Research and Perspectives in Endocrine Interactions

Paolo Sassone-Corsi
Yves Christen *Editors*

A Time for Metabolism and Hormones

Research and Perspectives in Endocrine Interactions

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A Time for Metabolism and Hormones

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ISSN 1861-2253 ISSN 1863-0685 (electronic)
Research and Perspectives in Endocrine Interactions
ISBN 978-3-319-27068-5 ISBN 978-3-319-27069-2 (eBook)
DOI 10.1007/978-3-319-27069-2

Library of Congress Control Number: 2015957954

Springer Cham Heidelberg New York Dordrecht London
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Preface

Each morning we wake up from a night of sleep, and each day we eat our regularly timed meals, go through our normal routines, and fall asleep again for another night. This rhythm, so-called circadian—after the Latin words circa diem ("about a day")—underlies a wide variety of human physiological functions, including sleep–wake cycles, body temperature, hormone secretion, exercise activity, and feeding behavior. Circadian rhythms are remarkably conserved throughout evolution, and it is becoming commonly appreciated that circadian rhythms represent an exquisite example of systems biology.

At the heart of all cyclic biological functions is the circadian clock, a highly conserved molecular system that enables organisms to adapt to common daily changes, such as the day–night cycle and food availability. The mammalian anatomical structure in the brain that governs circadian rhythms consists of a small area of the anterior hypothalamus, called the suprachiasmatic nucleus (SCN). For decades, this "central pacemaker" was thought to be the unique circadian clock of the organism. This dogma was challenged when peripheral tissues were also found to contain functional circadian oscillators that are self-sustained at the single cell level. This notion, together with the discovery that a remarkable fraction of the genome is transcriptionally controlled by the clock, illustrated that circadian control must play a key role in governing the metabolism and physiology of all organisms. This concept was recently validated by studies of the metabolome revealing that a large fraction of metabolites oscillate in a given tissue.

Recent years have seen spectacular advances in the field of circadian biology. These have attracted the interest of researchers in many fields, including endocrinology, neurosciences, cancer, and behavior. By integrating a circadian view within the fields of endocrinology and metabolism, researchers will be able to reveal many, yet-unsuspected aspects of how organisms cope with changes in the environment and subsequent control of homeostasis.

The concept behind the Fondation IPSEN *Colloque Medecine et Recherche* on "A Time for Metabolism and Hormones," held in Paris on December 5, 2014, was to capture the excitement of this field as it is opening new avenues in our

understanding of metabolism and endocrinology. A panel of the most distinguished investigators in the field gathered together to discuss the present state and the future of the field. These proceedings constitute a compendium of the most updated views by these investigators. We trust that it will be of use to those colleagues who will be picking up the challenge to unravel how the circadian clock can be targeted for the future development of specific pharmacological strategies toward a number of pathologies.

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Acknowledgements

The editors wish to express their gratitude to Mrs. Mary Lynn Gage for her editorial assistance and Mrs. Astrid de Gérard for the organization of the meeting.

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The Epigenetic and Metabolic Language of the Circadian Clock

Paolo Sassone-Corsi

Abstract The circadian clock controls a large variety of neuronal, endocrine, behavioral and physiological responses in mammals. This control is exerted in large part at the transcriptional level on genes expressed in a cyclic manner. A highly specialized transcriptional machinery based on clock regulatory factors organized in feedback autoregulatory loops governs a significant portion of the genome. These oscillations in gene expression are paralleled by critical events of chromatin remodeling that appear to provide plasticity to circadian regulation. Specifically, the NAD^+-dependent deacetylases SIRT1 and SIRT6 have been linked to circadian control of gene expression. This and additional accumulating evidence shows that the circadian epigenome appears to share intimate links with cellular metabolic processes and has remarkable plasticity, showing reprogramming in response to nutritional challenges. In addition to SIRT1 and SIRT6, a number of chromatin remodelers have been implicated in clock control, including the histone H3K4 tri-methyltransferase MLL1. Deciphering the molecular mechanisms that link metabolism, epigenetic control and circadian responses will provide valuable insights towards innovative strategies of therapeutic intervention.

Introduction

Metabolism, homeostatic balance and behavior follow the 24-h daily cycle (Eckel-Mahan and Sassone-Corsi 2013). Circadian rhythms are virtually present in all life forms on our planet, including mammals, insects, plants, fungi and cyanobacteria. In higher organisms, circadian rhythms have evolved into a complex physiological and molecular system demonstrated by sleep-wake cycles, daily fluctuations in body temperature, blood pressure, cellular regeneration and behavior such as food

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P. Sassone-Corsi, Y. Christen (eds.), *A Time for Metabolism and Hormones*, Research and Perspectives in Endocrine Interactions,
DOI 10.1007/978-3-319-27069-2_1

intake and alertness levels (Asher and Sassone-Corsi 2015). Metabolism, nutritional intake and body homeostasis are also under circadian control, displaying rhythms in the levels of circulating hormones and metabolites, as well as enzymes within the biochemical pathways participating in their biosynthesis (Eckel-Mahan and Sassone-Corsi 2013; Gamble et al. 2014). Circadian rhythms are so intimately linked to biological processes that their misregulation may lead to a number of pathologies such as obesity, metabolic syndrome, diabetes, cardiovascular diseases, inflammation, sleep disorders and some cancers (Eckel-Mahan and Sassone-Corsi 2013).

The molecular bases of circadian rhythms have been explored, revealing a remarkable variety of molecular mechanisms that underlie clock function. An important system of circadian control utilizes the core clock molecular machinery that consists of transcription factors and regulators, both activators and repressors, that act in concert to drive circadian expression of an important fraction of the genome. A number of high-throughput transcriptome profiling studies have established that 15–30 % of all transcripts are controlled by the clock, depending on the tissue or cell type (Duffield et al. 2002; Panda et al. 2002; Storch et al. 2002; Ueda et al. 2002). Accumulating evidence has shown that this global program of gene expression is achieved through events of cyclic chromatin remodeling and epigenetic control.

Chromatin Remodeling, Cyclic Transcription and the Clock

The molecular organization of the circadian system relies on a network of cellular oscillators present in virtually every cell of the organism. An intricate network of transcriptional-translational feedback loops constitutes the molecular clock (Eckel-Mahan and Sassone-Corsi 2013; Zhang and Kay 2010). The basic helix-loop-helix (b-HLH)-PAS proteins CLOCK and BMAL1 are core elements of this system and function as transcriptional activators to drive the expression of many clock-controlled genes (CCGs). CLOCK and BMAL1 heterodimers bind E-boxes in CCG promoters and activate their expression. Among the CCGs there are genes encoding other core clock protein repressors Period (PER1-3) and Cryptochromes (CRY1-2). PER and CRY proteins heterodimerize in the cytoplasm and translocate to the nucleus to inhibit CLOCK:BMAL1-mediated transcription. The stability of PER:CRY complexes is regulated by posttranscriptional modifications (Lee et al. 2009) and ubiquitination events (Busino et al. 2007; Hirano et al. 2013; Siepka et al. 2007; Yoo et al. 2013). The time-controlled clearance of the repressors primes for a the next cycle of CLOCK:BMAL1-driven gene activation. This system then leads to the cyclic activation of other regulatory pathways generating interconnected transcriptional feedback loops. These provide remarkable plasticity to the circadian system, eliciting multiple daily oscillations in the transcriptome (Masri and Sassone-Corsi 2010).

Specific cyclic chromatin transitions occur in a genome-wide scale and are associated with circadian waves of transcription (Masri and Sassone-Corsi 2010). Several chromatin remodelers have been found to be involved in circadian control. The protein CLOCK was found to operate as an acetyltransferase on histone H3 at K9 and K14 (Doi et al. 2006), modifications associated with a chromatin state permissive for transcription. CLOCK acts in concert with other histone acetyltransferases (HATs) (Etchegaray et al. 2003), such as CBP (CREB binding protein), p300 and with the CBP-associated factor PCAF (Lee et al. 2010; Curtis et al. 2004; Takahata et al. 2000). A number of histone deacetylases (HDACs) have been found to counterbalance these HATs. For example, the circadian repressor PER recruits SIN3A-HDAC1 (Duong et al. 2011), whereas the protein CRY1 associates with the complex SIN3B-HDAC1/2 (Naruse et al. 2004). The circadian regulator REV-ERBα recruits the NCoR-HDAC3 complex in a rhythmic manner to chromatin via a process that has been linked to the control of lipids metabolism in the liver (Sun et al. 2011). Thus, a variety of circadian repressive complexes appear to exist that may elicit distinct functions at unique times of the circadian cycle. The nicotinamide adenine dinucleotide (NAD^+)-dependent class III of HDACs was found to play a critical role in connecting cellular metabolism to circadian physiology. The founding member, SIRT1, gives the name to this class of enzymes, collectively known as sirtuins. There are seven sirtuins, all involved in various aspects of metabolism, inflammation and aging; their intracellular localization is nuclear, cytoplasmic or mitochondrial. The nuclear proteins SIRT1 and SIRT6 have been shown to contribute to circadian transcription (Nakahata et al. 2008; Masri et al. 2014).

A number of chromatin post-translational modifications have been linked to clock function in addition to acetylation. The first evidence that a histone modification may play a role in circadian transcription was the light-inducible phosphorylation at H3-S10 in SCN neurons (Crosio et al. 2000). The activating histone methylation H3K4me3 has also been linked to clock control and it seems to be essential to permit circadian chromatin transitions that lead to activation of CCG expression (Ripperger and Schibler 2006). MLL1, a H3K4 histone methyltransferase (HMT), was shown to elicit CLOCK:BMAL1 recruitment to chromatin at specific circadian promoters and for the cyclic tri-methylation at H3K4 (Katada and Sassone-Corsi 2010). Also the repressive mark H3K27me3 is clock controlled at the *Per1* promoter through a mechanism that involves the methyltransferase EZH2 (Etchegaray et al. 2006). Additional chromatin remodelers involved in circadian function include the demethylase JARID1a that appears to inhibit HDAC1, thereby enhancing CLOCK:BMAL1-mediated transcription (DiTacchio et al. 2011), and the FAD (Flavin Adenine Dinucleotide)-dependent demethylase LSD1 whose function is controlled by PKCα-mediated circadian phosphorylation (Nam et al. 2014).

Cellular Metabolism and the Circadian Clock Converge

A large number of human studies and animal models provide solid evidence of the reciprocal regulation between the circadian clock and cellular and organismal homeostasis (Eckel-Mahan and Sassone-Corsi 2013; Dallmann et al. 2012; Eckel-Mahan et al. 2012, 2013; Hatori et al. 2012; Kasukawa et al. 2012). The clock regulates metabolism by controlling the expression of a large fraction of the genome. Moreover, the oscillator appears to sense the cellular energy state and consequently adapts its function accordingly.

Several levels of interplay exist between cellular metabolism and chromatin remodeling (Masri and Sassone-Corsi 2010; Feng and Lazar 2012; Katada et al. 2012). Acetylation of histones or non-histone nuclear proteins depends on the supply of acetyl-CoA in the nuclear compartment. The main carbon source in mammals is glucose, which generates acetyl-CoA because of the enzyme adenosine triphosphate (ATP)-citrate lyase (ACLY). ACLY protein levels are cyclic in the liver (Mauvoisin et al. 2014), and ACLY activity controls global histone acetylation depending on glucose availability (Wellen et al. 2009). Thus, circadian changes in histone acetylation are controlled not only by specific HATs but also by interconnected metabolic pathways and enzymes supplying nuclear acetyl-CoA. A similar regulation involves S-adenosyl methionine (SAM), the metabolite used by methyltransferases to deliver methyl groups. Changing SAM levels directly influence H3K4me3 levels in mouse pluripotent stem cells (Shyh-Chang et al. 2013). Also, treatment with 3-deazaadenosine (DAA), an inhibitor of SAH (S-adenosylhomocysteine) hydrolysis that hinders transmethylation, elongates the circadian period (Fustin et al. 2013). Further research is necessary to decipher the impact of one carbon metabolism in the circadian transcriptome.

Nicotinamide adenine dinucleotide (NAD^+) is a pivotal metabolite for the circadian epigenome. NAD^+ shows robust diurnal rhythms in synchronized cells and mice (Bellet et al. 2013; Nakahata et al. 2009; Ramsey et al. 2009), and operates as a cofactor for class III of HDACs, the sirtuins (see next section).

The core machinery may be directly influenced by changing metabolic states. Specifically, the DNA-binding function of NPAS2:BMAL1 and CLOCK:BMAL1 heterodimers was shown to be influenced by the redox states of NAD(H) or NADP (H) (Rutter et al. 2001). This finding implied that CLOCK:BMAL1 transcriptional activity should be sensitive to the levels of cellular redox. While a causal evidence for this regulation has not been explored, circadian oscillations in intracellular redox potentials are evolutionary conserved (Eckel-Mahan and Sassone-Corsi 2013; Asher and Sassone-Corsi 2015). Thus, while the ability of NPAS2 or CLOCK to sense the intracellular redox state in vivo remains to be proven, independent evidence provides interesting information. Indeed, crystallographic analyses of the CRY1-PER2 complex indicate that a disulfide bond between two cysteine residues in CRY1 weakens its interaction with PER2, whereas a reduced state of CRY1 stabilizes the complex and facilitates transcriptional repression (Schmalen et al. 2014). In this scenario, CRY2 would retain specific FAD (Flavin

Adenine Dinucleotide) binding activity, and FAD competes for CRY2 binding pocket with the ubiquitin ligase complex SCF^{FBXL3}, which has been shown to control period length by regulating CRYs stability (Xing et al. 2013). Interestingly, this finding provides a possible approach to pharmacologically adjust circadian period length by using small molecules resembling FAD (Hirota et al. 2012).

Posttranslational modifications of clock proteins have been shown to modify their regulatory capacity. For example, CLOCK, BMAL1 and PER2 can be O-linked *N*-acetylglucosamine (GlcNAc)-modified by the enzyme O-GlcNAc transferase (OGT), which results in a change in their activities (Kaasik et al. 2013; Li et al. 2013). Importantly, liver-specific ablation of OGT leads to dampened oscillation of *Bmal1* and gluconeogenic genes. Thus, glucose levels dictate the availability of GlcNAc, OGT serving as a signal transducer between cellular metabolism and circadian components. Along the same lines, phosphorylation of CRY1 by the nutrient sensor kinase AMPK (AMP-activated protein kinase) connects cellular energy levels with the circadian clock by adjusting it to the changing intracellular ratio of AMP/ATP (Jordan and Lamia 2013; Gomes et al. 2013).

The Central Role of Sirtuins

The intracellular availability in time and space of specific metabolites constitutes an intriguing level of control for their protein sensors (Katada et al. 2012). In this respect, the circadian oscillation in NAD^+ concentration represents a revealing paradigm. The NAD^+ biosynthetic salvage pathway controls the conversion of nicotinamide (NAM) to β-nicotinamide mononucleotide (NMN); this step is catalyzed by a rate-limiting step enzyme, the nicotinamide phosphoribosyltransferase (NAMPT, also known as visfatin). The circadian machinery controls the transcription of the *Nampt* gene through direct binding of CLOCK:BMAL1 to E-boxes in the promoter (Nakahata et al. 2009; Ramsey et al. 2009). NMN is converted to NAD^+ by the enzymes nicotinamide mononucleotide adenylyltransferase 1-3 (NMNAT1-3) (Fig. 1). Thus, a transcriptional-enzymatic feedback loop controls NAD^+ biosynthesis and availability that in turn could result in circadian function of a variety of NAD^+-dependent enzymes. Moreover, there is a differential regulation of NAD^+ levels and NAD^+-consuming enzymes in various cell compartments (Gomes et al. 2013; Yang et al. 2007). In this respect the sirtuins deserve special attention. Indeed, of the seven mammalian sirtuins, three (SIRT1, SIRT3 and SIRT6) have been functionally linked to circadian control and found to modulate cyclic outputs in response to metabolic cues.

SIRT3 is a mitochondrial enzyme that displays robust changes in its deacetylase activity in response to NAD^+ levels (Hebert et al. 2013; Peek et al. 2013; Masri et al. 2013). SIRT3 controls mitochondrial function, including fatty acid oxidation and intermediary metabolism, by directly targeting rate-limiting enzymes for mitochondrial biochemical processes (Peek et al. 2013). As mitochondrial fatty acid

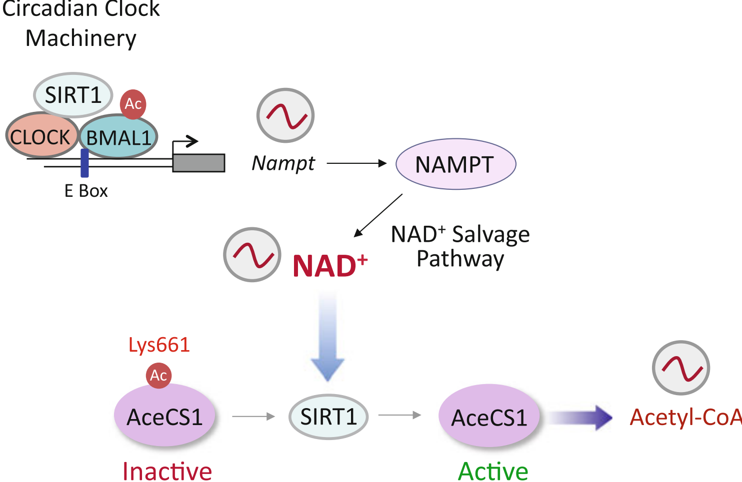

Fig. 1 Metabolism and the circadian clock converge. A paradigm example is represented by the role of SIRT1 and other sirtuins in clock regulation. The circadian machinery controls a large fraction of the genome through the transcriptional regulation of CCGs. One of the CCGs is the gene encoding the protein NAMPT, the rate-limiting enzyme in the NAD^+-salvage pathway. Cyclic transcriptional control of the *Nampt* gene results in the cyclic synthesis of NAD^+, which in turn is consumed rhythmically by enzymes such as SIRT1, whose deacetylase activity is consequently cyclic. One of the non-histone targets is the enzyme AceCS1, which contributes to the synthesis of Acetyl-CoA. AceCS1 is acetylated at one residue, Lys661, and its cyclic deacetylation by SIRT1 activates the enzyme, resulting in cyclic synthesis of Acetyl-CoA and thereby oscillating availability of acetyl groups required for global acetylation

oxidation and protein acetylation show circadian rhythmicity (Masri et al. 2013), the link with NAD^+ availability through SIRT3 is of particular interest. Also, mitochondria from *Bmal1*$^{-/-}$ mice display reduced oxidative ability and decreased mitochondrial NAD^+ levels (Peek et al. 2013). These findings, together with the implication of SIRT1 in circadian control, raise the possibility that the sirtuins-NAD^+ link with the clock may represent a critical molecular pathway to govern the process of aging.

The implication of nuclear sirtuins in clock function is multiple. SIRT1 is both nuclear and cytoplasmic whereas SIRT6 is exclusively nuclear and mostly chromatin bound, localized at transcriptionally active genomic loci. SIRT1 and SIRT6 operate through distinct mechanisms to coordinate the clock machinery in a differential manner and thereby delineate the circadian transcriptional output (Masri et al. 2014). Because of these different mechanisms of action, in the liver these two sirtuins coordinate circadian expression of distinct groups of genes. SIRT6 exerts its function by coordinating CLOCK:BMAL1 recruitment to specific chromatin sites (Masri et al. 2014). SIRT1, which is mostly nucleoplasmic and is recruited to chromatin only 'on demand', deacetylates histones and non-histone proteins. Among the non-histone targets of SIRT1 there are the clock proteins BMAL1 and PER2 (Asher et al. 2008; Hirayama et al. 2007). SIRT1 is also able

to deacetylate MLL1, thereby controlling its methyltransferase activity. Thus, there is control in H3K4 tri-methylation through the cyclic oscillation of NAD^+ levels (Aguilar-Arnal et al. 2015).

SIRT1-mediated deacetylation also affects circadian levels of other metabolites besides NAD^+. Specifically, intracellular acetyl-CoA levels are controlled by the clock through SIRT1-controlled deacetylation of the enzyme acetyl-CoA Synthetase 1 (AceCS1) (Sahar et al. 2014). This acetylation switch controls AceCS1 activity, leading to cyclic synthesis of acetyl-CoA (Fig. 1), that then is likely to influence the acetylation levels of histones and non-histone proteins (Sahar et al. 2014). In contrast, SIRT6 deacetylase activity seems to be efficient in removing long chain fatty acids from lysine residues (Jiang et al. 2013). In this respect it is noteworthy that not only on NAD^+, but also on fatty acids, control the activity of SIRT6 (Feldman et al. 2013). Thus, SIRT6 appears to occupy a key position in the control of fatty acids metabolism by the clock. Indeed, CLOCK: BMAL1-driven activation of genes involved in fatty acid biosynthesis is modulated by SIRT6 (Masri et al. 2014).

High-throughput analysis of the transcriptome and metabolome along the circadian cycle has revealed notable differences in the metabolic functions of SIRT1 and SIRT6. Using mice with liver-specific deletion of either SIRT1 or SIRT6, a specific role for SIRT6 was shown in dictating the synthesis and breakdown of fatty acid pathways, as well as their storage into triglycerides. SIRT6 operates at least in part through the control of alternative circadian transcriptional pathways, specifically because of the chromatin recruitment of the sterol regulatory element-binding protein 1 (SREBP1) (Masri et al. 2014). Thus, it is through genomic partitioning that the two deacetylases contribute to a parallel segregation of cellular metabolism (Masri et al. 2014).

