in Petri dishes were immediately frozen on dry ice and liquid nitrogen respectively and maintained at -80°C until protein extraction. Whole cell extracts were prepared in Extraction Reagent Buffer (Pierce, Rockford, IL, USA) containing protease and phosphatase cocktails inhibitors (Sigma, Saint-Louis, MO, USA). The protein content of the extracts was determined using the Bio-Rad protein assay reagent (Hercules, CA, USA). Twenty-five and 30 µg of PG and rat-1 cells protein extracts respectively were separated on SDS-polyacrylamide 4-12% Bis-Tris Gel (Invitrogen) in MES Buffer (Invitrogen) and further transferred to a nitrocellulose membrane in Transfer Buffer (Invitrogen).

Each blot was successively incubated with primary antibodies recognizing phosphorylated forms of JNK at Thr-183 and Tyr-185 (P-JNK, Cell Signaling Technology, Danvers, MA, USA), total JNK (total-JNK, Cell Signaling Technology), and ACTIN (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After blocking the membranes in hybridization buffer constituted of 0.01 M PBS pH 7.4, 5% milk and 0.05% Tween20 (Bio-Rad) for 1 h, blots were incubated at 4°C overnight with primary antibodies diluted at 1:1000 in hybridization buffer. Membranes were rinsed 3 times for 10 min in 0.01 M PBS and incubated for 1 h with the secondary antibodies mouse anti-rabbit horseradish peroxidase conjugated (for P-JNK and total-JNK primary antibodies) or donkey anti-goat horseradish peroxidase conjugated (for ACTIN antibodies) (both from Santa Cruz Biotechnology), diluted at 1:5000 in hybridization buffer. After 3 additional washes in 0.01 M PBS, immuno-positive bands were detected by chemiluminescent detection using Western Blotting Luminol Reagent (Santa Cruz Biotechnology). Each image was subjected to quantification using the NIH ImageJ analysis software (version 1.33u) and the intensity of each P-JNK or total-JNK band was normalized towards the corresponding ACTIN band intensity.

4. IMMUNO-HISTOCHEMISTRY

PG were fixed at 4°C in 4% paraformaldehyde in phosphate buffer (0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4) pH 7.4 for 30 min and 20% sucrose in phosphate buffer for 24 h. Twenty µm-thick sections were prepared using a cryostat. After the sections were blocked, they were incubated with P-JNK antibody at 1:2000 dilutions or total-JNK antibody at 1:200 dilutions in 0.01 M PBS containing 1% fetal bovine serum and 0.3% Triton X-100 at 4°C. All slices were washed three times for 10 min with 0.01 M PBS and incubated for 1 h with biotinylated anti-rabbit antibodies diluted to 1:200 with PBS containing 1% fetal bovine serum and 0.3% Triton X-100. The slices were again washed three times with 0.01 M PBS and incubated for 1 h in an avidin–biotin complex solution. Slices were stained using 1% diaminobenzidine (DAB) chromogen solution (Vector Laboratories, Inc., Burlingame, CA, USA) for 10 min and mounted on gelatin-coated glass slides. The image from each section was captured on a CCD video camera (Carl Zeiss MicroImaging, Inc. Thornwood, NY, USA) attached to a light microscope. Protein expression was quantified by measuring the signal density using the NIH ImageJ analysis software (version 1.33u).

5. REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (Q-PCR)

Total RNA were extracted from PGs using Trizol reagent (Life Technologies, Grand Island, NY, USA) after sonication. DNA was degraded by DNase I. First-strand cDNA was synthesized using moloney murine leukemia virus reverse transcriptase (MMLV-RT, Invitrogen). Each set of samples was simultaneously processed for RNA extraction, DNase I treatment, cDNA synthesis, and PCR reaction. Q-PCR was performed using an *i*Cycler (Bio-Rad) in the presence of SYBR green I (Bio-Rad) with primers to rat *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) (5'-AGACAGCCGCATCTTCTT GT-3'; 5'- TGATGGCAACAATGTCCACT-3'), rat *Aa-Nat* (5'-TCACTGAGCTGCA ATGTTCC-3'; 5'-GATGTCAGCCTGGCCTAGAG-3'), mouse *Per1* (5'-CCTCTGCAG GTTCAGGTCTC-3'; 5'-ATAGGGGAATGGTCAAAGGG-3') and rat *Mkp-1* (5'-ACT TCCCTGTCTCCATCCCT-3'; 5'-ACGTGAAACTCCACACCTCC-3'). Forty cycles of amplification were performed after 30 sec of denaturation at 95°C. Once the temperature

reached 95°C, it was decreased to 60°C, maintained for 20 sec, and raised to 72°C for 30 sec. Fluorescence was measured at the melting temperature after each cycle, allowing comparison of fluorescence intensities among samples while their increase is within the linear range. Melting temperatures used were 86°C for both *Gapdh* and *Mkp-1*, and 89°C for both *Aa-Nat* and *Per1*. Quantification of *Gapdh*, *Aa-Nat*, *Per1*, and *Mkp-1* cDNAs was accomplished by comparing the threshold cycles for amplification of the unknowns with those of four concentrations of standard using the *i*Cycler software (version 3.1). After 40 cycles of reactions, the PCR products were run on an agarose gel to verify that a single amplicon of appropriate size was amplified. *Mkp-1*, *Per1*, and *Aa-Nat* mRNA were normalized using *Gapdh* mRNA levels. In each experimental set, maximum value was represented as 100 and each value was normalized relative to the maximum value.

6. BIOLUMINESCENCE ASSAY

6.1. REAL-TIME BIOLUMINESCENCE MONITORING APPARATUS

The real-time bioluminescence monitoring system (LumiCycle, Actimetrics, Wilmette, IL, USA) allows luminometry on self-luminous tissues. It is constituted of a light-tight chamber placed in a standard air incubator and equipped with 4 photon-counting photomutliplier tubes (PMT) (Figure 28). Each PMT is characterized by low dark counts (defined as the number of counts registered by the PMT in complete darkness) and a high sensitivity in green portion of the spectrum at which Luciferase emits light. Inside the light-tight box, an automated turntable can receive 32 tissue or cell samples at a time, each in a 35 mm Petri dish. Eight dishes share each photodetector. Based on our experience, we set the apparatus so that every 10 min, the turntable brings 4 dishes under each PMT that counts the bioluminescence for 75 sec. Data are acquired using a PCI-6601 card and a NI-DAQ 7.1 acquisition driver (National Instruments, Austin, TX, USA) associated with an acquisition software (LumiCycle Software, Actimetrics).

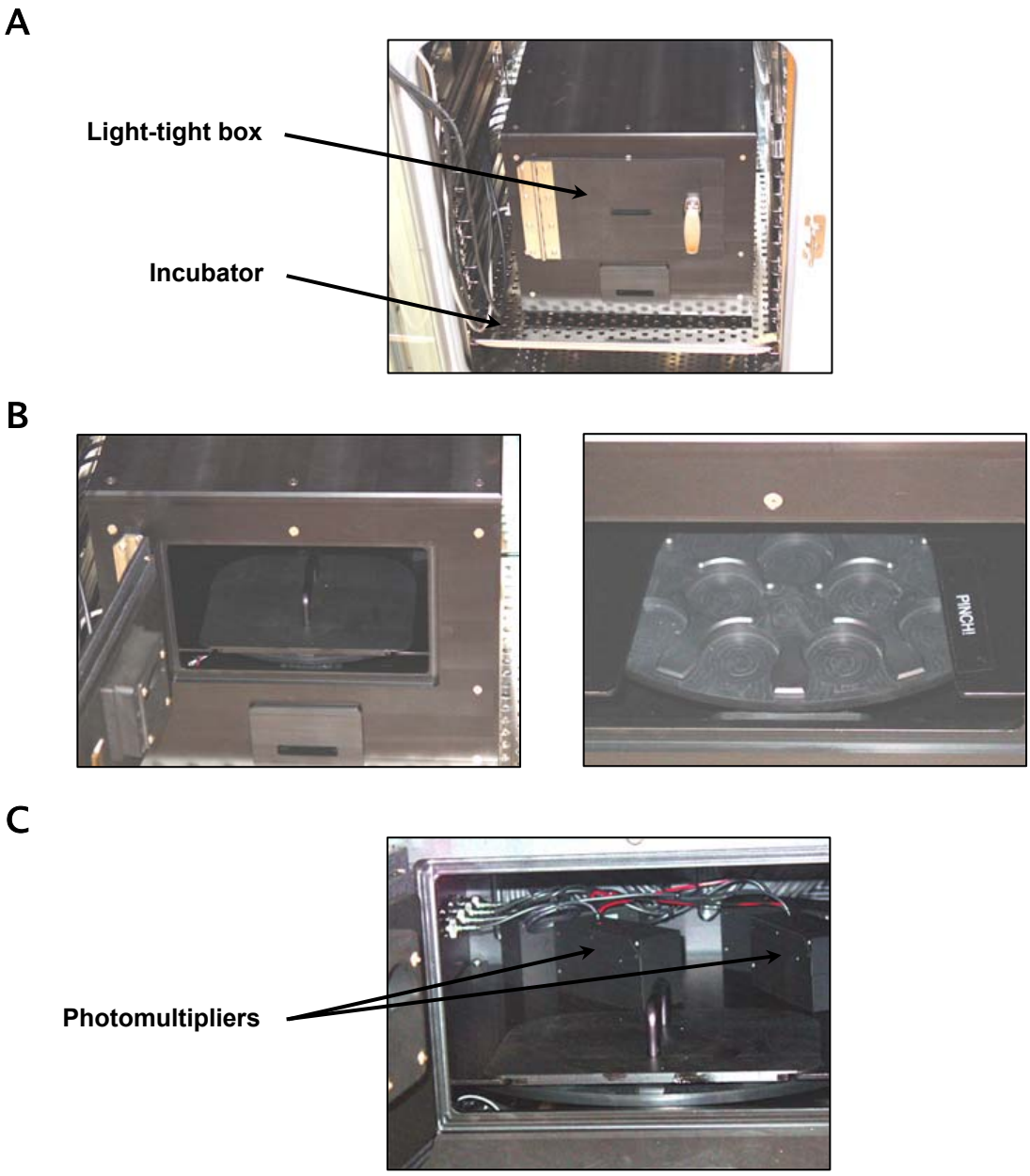

Figure 28. Real-time bioluminescence monitoring apparatus.

The real-time bioluminescence monitoring apparatus is constituted of a light-tight box placed in a standard air incubator (**A**). Inside the dark chamber, an automated turntable can receive 32 tissue or cell samples at a time, each in a 35 mm Petri dish (**B**). The chamber is equipped with 4 photon-counting photomutliplier tubes (**C**) that detect the emission of photons. Eight dishes share each photomultiplier and we set the apparatus so that the bioluminescence emitted from each dish is registered for 75 sec, every 10 min.