Finally, these findings suggest a role for genome topology in circadian control (Aguilar-Arnal et al. 2013). Our studies have identified the presence of circadian interactomes where co-regulated genes are physically associated in the circadian epigenome. Nuclear sirtuins may constitute a paradigm for other chromatin remodelers that could contribute in the cyclic control of the nuclear landscape. Also, specific changes in the nuclear localization of NAD^+ may provide the possibility of restricting the distribution of this metabolite to “niches” of activity (Katada et al. 2012).

Conclusion

The ability of the circadian clock machinery to sense the metabolic state of the cell in a time-specific manner places it in a strategic position. Indeed, fascinating findings reviewed in this article demonstrate the direct implication of the clock in the maintenance of cellular homeostasis. The clock machinery appears to integrate environmental and metabolic signals to directly translate them in plasticity in gene expression so to favor the adaptation of the organism to specific conditions. As the

circadian transcriptional landscape is highly complex, including dynamic changes in nuclear organization (Katada et al. 2012; Aguilar-Arnal et al. 2013), it becomes critical to decipher how the nuclear landscape integrates metabolic cues and shapes the transcriptional output. It is through the analysis of the specific coordination that key chromatin remodelers have with clock transcription factors that we will gain insights into how the intracellular metabolic state communicates with the clock machinery. As disruption of clock function has been linked to a variety of pathological conditions, revealing the clock mechanisms will lead to innovative strategies towards the pharmacological treatment of metabolic syndromes, obesity, diabetes, inflammation and even cancer.

Acknowledgments Studies in the Center for Epigenetics and Metabolism are supported by the National Institute of Health, Merieux Research Grants, and INSERM (Institut National de la Sante et Recherche Medicale, France).

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Molecular Architecture of the Circadian Clock in Mammals

Joseph S. Takahashi

Abstract The circadian clock mechanism in animals involves an autoregulatory transcriptional feedback loop in which CLOCK and BMAL1 activate the transcription of the *Period* and *Cryptochrome* genes. The PERIOD and CRYPTOCHROME proteins then feed back and repress their own transcription by interaction with CLOCK and BMAL1. We have studied the biochemistry of the CLOCK:BMAL1 transcriptional activator complex using structural biology as well as the genomic targets of CLOCK and BMAL1 using ChIP-seq methods. We describe the dynamics of the core circadian clock transcriptional system. CLOCK and BMAL1 interact with the regulatory regions of thousands of genes. The gene network and dynamics of the system will be discussed. A mechanistic description of the core circadian clock mechanism should promote our understanding of how the circadian clock system influences behavior, physiology and behavioral disorders.

Introduction

Over the last 20 years, my laboratory has been focused on understanding the molecular mechanism of circadian clocks in mammals. We have used mouse genetics as a tool for discovery of the critical genes involved in the generation of circadian rhythms of mammals (Takahashi et al. 1994; Lowrey and Takahashi 2011). Our initial discovery of the *Clock* gene using forward genetic screens and positional cloning (Vitaterna et al. 1994; Antoch et al. 1997; King et al. 1997), and the identification of BMAL1 as the heterodimeric partner of CLOCK (Gekakis et al. 1998), led to idea that the CLOCK:BMAL1 transcriptional activator complex

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P. Sassone-Corsi, Y. Christen (eds.), *A Time for Metabolism and Hormones*, Research and Perspectives in Endocrine Interactions,
DOI 10.1007/978-3-319-27069-2_2

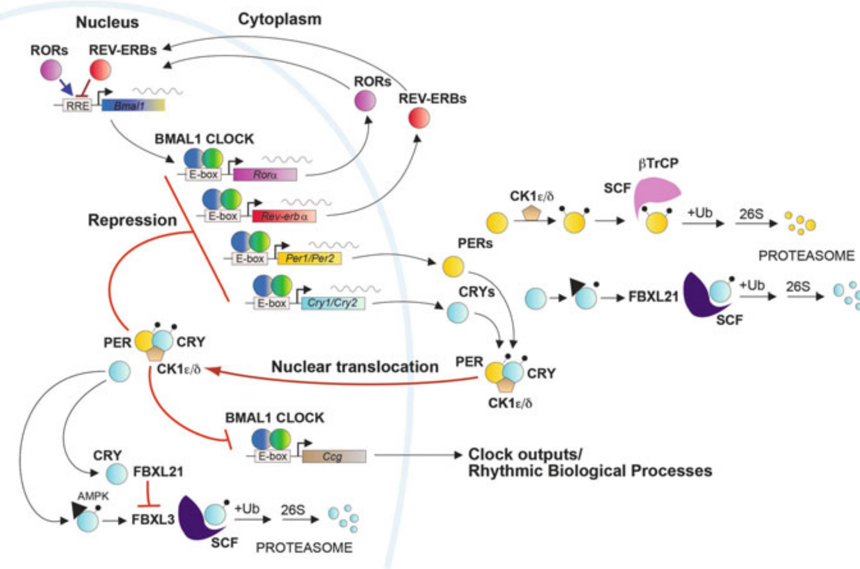

Fig. 1 Model of the circadian clock in mammals. CLOCK and BMAL1 act as master transcription factors to regulate: (1) the *Per* and *Cry* genes in the core feedback loop of the clock; (2) the REV-ERB/ROR feedback loop regulating *Bmal1* transcription; and (3) thousands of target genes that are clock outputs. The stability of the PER and CRY proteins is tightly regulated by E3 ubiquitin ligases in both the cytoplasm and nucleus that determine circadian period (Adapted from Mohawk et al. 2012 and Yoo et al. 2013)

was upstream of the *Period* and *Cryptochrome* genes, whose gene products then repressed CLOCK:BMAL1 to form an autoregulatory transcriptional feedback loop (Lowrey and Takahashi 2000). Since the identification of these "core circadian clock genes" (i.e., *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1* and *Cry2*), additional feedback loops driven by CLOCK:BMAL1, such as the loop involving *Rev-erbα* to repress *Bmal1* transcription, have been described (Preitner et al. 2002). In addition, the regulation of the stability of the PER and CRY proteins by specific E3 ubiquitin ligase complexes has been found to be important for determining the periodicity of the circadian oscillation (Busino et al. 2007; Gallego and Virshup 2007; Siepka et al. 2007; Meng et al. 2008; Yoo et al. 2013). Together, this work has led to a description of a model of the circadian clock in mammals (Fig. 1).

With the discovery and cloning of clock genes came the realization that their expression was ubiquitous (Lowrey and Takahashi 2004). We now accept that clock genes are housekeeping genes and are expressed in essentially all cells. What was perhaps even more surprising was the observation made using circadian gene reporter technology that essentially every peripheral organ system and tissue has the capacity to express autonomous circadian rhythms (Yoo et al. 2004). Thus the ubiquitous expression of clock genes is a reflection of the ubiquitous capacity of most tissues and cells to express circadian oscillations. These distributed circadian oscillators are cell autonomous and can function independently of the central clock

located in the suprachiasmatic nucleus (SCN) (Nagoshi et al. 2004; Welsh et al. 2004; Yoo et al. 2004). The realization that the body is composed of a multitude of cell-autonomous clocks has raised a number of questions concerning the organization of the clock system and the role of the SCN clock in "circadian organization." Elsewhere, we have also explored the role of the SCN as a master pacemaker to synchronize peripheral oscillators (Yoo et al. 2004; Hong et al. 2007; Kornmann et al. 2007; Buhr et al. 2010; Hughes et al. 2012), as well as the role of intercellular coupling in the robustness of the SCN oscillator (Liu et al. 2007; Buhr et al. 2010; Ko et al. 2010; Welsh et al. 2010).

Structural Biology of Clock Proteins

Despite our general knowledge of clock components and their interactions, the biochemical mechanisms of circadian clock proteins and how they function within the circadian feedback loop are largely unknown. For example, many coding mutations have been described for mammalian clock proteins but, at a macroscopic level, we have little hope of understanding how they exert their phenotypic effects without a deeper understanding of their molecular mechanism. For these reasons, we have turned to structural biology to understand circadian proteins at an atomic level of resolution. Recently, we have solved the three-dimensional structure of the CLOCK:BMAL1 heterodimeric transcriptional activator complex using X-ray crystallography (Huang et al. 2012). The CLOCK:BMAL1 structure reveals an asymmetric heterodimer in which the bHLH, PAS-A and PAS-B domains of each subunit interact with their complementary domains but do so in an unexpected manner (Fig. 2). The PAS-A domains dimerize via symmetrical interactions involving α-helical domains (that are N-terminal to the canonical PAS fold) that pack against the β-sheet surfaces of the PAS-A domains (Fig. 3a). In contrast, the PAS-B domains dimerize in an asymmetric, head-to-tail fashion so that the β-sheet surface of BMAL1 interacts with the α-helical surface of CLOCK (Fig. 3b). A conserved BMAL1 Trp427 residue on an H-I loop (connecting the Hβ and Iβ strands) inserts into a hydrophobic pocket on the α-helical surface of CLOCK that resembles the co-factor binding pocket in other PAS proteins. Interestingly, a Trp residue is also conserved on the H-I loops of CLOCK, PER1 and PER2 PAS domains, suggesting that an aromatic residue inserting into the PAS receiver pocket may represent a common motif for PAS domain interactions (Crane 2012).

The structure of CLOCK:BMAL1 represents a starting point for understanding at an atomic level the mechanism driving the mammalian circadian clock. Many of the previously identified mutations on CLOCK and BMAL1 can be mapped onto the structure and, for example, predict regions of interaction of CLOCK with the CRY proteins (Huang et al. 2012). The crystal structures for the PAS-A/PAS-B domains of the mammalian PERIOD proteins (Hennig et al. 2009; Kucera et al. 2012), for the photolyase homology domains of the mammalian CRY1 (Czarna et al. 2013) and CRY2 (Xing et al. 2013) proteins, and for the CRY2/PER2-CRY binding domain complex (Nangle et al. 2014) beg the question of how

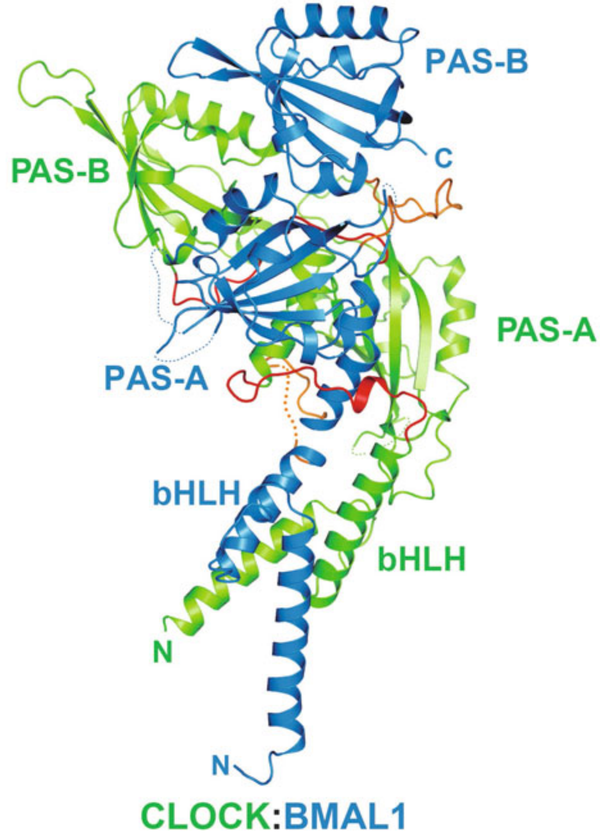

Fig. 2 CLOCK:BMAL1 structure showing bHLH, PAS-A and PAS-B domains. Linker regions shown in *red* or *orange* (From Huang et al. 2012)

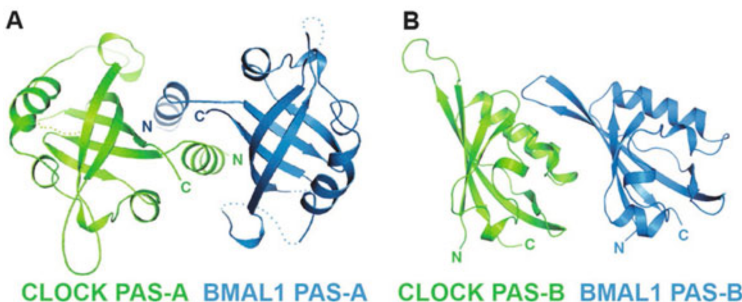

Fig. 3 PAS domains of CLOCK:BMAL1. (**a**) PAS-A interactions shown looking down the axis of the complex. (**b**) PAS-B interactions shown from a *side view* (From Huang et al. 2012)

PER and CRY interact with CLOCK:BMAL1 to repress their function. Because the native CLOCK:BMAL1/PER:CRY quaternary complexes are megadalton in size and involve other interacting proteins, and because important domains of these proteins are flexible, the solution of these complexes likely will require a combination of crystallography, NMR, and cryo electron microscopy methods in future work.

Transcriptional Architecture and Chromatin Dynamics of the Clock

To define the *cis*-acting targets of the core circadian transcriptional regulators, we used chromatin immunoprecipitation followed by sequencing (ChIP-seq) to locate DNA binding sites for BMAL1, CLOCK, NPAS2, PER1, PER2, CRY1 and CRY2 in vivo in murine liver at six times during the circadian cycle. Figure 4 shows a browser view of the *Dbp* locus, a major target gene of CLOCK-BMAL1 (Ripperger and Schibler 2006). The activators BMAL1, CLOCK and NPAS2 bind in a cyclic manner between CT0 and CT12 (CT = circadian time; CT0 is the beginning of the subjective day; CT12 is the beginning of the subjective night) at three locations in the promoter, intron 1 and intron 2. PER1, PER2 and CRY2 bind the same sites with an opposite phase at CT12-20. CRY1 exhibits a third pattern that peaks at CT0.

In genome-wide analysis, CLOCK and BMAL1 bind to over 4600 and 5900 sites, respectively, corresponding to ~3000 unique genes (Koike et al. 2012). The repressors CRY1 and CRY2 bind to significantly more sites, and many thousands of these sites are independent of CLOCK:BMAL1 and reveal DNA binding motifs for nuclear receptors (Koike et al. 2012), including the glucocorticoid receptor consistent with recent work (Lamia et al. 2011). To examine functional readouts, we used whole transcriptome RNA-seq to profile cycling genes in the liver using samples taken every 4 h over 48 h (Koike et al. 2012). Using the intron RNA signal as a proxy for pre-mRNA, we found ~1300 cycling genes and, surprisingly, they were clustered in time with a peak at CT15 (Fig. 5). To explore the possible origins of the global rhythms in nascent transcription, we analyzed the genome-wide occupancy of RNA polymerase II (RNAPII) as a function of the circadian cycle. The large subunit of RNAPII contains a C-terminal domain (CTD) that is modified at various stages of transcription (Sims et al. 2004; Fuda et al. 2009). RNAPII is recruited into the pre-initiation complex with a hypophosphorylated CTD that is recognized by the 8WG16 antibody (Jones et al. 2004). Again to our surprise, we found that RNAPII-8WG16 occupancy was highly circadian across the genome in the liver, with a peak at CT14.5, which preceded the intron RNA peak by 0.5 h (Fig. 5). Initiation of RNAPII involve phosphorylation on serine 5 (Ser5P) on the CTD of RNAPII and is recognized by the 3E8 antibody (Chapman et al. 2007). We found that RNAPII-Ser5P occupancy was also circadian, with over 13,000 sites that were significant for cycling. The timing of RNAPII-Ser5P peaked at CT0 and coincided with the peak of CRY1. At this time we found an association of CRY1, CLOCK,

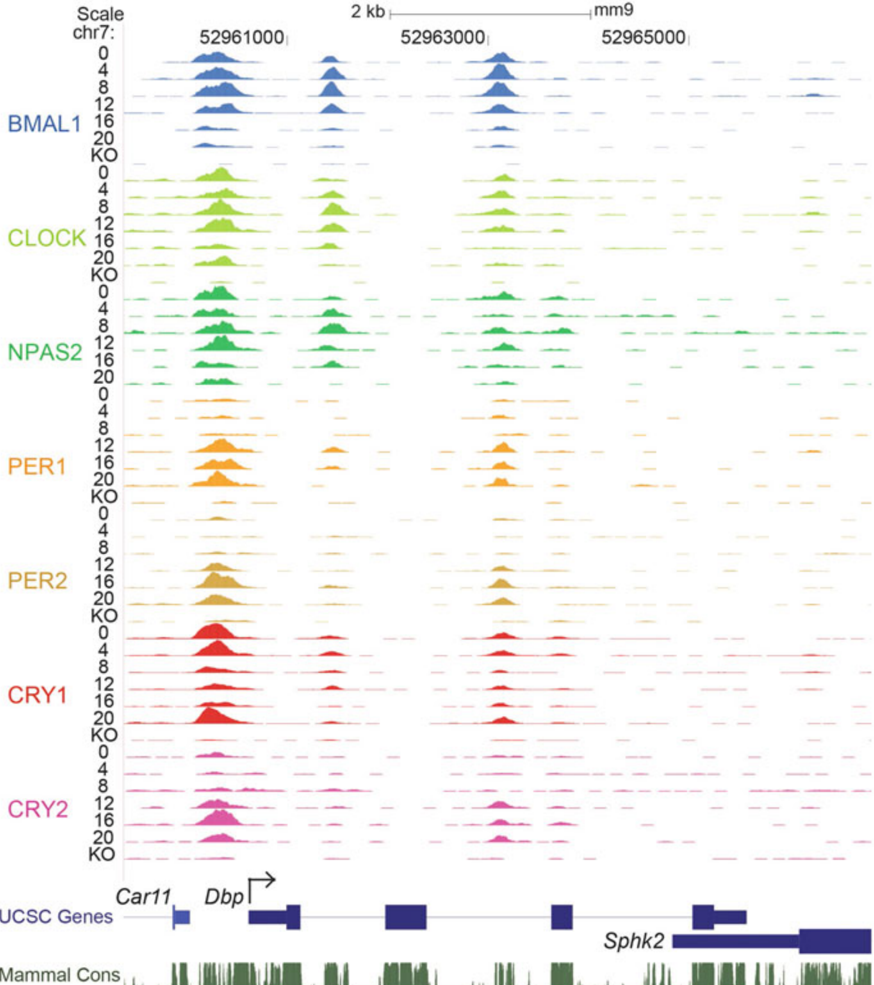

Fig. 4 UCSC genome browser view of ChIP-seq profiles of circadian transcription factors at the *Dbp* gene at six circadian times of day. BMAL1 (*blue*), CLOCK (*green*), NPAS2 (*dark green*), PER1 (*orange*), PER2 (*gold*), CRY1 (*red*), CRY2 (*pink*). 0, 4, 8, 12, 16, 20 CT (h). *KO* knockout. (From Koike et al. 2012)

BMAL1 and RNAPII-Ser5P binding sites, suggesting that CLOCK:BMAL1 could recruit and initiate RNAPII but CRY1 repressed the complex leading to a "poised" state.

Given the genome-wide circadian rhythms of RNAPII occupancy, we assessed chromatin states associated with transcription initiation and elongation during the circadian cycle. Figure 6 shows a browser view of six histone modifications that are characteristic of promoters, enhancers and transcription elongation (Kim et al. 2005; Barski et al. 2007; Guenther et al. 2007; Li et al. 2007; Creyghton

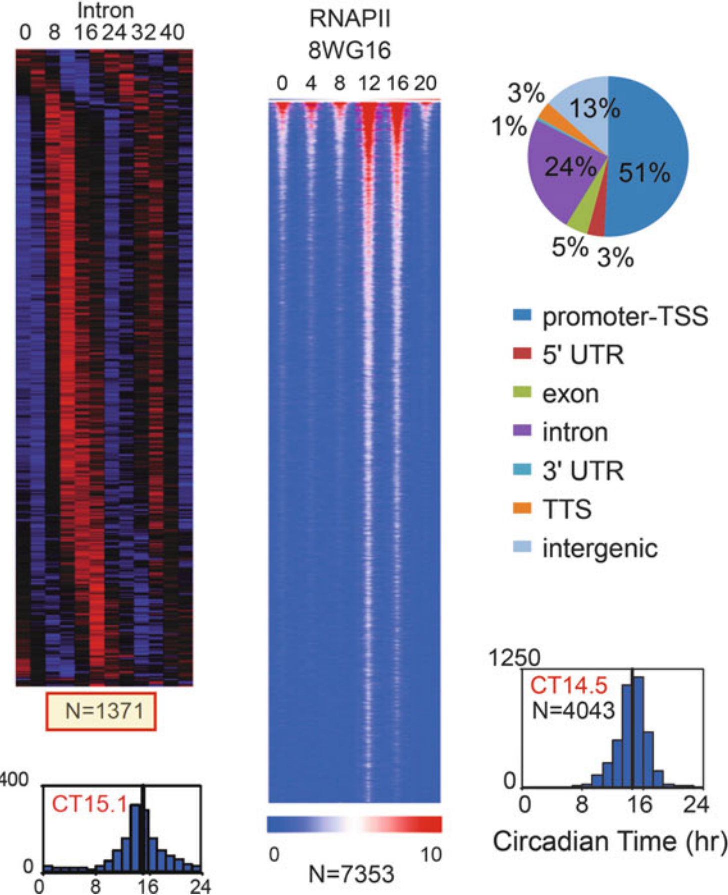

Fig. 5 Heatmap views of cycling intron RNA genes (*left*) and RNAPII-8WG16 occupancy (*right*). More than 4000 peaks had significant circadian RNAPII binding (From Koike et al. 2012)

et al. 2010; Ong and Corces 2011; Rada-Iglesias et al. 2011). Histone H3K4me3, H3K9ac and H3K27ac are enriched at promoters and show robust circadian rhythms in occupancy at the *Dbp* gene. When examined across the genome, we found that circadian rhythms in RNAPII occupancy as well as histone H3K4me3, H3K9ac and H3K27ac modifications occurred in the majority of expressed genes, even in cases where cycling RNA could not be detected. Thus a third surprise in this work was the observation that chromatin states were being modulated in a circadian manner across the genome in the liver.