6.2. DATA ANALYSES

Acquired raw data were analyzed using the LumiCycle Data Analysis Software (Actimetrics). This software allows the user to visualize and analyze raw data. The LumiCycle analysis program first attempts to remove any baseline drift from the data. This baseline correction of the data is performed by fitting a polynomial curve to the data (Figure 29). The polynomial is of the order specified by the user. This number roughly corresponds to the number of inflections or bends that are allowed in the fitted curve and usually matches the number of days included in the data. The baseline fit curve obtained is subtracted from the raw data and the resulting baseline-subtracted data is then used by the software to determine the period. A chi-square periodogram analysis was performed on each baseline-subtracted data to establish whether or not there was a significant circadian rhythm. The estimation of the period was performed through a Fourier transform of the records: the software fits the data to a sine wave (Figure 29) multiplied by an exponential decay which can be written as follows:

$$L = A\sin(2\pi/P\text{-}t0)\times e^{-t/d}$$

where L is the bioluminescence in counts/sec, i.e., the photon counts emitted per one second, P is the period in hours, t0 is the phase in cycles relative to the start of the fitted portion of the data, A is the amplitude in counts/sec and d is the exponential damping time constant in days. The time points of the peaks and troughs of the baseline-subtracted data are fitted to the dominant sine wave and then fitted to an exponential decay. The damping rate was defined as the number of days necessary for the amplitude of the rhythm to decrease to 1/e (~36.79 %) of its initial value. Note that for oscillations with period different from 24 h, the phase parameter, which is the time of day during the first cycle at which the fitted curve crosses 0, has little meaning. Indeed, in that case, the zero-crossing time will be different for each cycle.

Figure 29. Bioluminescence data analysis.

A. The LumiCycle data analysis software removes the baseline drift from the data (white line) by fitting a polynomial curve (red line) to the data. The polynomial is of the order specified by the user and roughly corresponds to the number of days included in the data. In this example, 6 days of record are analyzed and the polynomial order has been set to 6.
B. The baseline fit curve obtained in A is subtracted from the raw data and the resulting baseline-subtracted data (white line) is then used by the software to determine the period. The software performs a Fourier transform of the data: the time points of the peaks and troughs of the baseline-subtracted data are fitted to the dominant sine wave and then fitted to an exponential decay (red line).
C. The Fourier transform of the data provides an estimation of the period, amplitude, damping rate and phase of the data.

RESULTS

D. RESULTS

1. DO JNK PARTICIPATE TO THE REGULATION OF THE MOLECULAR CLOCKWORK?

ERK, **p38^{MAPK}** and **JNK** are **rhythmically activated** in the **SCN** of Syrian hamster (Pizzio et al., 2003). Similarly, ERK and p38^{MAPK} present higher activity levels at night as compared to daytime in the **PG** of rats (Ho et al., 2003a; Chik et al., 2004). Up to now, the studies that focused on the functions that MAPK rhythmic activations may play in the molecular clockwork have mostly concerned ERK. It appears clear now that **ERK participate to the photic entrainment** by conveying the light-induced signal that leads to *Per1*/*2* gene expression in the SCN. An important indication of the role of the **SAPK** activation in the molecular mechanism of circadian clocks comes from a study performed on chick PGs (Hayashi et al., 2003). In birds and lower vertebrates, the PG constitutes a self-sustained circadian clock similar to the mammalian SCN. Hayashi and colleagues demonstrated that the chronic blockade of p38^{MAPK} activation in the pineal dramatically **lengthens the period of the cyclic circadian release of MEL**. This suggests a critical role of p38^{MAPK} in the **determination of the pace of the clock**. To investigate whether JNK might have a similar function, we altered JNK activation and analyzed the effects on the molecular clockwork.

1.1. PUBLISHED ARTICLE

C-JUN N-TERMINAL KINASE INHIBITOR SP600125 MODULATES THE PERIOD OF MAMMALIAN CIRCADIAN RHYTHMS

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Neuroscience (2007)

c-Jun N-TERMINAL KINASE INHIBITOR SP600125 MODULATES THE PERIOD OF MAMMALIAN CIRCADIAN RHYTHMS

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Neuroscience, 2007, Vol. 145, Pages 812–823

Pages 812 à 823 :

1.2. SUPPLEMENTAL UNPUBLISHED DATA

We have shown that a tight regulation of JNK activation level is necessary to determine the pace of the oscillations in the SCN and several peripheral oscillators. To complement these data, we investigated the roles of $p38^{MAPK}$ and ERK activation in the regulation of the molecular clockwork. We used our model of rat-1 fibroblasts stably transfected with the reporter construct *Period1-luciferase*. Following treatment with inhibitors that block $p38^{MAPK}$ or ERK activation, we recorded the *Period1*-driven bioluminescence oscillations in the real-time bioluminescence monitoring system.

The effects of the $p38^{MAPK}$ inhibitor SB203580 on the period of Period1–luciferase *rhythms in rat-1 fibroblasts*

To prevent $p38^{MAPK}$ phosphorylation and analyze if it is necessary to maintain normal circadian oscillations, we treated rat-1 fibroblasts with the $p38^{MAPK}$ inhibitor SB203580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole, Calbiochem) (see *section D1.1* for full protocol description). As compared to the control condition, the period length was significantly extended by 30 µM SB203580 ($P < 0.0005$, Table 2, Figure 30). Although the amplitude of the oscillations was affected by the SB203580 treatment ($P < 0.05$, Table 2), the damping rate was not changed ($P > 0.1$, Table 2).

Treatment	Period (h)	Amplitude (counts/sec)	Damping rate (days)
control	24.12 ± 0.07	161.20 ± 13.88	1.66 ± 0.08
SB203580 30 µM	33.83 ± 0.47 ***	66.38 ± 19.34 *	2.24 ± 0.39 *ns*
SB202474 30 µM	24.07 ± 0.15 *ns*	248.38 ± 16.66 *	1.61 ± 0.19 *ns*

Table 2. Period, amplitude and damping rate analyses of bioluminescence patterns in rat-1 fibroblasts treated with the $p38^{MAPK}$ inhibitor SB203580 and its inactive analogue SB202474.

ns, not significant; *, $P < 0.05$; ***, $P < 0.0005$.

The effect of SB202474 (4-Ethyl-2(p-methoxyphenyl)-5-(4′-pyridyl)-IH-imidazole, Calbiochem), an inactive analog of the $p38^{MAPK}$ inhibitor (30 μM) was further tested. The period of *Period1*-driven bioluminescence was not affected by the inactive inhibitor ($P > 0.1$, Table 2, Figure 30). Amplitude was significantly changed by the SB202474 treatment ($P < 0.05$, Table 2) but the damping rate stayed unchanged ($P > 0.01$, Table 2).

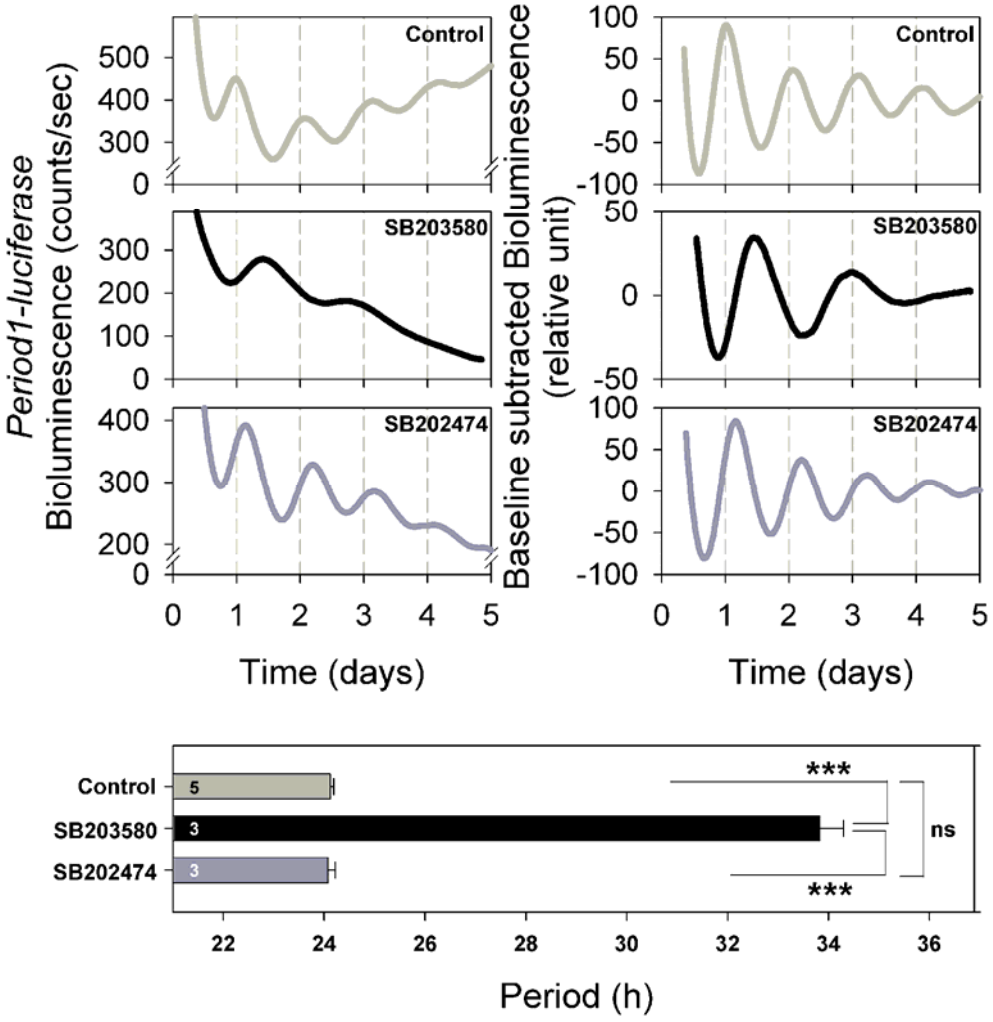

Figure 30. The effects of the $p38^{MAPK}$ inhibitor SB203580 on the period of *Period1-luciferase* bioluminescence rhythms in rat-1 fibroblasts.

Fibroblasts were synchronized by medium change and *Period1*-driven bioluminescence was recorded for several days. SB203580 (30 μM) extended the period of the bioluminescence rhythms. SB202474, an inactive analogue of SB203580 (30 μM) showed no significant effect. The left panels show the representative raw bioluminescence recordings and the right panels the baseline-corrected bioluminescence data. The columns in the lower panel show the estimated period length in each experimental condition. Values are means ± S.E.M. ***, $P <$ 0.005; ns, not significant. In all panels, the sample size is indicated in the columns.

The effects of the MEK inhibitor PD98059 on the period of Period1–luciferase *rhythms in rat-1 fibroblasts*

We treated rat-1 fibroblasts with the MEK inhibitor PD98059 (2′-Amino-3′-methoxyflavone, Calbiochem) to block ERK activation and investigate whether ERK phosphorylation is required to maintain circadian oscillations (see *section D1.1* for full procedure description). Two doses of MEK inhibitor were tested: 10 μM and 30 μM. In both conditions, no oscillations of bioluminescence were recorded, as opposed to control samples without inhibitor (Figure 31).

We then replaced the medium containing the MEK inhibitor by fresh medium devoid of PD98059 and examined whether oscillations were restored. In the presence of the inhibitor, no oscillations were recorded the first 3 days. After changing the medium on the fourth day, we monitored oscillations with a period of 23.95 ± 0.25 h (Figure 31).

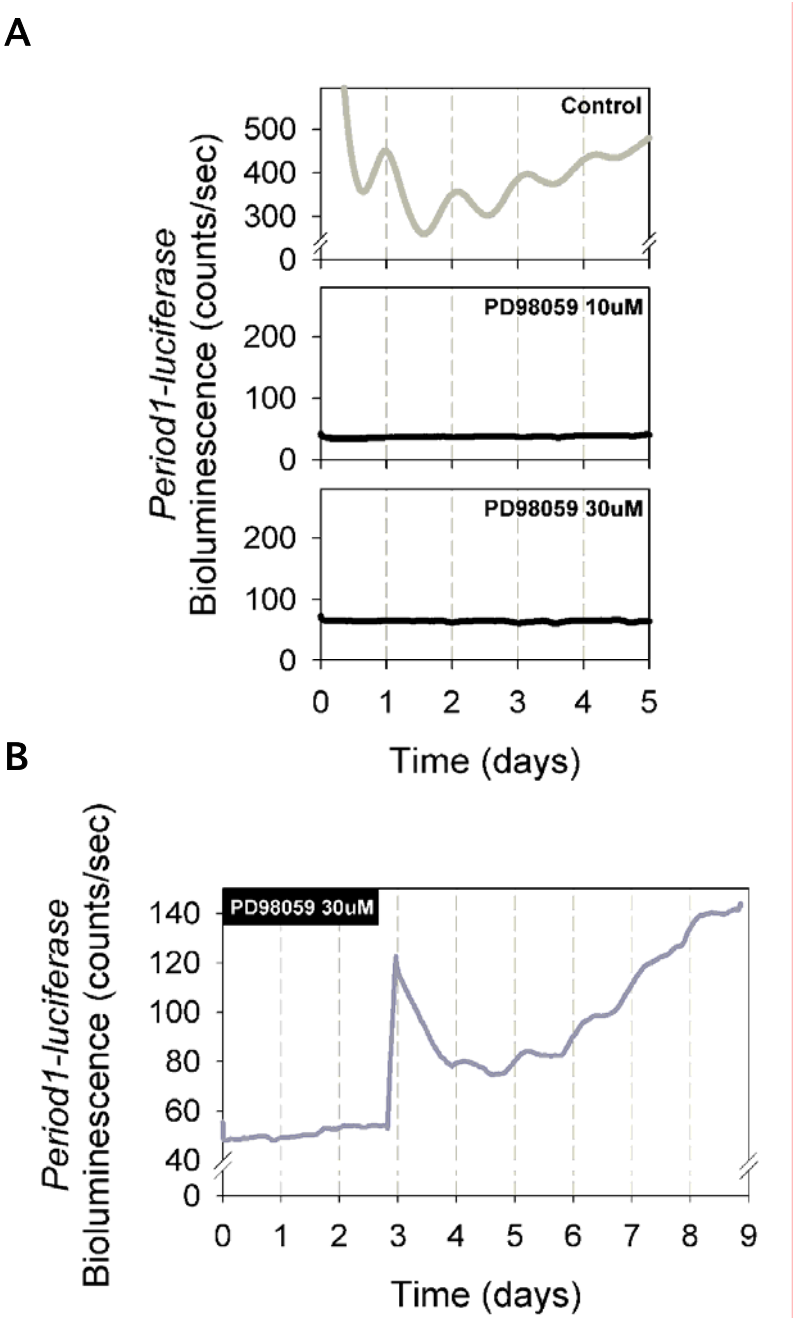

Figure 31. The effects of the MEK1 inhibitor PD98059 on the period of *Period1-luciferase* bioluminescence rhythms in rat-1 fibroblasts.

A. Fibroblasts were synchronized by medium change and *Period1*-driven bioluminescence was recorded for several days. No oscillations of the bioluminescence were recorded with both doses of PD98059 (10 μM and 30 μM).
B. When fibroblasts were initially treated for three days with PD98059 (30 μM, filled bar), no oscillations of the bioluminescence were recorded. Medium was replaced on the fourth day by assay medium without PD98059. After washout, robust oscillations of the bioluminescence could be monitored. (23.95 ± 0.25 h). n = 2. The panels show representative raw bioluminescence recordings for each condition.

2. DO SAPK REGULATE THE PINEAL GLAND PHYSIOLOGY?

The proper regulation of the clock genes-based apparatus of the SCN and peripheral oscillators appear essential to build up a time schedule that accurately spans 24 h. Nevertheless, the appropriate control of the mechanisms by which the time information is distributed within the organism is at least as much important. **MEL** is one major output of the SCN. The hormone is released each and every night by the PG in the bloodstream which allows it to reach every target tissue. The mechanisms of MEL regulation have been largely studied for years but it is only recently that MAPK implication has been suggested. Studies mostly performed by the group of Ho in the years 1999-2004 have shown that **ERK1/2 and p38^{MAPK} rhythmic activations are likely to modulate AA-NAT activity** levels during night-time.

At night, the SCN-driven NE release on the PG significantly changes the PG physiology to prepare the organ for MEL synthesis. Strikingly, NE signaling initiates the transcription of numerous genes including *Aa-Nat*, *Per1* and *Mkp-1* genes (Fukuhara et al., 2003). NE signaling also up-regulates the activity of ERK1/2 and p38^{MAPK} in the PG. Although no function of MKP-1 in the MEL synthesis has been revealed yet, MKP-1 is a DSP that may deactivate ERK1/2 and p38^{MAPK} and thus participate to the control of the AA-NAT activity. Since little is known on the roles of **JNK** in the PG, **we evaluated whether JNK isoforms are activated by NE and are mandatory to the NE-induced gene expression of *Aa-Nat*, *Per1* and *Mkp-1* in the PG**.

It should be noted that shortly after we initiated this study, the group of Ho published two related papers on MKP-1. They demonstrated that the transcription and translation of MKP-1 are quickly and largely induced by NE release at night (Price et al., 2004b) through the recruitment of both α_1- and β_1-AR (Price et al., 2004a). These data are consistent with the original work of Fukuhara and colleagues (2003) and support a role of MAPK signaling in the modulation of the pineal physiology.

REGULATION OF CAMP-INDUCED ARYLALKYLAMINE *N*-ACETYLTRANSFERASE, PERIOD1, AND MKP-1 GENE EXPRESSION BY MITOGEN-ACTIVATED PROTEIN KINASES IN THE RAT PINEAL GLAND

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Molecular Brain Research (2005) 139:333-340

Regulation of cAMP-induced arylalkylamine N-acetyltransferase, Period1, and MKP-1 gene expression by mitogen-activated protein kinases in the rat pineal gland

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Molecular Brain Research, 2005, Vol. 139, Pages 333 – 340

DISCUSSION AND PERSPECTIVES

E. DISCUSSION AND PERSPECTIVES

Studies performed during the past decade have greatly improved our understanding of the molecular mechanisms of the biological clock. Recent studies indicate that post-translational modifications of clock-relevant proteins seem to control the circadian system at different levels. These modifications implicate a series of protein kinases, including the multifunctional MAPK, as key components of the circadian system. However, the functions of the SAPK, especially JNK, are still largely unknown, as they have been less studied than other MAPK. The present study provides new evidence for the roles of JNK in the modulation of the circadian molecular machinery, and of SAPK ($p38^{MAPK}$ and JNK) in the regulation of the MEL synthesis, a major hormonal clock output.

1. ROLE OF JNK IN THE REGULATION OF THE CIRCADIAN MOLECULAR CLOCKWORK

Recent studies have demonstrated that rat-1 fibroblasts can be used as a model to study the molecular mechanisms of the circadian clock as they contain functional clocks similar to the SCN clock (Balsalobre et al., 1998; Balsalobre et al., 2000; Yagita et al., 2001; Izumo et al., 2003; Hastings, 2005). Therefore, we used rat-1 fibroblasts to investigate the role of MAPK in the regulation of the molecular clockwork. For this purpose, we utilized an elegant method by which bioluminescence levels driven by the promoters of *Per1* or *Per2* genes are monitored in real-time in rat-1 fibroblasts stably-transfected with a *Period1-luciferase* reporter construct, and in tissues of *Period1-luciferase*- and $Period2^{Luciferase}$- genetically modified mice.

1.1. A ROLE FOR EACH OF THE MAPK

1.1.1. THE THREE MAPK SUBFAMILIES ARE ACTIVE IN SEVERAL CLOCK/OSCILLATORS

Three main subfamilies of MAPK exist: ERK, $p38^{MAPK}$ and JNK. All MAPK are part of signal transduction modules, which are characterized by a cascade of phosphorylation of several kinases that eventually promotes MAPK activation. Importantly, activation occurs through phosphorylation of two specific phosphoacceptor sites located within the phosphorylation lip. If MAPK exert particular functions in the circadian system, they are likely to show particular patterns of phosphorylation. This has been previously studied for ERK1/2 and $p38^{MAPK}$ in several biological clock-related structures. ERK1/2 are rhythmically activated every night in the bullfrog retina (Harada et al., 2000), the chick PG (Sanada et al., 2000) and the rat PG (Ho et al., 2003a). However, other structures like the Syrian hamster SCN (Pizzio et al., 2003) and the mouse SCN (Obrietan et al., 1998) show rhythms with a maximum phosphorylation during the day. Interestingly, Nakaya and colleagues showed that the ERK1/2 phosphorylated levels may peak during the day in the shell of the mouse SCN while it peaks at night in the central part of the SCN (Nakaya et al., 2003). Additional investigations revealed that $p38^{MAPK}$ are diurnally phosphorylated in both the Syrian hamster SCN (Pizzio et al., 2003) and the rat PG (Chik et al., 2004), while they remain constitutively active in the chick PG (Hayashi et al., 2003). Recently, few studies noticed that JNK are diurnally phosphorylated in the Syrian hamster SCN (Pizzio et al., 2003) but constitutively active in *Xenopus* photoreceptor cells (Hasegawa & Cahill, unpublished observation). When we initiated this study, we first examined the JNK activation pattern in cultured rat-1 fibroblasts, our main model of study. We found that **p46 kDa and p54 kDa JNK isoforms are both acutely phosphorylated after synchronization of rat-1 fibroblasts, while only p46 kDa phosphorylation levels are rhythmic over the circadian cycle.** Remarkably, the absence of rhythmic activation of p54 kDa JNK does not preclude these isoforms from regulating the molecular clockwork as suggested by the role of $p38^{MAPK}$ in the regulation of the chick PG clock (Hayashi et al., 2003). There are indeed several possible cellular mechanisms by which MAPK pathways may regulate the circadian clock, such as rhythmicity in the protein kinase activity level, in substrate availability or in its subcellular localization. Although the amplitude of the p46 kDa rhythmic activation is

low in rat-1 cells, it is consistent with the limited diurnal activation reported in the SCN of Syrian hamsters (Pizzio et al., 2003).