What accounts for these genome-wide circadian rhythms in RNAPII occupancy and histone modifications? Examination of the relationship between circadian transcription factor occupancy and gene expression shows that approximately 90 % of genes bound by these factors are expressed whereas only 1–5 % of unexpressed genes are similarly bound (Koike et al. 2012). These results

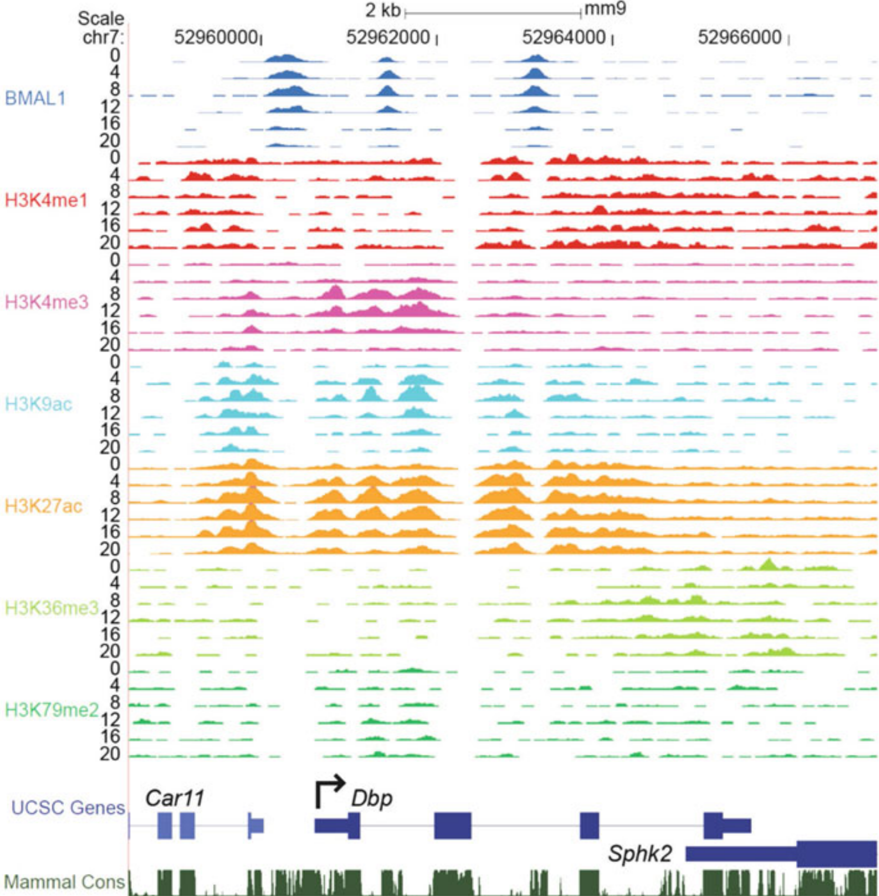

Fig. 6 UCSC genome browser view of histone methylation and acetylation at the *Dbp* gene. BMAL1 (*blue*), H3K4me1 (*red*), H3K4me3 (*pink*), H3K9ac (*aqua*), H3K27ac (*orange*), H3K36me3 (*green*), H3K79me2 (*dark green*) (From Koike et al. 2012)

demonstrate that gene expression per se rather than rhythmic gene expression is tightly correlated with circadian transcription factor binding. Rhythmic circadian transcription factor occupancy in turn could then be responsible for RNAPII recruitment and initiation on a genome-wide basis, which would then lead to the global rhythmic histone modifications seen here. Thus, circadian transcriptional regulators appear to be involved in the initial stages of RNAPII recruitment and initiation and the histone modifications associated with these events to set the stage for gene expression on a global scale, but additional control steps must then determine the ultimate transcriptional outputs from these sites.

In summary, we have defined the *cis*-regulatory network of the entire core circadian transcriptional regulatory loop on a genome scale and found a highly stereotyped, time-dependent pattern of core transcription factor binding, RNAPII

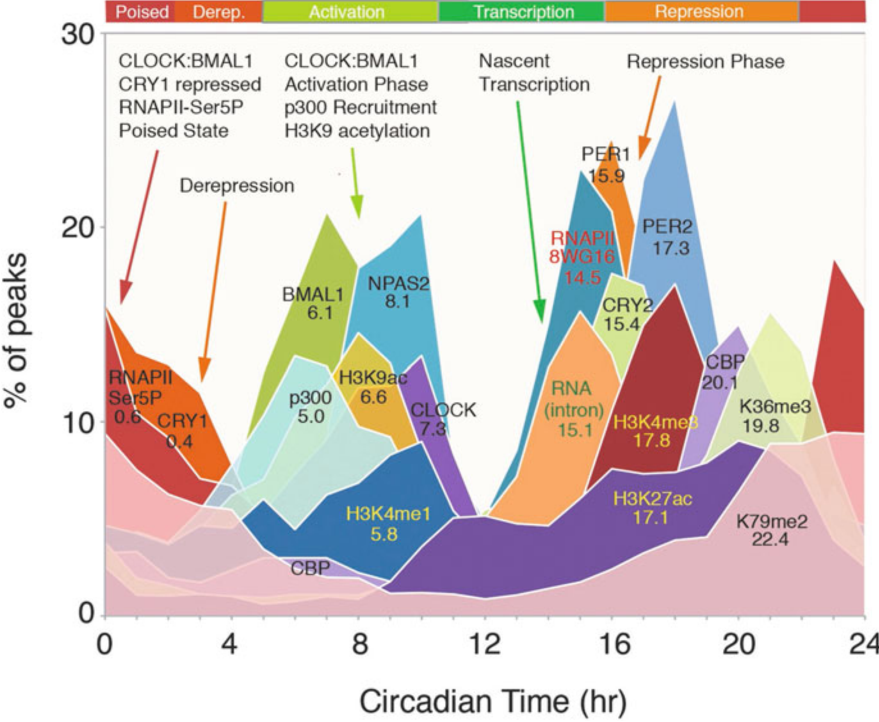

Fig. 7 Circadian transcriptional landscape in the liver. Histograms show the phase distributions of each factor as a function of time of day. *Derep* derepression (From Koike et al. 2012)

occupancy, RNA expression and chromatin states (Fig. 7). We defined three distinctive phases of the circadian cycle: (1) a poised phase in which CLOCK: BMAL1 and CRY1 bind to E-box sites in a transcriptionally silent state associated with RNAPII-Ser5P; (2) a temporally coordinated transcriptional activation phase in which RNAPII and p300 recruitment, pre-mRNA transcript expression, and H3K9ac, H3K4me3 and H3K27ac occupancy oscillate; and (3) a repression phase in which PER1, PER2 and CRY2 occupancy peaks. Circadian modulation of RNAPII recruitment and chromatin remodeling occurs on a genome-wide scale far greater than that seen previously by gene expression profiling. Thus, the circadian clock in the liver modulates the occupancy of RNAPII across the genome, leading at least in part to genome-wide circadian modulation of chromatin states that, in turn, poise the genome for transcription on a daily basis to act in concert with the daily metabolic demands of the organism.

Acknowledgment J.S.T. is an Investigator in the Howard Hughes Medical Institute. Research also supported by awards from the NIH.

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Circadian Mechanisms in Bioenergetics and Cell Metabolism

Joseph Bass

Abstract Circadian clocks are biologic oscillators present in all photosensitive species that produce 24-h cycles in the transcription of rate-limiting metabolic enzymes in anticipation of the light–dark cycle. In mammals, the clock drives energetic cycles to maintain physiologic constancy during the daily switch in behavioral (sleep/wake) and nutritional (fasting/feeding) states. A molecular connection between circadian clocks and tissue metabolism was first established with the discovery that 24-h transcriptional rhythms are cell-autonomous and self-sustained in cultured fibroblasts, and that clocks are present in most tissues and comprise a robust temporal network throughout the body. A central question remains: how do circadian transcriptional programs integrate physiologic systems within individual cells of the intact animal and how does the ensemble of local clocks align temporal harmonics in the organism with the environment? Our approach to studies of metabolic regulation by the molecular clock began with analyses of metabolic pathologies in circadian mutant animals, experiments that first became possible with the cloning of the clock genes in the late 1990s. A paradox in our early studies was that the effects of circadian clock disruption were both nutrient- and time-dependent, so that, under fed conditions, animals exhibited diabetes whereas during fasting, they decompensated and died. Application of a broad range of tissue-specific genetic and biochemical approaches has now begun to provide mechanistic insight into the circadian control of metabolism.

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P. Sassone-Corsi, Y. Christen (eds.), *A Time for Metabolism and Hormones*, Research and Perspectives in Endocrine Interactions,
DOI 10.1007/978-3-319-27069-2_3

Genetic Approaches to Dissecting Circadian Physiology

Glucose homeostasis is a dynamic process that is subjected to rhythmic variation throughout the daily light–dark cycle. Impaired glucose regulation arises from desynchrony in the integration of anabolic, catabolic, and incretin hormones across the circadian cycle and leads to metabolic syndrome and diabetes mellitus, disorders that are associated with over-nutrition, sedentary lifestyle, and sleep-wake disruption common in industrialized society. Individuals with diabetes must adjust their insulin levels differently every day and night even independently of how much they eat; however, the molecular underpinnings of circadian glucose regulation were previously not well understood. Genome-wide association and deep-sequencing studies have shown that variants of the *melatonin receptor 1b* and *cryptochrome 2* genes correlate with glucose variation in humans, suggesting a genetic linkage between the circadian system and glucoregulatory processes in man (Bouatia-Naji et al. 2009; Mulder et al. 2009; Dupuis et al. 2010). Against this backdrop, work from our laboratory using circadian clock mutant mice first revealed an essential role for the intrinsic beta cell clock in insulin secretion, beta cell development, and diabetes mellitus (Marcheva et al. 2010). Subsequent studies in three other groups have corroborated our observation that local function of the clock transcription factor in islets is crucial for normal glucose homeostasis (Sadacca et al. 2011; Lee et al. 2013; Pulimeno et al. 2013). Importantly, work from the Dibner laboratory has been the first to manipulate and monitor clock function in isolated human islet cells, raising the possibility that future investigation into circadian cell physiology will yield new understanding of beta cell failure in man (Pulimeno et al. 2013). In recently published work, we have developed tamoxifen-inducible Cre-LoxP technology to conditionally eliminate clock gene function in pancreas (PMID 26542580). Remarkably, our results establish that acute pancreatic clock ablation in the adult is sufficient to cause diabetes mellitus in the whole animal. These new genetic studies are the first to demonstrate an essential role for the adult circadian system in beta cell glucose regulation, although a gap remains in our understanding of the cell and molecular bases for clock function in the beta cell. Using conditional gene targeting and next-generation sequencing described in the following sections, we are presently poised to dissect the genomic, biochemical, and physiologic mechanism of the clock in beta cell failure. Moreover, since clock transcription factors impact both islet cell growth and stress response, we also seek to understand the role of the islet cell clock in susceptibility to beta cell apoptosis in type 1 diabetes, in islet regenerative capacity, and in islet cell survival in insulin resistant obesity.

Our analysis of the beta cell clock also opens broader insight into the role of transcription factor deregulation in beta cell failure and the unifying and distinct molecular events between tissues that culminate in diabetes mellitus. In this regard, positional cloning of genes causing Maturity Onset Diabetes of the Young (MODY) in humans has revealed that the hepatic nuclear factor (HNF) network of forkhead transcription factors plays a critical role in beta cell development and function,

although knowledge of the cell-context specific determinants of HNF action in liver and pancreas remains incomplete. By analogy, an important goal in understanding how beta cell transcription defects related to the clock pathway give rise to diabetes will be to elucidate differences in the clock-controlled enhancer network in liver and pancreas and to compare the cistromes and transcriptomes for these factors in each tissue. Interestingly, there is coincidence of CLOCK/BMAL1 binding sites in liver at loci marked by monomethylated H3K4 in pancreas, although it is not known whether the enhancer state (poised, H3K4me1 vs active, H3K4me1 with H3K27Ac) in pancreas varies over the 24-h cycle. In the long term it will be necessary to evaluate the cistromes and localization of clock factors with established transcription factors involved in beta cell function over the full circadian cycle in both liver and pancreas. Such studies will further elucidate the mechanism by which the clock controls gene transcription networks involved in insulin release, namely by determining the extent to which CLOCK/BMAL1 directly binds to promoters and/or enhancers or regulates epigenetic chromatin modifiers that determine accessibility to transcription factors and RNA polymerase genome-wide. Overall, studies of the beta cell molecular clock will elucidate how glucose homeostasis is coupled to the light/dark cycle and the transcriptional determinants of circadian physiology.

Clock-NAD$^+$-Sirtuin Pathway in Bioenergetics

A major step in understanding how the clock-NAD$^+$ cycle impacts physiology came from the observations that circadian mutant mice become hypoglycemic and die when subject to a prolonged fast (PMID 24051248, unpublished data) and also exhibit muscle and heart failure (PMID 20956306, 21452915), all hallmarks of mitochondrial disease, which prompted us to dissect the mechanisms of clock regulation of mitochondrial function. We began our investigation into the effect of NAD$^+$ deficiency on mitochondrial function in circadian mutant animals using a multi-faceted approach, including unbiased proteomics, which led to the identification of abnormal acetylation of enzymes involved in lipid oxidation, amino acid catabolism, tricarboxylic acid (TCA) cycle, electron transport chain (ETC), and superoxide dismutase pathways. Importantly, loss-of-function mutations in several of these oxidative enzymes have also been identified in the human metabolic myopathy syndrome and in both glioblastoma and renal cell carcinoma, indicating a broader effect of the clock-NAD$^+$ pathway on mitochondrial metabolism in both normal and transformed cells. Using tissue- and cell-based bioenergetics assays, we discovered that abrogation of the clock impairs electron transfer from lipid to the TCA cycle, in addition to increased mitochondrial production of superoxide free radical, increasing sensitivity to genotoxic stress. Our work also showed that cells exhibit an autonomous rhythm of oxygen consumption, glucose oxidation, and mitochondrial lipid catabolism. Importantly, the oxygen consumption cycle in muscle is directly linked to metabolism of NAD$^+$ and activity of the mitochondrial NAD$^+$-dependent deacetylase SIRT3 (Peek et al. 2013).

Although the aforementioned work has pinpointed specific defects in clock control of mitochondrial function, several unanswered questions remain in dissecting the effect of clock-NAD^+ rhythms on physiology and cell biology. First, it is not yet known how NAD^+ deficiency locally within skeletal muscle contributes to respiration or exercise tolerance in circadian mutant mice or in animals subjected to environmental circadian disruption. Though skeletal muscle ablation of the clock has been achieved in our group and others, the biochemical pathways through which clock abrogation impairs oxidative capacity remain largely unknown (Dyar et al. 2014).

Second, we still do not know whether clock abrogation and NAD^+ deficiency in liver or skeletal muscle impacts overall energy balance and alters the capacity to utilize carbohydrate and lipid as a fuel source. New pharmacologic (Wang et al. 2014) and genetic means to raise NAD^+ both globally in the whole animal and selectively within either liver or skeletal muscle are now available and will be powerful tools in evaluating the potential to boost NAD^+ as a therapeutic strategy in myopathy and liver defects of circadian mutant animals. Finally, in addition to its function as a cofactor for the class III histone deacetylases, NAD^+ is a cofactor for the poly-ADP-ribosylases, critical factors in DNA repair and stress response, though the possible interaction between rhythmic regulation of NAD^+ and PARP activity is not known. Lastly, NAD^+ functions as an electron transport molecule and, as such, it is a direct marker of cellular redox state and the balance between glycolytic and oxidative metabolism. Whether NAD^+ might participate in the bidirectional communication between metabolism and the clock system remains an area of intensive investigation. In summary, discovery of the clock as an upstream regulator of NAD^+ provides a wealth of opportunity to dissect the interrelationship between circadian rhythms, physiology, and epigenetics.

Reciprocal Control of the Clock by Nutrient

Circadian clocks are biologic oscillators that produce 24-h cycles in the transcription of rate-limiting metabolic enzymes in anticipation of the solar cycle. The molecular clock is programmed by a transcription-translation feedback loop that is comprised of activators (CLOCK/BMAL1) that induce the expression of their own repressors (CRYs/PERs) in a cycle that repeats itself every 24 h. The REV-ERB and ROR proteins form an ancillary loop that modulates *Bmal1* transcription. In animals, clocks are organized hierarchically, with brain pacemaker cells synchronizing peripheral tissue clocks, leading to a classical view of the central clock as the main driver of metabolism. However, circadian oscillations within both brain and peripheral tissues have recently been shown to be sensitive to timing of nutrient availability and can become uncoupled from the light–dark cycle, as demonstrated by experimentally restricting food access to the light cycle when mice are normally resting (Damiola et al. 2000; Stokkan et al. 2001). Further, simply substituting regular with high fat chow in mice fed ad libitum lengthens

periodicity of locomotor activity and alters peripheral metabolic rhythms, providing further evidence for a bidirectional relationship between clock function and metabolism (Kohsaka et al. 2007). Our discovery that diet-induced obesity reprograms both the cellular molecular clock and behavior revealed for the first time that a controlled change in nutritional environment leads to altered circadian rhythms. This idea, that circadian and metabolic systems reciprocally interact and that perturbation of the metabolic environment alters the homeostatic relationship between these systems, has been widely confirmed but still remains poorly understood at the mechanistic level. Human analyses, including genome-wide association studies, population based case–control investigation, and clinical research, have cumulatively indicated a strong interrelationship between circadian disruption, obesity, diabetes mellitus, and metabolic syndrome. Moreover, certain inflammatory and cardiovascular events, including thrombosis and nocturnal asthma, exhibit pronounced circadian variation. Surprisingly, dietary macronutrient directly impacts behavioral and molecular clock function, and circadian disruption itself exacerbates the progression of diet-induced obesity, exerting distinct effects within local metabolic organs. Moreover, limiting high-fat food to the incorrect circadian phase accelerates weight gain, whereas limiting high-fat feeding to the correct phase ameliorates hepatic steatosis, a hallmark of metabolic syndrome (Maury et al. 2010). While we previously demonstrated that diet-induced obesity reprograms the cellular molecular clock and circadian behavior, we have more recently sought to identify the macronutrient disruptor of circadian behavior by providing mice an isocaloric diet high in either saturated or unsaturated fats (SFD and UFD). Our goal is to identify the mechanism by which a macronutrient directly alters behavior and neuronal circadian pacemaker function. We propose that nutrient signaling plays a central role in inter-organ circadian communication and that circadian disruption induced by high saturated fat contributes to the rate of progression of metabolic syndrome.

Summary and Future Directions

A major window to understanding how the clock is coupled to metabolism was opened with discovery of metabolic syndrome pathologies in multi-tissue circadian mutant mice, including susceptibility to diet-induced obesity, mis-timed feeding rhythms, hypoinsulinemia, and energetic collapse upon fasting. Using Cre-LoxP conditional transgenesis and dynamic endocrine testing, we have pinpointed the tissue-specific role of the clock in energy and glucose homeostasis, with our most detailed understanding of this process in liver, muscle, and endocrine pancreas. In the post-prandial condition, the beta cell clock is essential for nutrient and adenyl cyclase-induced insulin exocytosis. In contrast, the hepatocyte and myocyte clocks are required for oxidative metabolism. Circadian mutant mice die upon prolonged fasting due to mitochondrial failure, a defect that we have tied to the bioavailability of NAD^+, a cofactor of the class III histone deacetylases and poly-ADP ribosylase

enzymes involved in adjusting metabolic and gene regulation in response to environmental change, including glucose deprivation, oxidative damage, and cell stress. Indeed, we have found that liver and myoblasts exhibit an autonomous rhythm of oxygen consumption, glucose oxidation, and mitochondrial lipid catabolism that is directly linked to an autonomous rhythm of NAD^+ metabolism and, consequently, to cyclic activity of the mitochondrial NAD^+-dependent deacetylase SIRT3. NAD^+ supplementation using the pro-drug NMN improves respiration in live animals, indicating that circadian control of NAD^+ metabolism plays a key role in cellular and organismal respiration. A future challenge will be to determine the cell and molecular basis for the interplay between nutritional and circadian processes important in metabolic health and disease states.