1.1.2. JNK MODULATE THE PACE OF THE CIRCADIAN OSCILLATIONS

Because we found that JNK isoforms are phosphorylated in rat-1 fibroblasts, we decided to evaluate their role by preventing their activation. We used the ATP-competitive inhibitor SP600125, effective on all isoforms of JNK, to test the effects of suppression of JNK phosphorylation in *Period1-luciferase* rat-1 fibroblasts. The main result of our study is the demonstration that **inhibiting JNK activation results in a dramatic dose-dependent lengthening of the period length of *Period1*-driven bioluminescence rhythms.** Strikingly, a 30µM dose of SP600125 extends the period by more than 7 h. This effect strongly suggests that **JNK activation may be required to properly set the pace of the molecular clockwork**. Although rat-1 fibroblasts in culture represent a good model of oscillator, recent studies suggest that clocks/oscillators in different tissues and cells are unique and different from the SCN clock (*section A2.3*). It thus cannot be excluded that JNK implication might be tissue-specific. To evaluate this possibility, we cultured SCN slices from *Period1-luciferase* and *Period2*^{Luciferase} mice as well as pieces of lungs and kidneys, two organs known to contain an oscillator (Yoo et al., 2004). PGs were also tested, although their identification as an oscillator is still controversial (Takekida et al., 2000; Abe et al., 2002; Simonneaux et al., 2004; Fukuhara et al., 2005). Interestingly, **we identified a tissue-specific effect of the JNK inhibitor, which lengthened the period of *Period1*- and *Period2*-driven bioluminescence rhythms in the SCN, lungs and PGs, but not in the kidneys**. Instead, the effect of SP600125 on the kidney oscillator is of two kinds: it disrupts the *Period1-luciferase* oscillations, whereas it does not affect the *Period2*^{Luciferase} rhythms. It is thus likely that **JNK are implicated in the regulation of the pace of the molecular clockwork in some, but not all, clock entities**. It is remarkable that in the SCN, lungs and the PGs, the magnitude of the effect of SP600125 is similar to what has been described in the rat-1 fibroblasts. Although this result does not constitute *per se* the demonstration that the mechanisms involved in these various tissues are similar, it does not disqualify this hypothesis.

It should be noted here that our real-time bioluminescence data on the control *Period1*-driven expression in the PG of *Period1-luciferase* transgenic mice correlate

with previous *in vitro* results on transgenic rats (Abe et al., 2002) and mice (Fukuhara et al., 2005): *Period1*-directed bioluminescence oscillations are maintained for few cycles *in vitro* and eventually damp. Besides, our *Period2*-driven bioluminescence records in *Period2*^{Luciferase} mice constitute to my knowledge the first report of persistence of *Period2* transcription in the PG of mice *in vitro*. Together with the *Period1-luciferase* results, this provides new evidence to support the recognition of the PG as a regular oscillator, which most likely requires the SCN-driven NE release to synchronize rhythms among individual pinealocytes, *in vivo*. This correlates with a recent study showing that the night-time gating of the *Aa-Nat* transcription, a species-dependent phenomenon notably found in human and Syrian hamster (Vaughan et al., 1976; Vaughan & Reiter, 1987; Garidou et al., 2003), persists in mouse PG *in vitro* (Fukuhara et al., 2005), which suggests that it is likely to depend upon the PG oscillator. An additional study we performed in parallel to my PhD (*section G2.2*) demonstrates that this PG oscillator is likely to control the sensitivity of the PG to adrenergic stimulants through a control of the basal calcium levels within the PG (Chansard et al., 2006).

1.1.3. p38^{MAPK} CONTROL THE PERIOD OF THE CIRCADIAN OSCILLATIONS

Using our model of *Period1-luciferase* rat-1 fibroblasts, we evaluated whether p38^{MAPK} modulate the molecular clockwork as well. We demonstrated that **inhibiting p38^{MAPK} activation by chronically treating fibroblast cultures with the pyridyl imidazol inhibitor SB203580 significantly increases the period of the *Period1*-directed bioluminescence**. The lengthening of the period, by more than 9 h with a 30µM dose of SB203580, is not reproduced by treating rat-1 fibroblasts with SB202474, an inactive analogue of SB203580. These results strongly suggest that **p38^{MAPK} activation, like JNK activation, is involved in the determination of the period of the clock**. This is consistent with the conclusions of a previous study which showed that a chronic treatment with SB203580 lengthens the period of the clock that controls the rhythmic release of MEL in the chick PG (Hayashi et al., 2003). The similitude between these and our results not only validate our model of study, but it also supports a role for both SAPK activations in the control of the period length. Hayashi and colleagues reported that a 4-h pulse of SB203580 phase-dependently delayed the phase of the MEL release, with a maximum effect during the day. This suggested a time-of-day dependent effect of

$p38^{MAPK}$ on the molecular clockwork. A similar phase-dependent effect of SB203580 was observed in cultures of *Xenopus* photoreceptor layers after a 6-h pulse treatment (Hasegawa & Cahill, 2004). Interestingly, Hasegawa and Cahill noticed that a 6-h pulse of SP600125 at ZT6 induced a dose-dependent phase delay similar to the SB203580-induced phase-delay at the same time. Likewise, a 4-h pulse of SP600125 phase-delays the *Bmal1-luciferase* transcription in rat-1 fibroblasts (Yamagishi et al., 2006). This strengthens the possibility that **both SAPK modulate the molecular clock in a comparable manner.**

1.1.4. ERK1/2 MAY PARTICIPATE TO THE SYNCHRONIZATION OF SINGLE-CELL CLOCKS

We completed our study by evaluating the effect of MEK inhibition, and as a consequence ERK1/2 inhibition, on the circadian clockwork by treating rat-1 cells with the MEK1 inhibitor PD98059. Contrary to SAPK, we found that **ERK1/2 inhibition is likely to prevent the synchronization of rhythms among individual fibroblasts since no oscillation is recorded at the two doses tested**. Moreover, removal of the inhibitor *via* a medium change allowed us to record *Period1*-directed bioluminescence oscillations at the level of the dish. This suggests that the **ERK1/2 effect is reversible**. Medium change is thus able to synchronize *Period1*-driven bioluminescence oscillations among individual fibroblasts previously treated with PD98059. Our results are consistent with the finding that the MEK1/2 inhibitor U0126 prevents the resetting of *Per1*, *Per2* and *Dbp* expression by tissue plasminogen activator (TPA) in NIH3T3 fibroblasts when administered before synchronization. In contrast, U0126 has no effect when the treatment starts 8 h after synchronization (Akashi & Nishida, 2000), supporting that MEK1/2 are likely involved in synchronization of rhythms, but unlikely implicated in the circadian rhythm generation.

1.1.5. SPECIFICITY OF MAPK INHIBITORS

All the results discussed above on the probable role of MAPK in the regulation of the molecular clockwork were obtained by treating tissues with a series of MAPK inhibitors. These inhibitors were all **initially described as highly specific** towards their target

MAPK (Alessi et al., 1995a; Cuenda et al., 1995; Bennett et al., 2001). Although more recent studies confirmed the high specificity of PD98059 for MEK1 (Davies et al., 2000), **others have questioned the specificity of SAPK inhibitors**. First, a kinase assay investigation showed that SB203580 has no inhibiting effect on JNK (Davies et al., 2000), while Hasegawa and Cahill (2004) noticed that **SB203580 is able to inhibit both $p38^{MAPK}$ and JNK isoforms** in *Xenopus* photoreceptor cells. Hence, Hasegawa and Cahill suggested that the SB203580-induced effects on the phase of the MEL release may result from the inhibition of the two kinases subfamilies. The functional balance of $p38^{MAPK}$ and JNK activations would therefore determine the phase and the period of the clock.

Likewise, initial reports described SP600125 as a highly specific inhibitor for JNK activation (Bennett et al., 2001) whilst a recent kinase assay study pointed out that **SP600125 not only alters JNK activation but also affects several other kinases** with various efficiencies (Bain et al., 2003). Among them, some such as adenylyl monophosphate-activated protein kinases (AMPK), the dual-specificity tyrosine-phosphorylated and regulated kinases (DYRK), the serum- and glucocorticoid-induced kinases (SGK), and the p38 regulated/activated kinases (PRAK) are not known to have any implication in the molecular clockwork. Interestingly, the checkpoint kinases 1 (CHK1), which activity is down-regulated by SP600125, seems to interact with the mammalian TIMELESS clock protein (Unsal-Kacmaz et al., 2005). Mammalian TIMELESS is a homologue of the *Drosophila* TIMELESS and is believed to participate in the mammalian circadian rhythmicity (Barnes et al., 2003; Gotter, 2006). However, no effect of the kinase on the pace of the molecular clockwork is currently known.

Importantly, **SP600125 was found to inhibit CKIδ** that is implicated in the degradation and subcellular localization of PER and CRY clock proteins (*section A2.2.1.3*). There is some controversy on the role of CKIε/δ in the modulation of the dynamic of the circadian cycles. Initial studies revealed that a mutation in CKIε or CKIδ may decrease their activity, leading to a reduced phosphorylation and a decreased degradation of PER proteins, and eventually to a short-period phenotype (Lowrey et al., 2000; Toh et al., 2001; Xu et al., 2005). It is thus tempting to speculate that the potential decrease in the CKIδ activity following SP600125 treatment would lead to a short-period phenotype. But, we obtained opposite results and consequently inhibition of CKIδ activity may not be responsible for SP600125-induced period lengthening in our system. However, Takano and colleagues (2004) demonstrated that phosphorylation of PER1 by

CKIε facilitates nuclear entry of the PERs-CKIε/δ-CRYs complexes. By reducing CKIδ activity, SP600125 may thus impede the nuclearization of the multiprotein complexes which would likely slow down the circadian oscillator. From these results, we cannot rule out the possibility that the **JNK inhibitor's effects on the period lengthening of the *Period1*-luciferase transcription may result from an unspecific action of SP600125 on CKIδ**.

Besides CKIδ, **CDK2** that controls the cell cycle by participating to the transition from the G_1 phase towards the S phase (Malumbres & Barbacid, 2005), is inhibited by SP600125. Two reports showed that olomoucine, an inhibitor of this kinase, dose-dependently lengthens and phase-delays the period of the rhythm of the optic nerve activity in the eye of the marine snail *Bulla gouldiana* (Krucher et al., 1997) and the eye of the *Aplysia californica* (Sankrithi & Eskin, 1999). Those effects on the circadian oscillations are similar to what we reported using SP600125. Therefore, **an unspecific action of the JNK inhibitor on CDK2 cannot be excluded**.

Altogether, this information suggests that we cannot rule out the possibility that the lengthening effect of SP600125 that we reported may be mediated by the inhibition of several kinases and not only JNK. The slowing down of the oscillations would therefore be the result of either additive effects of the inhibition of JNK and other kinases, or a substantial change in the balance of JNK and other target kinases phosphorylations. To start clarifying this point, we attempted to demonstrate that as opposed to SP600125, an increase in JNK activity levels would accelerate the circadian clockwork. Our first strategy consisted in stably-transfecting *Period1-luciferase* rat-1 fibroblasts with recombinant JNK1 and/or JNK2 included in constitutive mammalian expression vectors. However, the results were not conclusive. JNK are implicated in many cellular functions and maintaining constitutively high levels of JNK led to abnormal phenotypes, such as elongation of the cytoplasm, expansion of the cell size and enlarged nuclei. Among the four cell lines over-expressing JNK1, only one was showing a normal phenotype. When recording *Period1*-directed bioluminescence oscillations in the LumiCycle, we found that the clock was ticking ~1 h faster than in control rat-1 fibroblasts. Moreover, two out of five cell lines over-expressing JNK2 isoforms exhibited a normal phenotype but the period was inconsistently affected: one cell line showed a shortening of the period by ~1 h while the other did not exhibit any significant change as compared to a control cell

line. The inconsistency of the results as well as the poor number of normal cell lines that could be tested convinced us to abandon this low-throughput strategy.