Acknowledgments

Funding Sources P01AG011412 (National Institute on Aging), R01DK090625 (National Institute of Diabetes and Digestive and Kidney Disease), R01DK100814 (National Institute of Diabetes and Digestive and Kidney Disease), 17-2013-511 (Juvenile Diabetes Research Foundation with Helmsley Charitable Trust), 1-INO-2014-178-A-V (Juvenile Diabetes Research Foundation with Helmsley Charitable Trust). I thank the lab members who participated in our cited research and to KM Ramsey for helpful comments and suggestions on the manuscript.

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Control of Metabolism by Central and Peripheral Clocks in Drosophila

Amita Sehgal

Abstract Drosophila is a powerful system for the molecular analysis of circadian clocks, providing the first account of how such a clock is generated. It is also proving to be an excellent model to dissect the neural basis of circadian behavior. In addition, clocks are located in peripheral tissues in flies, but much less is known about these clocks and about the physiological processes they control. This chapter describes the use of Drosophila for understanding the circadian control of metabolism. While a clock in the fat body is critical for metabolic function, it is clear that neuronal clocks are also involved. Indeed, synchrony between these clocks is important for reproductive fitness. A complex interplay between circadian and metabolic signals is indicated by the finding that metabolic pathways can even impact rest:activity rhythms controlled by the brain clock. Drosophila may be an optimal system to dissect the nature of these interactions and their importance for organismal fitness and life span.

Genetic analysis of circadian rhythms started with the isolation of the *period (per)* mutants in the fruit fly, *Drosophila melanogaster*, followed by isolation of the *per* gene in the mid 1980s (Bargiello et al. 1984; Jackson et al. 1986; Konopka and Benzer 1971; Reddy et al. 1984; Zehring et al. 1984). Subsequent studies identified the *per* partner, *timeless (tim)*, and the transcriptional feedback loop that we now know lies at the heart of the clock mechanism in all species (Sehgal et al. 1994, 1995). In the Drosophila loop, the Clock (CLK) and cycle (CYC) transcriptional activators promote expression of *per* and *tim* mRNA during the mid to late day but are repressed by feedback activity of PER-TIM in the late night and early morning. Regulated expression and activity of clock proteins in this loop are sustained through post-translational mechanisms, in particular the action of multiple kinases and phosphatases (Zheng and Sehgal 2008, 2012).

Contrary to expectations that clocks would be localized largely, if not exclusively, in the brain, analysis of Drosophila *per* showed that it was expressed in multiple tissues throughout the body (Liu et al. 1988; Saez and Young 1988). Indeed, use of a reporter in which *per* was fused to firefly luciferase showed that

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P. Sassone-Corsi, Y. Christen (eds.), *A Time for Metabolism and Hormones*,
Research and Perspectives in Endocrine Interactions,
DOI 10.1007/978-3-319-27069-2_4

per was expressed cyclically in most tissues. Analysis of isolated tissues revealed that luciferase activity continued to cycle in the absence of neural connections or systemic signals, indicating the presence of tissue-autonomous clocks (Plautz et al. 1997). Subsequent studies showed that the degree of autonomy varied from tissue to tissue. The Malphigian tubules or fly kidneys, for instance, appeared to be completely autonomous, such that they retained their own "timing" even when transplanted into a host that was synchronized to a different day:night cycle (in other words, a different time zone; Giebultowicz et al. 2000). On the other hand, the clock in the prothoracic gland, which drives a circadian rhythm of eclosion (hatching of adult flies from pupae) in Drosophila, is "slave" to the "master clock in the brain (Myers et al. 2003). Thus, the brain clock is required for eclosion rhythms as well as for maintenance of the prothoracic clock (Myers et al. 2003). In addition, central nervous system signals, in particular the neuropeptide Pigment Dispersing Factor (PDF), modulate the clock in pheromone-producing oenocytes, which regulate mating (Krupp et al. 2013).

The emerging pattern is that of a network of clocks that control many aspects of physiology and depend upon neural function to varying extents. The question is the extent to which Drosophila can be used to study circadian regulation of these different physiological processes and provide an understanding of the circadian system as a whole. This chapter outlines studies directed towards circadian control of metabolism in Drosophila.

Use of Drosophila to Study Behavior and Metabolic Function

As noted above, Drosophila has proved to be an outstanding system to dissect the molecular basis of the clock. Genes first found in Drosophila are now known to be mutated in some human circadian disorders. It is now also clear that Drosophila can be exploited to provide a complete understanding of the neural circuits that drive rhythms in behavior. The *per* and *tim* mutants were isolated through screens that used eclosion behavior as an assay for circadian function. Eclosion is "gated" by the circadian clock to occur around dawn, so while it only occurs once in the life of every fly, it can be monitored as a rhythm in a population. In addition to eclosion, the *per* and *tim* mutants were found to affect rhythms of rest:activity, and subsequently, in particular with the development of high throughput systems for monitoring locomotor activity, the field shifted to almost exclusively using rest:activity as a readout of internal clock function. Through work done in several laboratories, we now have a fairly good understanding of the clock neurons in the brain that drive rhythms of rest:activity (Nitabach and Taghert 2008). Interestingly, different subsets of neurons are required for different aspects of the overt rhythm, for instance. for the morning and evening peaks of locomotor activity. In addition, we recently identified a neural circuit that connects the clock neurons to other brain cells required for rhythmic rest:activity (Cavanaugh et al. 2014). It seems likely that, in the near future, we will be able to trace the passage of time-of-day signals all the way from the clock to the motor neurons that drive activity.

Until ~2008, little to no work had been done on circadian metabolism in Drosophila. However, flies have been used for general studies of metabolism, and are particularly useful as a model for aging, which is influenced strongly by metabolic parameters (Katewa and Kapahi 2010). As circadian regulation may be relevant for aging, we undertook to address links between metabolism and the circadian system.

The Drosophila Fat Body Contains a Clock that Regulates a Rhythm of Feeding

As we were accustomed to monitoring behavior in Drosophila, our studies of metabolic function also started with measurements of a metabolism-influenced behavior. We assayed food intake at different times of day and found that flies display a circadian rhythm of feeding such that food intake occurs maximally in the morning hours (Xu et al. 2008). A later study identified an additional peak of feeding that occurs later in the day and confirmed that nighttime hours of quiescence are associated with reduced food intake. As required of an endogenously driven rhythm, the rhythm of feeding persists in the dark, i.e., in the absence of environmental cycles. Also, it is eliminated in the dark in flies lacking the *Clk* gene, demonstrating that it is under the control of the molecular clock mechanism described above (Xu et al. 2008).

To address the regulation of the feeding rhythm, we considered a role for the fat body, as this is a major metabolic tissue in Drosophila and is generally considered the functional equivalent of the liver. We found that clock genes, specifically *tim*, were expressed in the fat body and displayed a daily rhythm (Xu et al. 2008). To determine if this cycling was driven by a clock in the fat body, as opposed to signals from elsewhere, we disrupted the fat body clock by transgenically expressing a dominant negative version of the CLK protein. This manipulation abolished *tim* cycling, indicating that it depends upon a clock in the fat body. Interestingly, disruption of the fat body clock also affected the phase of the feeding rhythm, such that flies now showed maximal food consumption in the evening hours (Xu et al. 2008). The fact that the feeding rhythm was not abolished suggests that clocks in other tissues can also drive this rhythm.

Fat Body and Neuronal Clocks Coordinately Regulate Metabolic Parameters

We found that loss of the fat body clock did not just affect the feeding rhythm but also overall food intake (Xu et al. 2008). Food consumption was higher at all times of day relative to controls. Reasoning that increased food consumption increases sources of energy and therefore might be protective in adverse conditions of low

nutrient availability, we tested flies lacking a fat body clock in starvation assays. To our surprise, we found that they were actually more sensitive to starvation and so died earlier than their wild type counterparts. This finding suggested that the increased food consumption was not increasing nutrient stores but was perhaps occurring in response to low endogenous levels of nutrients. Indeed, we found that glycogen and triglyceride levels were low in flies that lacked a clock in the fat body.

These results were unexpected because clock mutants, in other words flies lacking clocks in all tissues, do not show obvious metabolic phenotypes. The defects seen when only the fat body clock was ablated suggested that clocks in other tissues might have opposing effects on metabolic parameters. Neurons appeared to be good candidates for housing such clocks, as the brain is known to regulate metabolic activity, and so we disrupted clock function in neurons. We used the same tool as for the fat body clock (dominant negative clock proteins) and confirmed that neuronal clocks were disrupted by monitoring rest:activity behavior. As expected, rest:activity was arrhythmic. Measurement of metabolic parameters showed that nutrient stores, triglycerides and glycogen, were higher in flies with disrupted neuronal clocks than in wild type controls (Xu et al. 2008). As might be predicted, loss of neuronal clocks also increased resistance to starvation.

These data indicate that the fat body and the neuronal clock oppose each other in the control of metabolic function (Fig. 1). Typically, the fat body clock suppresses feeding, promotes storage of nutrients and increases resistance to starvation. Thus, loss of the fat body clock results in increased feeding, lower nutrient stores and sensitivity to starvation. Conversely, neurons are very metabolically active, and so clocks in these promote feeding, depletion of energy stores and sensitivity to starvation. All these functions are likely reversed when neuronal clocks are lost.

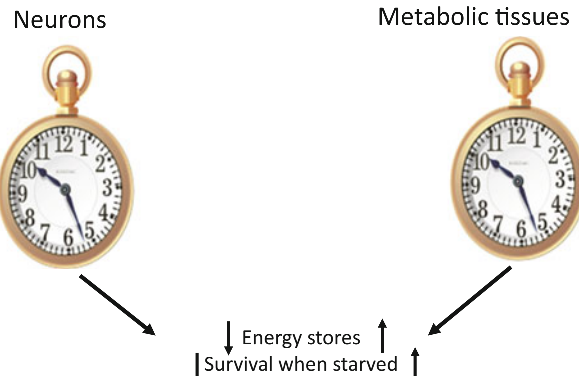

Fig. 1 Neuronal and metabolic clocks have opposing effects on metabolic parameters. These effects are predicted based upon phenotypes obtained by disrupting neuronal or fat body (metabolic) clocks. Disruption of neuronal clocks increases glycogen and trigyceride stores and promotes survival in response to starvation, whereas disruption of the fat body clock decreases glycogen and trigyceride stores, increases feeding and decreases survival upon starvation

We documented increased nutrients and resistance to starvation in the absence of neuronal clocks but were unable to reliably quantify food intake, as this was so low.

In a subsequent study, we identified a specific group of neuron that regulate triglyceride levels (DiAngelo et al. 2011). These are the central clock neurons in the brain, which are critical for rest:activity rhythms. Interestingly, though, the effects of these neurons on triglyceride levels are separable from their effects on rest: activity.

Rhythmic Gene Expression in the Fat Body Is Controlled Largely, but not Exclusively, by the Fat Body Clock

To address the mechanisms by which the fat body clock regulates metabolic homeostasis, we sought to identify the genes expressed rhythmically in this tissue (Fig. 2). To this end, we collected tissue every 2 h around the clock over a 2-day period and profiled gene expression using microarrays (Xu et al. 2011). Simultaneously, we collected samples every 4 h from flies lacking a fat body clock due to expression of a dominant negative form of the CLK protein. We found that expression of many genes is cyclic in the fat body. Interestingly, several of these continue to cycle when the fat body clock is ablated, suggesting the influence of other factors, either the light:dark cycle or clocks elsewhere. In recent work, we have found that clocks in other tissues are required for at least some of the rhythmic cycling in the fat body.

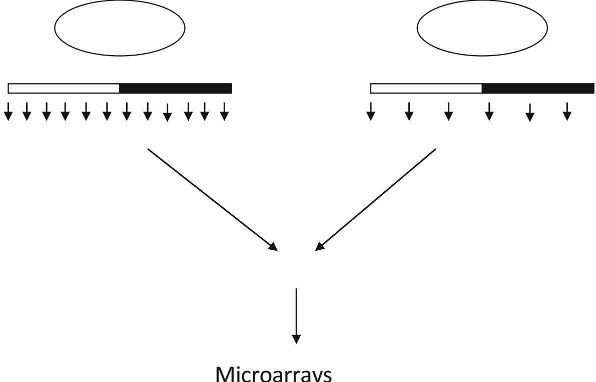

Fig. 2 Circadian gene expression in the fat body: The protocol shown was followed to assay circadian gene expression in the fat body. Fat bodies were collected at 2-h intervals over a 48-h cycle in wild type flies and at 4-h intervals in flies lacking a fat body clock. Several classes of genes were found to cycle

A Restricted Feeding Paradigm Resets the Phase of Cyclic Gene Expression in the Fat Body but not in the Brain

The genes expressed cyclically in the fat body fall into many different functional categories, including lipid synthesis (in particular, fatty acid elongation), lipid breakdown, steroid hormone metabolism and immune function. The peak of gene expression for these different processes tended to occur at different times of day. To determine if temporal separation of gene expression by the clock was important for metabolic physiology, we sought to disrupt this temporal relationship. Reasoning that the time of feeding might be important for the peak in metabolic gene expression, but perhaps not for expression of immune genes, we restricted food to a time of day when feeding was typically less (6 h in the early evening) and we examined circadian gene expression (Xu et al. 2011). We found that the time of feeding was indeed important, in fact even more than predicted. Thus, the clock in the fat body was reset by the time of feeding, which led to a reset of all downstream cycling genes.

Restricted feeding (RF) only changed the phase of gene expression if it occurred at the wrong time of day. If food was restricted to a time that corresponded to the normal daily peak of feeding, then the phase was maintained and the amplitude of the rhythm became stronger (note that normally the amplitude is low in constant darkness). On the other hand, RF had no effect on circadian gene expression in the brain (Xu et al. 2011).

Decoupling Peripheral and Brain Tissues Decreases Reproductive Fitness

As discussed above, a RF paradigm desynchronizes brain and fat body clocks as it resets the fat body, but not the brain clock. To determine if this process had physiological consequences, we monitored egg laying as a measure of reproductive fitness in animals maintained on RF. To exclude any influence of the duration of feeding, we compared egg production by flies fed for 6 h daily at the time they would normally eat with those fed for 6 h at the wrong time of day (Xu et al. 2011). Measurements of food intake showed equal food consumption in both groups, indicating that 18 h of starvation promoted equivalent feeding regardless of circadian time.

We found that flies fed at the wrong time laid fewer eggs than those fed at the correct time. However, these differences were not noted in a *Clk* mutant, indicating that they reflected an interaction of the time of feeding with endogenous clocks (Xu et al. 2011). We surmise that desynchrony of brain and peripheral clocks, achieved by an RF paradigm, reduced reproductive success.

Metabolic Signals Also Affect Clocks in the Brain

While this chapter focuses on the circadian control of metabolism, we have also uncovered effects of metabolic signals of central clock function and rest:activity behavior. We found that the FOXO protein, a well-known component of metabolic pathways, is expressed in the fat body but can influence the brain clock's response to oxidative stress (Zheng et al. 2007). We also found that manipulations of the TOR-Akt pathway alter periodicity of rest:activity rhythms in parallel with effects on the molecular clock in brain neurons (Zheng and Sehgal 2010). Thus, metabolism and circadian clock interact on multiple levels, with consequences in both directions.

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Circadian Post-transcriptional Control of Metabolism

Carla B. Green

Abstract Circadian clocks control thousands of genes, which ultimately generate rhythms in signaling pathways, metabolism, tissue physiology and behavior. Although rhythmic transcription plays a critical role in generating these rhythmic gene expression patterns, recent evidence has shown that post-transcriptional mechanisms are also important. Here we describe studies showing that regulation of mRNA poly(A) tail length is under circadian control and that these changes contribute to rhythmic protein expression independently of transcription. Nocturnin, a circadian deadenylase that shortens poly(A) tails, contributes to this type of circadian post-transcriptional regulation. The importance of tail-shortening by Nocturnin is evident from the phenotype of mice lacking Nocturnin, which exhibit resistance to diet-induced obesity and other metabolic changes.

Introduction

Circadian clocks regulate and coordinate rhythms in behavior, physiology, biochemistry and gene expression in mammals (Pittendrigh 1981a, b; Akhtar et al. 2002; Panda et al. 2002; Storch et al. 2002; Ueda et al. 2002; Duffield 2003; Welsh et al. 2004; Reddy et al. 2006), allowing animals to synchronize appropriately to the environmental light:dark cycles. The mammalian circadian clock is composed of an intracellular feedback mechanism in which interlocking transcriptional-translational feedback loops generate the 24-h rhythms (reviewed in Lowrey and Takahashi 2004; Takahashi et al. 2008) and drive rhythms of 5–10 % of genes in a cell type-specific manner (Duffield 2003; Rey et al. 2011; Koike et al. 2012; Menet et al. 2012). This extensive control over mRNA expression results in rhythmicity of many cellular pathways, including many aspects of metabolism. Mutations that alter the clock have broad negative effects on the

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P. Sassone-Corsi, Y. Christen (eds.), *A Time for Metabolism and Hormones*,
Research and Perspectives in Endocrine Interactions,
DOI 10.1007/978-3-319-27069-2_5

organism, including insulin resistance and obesity (Rudic et al. 2004; Shimba et al. 2005; Turek et al. 2005; Green et al. 2008), some types of cancer (Fu et al. 2002; Gorbacheva et al. 2005; Hoffman et al. 2009, 2010a, b; Ozturk et al. 2009; Kang et al. 2010), cardiovascular disease (Curtis et al. 2007; Reilly et al. 2007) and sleep and affective disorders (Toh et al. 2001; Kripke et al. 2009; Srinivasan et al. 2009; Touma et al. 2009). Therefore, an understanding of the molecular mechanism of clocks in mammals is critical for the understanding and treatment of human health.

The components of the central circadian clock are transcriptional activators and repressors, and cyclic activation and repression drive the oscillation that comprises the pacemaker and generates the 24-h periodicity. In addition, these proteins drive rhythms in many other genes, through both direct and indirect transcriptional mechanisms. Although this transcriptional control is a major contributor to the resulting rhythms in mRNA levels, a number of recent studies have demonstrated that post-transcriptional regulation also must play an important role. For example, a large percent of rhythmic mRNAs in liver do not have rhythmic pre-RNAs (Koike et al. 2012; Menet et al. 2012) and, in mouse liver, almost 50 % of the rhythmic proteins do not have rhythmic steady-state mRNA levels (Reddy et al. 2006). Moreover, circadian rhythms can exist in red blood cells devoid of nuclei (O'Neill and Reddy 2011; O'Neill et al. 2011). Therefore, regulatory mechanisms beyond transcription can also drive rhythmic physiology.

Post-transcriptional Mechanisms

Although transcription drives mRNA synthesis, the ultimate protein expression patterns also reflect regulation at many other levels (Fig. 1). Even as the mRNA is being transcribed, large complexes of proteins associate with the nascent transcript and regulate the efficiency and pattern of splicing, the choice of 3′-end cleavage site and polyadenylation (Pawlicki and Steitz 2010). The mature transcript undergoes further regulation during nuclear export, cytoplasmic localization, RNA stability and translation. The importance of post-transcriptional regulation has become clear over the last decade, with the discovery of many RNA binding proteins, specific types of ribonucleases, and the extensive machinery that conducts microRNA-mediated control of mRNA stability and translation. Although significant progress has been made in this area, understanding of post-transcriptional mechanisms still lags behind that of transcriptional processes.

The poly(A) tails at the 3′-end of most eukaryotic mRNAs are thought to be important for controlling translatability and stability, and one post-transcriptional regulatory mechanism is to modulate the length of these tails. Indeed, regulation of poly(A) tail length has been shown to play critical roles in many biological processes, including oocyte maturation, mitotic cell cycle progression, cellular senescence and synaptic plasticity (Gebauer et al. 1994; Groisman et al. 2002, 2006; Huang et al. 2002, 2006; Novoa et al. 2010). Changes in poly(A) tail length can occur at many points during the lifetime of an mRNA. Long poly(A) tails of

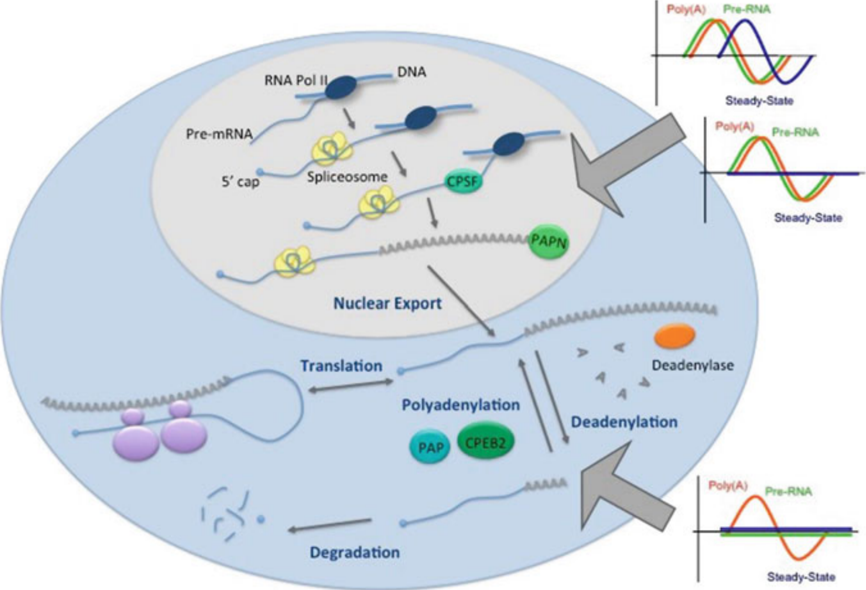

Fig. 1 Post-transcriptional regulation controls expression at many steps throughout the lifetime of the mRNA, and rhythms in poly(A) tail length can result from transcriptionally coupled mechanisms and cytoplasmic mechanisms (Kojima et al. 2012)

about 250 nt are initially added to the nascent transcript in the nucleus following the 3′-end cleavage (Kuhn and Wahle 2004). Following export out of the nucleus, a protein called cytoplasmic poly(A)-binding protein (PABPC) binds to the tail and stabilizes the mRNA. Through direct interactions with the translation-initiation factor eIF4G, which in turn binds to the cap-binding protein eIF4E, PABPC is thought to facilitate translation initiation by forming a “closed-loop” circular structure (Kuhn and Wahle 2004). Removal or shortening of the tail by a specific class of ribonucleases called deadenylases can, in turn, result in translational silencing and, in some cases, mRNA degradation. Alternatively, cytoplasmic polyadenylation can, in some cases, lengthen the tail of an mRNA that was previously shortened and stabilize it and render it translationally competent (Richter 2007).

Circadian Control of poly(A) Tail Length

Daily variations in poly(A) tail length were reported for two mRNAs (Robinson et al. 1988; Gerstner et al. 2012), causing us to wonder whether the circadian clock uses this mechanism more broadly to regulate gene expression post-transcriptionally. Therefore, we fractionated mRNAs from mouse livers collected

at various circadian times into pools of mRNAs with short (~50 nt) and long (>100 nt) poly(A) tails (Kojima et al. 2012) using a modification of an oligo (dT) affinity chromatography method with differential elution stringencies (Meijer et al. 2007). These pools of mRNAs, along with a non-fractionated total poly(A)+ control, were subjected to microarray analysis, and relative tail-length was determined by the ratio of expression of each mRNA in the long vs. short tail pools (normalized for expression level using the total poly(A)+ expression level). Using this method, followed by independent validation, we identified several hundred mRNAs that exhibited robust changes in poly(A) tail length over the course of the circadian day.

Further characterization of these mRNAs revealed that they fell into three general classes (Fig. 1). The first class contained mRNAs that were transcribed rhythmically and also exhibited rhythms in their overall steady-state levels. The second class was also transcribed rhythmically but these mRNAs had long half-lives and therefore were not rhythmic at the steady-state level. The third class of mRNAs with rhythmic poly(A) tails were not rhythmic at either the level of synthesis or at the steady-state level; these mRNAs appear to be long-lived and have poly(A) tails that are cyclically shortened and lengthened in the cytoplasm. Strikingly, in all the cases we tested, the poly(A) tail rhythms correlated with rhythmic protein levels, even in the cases where the steady-state levels of the mRNAs were not changing. These data suggest that circadian changes in poly (A) tail length can significantly contribute to rhythmic protein synthesis, independent of transcription.

Nocturnin Is a Circadian Deadenylase

The mechanism by which the clock controls poly(A) tail length is not well understood and appears to involve different mechanisms at different circadian phases (Kojima et al. 2012). However, one strong candidate is the deadenylase Nocturnin (gene name, *Ccrn4l*; Green and Besharse 1996; Baggs and Green 2003), which is robustly rhythmic in many mouse tissues, with peak expression in the middle of the night (Wang et al. 2001; Garbarino-Pico et al. 2007; Kojima et al. 2010). Nocturnin is a member of the superfamily of deadenylases that includes CCR4, Nocturnin, Angel, and 2′PDE (Goldstrohm and Wickens 2008; Godwin et al. 2013), but Nocturnin has a distinct amino-terminus from the other members. Nocturnin is also unique among all deadenylases in its characteristic high amplitude rhythms, with nighttime peaks (most of the other deadenylases are arrhythmic or have very low amplitude rhythms that peak in the day) (Kojima et al. 2012). In addition, Nocturnin is unique in that it is an immediate early gene that is acutely induced by many stimuli (Garbarino-Pico et al. 2007). Given the difference in temporal and spatial expression patterns of the deadenylases (Yamashita et al. 2005; Morita et al. 2007; Wagner et al. 2007; Kojima et al. 2012) and the different phenotypes caused by disrupting specific deadenylases (Molin and Puisieux 2005; Morris

et al. 2005; Green et al. 2007; Morita et al. 2007; Washio-Oikawa et al. 2007), it is likely that each deadenylase targets a specific set of transcripts, although the identities of these transcripts and the mechanisms by which they are targeted by a particular deadenylase are not well characterized.

Loss of Nocturnin Results in Broad Metabolic Changes

The importance of Nocturnin's contribution to circadian changes in poly(A) tail length was tested by generating mice lacking Nocturnin (*Noc−/−*; Green et al. 2007). These mice appeared normal and healthy when raised in standard conditions and bred well. However, when raised on a Western-style high fat diet, the *Noc−/−* mice did not gain weight at the same rate as the wild-type mice and remained lean whereas the wild-type mice became obese (Fig. 2). The *Noc−/−* mice had smaller fat pads and were protected from hepatic steatosis. Despite this resistance to diet-induced obesity, the *Noc−/−* mice did not eat less, were not more active, and did not show significant changes in whole body respiration as measured in metabolic cages (Green et al. 2007; Douris and Green 2008). These mice did, however, have changes in mRNA expression levels of many key metabolic regulators in the liver, often showing loss of rhythmicity of normally rhythmic genes. Nocturnin is likely not part of the core circadian mechanism, because the *Noc−/−* mice had normal circadian locomotor rhythms and normal expression of the core clock genes in the liver. However, it is directly regulated by the core circadian transcription factor heterodimer CLOCK/BMAL1 and is, therefore, a direct output of the intracellular core circadian loop. In addition, it is regulated by systemic circadian signals, likely originating directly or indirectly from the core circadian pacemaker in the suprachiasmatic nucleus in the hypothalamus, because Nocturnin is one of only a few dozen rhythmic genes that maintain rhythmicity following

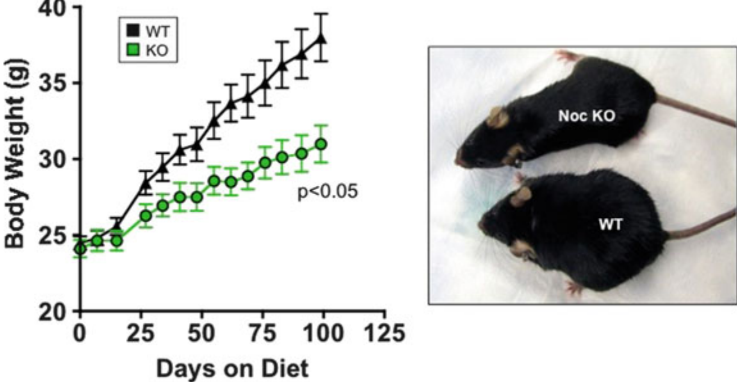

Fig. 2 Nocturnin knockout (KO) mice are resistant to diet-induced obesity. *WT* wild-type (Modified from Green et al. 2007)

genetic disruption of the clock, specifically in the liver of mice (Kornmann et al. 2007).

Some clues to the mechanism behind the lean phenotype observed in the *Noc−/−* mice came from examination of Nocturnin's role in the small intestine (Douris et al. 2011). Nocturnin is expressed throughout the digestive tract, but with particularly high levels in the upper part of the small intestine. As in other tissues, it is robustly rhythmic, peaking during the night—the time of maximal food intake in the nocturnal mouse. Pan and Hussain (2009) had previously shown that many of the transporters involved in macronutrient absorption by the intestinal enterocytes were under the control of the circadian clock. Accordingly, we found that lipid absorption in the wild-type mice was strongly circadian, with rapid appearance of newly ingested lipoprotein particles into the circulation when the mice were gavaged with olive oil at night, but slow and limited appearance when olive oil was administered during the day. In contrast, the *Noc−/−* mice had no rhythm in absorption and exhibited slow "daytime-like" absorption profiles following gavage given both night and day (Douris et al. 2011; Stubblefield et al. 2012). Furthermore, the enterocytes in the *Noc−/−* mice accumulated large cytoplasmic lipid droplets, suggesting that dietary lipids were stored in these cells when Nocturnin was not present—at all times in the *Noc−/−* cells or during the daytime in wild-type mice. The mechanism by which this deadenylase regulates dietary lipid absorption is not clear, but several mRNAs that encode proteins involved in lipid droplet formation, breakdown and chylomicron assembly are dysregulated in the *Noc−/−* intestine, and some of these may be direct targets of Nocturnin deadenylase activity.

Nocturnin also plays important roles in other metabolically relevant tissues. In bone, Nocturnin interacts with a specific long isoform of *Igf1* mRNA, suppressing its expression (Kawai et al. 2010a). In bone-marrow stromal cells, Nocturnin is acutely induced more than 30-fold by the peroxisome proliferator-activated receptor gamma (PPAR-gamma) agonist rosiglitazone, and *Noc−/−* mice have reduced marrow adiposity and high bone mass (Kawai et al. 2010b). In addition, overexpression of Nocturnin enhances adipogeneis in preadipocyte 3T3-L1 cells and negatively regulates osteogenesis in mouse osteoblastic MC3T3-E1 cells (Kawai et al. 2010b). Together these data suggest that Nocturnin plays an important role in the mesenchymal stem-cell lineage allocation that may ultimately influence adipogenesis and body composition.

Conclusions

The large contribution of post-transcriptional regulation to the generation and modulation of rhythmic mRNA and protein profiles has recently become apparent largely thanks to the use of genome-wide interrogation of rhythmic mRNA expression and transcriptional and post-transcriptional states. The ongoing development of innovative high-throughput methods for analyzing various nuances of gene expression (TAIL-seq, GRO-seq, CLIP-seq, and many more) will undoubtedly

yield ever more information about how the clock controls the many layers of gene expression that drive the complex rhythmic physiology and behavior of mammals. We expect that new modes of post-transcriptional regulatory mechanisms will be uncovered and that these will be shown to play an important role in shaping these rhythms.

Nocturnin is likely only one of many post-transcriptional modulators that contribute to circadian expression profiles, but the profound metabolic phenotype in the *Noc−/−* mice shows that it is playing an important role in regulating circadian metabolic profiles. However, to understand how loss of Nocturnin causes these phenotypes, it will be critical to identify the relevant Nocturnin target mRNAs and to uncover how these target mRNAs are recognized by Nocturnin. Finally, the exact function of deadenylation by Nocturnin is still not clear. Although removal of tails has long been thought to target them for decay, it has recently been discovered that many mRNAs are maintained in the cell in short-tailed states that are quite stable. Are these short-tailed mRNAs translationally silent and waiting to have their tails lengthened in response to the appropriate signal or do they have some other function? Only time will tell...

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Redox and Metabolic Oscillations in the Clockwork

Akhilesh B. Reddy

Abstract Daily (circadian) clocks have evolved to coordinate behaviour and physiology around the 24-h day. Most models of the eukaryotic circadian oscillator have focused principally on transcription/translation feedback loop (TTFL) mechanisms, with accessory cytosolic loops that connect them to cellular physiology. Recent work, however, questions the absolute necessity of transcription-based oscillators for circadian rhythmicity. The recent discovery of reduction-oxidation cycles of peroxiredoxin proteins, which persist even in the absence of transcription, have prompted a reappraisal of current clock models in disparate organisms. A novel mechanism based on metabolic cycles may underlie circadian transcriptional and cytosolic rhythms, making it difficult to know where one oscillation ends and the other begins.

Introduction

Daily biological clocks provide living organisms with temporal organisation over a 24-h timescale. Organisms from bacteria to humans have evolved these rhythms to adapt their physiology to the solar cycle and anticipate the availability of resources (e.g., food and light; Bass 2012). Despite their presence in evolutionarily disparate organisms, the molecules underlying the clockwork seemed to be different in these organisms. This finding has given rise to the identification of "clock genes" that oscillate with 24-h periods but that are not related in their DNA or protein sequences, except in some cases when comparing fruit flies and mammals. This has led to the notion of the divergent evolution of different clock circuits in various model organisms, the only link being the way in which the components are joined together in a negative feedback loop topology (Rosbash 2009; Fig. 1).

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P. Sassone-Corsi, Y. Christen (eds.), *A Time for Metabolism and Hormones*,
Research and Perspectives in Endocrine Interactions,
DOI 10.1007/978-3-319-27069-2_6

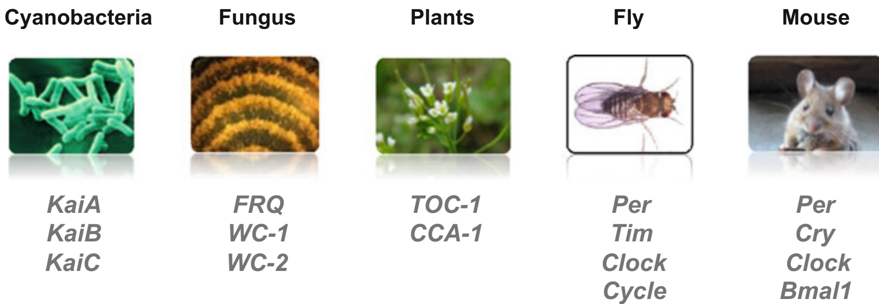

Fig. 1 The dominant model organisms used for the study of circadian rhythms are shown. Below are lists of the more important clock genes that are/were thought to mediate negative feedback loops in these organisms. At the DNA or protein sequence levels, there is no evolutionary conversation beyond some in fly and mammals

Challenging Transcriptional Models of the Clockwork

An increasing number of studies, both in higher and lower organisms, have questioned the necessity of a functional transcriptional oscillator for cellular rhythmicity. Circadian transcription is stochastic in mammalian cells (Suter et al. 2011); therefore, one would expect that perturbation of transcription during cell division would result in phase variability, which is not seen. In line with this finding, global inhibition of transcription with actinomycin D and α-amanitin has revealed the robustness of circadian oscillators to such severe perturbations, and single cells exhibit bioluminescence rhythms even when the transcription rate is reduced by ~70 % (Dibner et al. 2009).

Perhaps more importantly, studies showing that constitutive expression, or deletion, of "clock genes" does not abolish circadian rhythms call into question the importance of transcription in current clock models. In flies, expression of both *per* and *tim* under the control of a constitutive promoter can affect circadian rhythms. However, ~50 % of the flies still exhibited robust behavioral rhythms (Yang and Sehgal 2001). Similarly, fungi can exhibit conidiation (spore formation) rhythms in the absence of central components of their feedback loop [involving the *frequency* (*frq*) gene] (Lakin-Thomas 2006). Moreover, in some organisms, the dominant mechanism regulating circadian rhythms seems to be post-transcriptional, as exemplified by the circadian control of translation of luciferin binding protein (LBP) in the unicellular alga *Gonyaulax polyedra* (Morse et al. 1989; Mittag et al. 1994).

In mammals, the situation is difficult to dissect since circadian genes often have multiple homologues; therefore, double-mutant animals are generally needed to observe a behavioral phenotype. *Bmal1* was thought to be the only exception to this, with its suppression leading to clear behavioural arrhythmicity (Ko et al. 2006). Constitutive brain-specific expression of *Bmal1* in knock-out animals is, however, able to restore behavioral rhythmicity, questioning the necessity of rhythmic *Bmal1* transcription (McDearmon et al. 2006). In addition, brain-specific knockout of *Bmal1* expression produces gross pathology, with a striking abundance of activated microglia in the brains of mice, which gets progressively worse over the first 6 months of life. This finding makes it extremely difficult to dissociate the effects if BMAL1 as a generically important transcription factor from those specifically related to the malfunctioning of a biological clock (Musiek et al. 2013).

Even more importantly, imaging of suprachiasmatic nucleus (SCN) slices from arrhythmic Bmal1−/− and Cry1−/−Cry2−/− animals with bioluminescence reporters revealed the persistence of low amplitude rhythms in individual neurons (Ko et al. 2010; Maywood et al. 2011). As recently shown, it is likely that developmental effects underlie the apparent arrhythmicity that is observed when adult animals are assayed, as is the case in most experimental paradigms (Ono et al. 2013).

There is thus considerable evidence that current transcription-translation feedback loops cannot account for the multiple lines of experimental evidence that have revealed circadian oscillations in the presence of inactivated feedback loops or indeed in their absence.

Non-transcriptional Clock Mechanisms

The experimental anomalies highlighted above suggest that other mechanisms are required to fully explain the molecular basis of circadian timekeeping. It is indeed worth underscoring that transcriptional mechanisms were regarded as only one of the several possibilities that were investigated before the discovery of “clock genes” (Edmunds 1988).

An instructive outlier in clock research is the macroscopic unicellular alga *Acetabularia*, which can maintain self-sustained circadian rhythms in photosynthetic activity when its nucleus is removed by cutting off its nucleus-containing rhizoid process (Sweeney and Haxo 1961). Intriguingly, its nucleus is able to dictate the phase of oscillation but is dispensable for entrainment and phase shifting (Schweiger et al. 1964). Moreover, inhibition of transcription with Actinomycin D did not suppress rhythms in either nucleated or enucleated *Acetabularia* cells, although the former surprisingly lost rhythmicity after 2 weeks under these conditions (Mergenhagen and Schweiger 1975). Similarly, platelets were used to show that glutathione exhibited circadian oscillations relying on de novo synthesis of this important cellular reductant (Radha et al. 1985), again in the absence of a nucleus. These examples point to the fact that current circadian models cannot explain issues

raised almost 40 years ago, in some cases, suggesting the existence of non-transcriptional rhythms.

How can we reconcile these seemingly opposite views? One way is to view transcription and translation in the current models as having limited roles in setting the pace of the oscillator and to note that they are needed to maintain the levels of clock proteins and to control circadian output functions. Accordingly, post-translational modifications of known clock proteins could be the fundamental oscillator, but the transcriptional oscillator would be important for robustness and could amplify post-translational oscillations. In fact, such a model exists in cyanobacteria, in which the master transcriptional regulator KaiC is part of its post-translational oscillator.

An alternative point of view is that circadian timekeeping might have evolved more than one clock in the cell to meet the requirements of precision, robustness and stability. In this case, the known transcriptional oscillator would be coupled to a post-translational oscillator. Post-translational modifications are an integral feature of the current transcription-translation feedback models, but a definitive post-translational oscillator has not yet been identified in eukaryotic species. The recent discovery of oxidation cycles in peroxiredoxin proteins (PRDXs) offers a new window on non-transcriptional rhythms in higher organisms (O'Neill and Reddy 2011; O'Neill et al. 2011; Edgar et al. 2012; Olmedo et al. 2012). More importantly, this finding immediately suggests a common phylogenetic origin for circadian timekeeping mechanisms in virtually all species relying on oxygen for energy metabolism (Edgar et al. 2012).

PRDX Rhythms

PRDXs are an antioxidant protein family involved in hydrogen peroxide metabolism and signalling (Hall et al. 2009). Their catalytic mechanism involves the oxidation of a catalytic cysteine residue in the enzymes' active site to sulfenic acid (Cys-SOH), which then forms a disulfide bond with another non-catalytic (and so-called 'resolving') cysteine residue. The thioredoxin system usually completes the cycle by reducing this disulfide bond while oxidising a molecule of NADPH. This catalytic loop has rapid turnover and allows the maintenance of low levels of intracellular hydrogen peroxide.

So-called 'typical 2-Cys PRDXs,' a subclass of PRDXs whose basic functional unit is a homodimer in which catalytic and resolving cysteine residues belong to different molecules of PRDX, are the main players implicated in circadian cycles. These can undergo further oxidation of their catalytic cysteine to sulfinic and sulfonic acid forms (Cys-$SO_{2/3}H$). The 'over-oxidised' Cys-SO_2H residues can be slowly recycled through adenosine triphosphate (ATP)-dependent reduction by sulfiredoxin (Rhee et al. 2007), whereas further oxidation to Cys-SO_3H (termed 'hyper-oxidation') is thought to be irreversible.