For our second approach, we tested the effects of valproic acid, an anticonvulsant used to treat bipolar disorder patients and known to affect the period of activity (Rietveld & van Schravendijk, 1987; Klemfuss & Kripke, 1995; Dokucu et al., 2005), on the *Period1*-driven bioluminescence rhythms in rat-1 fibroblasts. Next, we evaluated whether this effect was correlated to changes in JNK phosphorylation levels. Strikingly, we found that **valproic acid simultaneously shortens the period of the *Period1*-directed light emission rhythms and over-phosphorylates JNK isoforms**. Although these data do not provide conclusive evidence, they are consistent with the results obtained after JNK inhibition and support the role of JNK in modulating the pace of the molecular clockwork. It is noticeable that **our results represent the first demonstration of the valproic acid effects on the molecular clockwork**. Patients suffering from bipolar disorder, a disease characterized by cycling periods of mania and periods of depression, experience unstable circadian rhythms and valproic acid remarkably stabilizes their mood (Goodwin & Jamison, 1990; Tsujimoto et al., 1990; Jones et al., 2005). Because valproic acid improves the mood status of those patients, it may act through an action on the molecular clockwork, and our results thus provide a potential pathway for the actions of valproic acid.

Several experiments may be performed to strengthen our results and possibly bring a definite evidence for the involvement of JNK in the control of the pace of the clock.

- All of our data have been obtained from *in vitro* studies and it will be interesting to analyze whether JNK also set the pace of the circadian rhythms *in vivo*. One of the most convenient methods to analyze the effect of a given parameter on the SCN is to monitor the locomotor activity, a major output of the SCN. It is now well-described that the SCN are most likely controlling the locomotor activity through a diffusible signal (Silver et al., 1996; Kramer et al., 2001; Cheng et al., 2002). If the JNK inhibitor SP600125 alters the molecular machinery of the SCN, the locomotor activity would then be likely affected. In 2004, Iwahana and colleagues analyzed the effect of lithium, an inhibitor of GSK3 activation, on the circadian clock of mice. They added lithium in the food pellets and monitored a lengthening of the circadian locomotor activity in mice (Iwahana et al., 2004). For this experiment, we propose to infuse locally and chronically the small molecule SP600125 in the SCN using the

reverse microdialysis method. By monitoring the global locomotor activity during the time of administration, we would have a good estimation of the *in vivo* function of SP600125 in the SCN. The use of this technique would also allow us to evaluate whether SP600125 exhibits a phase-dependent effect on the period length. To this aim, 4-h pulses of SP600125 would be performed at various times of the circadian cycle. Evidently, this protocol could also be applied to SB203580 and PD98059 to evaluate the roles of $p38^{MAPK}$ and ERK1/2 *in vivo*.

- A weak point of our study is the use of inhibitors that are known to block JNK activation but which may also affect others kinases (Bain et al., 2003; *section E1.1*). The use of valproic acid which increases JNK phosphorylation and shortens the period of *Period1-luciferase* transcription supports a role for JNK isoforms. However, valproic acid-induced acceleration of the circadian cycles may result from an action of the anticonvulsant on additional kinases like GSK3 (Gould & Manji, 2002; Kim et al., 2005), which participate to the regulation of the locomotor activity in mice (Iwahana et al., 2004; *section A2.2.2.3*). An explicit evidence for the role of JNK would be obtained through the specific up-regulation or oppositely down-regulation of the JNK signaling pathways. To that aim, it would be advantageous to modify our JNK1/JNK2 over-expression approach (*see above*) and stably transfect rat-1 fibroblasts with recombinant JNK1 and/or JNK2 inserted in inducible mammalian expression vectors. JNK1 and/or JNK2 expression would be initiated several hours before the start of the experiment. This would prevent rat-1 fibroblasts from developing abnormalities due to the constitutively high levels of JNK. The complementary experiment where dominant negative forms of JNK1 and/or JNK2 embedded in inducible expression vectors are stably transfected to rat-1 fibroblasts would strengthen the results. Importantly, these experiments could indicate to which extent each of the JNK1 and JNK2 isoforms are implicated in the determination of the period length.

These and previous results thus indicate that MAPK all control the circadian molecular clockwork, although at different levels. Especially, ERK1/2 may be part of the signaling pathway that leads to the synchronization of rhythms among individual cells, while SAPK activity could control or at least modulate the beat of

the clock. What could be the potential mechanisms used by SAPK and more specifically JNK?

1.2. POTENTIAL MECHANISMS USED BY JNK TO MODULATE THE PERIOD LENGTH OF THE CIRCADIAN CLOCK

The pathways by which SAPK activations regulate the circadian clock are still unclear. Although Hayashi et al. (2003) showed that $p38^{MAPK}$ inhibition affects MAPKAPK2 activation levels but not ATF-2 or CREB phosphorylation, the authors could not conclude for a role of MAPKAPK2 in the $p38^{MAPK}$-mediated control of the chick PG clock. Multiple regulations allow the determination of the 24-h period, and among them the **transcriptional control** of clock genes and the **post-translational regulation** of clock proteins appear to be critical (*section A2.2.2*). JNK are thus likely to act at one or both of these levels.

1.2.1. FIRST HYPOTHESIS: CLOCK GENE TRANSCRIPTIONAL CONTROL BY JNK ISOFORMS

One of the main targets of JNK is c-JUN, a transcription factor that is activated through phosphorylation on Ser-63 and Ser-73 by JNK (Hibi et al., 1993). Together with other transcription factors like c-FOS, FRA-2 and ATF-2, c-JUN forms the AP-1 transcription activator complex, which regulates the expression of numerous genes *via* its interaction with the TRE binding sites located within promoters (Hess et al., 2004). *Per1* and *Per2* promoters contain TRE motifs and we can thus predict that **JNK might affect the pace of the *Period1-luciferase* and *Period2*^{Luciferase} transcription by controlling the transcriptional activity of the AP-1 complexes**. A clear evidence for a role of AP-1 complexes in *Per1*/*2* transcription is still missing, but the group of Sassone-Corsi indicated in a recent study that *Cry1a* gene transcription in zebrafish is regulated by the light-induced AP-1 activation (Hirayama et al., 2005). A decrease in JNK activation levels (directed by SP600125 treatment) would diminish the amount of AP-1 complexes which is believed to down-regulate *Per1* and *Per2* expression. The accumulation of PER proteins within the cytoplasm would therefore be slowed down, and the nuclearization of the PERs-CKIε/δ-CRYs protein complexes would be delayed, leading to period lengthening. Further experiments will support or invalidate this hypothesis. We cannot

rule out the possibility that other clock genes might be regulated by AP-1 complexes. Nevertheless, the experiments proposed below are designed to evaluate whether AP-1 complexes affect *Per1* or *Per2* transcription.

- First, to evaluate whether JNK isoforms modulate the period length through a control of the AP-1 complexes on *Per1* or *Per2* promoters, we could analyze the AP-1 binding activity. This could be achieved by performing an electrophoretic mobility shift assay (EMSA) using a double-strand oligonucleotide probe that contain a single TRE motif, and protein extracts from SP600125- or valproic acid-treated rat-1 fibroblasts. If our hypothesis is validated, AP-1 activity would be likely reduced in extracts from cells treated with the JNK inhibitor as compared to untreated cells, while it would be increased by a valproic acid treatment.
- Besides, if the pace of the circadian clock is regulated by a JNK-induced AP-1 control of *Per1* or *Per2* transcription, activation of c-JUN by JNK is likely to be an intermediate step between JNK and AP-1 activation. If this hypothesis is correct, the over-expression of c-JUN in rat-1 fibroblasts through an inducible mammalian expression vector would lead to an increased amount of AP-1 complexes and an increased *Per1* or *Per2* transcription. The accumulation of PER proteins and subsequently the nuclearization of the PERs-CKIε/δ-CRYs multiprotein complexes would be faster. In the LumiCycle, this would lead to a shortening of *Period1*- or *Period2*-directed bioluminescence rhythms. Conversely, an over-expression of a dominant-negative form of c-JUN that blocks c-JUN-controlled signaling pathways is likely to extend the period of the oscillations. The implication of c-JUN in JNK control of the period length would be strongly strengthened if a SP600125 treatment counterbalanced the effect of the c-JUN over-expression in transfected rat-1 cells.
- An additional way to investigate whether AP-1 control of *Per1* or *Per2* transcription is involved would be to stably transfect rat-1 fibroblasts with a *Period1*- or *Period2*-*luciferase* construct in which TRE motifs of the *Per1* or *Per2* promoters are selectively mutated (*ΔTRE-Period1* or *ΔTRE-Period2*). Upon treatment of these rat-1 cell cultures with either SP600125 or valproic acid, it would be possible to record *ΔTRE-Period1*- or *ΔTRE-Period2*-driven bioluminescence rhythms in the LumiCycle and determine which of the TRE, or how many TRE, is responsible for the lengthening or the shortening of the period. In the case none of the TRE appears to mediate the effects of SP600125 and valproic acid, it would be possible to extend the

study to other transcriptional regulatory motifs involved in the expression of *Per1* and *Per2*, like E-box elements and CRE motifs (*section A2.2.2.1*).

1.2.2. SECOND HYPOTHESIS: POST-TRANSLATIONAL CONTROL OF CLOCK PROTEINS BY JNK ISOFORMS

In addition to the transcriptional regulation of clock genes, **JNK might regulate the pace of the clock at the post-translational level**. Recent studies suggested that several protein kinases, including CKIε/δ, GSK3α/β and ERK1/2, help to define the 24-h period by phosphorylating specific clock proteins, and therefore **regulating their stability and subcellular localization** (*section A2.2.2.3*). Considering that the MAPK ERK1/2 interact with and phosphorylate BMAL1 and CRY1/2 clock proteins, I suspect that **JNK isoforms may phosphorylate clock proteins** as well. However, no evidence of such an interaction is found in the literature.

- The identification of the clock protein targeted by JNK isoforms could be achieved through an immunoprecipitation experiment. The lysates from rat-1 fibroblasts subjected to a medium change would be immunoprecipitated with JNK antibodies and subsequently immunoblotted with clock proteins antibodies. The reverse experiment, where the antibody for the clock protein candidate is used to immunoprecipitate protein complexes and JNK antibodies are used to detect these associations, would support the initial results. Besides, the interaction between JNK isoforms and the clock protein candidate could be searched in other oscillators like the liver, the PG or the kidney to evaluate whether JNK-clock protein candidate association is ubiquitous. Considering that SP600125 does not have the same effect on kidney that it does on other oscillators, we can hypothesize that JNK may have a different partner in that tissue. Alternatively, the clock protein candidate may be the same, which would imply different downstream mechanisms.
- Once the clock protein candidate is identified, we could evaluate whether JNK isoforms phosphorylate this protein. A kinase assay could be performed on immunoprecipitated clock protein candidate from rat-1 cells. Using a recombinant JNK and radiolabeled ATP, we would be able to detect any JNK-induced phosphorylation. Strikingly, the comparison between a control assay and an assay performed in the presence of either SP600125 or valproic acid should reveal a lower and higher phosphorylation respectively.

Several studies indicated that GSK3α/β (Harada et al., 2005; Iitaka et al., 2005) and CKIε/δ (*section A2.2.1.3*) control the period length by modulating the **degradation** of clock proteins which regulates their accumulation, and by regulating the **nuclear entry** of clock proteins. Several experiments can be considered to evaluate whether JNK-mediated phosphorylation of the clock protein candidate affect the pace of the oscillations by such means.