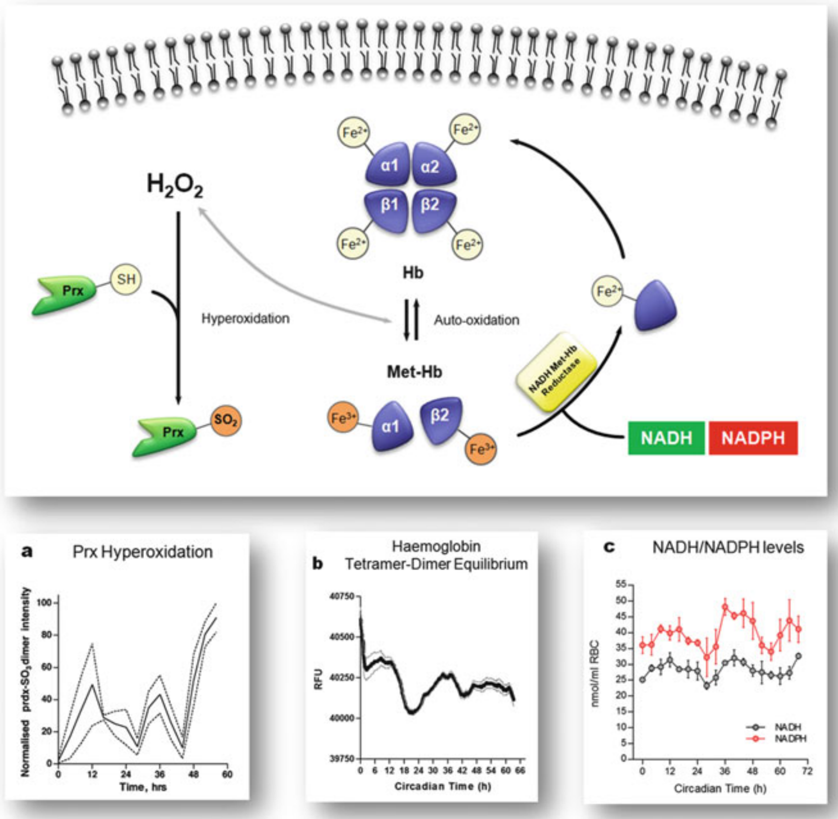

Fig. 2 A range of circadian oscillations in human red blood cells. (**a**) Oxidation of PRDXs occurs on a circadian basis in cells maintained in constant conditions (in the absence of external temporal cues) for at least 3 consecutive days. (**b**) Dynamic changes in the equilibrium of haemoglobin forms (tetramer vs. dimer states) oscillate according to a 24-h rhythm in vitro. (**c**) Oscillation of the key cellular reductants, NADH and NADPH, in red blood cells

Human red blood cells do not undergo transcription since they lack nuclei in their mature form. In these unique cells, PRDXs exhibit circadian accumulation of their dimeric over-oxidised form (PRDX-SO_2H) over several days (O'Neill and Reddy 2011). Such rhythms fulfil all criteria for circadian rhythms: (1) persistence in constant conditions; (2) the ability to be entrained (via temperature cycles in this case); and (3) temperature compensation (the clock does not run faster in higher temperatures). In addition, these redox rhythms are accompanied by oscillations in haemoglobin oxidation and metabolic variables, including NADH and NADPH (Fig. 2). Similar results have also recently been found in mouse red blood cells (Cho et al. 2014).

Rhythms similar to these are also present in the unicellular alga *Ostreococcus tauri*, even when transcription is inhibited by prolonged darkness (O'Neill et al. 2011); they are autophototrophic, requiring light for synthesis of most cellular substrates including RNA. Moreover, the deep phylogenetic conservation of PRDX redox rhythms extends to include fungal, plant, bacterial and even archaeal species. Critically, such rhythms are not dependent on previously identified clock genes, since mutants lacking circadian components maintain redox oscillations, albeit slightly phase-shifted (Edgar et al. 2012).

The phylogenetic conservation of PRDX rhythms suggests that primordial redox oscillators probably evolved following the Great Oxidation Event 2.5 billion years ago. At this time, photosynthetic bacteria are thought to have acquired the ability to produce oxygen from water, which caused a dramatic rise in Earth's atmospheric oxygen. Rhythmic production of oxygen and reactive oxygen species (ROS) by sunlight may therefore have been a critical driving force in the co-evolution of clock mechanisms and ROS removal systems that could anticipate, and thus resonate with, externally driven redox cycles (Bass 2012; Edgar et al. 2012; Fig. 3).

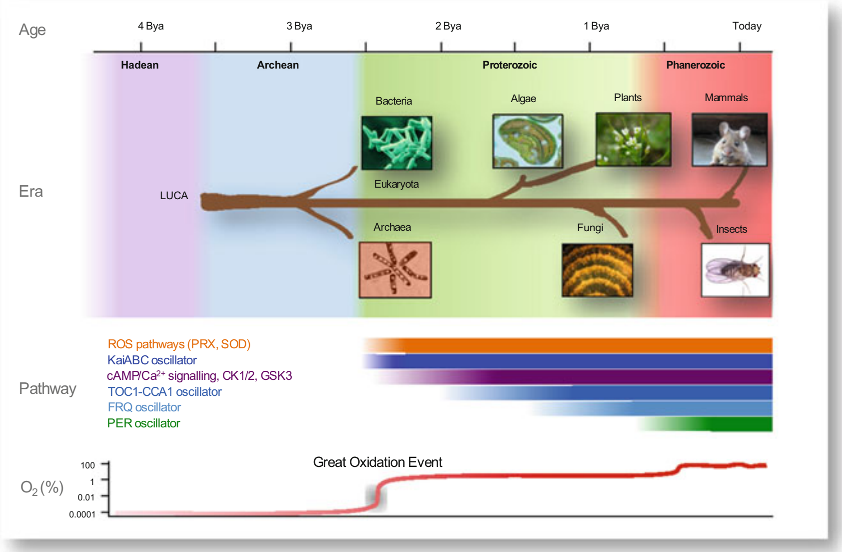

Fig. 3 Phylogenetic origins of circadian oscillatory systems. A timeline is shown at the *top* of the schematic, with the geological era illustrated. A schematic phylogenetic tree shows the origins of each organism studied, stemming from the last universal common ancestor (LUCA). The putative epoch over which each oscillator system has existed is illustrated by the labeled bars. CK1/2, casein kinase 1 or 2; GSK3, glycogen synthase kinase 3; SOD, superoxide dismutase (Adapted from Edgar et al. 2012)

Redox and Metabolic Clocks in Eukaryotes

There is clearly an interplay between circadian and metabolic cycles, and there is good evidence of reciprocal effects that disruption of one cycle has on the other at physiological and molecular levels (Bass 2012; Rey and Reddy 2013). High-fat diet, for example, lengthens the behavioral period of rhythms in mice and changes the expression pattern of clock genes (Kohsaka et al. 2007). Conversely, healthy patients subjected to 3 weeks of circadian disruption exhibit pre-diabetic symptoms (Buxton et al. 2012). The growing evidence suggesting that circadian rhythms are fundamentally metabolic requires that currently understood transcriptional oscillations are tightly coupled to metabolic cycles. This hypothesis is strongly supported by the numerous examples of accessory loops embedding the circadian transcriptional clock within cellular metabolism (Fig. 4).

An accessory loop involving NAD^+/NADH is likely to play an important role in connecting cytosolic and compartment-specific redox states to transcriptional clock components such as PER2 (Asher et al. 2008) and CLOCK/BMAL1 (Rutter et al. 2001; Nakahata et al. 2008; Asher et al. 2010; Yoshii et al. 2013). In addition, other redox-sensitive mechanisms have been identified in the clockwork and, in particular, the heme-sensing transcriptional regulators (Dioum et al. 2002; Yin et al. 2007; Gupta et al. 2011).

Even in early molecular studies of the circadian clock, before "clock genes" had been discovered in any model organism, rhythms in redox had been reported. For example, in plants, $NADP^+$:NADPH ratio exhibited circadian cycles in seedlings kept in constant darkness (Wagner and Frosch 1974). Several studies in rodents

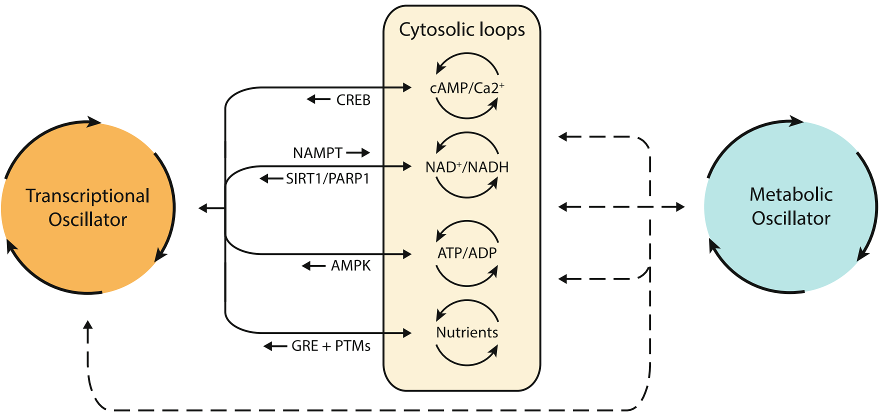

Fig. 4 Links between transcriptional, cytosolic, and metabolic cycles. Cytosolic processes are thought to be part of the transcription/translation feedback loop (TTFL). The latter are involved mainly in redox and energy metabolism and form accessory loops that are controlled by the TTFL oscillator and, in turn, feed back to it. Abbreviations: *CREB* cAMP response element-binding protein, *GRE* glucose response element, *PARP* poly(ADP-ribose) polymerase, *PTMs* posttranslational modifications (Adapted from Reddy and Rey 2014)

showed that redox parameters, including the glutathione redox ratio, were diurnally regulated in the liver, although it is possible that these oscillations might have been partially driven by food intake (Isaacs and Binkley 1977a, b; Robinson et al. 1981; Kaminsky et al. 1984; Belanger et al. 1991). Nevertheless, human platelets kept in vitro showed circadian rhythms in glutathione content (Radha et al. 1985), suggesting that feeding cycles might internally resonate with these cell-autonomous biochemical rhythms.

The hypothesis that metabolic cycles might be a fundamental mechanism underlying biological clocks has been proposed based on both theoretical and experimental observations (Roenneberg and Merrow 1999). Potential evidence for this hypothesis in mammals has come from the McKnight and Sagami groups, who have shown that BMAL1/CLOCK DNA-binding activity can be modulated in vitro by the redox poise of $NAD(P)^+$/NAD(P)H coenzymes (Rutter et al. 2001; Yoshii et al. 2013). In addition, the action of BMAL1/CLOCK on the NAD^+-producing enzymes lactate dehydrogenase (*Ldh*) and *Nampt* could potentially feed back onto intracellular redox balance (Rutter et al. 2001; Nakahata et al. 2009; Ramsey et al. 2009). These results still require in vivo confirmation, given the relatively high concentration (millimolar range) of the coenzymes used in in vitro assays previously (Rutter et al. 2001; Yoshii et al. 2013). The recent discovery of PRDX oscillations in non-transcriptional systems, however, offers supportive evidence that redox cycles can function as circadian oscillators in their own right.

It is evident that, in organisms in which metabolic oscillations have been found but transcription-translation feedback loops have not, as in the worm *Caenorhabditis elegans*, insights into metabolic oscillatory mechanisms may be easier to come by. It is thus possible that metabolic oscillations could drive PRDX oscillations in the absence of known transcriptional feedback oscillators (Olmedo et al. 2012). So-called accessory loops, including NAD^+/NADH and $NADP^+$/NADPH cycles, are potential candidates for self-sustained metabolic oscillators, but further studies of their oscillatory properties in clock mutant backgrounds will be of great interest to identify bona fide components of metabolic oscillators. However, this assumes that deletion of important circadian-relevant transcription factors itself does not lead to abhorrent redox changes in cells and tissues, as is the case in *Bmal1−/−* animals (Kondratov et al. 2006), which could compromise redox oscillations indirectly.

Conclusion

Metabolic non-transcriptional cycles clearly interlock with transcriptional processes in the circadian system. The peroxiredoxin system could be part of an uncharacterised metabolic oscillator, given its broad phylogenetic conservation and its slow kinetics, which is compatible with 24-h rhythmicity. Establishing the molecular links between fundamental cellular redox metabolism and transcriptional components of the clockwork remains an exciting challenge in the field.

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Rev-erbs: Integrating Metabolism Around the Clock

Mitchell A. Lazar

Abstract Mammalian circadian and metabolic physiologies are intertwined, and the nuclear Rev-erbα is a key transcriptional link between them. Rev-erbα, and the highly related Rev-erbβ, are potent transcriptional repressors that are required for the function of the core mammalian molecular clock. The Rev-erbs are also critical regulators of clock output in metabolic cells and tissues. This chapter focuses on the physiological functions of Rev-erbα and β in regulating circadian rhythms and metabolism in mammalian tissues.

Introduction

Much of biology is conducted with rhythms that have a phase of approximately 24 h, matching the duration of a day on planet Earth (Huang et al. 2011). The genetic basis of these circadian rhythms was unveiled in the fruit fly, *Drosophila melanogaster*, where the clock mechanism involves feedback regulation by factors whose own expression exhibit circadian rhythmicity (Rosbash et al. 1996). These factors function as transcriptional regulators, and it is now recognized that most genomes, including those of all mammals that have been evaluated, are transcribed in a rhythmic manner (Schibler 2006).

The mammalian clock mechanism involves interconnected transcriptional and translational feedback loops, where the most well-understood positive regulator is a heterodimer of the basic helix-loop-helix (HLH) transcription factors BMAL1 and CLOCK (King and Takahashi 2000). In addition to positively regulating clock output genes, the BMAL1/CLOCK heterodimer activates the expression of two negative regulators. One is another bHLH heterodimer, comprised of the proteins PERIOD (PER) and CRYPTOCHROME (CRY), which interact with BMAL1/CLOCK to

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P. Sassone-Corsi, Y. Christen (eds.), *A Time for Metabolism and Hormones*, Research and Perspectives in Endocrine Interactions,
DOI 10.1007/978-3-319-27069-2_7

interfere with its activity (King and Takahashi 2000). The second repressive loop is mediated by the Rev-erb nuclear receptors (NRs) α and β, of which Rev-erbα is the more highly functional (Everett and Lazar 2014). This chapter will focus on the Rev-erbs, particularly on the more well-studied Rev-erbα.

Repression of Transcription by Rev-erbs

Rev-erbs belong to a large NR superfamily of ligand-regulated transcription factors (Evans 2013). Discovered in 1989 (Lazar et al. 1989; Miyajima et al. 1989), Rev-erbα was one of the first identified orphan NRs, i.e., a member of the family whose ligand was not predicted from earlier physiology and biochemical studies (Mullican et al. 2013). The highly related Rev-erbβ was identified in 1994 (Bonnelye et al. 1994; Dumas et al. 1994; Forman et al. 1994; Retnakaran et al. 1994). Molecular heme has been identified as the endogenous ligand for Rev-erbα and Rev-erbβ (Raghuram et al. 2007; Yin et al. 2007). Although the physiological function of this regulation is not well understood, the ability to sense heme levels may position Rev-erb as a mediator of metabolic effects on metabolism.

Rev-erbs bind sequence-specifically to DNA, with the preferred binding site consisting of the classical NR half-site AGGTCA flanked by an A/T-rich 5′ sequence (Harding and Lazar 1993). This binding site is referred to as the RevRE or as the RORE, as it is also bound by the Retinoic Acid Receptor-related Orphan Receptor (ROR; Giguere et al. 1994). The DNA-binding domain (DBD) of Rev-erbα binds in the major groove of the AGGTCA half-site, whereas a C-terminal extension makes minor groove contacts with the A/T-rich 5′ sequence (Zhao et al. 1998). Rev-erbs bind as a monomer to this site but bind even more tightly as a dimer to a direct repeat with a 2 base pair spacer, referred to as the RevDR2 (Harding and Lazar 1995).

Rev-erbs lack the C-terminal region that is required for ligand-dependent transcriptional activation by other NRs (Glass and Rosenfeld 2000). Thus, they function primarily as potent repressors of transcription when bound to DNA (Zamir et al. 1997), interacting constitutively with the Nuclear Corepressor 1 (NCoR; Horlein et al. 1995; Zamir et al. 1996). NCoR is a large protein (~270 kDa) with inherent repressive function as well as several short helical domains that specifically interact with NRs, called the corepressor-NR (CoRNR) boxes (Hu and Lazar 1999). Heme further stabilizes its interaction with full-length, endogenous NCoR (Raghuram et al. 2007; Yin et al. 2007). In addition to serving as a heme sensor, the Rev-erb activity may also be sensitive to the oxidation state of the heme iron (Marvin et al. 2009). To bind NCoR stably enough to actively repress transcription, two Rev-erbα molecules must interact with CoRNR peptides from NCoR; this interaction can occur at the RevDR2 site, which the Rev-erbs bind cooperatively as a dimer, or at two Revre/RORE sites bound independently by Rev-erb monomers (Zamir et al. 1997).

NCoR represses transcription by nucleating a large multiprotein repressor complex, which impacts the epigenome and the function of core transcriptional factors and RNA polymerase II (Guenther et al. 2000; Yoon et al. 2003). Stoichiometric components of the NCoR complex include Transducin Beta-Like 1 (TBL1), G-protein Pathway Suppressor 2 (GPS2), and Histone Deacetylase 3 (HDAC3; Guenther et al. 2000; Zhang et al. 2002; Yoon et al. 2003). HDAC3 is of particular interest, because it is an epigenomic modulator that deacetylates lysine residues in the tails of nucleosomal histone proteins to create a repressive chromatin environment (Haberland et al. 2009). NCoR and HDAC3 are both required for Rev-erbα to repress *Bmal1* gene transcription (Yin and Lazar 2005), and both NCoR and HDAC3 are associated with Rev-erbα at thousands of DNA binding sites genome wide in the mouse liver (Feng et al. 2011).

Circadian Biology of Rev-erbs

In 1998, Rev-erbα was noted to one of the genes that oscillates within the circadian transcriptome of mammalian cells cycling in tissue culture (Balsalobre et al. 1998). In mice Rev-erbα mRNA expression is robustly circadian in multiple tissues (Yang et al. 2006), and genetic deletion of Rev-erbα shortens the period of behavioral rhythms by ~30 min in the absence of daily light cues (Preitner et al. 2002). Rev-erbα modulates the rhythmicity of additional circadian regulators, including *Clock* (Crumbley and Burris 2011), *Cry1* (Ukai-Tadenuma et al. 2011), *Nfil3/E4Bp4* (Duez et al. 2008), and *Npas2* (Crumbley et al. 2010) and thus has a major influence on the cell-autonomous molecular timing system. Indeed, constitutive expression of Rev-erbα in mouse liver represses the majority of cycling transcripts (Kornmann et al. 2006). Importantly, ablation of both Rev-erbα and β abrogates circadian gene expression in mouse embryonic fibroblasts, demonstrating a fundamental requirement for the Rev-erbs (Bugge et al. 2012). Moreover, genetic mutation of Rev-erbα and β caused arrhythmic behavior in mice (Cho et al. 2012). Therefore Rev-erbα and β are both required components of the core clock machinery. Loss of either Rev-erb alone is insufficient to abolish circadian rhythms, indicating that their clock functions are redundant, although Rev-erbα is more critical because its absence modestly disrupts normal circadian rhythms whereas the loss of Rev-erbβ does not.

Rev-erbα and Metabolism

Circadian rhythms and metabolism are highly intertwined (Eckel-Mahan and Sassone-Corsi 2013), and indeed Rev-erbα regulates metabolic function in many tissues. In the liver, Rev-erbα regulates cholesterol and bile acid metabolism (Duez et al. 2008; Le Martelot et al. 2009), and more recently has been observed to play a

key role in the circadian regulation of triglyceride metabolism (Feng et al. 2011). Rev-erbα binds widely and robustly to the genome at ZT10, when its expression is maximal; however, it binds to very few sites when its expression is at a nadir, such as at ZT22. This genomic binding is enriched at genes involved in lipid metabolism and, indeed, mice lacking Rev-erbα have mild fatty liver, or hepatic steatosis (Feng et al. 2011). The oscillatory expression of Rev-erbα regulates circadian gene expression directly at target genes with strong binding motifs, whose circadian expression is antiphase to that of Rev-erbα, as well as indirectly by repression of another circadian repressor called E4BP4, whose target genes are expressed in phase with Rev-erbα (Fang et al. 2014). The liver cistrome of Rev-erbβ is quite similar, and knockdown of Rev-erbβ in livers of Rev-erbα null mice caused a more markedly fatty liver (Bugge et al. 2012). NCoR and HDAC3 bind to the genome at the vast majority of Rev-erb sites and, indeed, ablation of either NCoR or HDAC3 in mouse liver leads to marked hepatic steatosis (Knutson et al. 2008; Sun et al. 2012, 2013).

Studies of adipocyte differentiation in cultured cell lines have suggested that Rev-erbα plays an important role in adipocyte differentiation (Chawla and Lazar 1993; Fontaine et al. 2003; Wang and Lazar 2008), yet white adipose tissue (WAT) mass was not reduced in mice lacking Rev-erbα (Chomez et al. 2000; Delezie et al. 2012), indicating that Rev-erbα is not absolutely required for adipocyte formation in vivo. Rev-erbα may play a role in brown adipose tissue (BAT), which is a major site of thermogenesis (Gerhart-Hines et al. 2013). Circadian expression of Rev-erbα in BAT peaks at ZT10, which is antiphase to the circadian rhythm of body temperature. Mice lacking Rev-erbα have a higher nadir in body temperature, at least in part due to derepression of Uncoupling Protein 1 (UCP1), which is a circadian target of Rev-erbα and constitutively high in the BAT of mice genetically lacking Rev-erbα (Gerhart-Hines et al. 2013). Mice also have an increased vulnerability to cold temperature at times of day when Rev-erbα levels are high; this vulnerability is ameliorated in the absence Rev-erbα (Gerhart-Hines et al. 2013).

A role for Rev-erbα in skeletal myocytes was first identified in C2C12 cultured myoblasts, where Rev-erbα represses the expression of genes involved in muscle cell differentiation (Downes et al. 1995). Rev-erbα mRNA expression is circadian manner in mouse skeletal muscle (Yang et al. 2006), and loss of Rev-erbα function reduces mitochondrial content and function, leading to an impaired exercise capacity (Woldt et al. 2013). It should be noted that the transcriptomic changes in muscle are not observed in liver or BAT and thus reflect tissue-specific functions of Rev-erbα.

Rev-erbα is also expressed in a circadian manner in the pancreatic islets and plays a role in the function of insulin-producing β-cells and glucagon-producing α-cells (Vieira et al. 2012, 2013). Islets isolated at the peak of Rev-erbα expression have higher levels of glucose-stimulated insulin secretion (Vieira et al. 2012), and Rev-erbα also promotes glucagon secretion in α-cells of the pancreas (Vieira et al. 2013).

Inflammatory cells are increasingly linked to metabolic function (Osborn and Olefsky 2012), and Rev-erbα mediates the circadian gating of the LPS-induced endotoxic response (Gibbs et al. 2012). Genome-wide studies of Rev-erbα and Rev-erbβ cistromes and transcriptomes suggest that Rev-erbα influences macrophage gene expression at bindings sites marked by hematopoietic transcription factors, including PU.1 (Lam et al. 2013).

Conclusions

The nuclear receptor Rev-erbα acts in a tissue-specific manner to regulate circadian rhythms as well as metabolism, in some cases acting redundantly with Rev-erbβ. A critical question is whether Rev-erbα can be targeted for therapeutic purposes. Synthetic pharmacological agonists have been developed (Grant et al. 2010; Solt et al. 2012), yet the tissue-specific complexity of Rev-erb biology raises major challenges to human therapeutics. Perhaps the dramatic circadian expression of Rev-erbs can be exploited by timing drug delivery to selectively impact their specific and beneficial functions in integration of metabolism and the circadian clock.

Acknowledgments I thank members of my research group, particularly Logan Everett, for helpful discussions. Research on Rev-erb in my laboratory is supported by NIH grant R01 DK45586 (MAL).

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Control of Sleep-Wake Cycles in Drosophila

Abhishek Chatterjee and François Rouyer

Abstract Inter-oscillator communication modulates and sustains the circadian locomotor rhythms in flies and rodent animal models. In Drosophila, the multi-oscillator network that controls sleep-wake cycles includes about 150 clock neurons. A subset of lateral neurons (LNs) expressing the Pigment-dispersing factor (PDF) appears to act as a master clock in constant darkness (DD). In light–dark (LD) cycles, flies show a bimodal distribution of their activity, and the PDF-expressing LNs play a major role in the control of the morning bout of activity. In contrast, a subset of PDF-negative LNs can generate evening activity in the absence of other functional oscillators. How these oscillators interact in a fully functional network to shape the sleep-wake cycle remains debated. The PDF neurons strongly influence the PDF-negative ones in DD and, to a lesser extent, in LD. The extent of hierarchy depends on environmental conditions and the way the dominance of PDF neurons is exerted on the different types of PDF-negative neurons is unclear. The recent discovery of light- and temperature-dependent oscillators in the dorsal neurons (DNs) sheds new light on the circuits that control the Drosophila diurnal behavior and its adaptation to environmental changes.

Background

The fruit fly *Drosophila melanogaster* displays rest-activity rhythms that rely on a circadian clock located in the brain. In light–dark (LD) cycles, adult flies show a bimodal activity with morning and evening peaks at dawn and dusk. Activity rhythms persist in constant darkness (DD), indicating the circadian nature of this behavior. Like peripheral clocks, the brain clock depends on a molecular feedback loop where the CLOCK (CLK) and CYCLE (CYC) transcriptional factors drive the expression of the PERIOD (PER) and TIMELESS (TIM) proteins that repress CLK/CYC activity. The negative feedback loop operates in about 150 neurons,

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P. Sassone-Corsi, Y. Christen (eds.), *A Time for Metabolism and Hormones*,
Research and Perspectives in Endocrine Interactions,
DOI 10.1007/978-3-319-27069-2_8

the so-called clock neurons, which account for about 0.1 % of the total CNS neural population. The numerical simplicity of these 150 neurons that form a network is remarkable in comparison to central circuits for other hardwired behaviors such as courtship or learning and memory. The Drosophila clock neuronal network is also remarkably simple in comparison to circadian control circuits in vertebrates, where several brain areas, including the hypothalamus, pituitary gland, pineal gland, olfactory bulb, etc., harbor numerous bona fide clock neurons. This smaller number of neurons offers the potential to manipulate oscillators at the single-cell level in vivo, through well-defined genetic handles.

There are two broad populations within the 150 clock neurons of the fly brain; one population is laterally placed and another is located along the dorsal margin of the brain. The lateral neurons (LNs) lie near the interface of the central brain and the optic lobe and are organized into a ventral cluster that include small (s-LNvs) and large (l-LNvs) cells, a dorsal cluster (LNds) and a posterior cluster (LPNs). The dorsal neurons (DNs) are in turn subdivided into three clusters designated as DN1, DN2 and DN3 (Fig. 1). Such anatomical categorization frequently has neurochemical and functional bases; for example, the four most ventral s-LNvs express the Pigment-dispersing factor (PDF) neuropeptide and promote morning activity in LD. Based on strong functional data, mostly behavioral and some neurophysiological in nature, a wiring diagram of these differentiated clusters of brain clock neurons has begun to materialize over the past 10 years. In the following section we will summarize the logic of organization of this circuit.

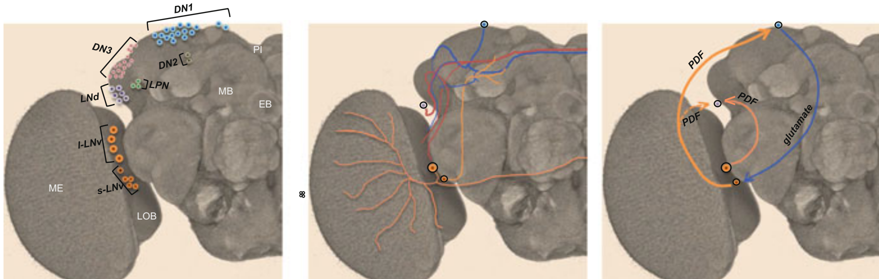

Fig. 1 The clock neurons of the Drosophila brain. *Left panel*: seven groups of clock neurons have been defined on an anatomical basis. The lateral neurons are organized into a ventral cluster that include small (s-LNvs) and large (l-LNvs) cells, a dorsal cluster (LNds) and a posterior cluster (LPNs). The dorsal neurons include three clusters designated as DN1, DN2 and DN3. Several neuropiles are indicated: Medulla (ME) and Lobula (LOB) in the optic lobe and Pars Intercerebralis (PI), Mushroom Bodies (MB) and Ellipsoid Body (EB) in the central brain. *Central panel*: projections of the different clock neuron subsets: s-LNvs and l-LNvs (*orange*), LNds and fifth PDF-negative s-LNv (*red*), DN1s (*blue*). *Right panel*: communication between neuronal clusters involves PDF from s-LNvs to LNds and DN1s as well as from l-LNvs to LNds, and glutamate from DN1s to s-LNvs

Layout of the Network in Constant Conditions

In the absence of cycling environmental cues, the fly clock circuit has been shown to adopt a functionally minimalist organization. In flies that experienced light–dark cycles and were subsequently kept in constant darkness, the presence of PDF-expressing s-LNvs was required to drive robust rhythmic behavior (Helfrich-Förster 1998; Renn et al. 1999) and a clock restricted to the PDF-expressing cells was sufficient to drive 24-h rhythms (Grima et al. 2004). In contrast, PDF-negative neurons drove behavioral rhythmicity under constant light if light inputs were reduced by the absence of the cell-resident photopigment cryptochrome (CRY). The precise location of these neurons that act as the pacemaker in constant light (LL) has been suggested to be either within the LNd cluster (Picot et al. 2007) or within the DN1s (Murad et al. 2007; Stoleru et al. 2007). In spite of running a functioning oscillator, the s-LNv neurons fail to influence the behavioral period in LL (Picot et al. 2007; Stoleru et al. 2007). The predominant contribution of the s-LNvs to behavioral rhythmicity, as evident in DD, becomes dramatically corroded in LL as ambient light inhibits their behavioral output (Picot et al. 2007). Under constant conditions the operation of the clock circuit remains highly centralized, but depending on the sustained presence or absence of light, this central position is occupied by either the PDF-negative clock neurons or the PDF-positive s-LNv neurons, respectively. Notably, in DD, the s-LNvs operate at the pinnacle of a hierarchy as they enforce a majority of other oscillators to realign their clock program in accordance with the sLNv pace (Stoleru et al. 2005). In contrast, messages from the non-PDF clocks have considerably subdued influence on the running of the master pacemaker in DD (Stoleru et al. 2005; Picot et al. 2007; Collins et al. 2014).

Recently, however, the existence of a centralized monopolar circuit organization in DD has been seriously challenged. The PDF clock has been shown to coherently change behavioral period only over a limited range, which is distributed asymmetrically around the 24-h focal point (Yao and Shafer 2014; Beckwith and Ceriani 2015). When the PDF neurons were forced to run at a pace beyond this specified range, multiple peaks of behavioral period emerged within a single fly, likely as a result of internal desynchronization among multiple oscillators (Yao and Shafer 2014; Beckwith and Ceriani 2015). Because these oscillators are coupled to the PDF clock with differing strength and range of entrainment, they are differentially affected by speed changes in the PDF clock (Yao and Shafer 2014). Thus, the behavioral period in DD is determined by the pace of not only the s-LNv clock but also by other oscillators enjoying different degrees of independence, although they were formerly thought to uniformly behave as slaves of the s-LNv pacemaker. As opposed to direct manipulation of individual oscillator pace, a parallel line of research was to putatively increase the excitability of different subsets of clock neurons to enhance their contribution in the network. This study raised the interesting possibility that the CRY-negative clock neurons, e.g., the DN2s, may have the potential to affect behavioral period like the well-known s-LNvs (Dissel

et al. 2014). Going one step further, it was proposed that the DD behavioral period is constructed by integrating the slightly longer period dictated by the s-LNvs and the slightly shorter period imposed by the DN2s, with other clock neurons modulating the contributions of these two oscillators (Dissel et al. 2014). The behavioral period would thus depend on the interactions between differently paced oscillators whose endogenous period and influence in the network vary according to environmental conditions. However, speed changes in all clock neurons excluding the PDF cells fell short of altering the behavioral period (Yao and Shafer 2014), in fact bolstering the older idea that PDF oscillators are the predominant determinant of the behavior period in DD. In absence of PDF signaling, the output from the s-LNvs was compromised, thereby allowing secondary oscillators to strongly influence the behavior period (Yao and Shafer 2014). Precisely which oscillators are coupled, whether coupling is directional, how the coupling strength is determined and what are the relative weights of different oscillators to behavioral period according to environmental conditions are some of the questions that fly chronobiologists will probably resolve in next few years. We predict that the existing momentum on neuronal mechanisms of behavioral period determination will be extended to understand the other fundamental parameters of rhythm, such as phase and waveform.

Network Architecture Under LD Cycles

Depending on the constraints of physiological thermal limit and light availability, animals evolved few basic patterns of diel activity: diurnal, nocturnal, crepuscular or cathemeral (Bennie et al. 2014). In mammals, a given animal can stably and predictably switch back and forth between different patterns in context-dependent ways (Kas and Edgar 1999; Mrosovsky 2003). The choice of a temporal niche takes place downstream of the suprachiasmatic nucleus (SCN) clock and is strongly influenced by light inputs (Mrosovsky and Hattar 2005; Doyle et al. 2008). A comparable plasticity is observed in flies. For example, a typically crepuscular male fly will become nocturnal in the presence of a mate or during moonlit nights (Bachleitner et al. 2007; Fujii et al. 2007) and will be more diurnal when daylight is low (Schlichting et al. 2015). At first glance, the similar phasing of molecular oscillations in the different clock neuron subsets of the brain suggests that shaping the sleep-wake cycle occurs downstream of the clock, but results obtained from manipulating these different subsets support a more complex model.

Flies in the standard laboratory condition of 12:12 LD cycles show a bimodal profile with peaks of activity coinciding with the putative twilight transitions. Very nicely, the behavioral sub-routines of generating a morning peak and an evening peak are orchestrated by two separable subsets of oscillator neurons, the s-LNvs and the LNds, respectively, providing concrete experimental support for the dual-oscillator model of Daan and Pittendrigh (Pittendrigh and Daan 1976; Grima et al. 2004; Stoleru et al. 2004) (Fig. 2). Of note, this dual-oscillator ground plan

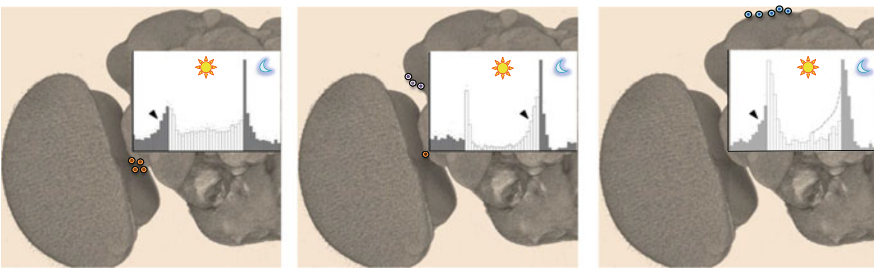

Fig. 2 Contribution of different clock neuron subsets to the LD behavior. Activity plots show the contribution of neuronal groups to morning and evening LD behavior. s-LNvs promote morning activity (*left*) whereas LNds and the fifth PDF-negative s-LNv promote evening activity (*center*). DN1s can promote morning activity and evening activity (*right*). The evening output of the DN1s is very weak in high light but strongly increases in low light (*dashed line*)

contrasts with the predominantly monopolar hierarchical organization prevailing in DD. The rather autonomous operation of two oscillators under LD cycles is abetted by their independent access to light information through redundant pathways—in-house CRY signaling and the visual system—whose output impinges on the clock circuit probably at multiple nodes, including the LNvs (Cusumano et al. 2009; Zhang et al. 2009). However, network interaction between oscillator neuron clusters could still be evident in LD. In the absence of PDF secretion by the LNvs, the evening peak of activity is advanced by a couple of hours (Renn et al. 1999). In the absence of both CRY and PDF, the evening peak vanishes and the phase of the molecular oscillations in the LNds is strongly altered (Cusumano et al. 2009; Zhang et al. 2009; Im et al. 2011). The phasing of the evening activity thus increasingly depends on PDF signaling when autonomous CRY-dependent photoreception decreases at the end of the day because of less intense and more reddish light. So far, the role of DNs in shaping the LD activity pattern seems to be largely secondary to the LNs (Grima et al. 2004; Stoleru et al. 2004; Zhang et al. 2010a, b). A functional clock restricted to the DN1s is sufficient to drive both morning and evening activity bouts in low light LD conditions, whereas high light permits morning activity only (Zhang et al. 2010b). These outputs are affected by temperature, and the DN1 neurons thus appear to be capable of integrating certain light and temperature information from the ambient environment (Zhang et al. 2010a, b). Although the expression of PDFR in the DN1s is important for their proper function (Zhang et al. 2010a), how they modulate the clock network's collective output remains unknown.

In summary, under periodic environmental cues, multiple, highly autonomous oscillators with distinct behavioral contributions collaboratively sculpt the organism's activity profile. In line with the 'internal coincidence model' of photoperiodism (Pittendrigh and Minis 1964), flexible changes in the clock network favoring the contribution of particular oscillators under certain ambient environmental conditions have been put forward as the mechanistic basis of seasonal adaptation in flies (Stoleru et al. 2007). The Daan/Pittendrigh model proposed that light

accelerates morning oscillators and decelerates evening oscillators to adapt the bimodal activity to the changing photoperiod. Fast- and slow-running neuronal oscillators were described in flies displaying split rhythms in LL (Yoshii et al. 2004). However, short and long period components were observed to derive from the LD evening bout, suggesting that light-accelerated clock neurons contribute to the evening activity (Yoshii et al. 2004; Rieger et al. 2006), in contrast to the prediction of the model. As indicated above, light was shown to promote the output of the LNd-based evening oscillator while inhibiting the morning oscillator carried by the PDF-expressing s-LNvs (Picot et al. 2007). The importance of PDF-negative cells in the presence of light is also shown by experiments comparing the relative influence of PDF-positive and PDF-negative neuronal subsets in different photoperiods. This work was done by looking at morning and evening activity peaks of flies with accelerated PDF-positive or PDF-negative neurons. Under long photoperiods, the evening oscillator located in PDF-negative cells was proposed to control the speed of the morning oscillators, whereas in short photoperiod conditions the morning oscillator of PDF cells would take the lead (Stoleru et al. 2007). The discovery of other subsets contributing to morning and evening activity bouts, in particular the DN1s contributing to evening activity in low light only, suggest that the adaptation to photoperiod changes might be more complex. Indeed, we have data indicating that new groups of oscillators are recruited when flies are confronted with summer-like conditions. Such laboratory-based simplified environmental parameters are probably inadequate to explain the working of the network under the complex natural conditions that exist in the spatiotemporal niche inhabited by Drosophila in the wild (Menegazzi et al. 2012, 2013; Vanin et al. 2012; De et al. 2013). In particular, daily temperature variations have a strong impact on the sleep-activity pattern and can even induce some morning and evening anticipatory activity in clockless flies (Vanin et al. 2012; Menegazzi et al. 2013). But the principles and logic of circuit operation learned from a severely artificial set-up could well be valid and applicable for the same network's functionality under more complex natural conditions.

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Circadian Metabolomics: Insights for Biology and Medicine

Steven A. Brown and Ludmila Gaspar

Abstract A biological "circadian" clock governs nearly all aspects of mammalian behavior and physiology. This control extends from activities of entire organ systems down to individual cells, all of which contain autonomous molecular clocks. Under this control, a significant fraction of the cellular metabolome—the collection of all small-molecule metabolites—varies in abundance according to time of day. Comparing the rhythmic expression of transcripts, proteins, and metabolites has yielded valuable insights into clock-controlled physiological mechanisms. In the future, their analysis could provide a glimpse of instantaneous clock phase, even providing notions of clock time based upon molecules within a single breath. Such knowledge could be important for disease diagnosis and for chronopharmacology.

Introduction: A Many-Clock Problem

A "master clock" tissue in mammals has been identified in the suprachiasmatic nuclei (SCN) of the hypothalamus, about 20,000 neurons distributed into bilateral nuclei just above the optic chiasma. Lesioning of this region results in loss of circadian behavior and physiology under constant environmental conditions (Eastman et al. 1984), and transplantation results in circadian behavior corresponding to that of the donor animal (Ralph et al. 1990). Although the SCN directs circadian timing, the circuitry of which circadian clocks are composed is in fact much more widespread: nearly every cell in the body contains an autonomous molecular oscillator driven by feedback loops of transcription and translation of dedicated "core clock" proteins (Brown and Azzi 2013). Therefore, circadian control of complex physiology is at least in part a question of orchestration: on the one hand, circadian signals from the SCN must synchronize peripheral oscillators

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P. Sassone-Corsi, Y. Christen (eds.), *A Time for Metabolism and Hormones*, Research and Perspectives in Endocrine Interactions,
DOI 10.1007/978-3-319-27069-2_9

elsewhere in the brain and body and, on the other, peripheral clocks must themselves direct circadian processes at a cellular level.

The ultimate consequences of this pervasive circadian control are that, in most mammalian tissues, 6–20 % of all transcripts and proteins are expressed in circadian fashion, i.e., with higher expression at one time of day and lower expression at another (Panda et al. 2002; Storch et al. 2002; Reddy et al. 2006; Robles et al. 2014). It is therefore not surprising that about 20 % of the mammalian metabolome shows circadian variation in both mice and men (Minami et al. 2009; Dallmann et al. 2012; Eckel-Mahan et al. 2012). Given the lower complexity of the metabolome and its extremely high conservation across species compared to the genome, an increasing number of studies have turned to metabolomics analyses to understand circadian biology.

Normally, circadian clocks throughout the body remain in relative synchrony with defined phase relationships. However, during timing shifts provoked by travel and shiftwork, in pathological cases such as inflammation and disease, or even due to the abnormal timing of food intake, this synchrony can be disrupted. For example, if normally nocturnal rodents are fed only during the day, clock phase in peripheral organs like liver and heart will change phase by nearly 12 h, while the SCN remains unaltered (Damiola et al. 2000; Stokkan et al. 2001). On the other hand, during a sudden change in light timing, the SCN will quickly alter its phase whereas peripheral organs require multiple days to do so (Davidson et al. 2009). Disease-mediated inflammation provides another example of peripheral clock dampening or dephasing: in response to infection, the circadian amplitude of transcription for multiple clock and clock-controlled genes decreases markedly (Cavadini et al. 2007). Finally, in both brain and peripheral tissues, sleep-related cellular signals can conflict with clock-related ones, leading to a dampening of circadian amplitude of clock-controlled genes (Maret et al. 2007; Moller-Levet et al. 2013; Archer et al. 2014). Both immediate and long-term consequences of such “clock desynchrony” are only beginning to be understood. For example, circadian amplitude in human subjects is directly correlated with survival time in some cancers (Innominato et al. 2012), and multiple studies in both humans and animals have linked shiftwork to increased disease and mortality (Viswanathan and Schernhammer 2009; Evans and Davidson 2013). As we discuss further below, metabolomics analyses could provide a powerful tool to study circadian phase and amplitude in both humans and animal models, potentially linking these parameters to human health in a wide variety of contexts.