- Harada and colleagues (2005) revealed that the GSK3β-mediated phosphorylation of Ser-557 of CRY2 promotes CRY2 degradation by the 26S proteasome. GSK3β is thus believed to participate to the CRY proteins clearance prior to the start of a new cycle. We can hypothesize that JNK-induced phosphorylation of the clock protein candidate could stabilize the protein and prevent it from being degraded. The candidate clock protein would then be able to be nuclearized and affect clock gene transcription in the nucleus. In the presence of SP600125, the defect in the candidate phosphorylation would lead to its degradation by the proteasome and an apparent delay in its nuclearization. This could support the lengthening effect of the JNK inhibitor on the circadian period. To assess whether JNK affect the stability of the candidate, we could carry out an assay where the immunoprecipitated candidate protein is incubated with or without a recombinant JNK in the presence or absence of the 26S proteasome inhibitor MG132, or the proteasome inhibitor clasto-lactacystin β-lactone which blocks the catalytic core (20S proteasome) of the 26S proteasome. An additional condition where the JNK inhibitor SP600125 is added to the recombinant JNK should provide a clear indication for the role of JNK-mediated phosphorylation on the stability of the candidate protein.
- A recent study revealed that CKIε promotes the nuclearization of PER1 proteins through their phosphorylation at Ser-661 and Ser-663 (Takano et al., 2004). I propose that the JNK modulation of the period of the clock might take place at the level of the nuclearization of the clock protein candidate as well. To verify this hypothesis, vectors containing a recombinant JNK or an empty vector could be co-transfected with a vector expressing the candidate protein in rat-1 cells. The same experiment could be performed in the presence of the JNK inhibitor SP600125 and we could evaluate differences in the candidate nuclearization by immuno-histochemistry directed against the candidate protein. If the hypothesis is validated, JNK would promote nuclearization. On the contrary, SP600125 treatment would block it which

would be likely to participate to the slowing down effect of the inhibitor on the molecular clockwork.

The first part of this study allowed the characterization of JNK as a likely regulator of the period of the circadian clock. This new finding completes ours and previous results that showed that p38^{MAPK} and ERK1/2 MAPK subfamilies also participate in the control of the molecular clockwork. Although this work does not provide any evidence related to the mechanisms involved in JNK effect, we hypothesized that JNK isoforms might affect either the transcription of clock genes or the degradation or nuclear entry of some clock proteins targets. We proposed several experiments to validate or refute these hypotheses.

2. ROLE OF SAPK IN THE REGULATION OF THE MEL SYNTHESIS

Over the years, it has become clear that MAPK are involved in numerous cellular functions, ranging from gene expression, growth and differentiation, to cell survival and death (Barr & Bogoyevitch, 2001; Pearson et al., 2001). The highly interactive nature of the MAPK signaling cascades as well as the diversity in MAPK targets correlate with their omnipresent function within cells. Remarkably, we and others found that MAPK are involved in the regulation of several aspects of the circadian system. Our data discussed in the previous section (*section E1*) suggest that JNK, like p38^{MAPK}, participate to the control of the pace of the clock and we proposed that JNK may act either at the transcriptional level or the post-translational level. Interestingly, recent studies reveled that MAPK, especially ERK1/2 and p38^{MAPK}, also participate to the modulation of the PG physiology by modulating the activity of the AA-NAT, the penultimate enzyme in the MEL synthesis pathway (*section A2.4.3.3*). We intended to determine new functions for SAPK, especially JNK, in the PG.

2.1. JNK CAN BE ACTIVATED IN THE PG

Recent studies revealed that ERK1/2 and $p38^{MAPK}$, are activated in the PG by a NE stimulation. Although the ERK1/2 activation depends upon a NE-cGMP pathway (Ho et al., 1999), $p38^{MAPK}$ activation is directed by a NE-cAMP pathway (Chik et al., 2004). In rodents, this latter one is essential to promote the expression of enzymes that contribute to the MEL synthesis (*section A2.4.3.3*). We thus investigated whether JNK activation, which had not been studied yet, might be under the control of a NE-cAMP signal, similar to the related $p38^{MAPK}$. We found that **JNK are indeed transiently phosphorylated by a cAMP analogue in rat pinealocytes**. Our results show some differences with the activation pattern of $p38^{MAPK}$ in the PG: **JNK activation peaks 20 min after cAMP analogue stimulation** while $p38^{MAPK}$ phosphorylation is still elevated 4 h after the stimulation (Chik et al., 2004). These results suggest **JNK and $p38^{MAPK}$ activations in the PG may serve different functions**. In the PG, $p38^{MAPK}$ activation by NE requires the recruitment of the GPCR α_1- and β_1-AR and the implication of Ca^{2+}, cAMP and PKA (Chik et al., 2004). Interestingly, the mechanisms of $p38^{MAPK}$ and JNK activation in various system show some similarities in that they can both be phosphorylated upon GPCR recruitment (Kyriakis & Avruch, 2001). It thus seems possible that JNK isoforms activation in the PG follows the night-restricted NE release and necessitates both α_1- and β_1-AR *in vivo*. Moreover, the cAMP-dependent protein kinase PKA may mediate JNK activation since it is able to activate TAK1, a MAPKKK upstream of JNK (Kyriakis & Avruch, 2001; Kobayashi et al., 2005). Detailing the JNK activation pathway may clarify the roles of JNK isoforms in the PG.

- The immunodetection of phosphorylated levels of JNK by Western blot following the treatment of rat pinealocytes or rat PG with a large set of chemicals would allow the identification of intermediates that lead to JNK activation in the PG. At first, it would be interesting to evaluate whether NE is able to induce JNK activation as it does for $p38^{MAPK}$. The use of specific α_1- and β_1-AR agonists (phenylephrine, isoproterenol) and antagonists (prazozin, propranolol) would identify which receptors are likely involved. It would also enable to evaluate whether the recruitments of both receptors potentiates JNK activation as compared to the enrollment of individual subtypes, as it has been suggested for $p38^{MAPK}$ activation. The identification of downstream intermediates would be achieved by treating the pinealocytes or the PG organ culture with various chemicals, such as a cGMP analogue, ionomycin that increases the level

of Ca^{2+}, and inhibitors of PKA, PKG, protein kinase C and CaMK. To complement this study, it would be interesting to consider the activation levels of several of the upstream kinases which could lead to JNK activation, like MAPKK4, MAPKK7 and TAK1 (*section A1.4.1.2*).

- Another important feature of MAPK in the mammalian PG is their rhythmic pattern of phosphorylation. This has been demonstrated for ERK1/2 (Ho et al., 2003a) and $p38^{MAPK}$ (Chik et al., 2004) which exhibit low activation levels during the day and high levels at night in the rat PG. This is likely correlated to the SCN-driven nocturnal NE release. If it is demonstrated that JNK are activated by a NE stimulation *in vitro*, JNK would be likely rhythmically activated. This could be demonstrated by performing Western blotting on rat PG extracts obtained from rats housed under a LD and DD light regimen and sacrificed at various time over 24 h.

2.2. $p38^{MAPK}$ AND JNK CONTROL THE GENE TRANSCRIPTION IN THE PG

The role of the ubiquitous ERK1/2 and $p38^{MAPK}$ in the physiology of the PG has been studied. Ho and colleagues revealed that the NE-induced activation of ERK1/2 at night (Ho et al., 1999; Ho & Chik, 2000; Ho et al., 2003a) is likely stimulating AA-NAT activity since the specific MEK1 inhibitor U0126 inhibits AA-NAT activity in rat pinealocytes (Ho et al., 2003b). Besides, a similar inhibition of $p38^{MAPK}$ activation (Chik et al., 2004) by the pyridinyl imidazole inhibitor SB202190 enhanced the long-term NE-stimulated AA-NAT activity which suggests that $p38^{MAPK}$ activation may limit the activation of AA-NAT *in vivo* (Man et al., 2004). Interestingly, the *Aa-Nat* transcription was not affected by $p38^{MAPK}$ inhibition and Man et al. proposed that the kinase may either up-regulate the degradation of the AA-NAT or decrease its translation. Hence, these initial results indicated that **ERK1/2 and $p38^{MAPK}$ modulate the time profile of the NE-stimulated MEL synthesis**.

The nocturnal NE release on the PG also drives the expression of a large set of genes (Humphries et al., 2002; Fukuhara et al., 2003). Several of these genes may be involved in the control of the MEL synthesis. This is the case of the well-described *Aa-Nat* which codes for the MEL rhythm-generating enzyme and is largely expressed at night (Baler et al., 1997). Microarray studies showed that *Mkp-1* expression is also higher at night than

during the day (Humphries et al., 2002; Fukuhara et al., 2003). Like *Aa-Nat*, *Mkp-1* is under the control of the photoneuronal system in the PG through a mechanism involving both α_1- and β_1-AR, and cAMP signaling (Price et al., 2004a; Price et al., 2004b). Although a clear demonstration is still missing, the DSP MKP-1 is likely to modulate the MEL synthesis by its control on the ERK1/2 and $p38^{MAPK}$ phosphorylation levels. In addition to *Aa-Nat* and *Mkp-1*, the PG, which is likely to be an oscillator, express the clock gene *Per1*. Remarkably, *Per1* is potently increased by adrenergic receptors at night (Fukuhara et al., 2000; Takekida et al., 2000; Fukuhara et al., 2002; Simonneaux et al., 2004).

Our results on cAMP-induced JNK activation in the PG discussed previously (*section E2.1*) suggest that **the activity of the three subfamilies of MAPK may be up-regulated upon NE stimulation**. Since NE acts on both gene transcription and MAPK activation, we wondered whether MAPK and more specifically SAPK, may participate to the NE-cAMP-induced gene expression in the PG. Our results first demonstrated that **JNK activation is mandatory to the cAMP-induced *Aa-Nat*, *Mkp-1* and *Per1* expression**, since the JNK inhibitor SP600125 strongly reduces cAMP-directed expression of these genes. On the contrary, the $p38^{MAPK}$ inhibitor SB203580 only reduced *Mkp-1* expression which suggests that in addition to JNK, **$p38^{MAPK}$ is required for *Mkp-1* transcription**. The finding that *Aa-Nat* expression is not affected by $p38^{MAPK}$ inhibition mediated by SB203580 is consistent with the previous demonstration that another pyridinyl imidazole inhibitor, SB202190, has no effect on *Aa-Nat* transcription (Man et al., 2004).

2.2.1. JNK control Aa-Nat, Mkp-1 and Per1 expression

It is remarkable that the **timing of JNK activation** is consistent with a role of the kinase in the control of *Aa-Nat*, *Mkp-1* and *Per1* expression. Indeed, JNK activation peaks 20 min after cAMP stimulation *in vitro* while consequently, *Mkp-1*, *Per1, and Aa-Nat* mRNA levels start to rise (Takekida et al., 2000; Fukuhara et al., 2002; Price et al., 2004b; Ho et al., 2006). It is thus likely that the gene transcription begins only when JNK activation, possibly mediated by the cAMP-PKA pathway (*section E2.1*) (Chik et al., 2004), reaches sufficient levels. As suggested earlier for the JNK control of the pace of

the circadian clock (*section E1.2.1*), JNK could positively regulate *Aa-Nat*, *Mkp-1* and *Per1* transcription through the modulation of the activity of transcription factors.

2.2.1.1. First hypothesis: control of gene transcription by AP-1 or SP1 activation.

The complex **AP-1** is a potential candidate since it is one of the main downstream targets of JNK in many systems (*section A1.4.2*). Interestingly, the promoters of *Aa-Nat* (Baler et al., 1997), *Mkp-1* (this study) and *Per1* (this study) all contain at least one **TRE**. In addition, rhythmic AP-1 binding activity has been shown in the rat PG with a zenith at night, supporting a possible role of AP-1 in the control of the nocturnal gene transcription (Guillaumond et al., 2000). If this hypothesis is correct, **both AP-1 and CREB may be required to promote the transcription of *Aa-Nat*, *Mkp-1* and *Per1***. It suggests that transcription would not occur without JNK phosphorylation and subsequent c-JUN and AP-1 activation.

Alternatively, another transcription factor, **SP1** (Chu & Ferro, 2005), **may be recruited by JNK to regulate *Aa-Nat*, *Mkp-1* and *Per1* gene expression**, which promoters all contain SP1 binding sites (GC-rich domains) (Baler et al., 1997; Hida et al., 2000; Ryser et al., 2004). Several recent studies revealed that JNK isoforms are able to activate SP1 in some mammalian systems. Moreover, a JNK inhibitor SP600126 treatment prevents the phosphorylation of SP1 (Higuchi et al., 2004; Chu & Ferro, 2006).

Although these hypotheses are attractive and fit with the SP600126-induced blockade of the cAMP-stimulated gene transcription we reported, several results may invalidate them. First, the **timing of AP-1 activation** in the PG may not support an *in vivo* effect of AP-1. Indeed, both c-JUN protein levels (Guillaumond et al., 2000; Sinitskaya et al., 2006) and AP-1 binding activity (Guillaumond et al., 2000) are still low an hour after the beginning of the subjective night, when gene transcription is initiated *in vitro*.

Besides, an *in vitro* study revealed that the presence of a **protein synthesis inhibitor does not prevent the NE-induced *Aa-Nat* synthesis in the rat PG**, suggesting that the translation of transcription factors, such as AP-1 and SP1, is not necessary to promote *Aa-Nat* transcription *in vitro* (Fukuhara et al., 2002) and *in vivo* (Sinitskaya et al., 2006). Similarly, ***Per1*** transcription is not inhibited by a protein synthesis inhibitor (Fukuhara et al., 2002).

Nevertheless, no data in the literature seem to invalidate a possible involvement of newly-synthesized transcription factors in ***Mkp-1*** expression, and it can be assumed that **SP1 could regulate *Mkp-1* transcription**. This implies that the transcriptional regulation of *Aa-Nat*, *Per1*, and *Mkp-1* may be distinct. Further experiments will determine whether *Mkp-1* might be regulated by SP1.

- At first, it would be necessary to test whether *Mkp-1* transcription is controlled by newly-synthesized transcription factors. To this aim, rat PGs in culture could be pre-incubated with a protein synthesis inhibitor, such as cycloheximide, and further stimulated with NE for several hours. At determined intervals, PGs would be harvested, and the level of *Mkp-1* mRNA could be tested by Q-PCR.
- If *Mkp-1* transcription is affected by cycloheximide, we could attempt to identify if SP1 is involved. I propose to block *Sp1* expression using siRNA-bearing adenovirus directed against *Sp1* mRNA of cAMP-stimulated rat pinealocytes in culture. Using the Q-PCR technique, we would be able to determine the effect of the blockade of SP1 translation on *Mkp-1* expression levels. If SP1 are involved, their blockade should reduce cAMP-induced gene transcription.

It should be noted that as opposed to the rat model, it has been shown that neosynthesized transcription factors are needed to promote the transcription of *Aa-Nat* in the Syrian hamster. A protein synthesis inhibitor administered prior to the light offset indeed blocks *Aa-Nat* transcription at night (Garidou et al., 2003). It would be interesting to evaluate whether JNK activation in the Syrian hamster PG participate in the stimulation of newly-synthesized transcription complexes to promote *Aa-Nat* expression in this species.

2.2.1.2. Second hypothesis: control of gene transcription through CREB phosphorylation

Aa-Nat (Baler et al., 1997), and possibly *Per1* (Travnickova-Bendova et al., 2002), transcription in the PG require phosphorylation of the widely expressed cAMP-dependent transcription factor CREB, which initiates gene transcription through promoter activation at the level of CRE motifs. *Mkp-1* promoter also contains two CRE motifs (Ryser et al., 2001; Ryser et al., 2004). Most studies, including the one performed on the circadian system (Obrietan et al., 1998; Obrietan et al., 1999; Gau et al., 2002),

demonstrated that CREB becomes activated upon phosphorylation at Ser-133. However, few studies indicated that Ser-133 phosphorylation may not be sufficient to promote CREB-mediated transcription and that phosphorylation of additional phosphoacceptor sites may be functionally relevant (Bito et al., 1996; Deisseroth & Tsien, 2002). Remarkably, Gau et al. (2002) demonstrated that phosphorylation of CREB on both Ser-133 and Ser-142 is required for appropriate light-induced phase-shifts in mice. Hence, **it is possible that the cAMP-mediated gene transcription of some genes necessitates the phosphorylation of CREB at multiple sites**. I propose that **cAMP-activated PKA phosphorylate CREB at Ser-133** (Roseboom & Klein, 1995) **while cAMP-activated JNK might phosphorylate the transcription factor on an additional site**. Since both phosphorylations would be required to permit gene expression, the inhibition of JNK activation by SP600125 would prevent CREB-mediated transcription, which is consistent with our results.

- The relationship between JNK isoforms and CREB has been little studied and the results are not consistent in the literature. Depending on the system, JNK have no effect (Bebien et al., 2003) or down-regulate CREB phosphorylation (Vaishnav et al., 2003). To evaluate whether JNK is involved in the regulation of CREB phosphorylation, rat PG or pinealocytes in culture should be stimulated by a cAMP analogue with or without the JNK inhibitor SP600125. An analysis by Western blot, using antibodies for the known phosphoacceptor sites of CREB (Kornhauser et al., 2002), would determine whether JNK activate CREB phosphorylation.
- After the demonstration that JNK activate CREB and the identification of the targeted phosphoacceptor site(s), it will be necessary to evaluate whether this JNK-mediated phosphorylation of CREB is mandatory for *Aa-Nat*, *Mkp-1* and *Per1* expression. A recombinant mutated form of CREB, with a substitution of the phosphoacceptor site(s), could be constitutively expressed in transfected rat pinealocytes. Few hours after cAMP treatment, a Q-PCR analysis would determinate whether the amino-acid substitutions which prevented the JNK-mediated phosphorylation of CREB affected the expression of *Aa-Nat*, *Mkp-1* and *Per1*. The same experiment performed with a recombinant CREB leading to a substitution of the Ser-133 site would provide useful information on the implication of each phosphorylation site on the CREB-mediated transcription.

2.2.2. p38^{MAPK} CONTROL MKP-1 EXPRESSION

Our results indicate that **in addition to JNK isoforms, the transcription of *Mkp-1* requires the activation of p38^{MAPK}**. This result was surprising, considering that p38^{MAPK} activation by cAMP is slow and peaks 2-3 h after cAMP analogue stimulation (Chik et al., 2004). In fact, *Mkp-1* expression peaks 1 h after stimulation (Price et al., 2004b) when activated p38^{MAPK} levels only start to rise (Chik et al., 2004; Ho et al., 2006). It suggests that a slight increase in the p38^{MAPK} activation is sufficient to promote *Mkp-1* expression. It should be noted however that a recent study challenges our results by showing that SB203580 does not affect NE-induced *Mkp-1* expression in rat pinealocytes (Price et al., 2007). cAMP is situated downstream of NE in the signaling cascade that leads to *Mkp-1* expression and the results of Price and colleagues suggest that an additional intermediate activated by NE may substitute for p38^{MAPK}-mediated regulation of *Mkp-1*.

The mechanisms by which p38^{MAPK} promote *Mkp-1* transcription are unknown. Like JNK, p38^{MAPK} are likely to exert their effect through the modulation of the activity of transcription factors. The specificity of the regulation of *Mkp-1* by p38^{MAPK} suggests that the transcription factors implicated are likely different from the ones recruited by JNK. The identification of the transcriptional regulator involved will require several steps.

- At first, it will be necessary to identify the region of the promoter that is likely involved in the p38^{MAPK}-mediated control of *Mkp-1* expression. To that aim, different length of *Mpk-1* promoter fragments (Ryser et al., 2001; Ryser et al., 2004) will be subcloned in a luciferase reporter plasmid transfected to rat pinealocytes. Upon stimulation with a cAMP analogue, the expression of the luciferase reporter will be assayed. The comparison between SB203580 treated and non-treated samples should allow the determination of a shorter region responsible for the p38^{MAPK}-induced *Mkp-1* transcription.
- Several potential transcription factors binding sites have been identified in the rat *Mkp-1* promoter (Ryser et al., 2001; Ryser et al., 2004). Among them, AP-1 (Kyriakis, 1999), SP1 (D'Addario et al., 2006), and E2F (Wang et al., 1999) may be regulated by p38^{MAPK} in various systems. After determining the potential transcription factors binding sites localized within the main regulatory fragment of the *Mkp-1* promoter, targeted mutagenesis could be performed to disrupt specifically each of these sites. The reporter plasmids made with these different mutated

fragments could be transfected to rat pinealocytes and a luciferase assay following cAMP analogue stimulation should identify the specific transcription factor mediating the $p38^{MAPK}$ control of *Mkp-1* expression.

The second part of our study supports a role for the activated SAPK in the regulation of the cAMP-induced transcription of several genes in the PG. Because AA-NAT, and possibly MKP-1, regulate the MEL synthesis, the control of *Aa-Nat* and *Mkp-1* gene expression by JNK and $p38^{MAPK}$ suggests that both SAPK might affect the MEL synthesis. Moreover, the distinct regulations of *Mkp-1* transcription, as compared to *Aa-Nat* and *Per1*, suggest that JNK and $p38^{MAPK}$ functions may be distinct. The experiments proposed may permit to identify the signaling pathways involved.

3. CONCLUSIONS.

The critical roles of MAPK are well-accepted in many systems. However, their implication in the circadian system has only started to be elucidated. The circadian organization developed by most organisms to anticipate and adapt to the changes in environmental conditions, is characterized by three major components: **1)** an **afferent system** that allows the **synchronization** of the clock/oscillator to an exact period of 24 h, **2)** a **self-sustained endogenous circadian clock/oscillator** which oscillates with an **approximate 24-h period**, and **3)** an **efferent system** which **distributes** the circadian information to defined targets. In the case of the SCN clock, the afferent pathways are mostly constituted of photic inputs, and the control of the MEL synthesis in the PG represents a main efferent pathway. Strikingly, our and previously published results indicate that **MAPK are likely to regulate the circadian system at the three organizational levels**.

- Initial studies on MAPK demonstrated that ERK1/2 are implicated in the photic synchronization of the SCN clock. Remarkably, ERK1/2 are over-phosphorylated upon light exposure, or treatment with its neurotransmitter GLU, at times where the light triggers behavioral phase-shifts (Obrietan et al., 1998; Butcher et al., 2002; Dziema et al., 2003), and ERK1/2 inhibition prevents these light-induced phase-shifts

in vivo (Butcher et al., 2002). Additional studies on the chick PG, which also contains a circadian clock, support a role of ERK1/2 in the photic entrainment by demonstrating that blocking the night-induced ERK1/2 activation in the PG induces a phase-delay in the rhythmic release of MEL (Sanada et al., 2000; Hayashi et al., 2001). In addition to the photic entrainment, ERK1/2 also seem to synchronize rhythms within individual cells. This has been initially suggested *in vitro* in NIH3T3 fibroblasts in culture that fail to exhibit synchronized rhythmic patterns of clock genes when the synchronizing cue is administered in the presence of a MEK1/2 inhibitor (Akashi & Nishida, 2000). Our data confirm these results by showing that ERK1/2 inhibition prevents the synchronization of *Period1*-driven bioluminescence oscillations among individual rat-1 fibroblasts. Altogether, these results support a role of **ERK1/2 in the afferent system that allows entrainment and synchronization** of circadian clock and oscillators.

- Recently, it has been demonstrated that $p38^{MAPK}$ participate to the definition of the period of the MEL rhythmic release from the chick PG (Hayashi et al., 2003). Our data not only confirm this role of $p38^{MAPK}$ in a mammalian model of oscillator, but provide evidence that JNK isoforms are also involved in the control of the period length of several mammalian clock/oscillators. Together, these results suggest that **the level of SAPK activation is critical in regulating the clock machinery**. Even though ERK1/2 inhibition after synchronization does not affect the period length (Akashi & Nishida, 2000), these kinases are able to phosphorylate BMAL1 and CRY1/2 proteins *in vitro* which suggests a potential impact on the molecular clockwork as well (Sanada et al., 2002; Sanada et al., 2004).
- Finally, our data and previously published results indicate that the three subfamilies of MAPK are likely involved in the regulation of the pineal physiology. First, we show that activations of JNK and $p38^{MAPK}$ are needed to participate to the night-induced transcription of multiple genes, including *Aa-Nat* and *Mkp-1*. Besides, $p38^{MAPK}$ and ERK1/2 modulate the AA-NAT activity at night (Ho et al., 2006). Considering that the AA-NAT is the rhythm-generating enzyme of the MEL synthesis, it can be hypothesized that **MAPK modulate the synthesis of MEL, a major output of the SCN clock**.

The implication of MAPK at all levels of the circadian system demonstrates their importance in the time-keeping mechanisms of mammals. The possibility to

control MAPK activation through a wide variety of signals, such as cytokines, pheromones, hormones or neurotransmitters, suggest that the physiological state of organisms may impact the circadian system at all levels. Hypothetically, an inflammatory situation could for instance activate SAPK, which would likely lead to a shortening of the period of the biological clock. An attractive hypothesis is that MAPK may also constitute a link between the etiology of some diseases, like bipolar disorder, and the circadian rhythms disturbances that have been associated to the pathology (Goodwin & Jamison, 1990; Tsujimoto et al., 1990; Jones et al., 2005). Hence, targeting MAPK may offer new solutions for the treatment of those diseases.

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APPENDIXES

G. Appendixes

1. Appendix 1 – List of Tables and figures

1.1. Tables

1.2. Figures

Introduction

2. APPENDIX 2 – ADDITIONAL PEER-REVIEWED ARTICLES

Besides the study of the implication of JNK in the circadian organization, I participated to several side projects during my PhD. Two peer-reviewd articles resulted from these collaborations.

2.1. ANALYSIS OF THE ADRENERGIC REGULATION OF THE MELATONIN SYNHTESIS IN THE SIBERIAN HAMSTER PINEAL GLAND

The seasonal variations of the light duration are translated into seasonal changes in the pattern of MEL synthesis in the PG (Reiter, 1993). These fluctuations are of particular import for seasonal animals, which use the variations in the MEL content to properly regulate numerous physiological functions, such as reproduction (Pevet, 1988; Goldman, 2001). The Siberian hamster is a seasonal rodent which displays great seasonal variations in both duration and amplitude of the MEL synthesis and release (Illnerova et al., 1984; Ribelayga et al., 2000). In the present study, we evaluated the adrenergic regulation of both AA-NAT and HIOMT activities and attempted to identify the role of each enzyme in the control of both the duration and the amplitude of the nocturnal peak of MEL.

ANALYSIS OF ADRENERGIC REGULATION OF MELATONIN SYNTHESIS IN SIBERIAN HAMSTER PINEAL EMPHASIZES THE ROLE OF HIOMT

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Neurosignals (2004) 13:308-317

ANALYSIS OF ADRENERGIC REGULATION OF MELATONIN SYNTHESIS IN SIBERIAN HAMSTER PINEAL EMPHASIZES THE ROLE OF HIOMT

Rosa M. Ceinos, **Mathieu Chansard,** Florent Revel, Christiane Calgari, Jesús M. Míguez, Valérie Simonneaux

Neurosignals, 2004, Vol 13, Pages 308-317

Pages 308 à 317 :

2.2. ANALYSIS OF THE GATING OF *AA-NAT* GENE EXPRESSION IN THE MOUSE PINEAL GLAND

The regulation of the MEL synthesis shows some species-dependent differences (Simonneaux & Ribelayga, 2003). Strikingly, the *Aa-Nat* synthesis in some species, like the human, Syrian hamster and mouse, present a time-of-day specific sensibility to adrenergic stimulants (Vaughan et al., 1976; Vaughan & Reiter, 1987; Garidou et al., 2003; Fukuhara et al., 2005). In mice, it has been recently demonstrated that this time-dependent sensitivity is maintained *in vitro*, which suggests that the gating mechanism may be controlled by the PG oscillator (Fukuhara et al., 2005). In the present study, we attempted to identify the potential gating mechanisms to confirm the involvement of the mouse PG oscillator in the time-of-day dependent control of *Aa-Nat* synthesis.

ROLE OF CALCIUM IN THE GATING OF ISOPROTERENOL-INDUCED ARYLALKYLAMINE N-ACETYLTRANSFERASE GENE EXPRESSION IN THE MOUSE PINEAL GLAND.

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J Pineal Res. (2006) 41:85-94

ROLE OF CALCIUM IN THE GATING OF ISOPROTERENOL-INDUCED ARYLALKYLAMINE N-ACETYLTRANSFERASE GENE EXPRESSION IN THE MOUSE PINEAL GLAND

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Journal of Pineal Research, 2006, Vol. 41, Pages 85-94

Pages 85 à 94 :

3. APPENDIX 3 – LIST OF COMMUNICATIONS

3.1. PUBLISHED PEER-REVIEWED ARTICLES

Chansard M.[1,3], Molyneux P.[2], Nomura K.[1], Harrington M.E.[2] & Fukuhara C.[1] (2007) c-JUN N-Terminal kinase inhibitor SP600125 modulates the period of mammalian circadian rhythms. *Neuroscience*. 145:812-823.

[1]Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA, USA.

[2]Neuroscience Program, Smith College, Northampton, MA, USA.

[3]INCI LC2 Département de Neurobiologie des Rythmes, CNRS/ULP UMR 7168, Strasbourg, France.

Chansard M.[1,2], Liang J.[1,2], Iwahana E.[1,2,3], Baker T.[1], Whittaker J.[1] & Fukuhara C.[1,2] (2006) Role of calcium in the gating of isoproterenol-induced *arylalkylamine* N-*acetyltransferase* gene expression in the mouse pineal gland. *J Pineal Res*. 41:85-94.

[1]Department of Anatomy and Neurobiology, Morehouse School of Medicine, Atlanta, GA, USA.

[2]Center for Behavioral Neuroscience, Morehouse School of Medicine, Atlanta, GA, USA.

[3]Department of Pharmacology, School of Science and Engineering, Waseda University, Tokyo, Japan.

Chansard M., Iwahana E., Liang J. & Fukuhara C. (2005) Regulation of cAMP-induced *arylalkylamine* N-*acetyltransferase*, *Period1*, and *MKP-1* gene expression by mitogen-activated protein kinases in the rat pineal gland. *Brain Res Mol Brain Res*. 139, 333-340.

Department of Anatomy and Neurobiology, Center for Behavioral Neuroscience, Morehouse School of Medicine, Atlanta, GA, USA.

Ceinos R.M.[1], Chansard M.[2], Revel F.[2], Calgari C.[2], Miguez J.M.[1] & Simonneaux V.[2] (2004) Analysis of adrenergic regulation of melatonin synthesis in Siberian hamster pineal emphasizes the role of HIOMT. *Neurosignals*. 13:308-317.

[1]Laboratorio de Fisiologia Animal, Facultad de Biologia, Universidad de Vigo, Vigo, Spain.

[2]Neurobiologie des Rythmes, UMR-CNRS 7518, Université Louis-Pasteur, Strasbourg, France.

3.2. PRESENTATIONS

3.2.1. ORAL PRESENTATIONS

SNRP Conference, Washington, June 2005
Chansard M. & Fukuhara C. Inhibition of c-JUN N-terminal kinase lengthens the period of *Period1-luciferase* rhythm in rat-1 fibroblasts.
Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA, USA.

3.2.2. POSTERS

SFN Congress of Neurosciences, Atlanta, October 2006
Chansard M.[1], Molyneux P.[2], Harrington M.E.[2] & Fukuhara C.[1] Modulation of period length by c-JUN N-terminal kinases in mammals.
[1]Department of Anatomy and Neurobiology, Morehouse School of Medicine, Atlanta, GA, U.S.A.
[2]Neuroscience Program, Smith College, Northampton, MA, U.S.A.

SRBR Biennial meeting, Sandestin, May 2006
Chansard M. & Fukuhara C. c-JUN N-terminal kinase inhibition lengthens the period of *mPeriod1-luciferase* rhythm in rat-1 fibroblasts.
Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA, USA.

Gordon Conference – Chronobiology, Newport, August 2005
Chansard M. & Fukuhara C. c-JUN N-terminal kinase inhibition lengthens the period of *mPeriod1-luciferase* rhythm in rat-1 fibroblasts.
Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA, USA.

SNRP Conference, Washington, June 2005
Chansard M. & Fukuhara C. Inhibition of c-JUN N-terminal kinase lengthens the period of *Period1-luciferase* rhythm in rat-1 fibroblasts.
Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA, USA.

Annual South East Nerve Net SENN, Atlanta, March 2004
Chansard M., Liang J. & Fukuhara C. Regulation of melatonin in the mammalian pineal gland: modulation by melatonin.
Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA, USA.

Annual South East Nerve Net SENN, Atlanta, March 2004

Liang J., Chansard M. & Fukuhara C. Melatonin synthesis gates in mouse pineal gland.

Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA, USA.

Neurogenetics: from the molecule to man, NEUREX, Basel, April 2003

Ceinos R.M.[2], Calgarie C.[1], Chansard M.[1] & Simonneaux V.[1] Adrenergic regulation of melatonin synthesis in Siberian hamster pineal gland. *In vivo* and *in vitro* studies.

[1]Laboratorio de Fisiologia Animal, Facultad de Biologia, Universidad de Vigo, Vigo, Spain.

[2]Neurobiologie des Rythmes, UMR-CNRS 7518, Université Louis-Pasteur, Strasbourg, France.