An Overview of Circadian Metabolomics

Typically, comprehensive metabolomics analyses are conducted by flow injection mass spectrometry. Thus, in a single assay lasting a few seconds, thousands of peaks corresponding to individual metabolites can be detected. At the moment, a significant limiting factor for these studies is the identification of the metabolites

corresponding to each peak. Most commercially accessible platforms can discretely identify a few hundred different substances, including lipids, amino acids, sugars, enzymatic cofactors, and peptides and hormones. In at least one study, these circadian metabolites have been compared to circadian transcripts in the same tissues in rodents, allowing a direct and comprehensive look at cellular pathways regulated in circadian fashion (Eckel-Mahan et al. 2012).

From this study, it was clear that the circadian clock exerts coordinated control over a large number of metabolic pathways, including those controlling the abundance of lipids, carbohydrates, and amino acids. Of course, given that food is itself consumed in time-of-day-dependent fashion, it would be formally possible that these variations could be indirect consequences of rhythmic activity, rather than direct clock control. In mice, for example, without rhythmic feeding only a small percentage of circadian transcripts continued to show diurnal oscillations (Vollmers et al. 2009). In humans, however, a very different picture has emerged. By analyzing metabolomics parameters from saliva and blood taken from humans kept in a "constant routine" of immobile reclined posture, hourly isocaloric meals, and sleep deprivation, Dallmann et al. (2012) could definitively rule out food-dependent control: 17 % of metabolites in both matrices were rhythmic even in the absence of rhythmic feeding, sleep, and activity (Fig. 1). These included lipids, carbohydrates, and amino acids, the same pathways that demonstrated metabolic control in mice (Eckel-Mahan et al. 2012). The same study also showed that the abundance of some metabolites increased or decreased monotonically with sleep deprivation, implying that sleep pressure and circadian influences might independently regulate diurnal metabolic physiology.

Metabolomics: Applications for Circadian Medicine

Because various circadian metabolites show peak abundance at different times of day, it is possible to use these relative quantities as indicators of timing. The idea is analogous to the "chronological garden" of the Swedish botanist Carl Linnaeus, who used plants flowering at different times of day to determine geological time at any moment. In precisely the same fashion, Minami et al. (2009) used blood metabolites from mice as a way of detecting circadian body time, and Martinez-Lozano Sinues et al. (2014) used metabolites within human breath. While potentially quite powerful, these molecular timetable-based methods are hampered by the high inter-individual variability of metabolite abundance among different subjects, making single-time-point analyses relatively imprecise. So far, an accuracy of about 2 h in circadian time is the best that has been attained. As more individuals are metabolomically characterized into different endophenotypic subtypes, it is likely that this accuracy will increase substantially.

Major applications of such technology would be twofold. First and most simply, it would be possible to determine human body time prior to clinical intervention. For most drugs, both pharmacokinetics and pharmacodynamics vary in circadian

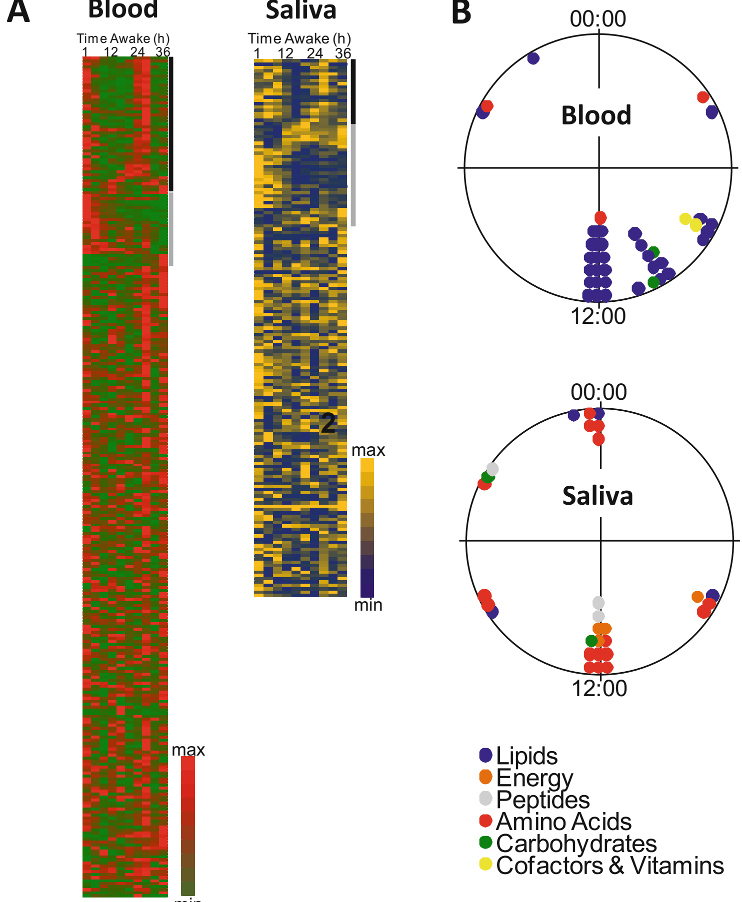

Fig. 1 (**a**) Heat map of circadian metabolites identified in human blood (*left*) and saliva (*right*) from subjects maintained in a constant routine of hourly isocaloric meals, immobile posture, constant dim light, and sleep deprivation. Rows: individual metabolites; columns: time relative to start of experiment. (**b**) Major classes of compounds identified in blood (*top*) and saliva (*bottom*), plotted in circadian time relative to theoretical dawn (Adapted from Dallmann et al. 2012)

fashion. In other words, not only is the metabolism of many xenobiotic substances strongly regulated but also the biological targets of the drugs themselves (Dallmann et al. 2014). Thus, potentially both increased efficacy and reduced toxicity could be obtained by precise timing of delivery, at least in the case of unstable compounds. Currently, multiple clinical trials have been run or are running, especially in the field of cancer, to test this concept (Innominato et al. 2014). Since cell division has been shown to be coordinated with circadian clock timing in both adult animals and cells (Matsuo et al. 2003; Nagoshi et al. 2004; Kowalska et al. 2013; Bieler et al. 2014; Feillet et al. 2014), and many chemotherapeutic agents are metabolized

by circadian isoforms of cytochrome P450 in the liver, it is logical to imagine that chemotherapeutic toxicity would itself be circadian. This concept has been demonstrated experimentally in mice (Gorbacheva et al. 2005), and human trials have also shown time-of-day-dependent effects (Innominato et al. 2014).

Secondly, metabolite timetable-based methods would be able to predict not only clock phase but also clock amplitude and possibly even circadian desynchrony. These parameters have been increasingly linked to disease both in humans and in animal models, as mentioned in the introduction, leading the World Health Organization to classify shiftwork as a suspected carcinogen. Even a simple indicator like the amplitude of circadian behavior correlates directly with survival during chemotherapy of metastatic colon carcinoma in humans (Innominato et al. 2012).

It is suspected that one of the main deleterious effects of shiftwork is circadian desynchrony among different organs. In mice, changes in daylight timing shift different organs at different speeds (Davidson et al. 2009). Potentially, metabolomics could give insight into this phenomenon, since various circadian components come from different tissues. For example, the hormone melatonin is secreted by the pineal gland of the hypothalamus and is thought to be a direct output of the SCN. By contrast, many other endocrine factors and metabolites detectable in blood or in breath arise primarily as byproducts of peripheral organ function (Gamble et al. 2014). Therefore, we propose that circadian metabolomics could be useful in elucidating desynchrony between clocks in brain and in other peripheral tissues. To date, no studies have used metabolomics methods in this fashion, but great potential exists.

Outlook and Conclusion

Questions of circadian desynchrony in health and disease are only beginning to be addressed. The idea that shiftwork might affect circadian clock function is easy to see. However, many other factors could play important and unsuspected roles. For example, recent studies have suggested that chronic sleep restriction alone, even without changed diurnal patterns of activity, could also disrupt circadian transcription (Moller-Levet et al. 2013). Similarly, depressive and affective disorders have long been known to be accompanied by dramatically different sleep–wake patterns (Lamont et al. 2007). Recent research has established a close tie between circadian dysfunction and metabolic disorders like obesity and diabetes (Maury et al. 2014). All of these syndromes are potentially explorable by circadian metabolomics. The conclusions that such studies might derive could both answer outstanding questions about circadian biology and improve human health.

Acknowledgments The research of SAB is supported by the Swiss National Science Foundation, the Velux Foundation, the Swiss Cancer League, and the Zürich Clinical Research Priority Project "Sleep and Health." He is a member of the Zürich Neurozentrum (ZNZ) and Molecular Life Sciences graduate programs within the Life Sciences Zürich Graduate School. LC is a member of

the ZNZ and has received support from the Zürich Clinical Research Priority Project “Sleep and Health”.

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Rhythms Within Rhythms: The Importance of Oscillations for Glucocorticoid Hormones

Stafford Lightman

Abstract The circadian activity of the hypothalamic-pituitary-adrenal (HPA) axis is made up from an underlying oscillatory rhythm of ACTH and glucocorticoid pulses that vary in amplitude but not frequency over the 24 h. This oscillatory activity is not the result of a hypothalamic oscillator but emerges as a natural consequence of the feedforward:feedback interaction between the pituitary corticotropes and the glucocorticoid-secreting cells of the adrenal cortex. This oscillatory activity has resulted in adaptations in the way tissues read their 'digital' ligand signal. The adrenal cortex is relatively insensitive to constant signals of ACTH but responds briskly to the equivalent amount of ACTH administered in a pulsatile manner. Similarly glucocorticoid-responsive tissues such as the brain and the liver are able to read the oscillating signals of cortisol or corticosterone secretion, with differential biochemical and functional responses to different patterns of ligand presentation. During a prolonged acute stress there is a major change in the pituitary-adrenal relationship, with a marked increase in the sensitivity of the adrenal to small changes in ACTH, so that following cardiac surgery small oscillations in ACTH result in massive swings in cortisol. This response appears to be due to a change both in the ACTH signalling pathway and in the endogenous activators and inhibitors of glucocorticoid synthesis.

Introduction

Oscillations are a basic characteristic of all matter. Atoms have their own characteristic oscillation frequencies, and the frequency of the oscillations of Cesium 133, for instance, is often chosen as the basis for atomic clocks. The kinetic theory of matter goes further to suggest that all matter is made up of particles that are constantly moving; in 1905, it was Albert Einstein who demonstrated how this

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P. Sassone-Corsi, Y. Christen (eds.), *A Time for Metabolism and Hormones*,
Research and Perspectives in Endocrine Interactions,
DOI 10.1007/978-3-319-27069-2_10

atomic activity explained the phenomenon of Brownian movement (Einstein 1905). It is therefore of no surprise that biological systems are also invariably dynamic, with both stochastic interactions and deterministic processes across multiple time scales ensuring the maintenance of homeostatic regulation and allowing the organism to adapt to changes in both internal and external environments.

The physical world has a direct impact on the one neuroendocrine system that is critical for life: the hypothalamic-pituitary-adrenal (HPA) axis. The daily rotation of the earth on its axis provides our planet with its regular 24-h day/night cycle and this is the cue for the circadian activity of the HPA axis, which ensures energy supplies are available prior to the daily phase of activity—day in man and night in rodents—by ensuring an anticipatory increase in plasma glucocorticoid levels. These glucocorticoid hormones—cortisol in man and corticosterone in the rodent (both called CORT in this manuscript)—do not simply organise the circadian aspects of metabolic, cognitive and immunological functions, they are also vital homeostatic regulators that are extremely responsive to any threat to the organism's internal stability. In addition to their circadian variation, they need to maintain exquisite sensitivity to both perceived and genuine stressors. It is this combined function of providing a day-to-day regulatory role together with a rapid response mode that requires a system that maintains its reactivity at all times, whatever the status of its circadian activity.

How can this be achieved? The circuitry for the HPA axis is shown in Fig. 1. As has been well described by other authors in this symposium, the suprachiasmatic nucleus (SCN) of the hypothalamus provides the circadian regulation via an inhibitory input to the corticotrophin-releasing hormone (CRH)-containing neurons of the hypothalamic paraventricular nucleus (PVN) (Vrang et al. 1995; Dickmeis 2009). These neurons in turn release CRH, which travels in the hypothalamic-pituitary portal blood system to corticotroph cells in the anterior pituitary, which then release adrenocorticotropic hormone (ACTH) into the systemic circulation. Surprisingly, the output from this system is not a simple analogue release of ACTH from the pituitary gland but a complex episodic series of pulses of hormone secretion (Jasper and Engeland 1991; Windle et al. 1998). In this chapter I shall describe the mechanism underlying the genesis of this oscillating hormone system and why it is so important for the ability of glucocorticoids to perform their multiple activities in so many different systems in the body.

The Origin of HPA Pulsatility

It had always been assumed that the pulsatility of both ACTH and CORT must be due to some hypothalamic oscillator resulting in pulses of CRH, which are then transcribed into pulses of ACTH and CORT. Indeed, there is evidence for episodic release of CRH from macaque hypothalamic explants (Mershon et al. 1992) and for rapid changes in CRH in the median eminence of rats (Ixart et al. 1991) and portal blood of sheep (Caraty et al. 1988). There is, however, a mismatch between the higher frequency of CRH pulses than the ACTH/CORT pulses, and Engler

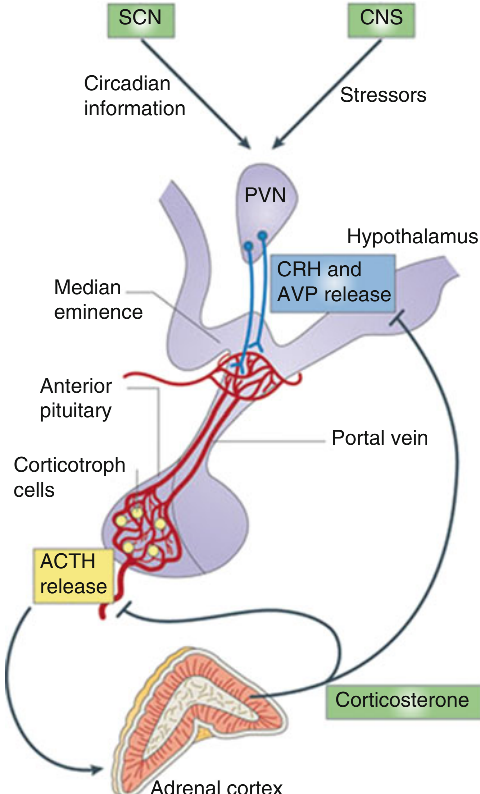

Fig. 1 The hypothalamic-pituitary-adrenal (HPA) axis. The hypothalamus receives circadian input from the suprachiasmatic nucleus (SCN) and stress-related inputs from the limbic system and brainstem. *PVN* paraventricular nucleus, *CRH* corticotrophin-releasing hormone, *AVP* arginine vasopressin. Reproduced with permission from Lightman and Conway-Campbell (2010)

et al. (1990) also demonstrated the maintenance of ACTH and cortisol pulses in the sheep even after disconnection of the pituitary from hypothalamic portal blood.

We therefore reconsidered what we knew about the interaction between pituitary corticotropes and adrenal cortical cells. Since the adrenal gland cannot store glucocorticoids—which are very lipophilic and thus cannot be stored in vesicles—every pulse of steroid released into the circulation must be newly synthesised. There must, therefore, be a delay between the signal from ACTH activation of adrenal MC2 receptors and the release of CORT. Indeed, this has been clearly demonstrated both in the rat and in man (Carnes et al. 1989; Henley et al. 2009). There is, in other words, a clear delay in the feedforward effect of ACTH on the release of CORT. What about the feedback of CORT on the pituitary corticotropes? A very rapid effect of CORT inhibiting ACTH release has been demonstrated both in the rat and in man (Jones et al. 1972; Rotsztejn et al. 1975; Hinz and Hirschelmann 2000; Russell et al. 2010). We therefore have a feedforward/feedback interaction between pituitary corticotroph-derived ACTH and adrenal cortical-derived CORT, with a built-in delay in the feedforward part of the loop. This is a system that mathematically must show endogenous oscillatory activity!

With this knowledge, we were able to collaborate with our mathematical colleagues who developed a mathematical model that predicted the ability of the pituitary adrenal system to support self-sustained ACTH and CORT oscillations at the frequency found in normal physiology, even in the face of a constant CRH drive (Walker et al. 2010). This model also predicted that these oscillations would not occur at very low CRH concentrations and would be damped at the high levels of CRH found after an acute stress, as we had found in rats following an acute stressor (Windle et al. 1998). We were then able to test this model experimentally using constant infusions of CRH into free running animals in the morning, a time when their endogenous CRH systems are suppressed by the SCN. Consistent with our mathematical model, a constant infusion of CRH produced normal ultradian oscillations of both ACTH and CORT, with the same pulse frequency as that found during normal nocturnal HPA activity (Walker et al. 2012). Furthermore, giving a constant infusion of a higher concentration of CRH resulted in a high and prolonged constant secretion of CORT, similar to that found following a severe stress.

Since circadian rhythmicity of the HPA axis is controlled by an inhibitory output from the SCN to the PVN, another prediction from our mathematical model was that interruption of this pathway would not only abolish circadian rhythmicity but would also allow unrestrained CRH secretion throughout the 24 h, resulting in ultradian secretion of ACTH and CORT throughout the 24 h and not just during times of peak activity. We tested this prediction both by lesioning of the SCN and by maintaining animals on a 24-h constant light cycle. Indeed, we found that in both cases there was a loss of circadian variability but maintenance of ultradian activity across the 24 h (Waite et al. 2012).

Implications of HPA Pulsatility

Since HPA pulsatility emerges as a natural consequence of the feedforward:feedback interaction between the pituitary and adrenal gland, it is not surprising that it has been reported to exist in all mammalian species that have been studied, including rat (Jasper and Engeland 1991; Windle et al. 1998), sheep (Fulkerson 1978), rhesus monkey (Holaday et al. 1977) and man (Weitzman et al. 1971; Henley et al. 2009). It would seem very likely, therefore, that physiological systems have adapted to read this digital ACTH and CORT signalling, and indeed this has proved to be the case.

Adrenal Adaptation to Pulsatile ACTH

Adrenal steroidogenesis is an extraordinarily dynamic process. Since steroid hormones cannot be stored for subsequent rapid release, each pulse of CORT seen in the plasma is the result of the very rapid intra-adrenal conversion of cholesterol to

CORT. Pituitary-derived ACTH binds to the melanocortin-2 receptor in adrenal fasciculata cells (Mountjoy et al. 1994), activating adenylyl cyclase and PKA-induced genomic and non-genomic steroidogenic pathways. CREB-induced transcription of the rate-limiting step of cholesterol transport into the mitochondrion (StAR) is enhanced by the binding of positive regulators (Sugawara et al. 1996; Caron et al. 1997; Song et al. 2001; Conkright et al. 2003; Takemori et al. 2007) and inhibition of the negative regulator DAX-1 (Song et al. 2004). PKA also modifies the rapid non-transcriptional modification of steroidogenic proteins, including phosphorylation of StAR itself (Arakane et al. 1997) and of hormone-sensitive lipase (HSL), which increases the intracellular levels of cholesterol itself.

So how does this complex system of different activators and inhibitors of CORT synthesis respond to different patterns of ACTH presentation? We have shown that, when endogenous ACTH is suppressed by administration of methylprednisolone, rats respond to pulsatile exogenous ACTH with a pulsatile release of CORT (Spiga et al. 2011). When the same dose of ACTH is infused at a constant rate, however, no CORT is secreted. Indeed, constant ACTH infusion actually results in a suppressed response to a subsequent stress amplitude pulse of ACTH, suggesting a dysregulation of the normal steroidogenic mechanisms (Spiga and Lightman 2014). The exact mechanism underlying this is unclear, but there is evidence that intra-adrenal GR can mediate local negative feedback on steroidogenesis via induction of DAX-1, perhaps accentuating the pulsatile characteristics of the response to a physiological pulse of ACTH while effectively inhibiting the response to a more constant exposure. Interestingly, by integrating our in vivo data with mathematical modelling of adrenal responses, we do find that rapid intra-adrenal inhibition must be an important factor in adrenal ultradian oscillations (Walker et al. 2014). This all suggests that the adrenal gland is beautifully adapted to respond to a pulsatile signal of ACTH, rather than showing a simple analogue response to different concentrations of this hormone.

During severe stress, there also seem to be special adaptations at the adrenal level. In a study of patients undergoing coronary artery bypass graft procedures (Fig. 2), we found an initial surge in both ACTH and CORT, followed by a fall in ACTH back to baseline levels but maintenance of the high levels of CORT, with continued but amplified ultradian responses of CORT to small changes in basal ACTH (Gibbison et al. 2014). The initial rise in both hormones was delayed after the actual surgery itself, suggesting it was the result of inflammatory cytokine production (from the sternotomy), which is known to go up at this time (Lahat et al. 1992; Roth-Isigkeit et al. 1999; de Mendonca-Filho et al. 2006). Therefore, in a reverse translation approach, we used a model of severe stress both with (LPS) and without (depot ACTH) associated systemic inflammation. ACTH and CORT followed each other closely in the depot ACTH-induced response, but after LPS we had the same findings as after cardiac surgery: maintenance of high CORT even after ACTH had fallen to normal levels. Furthermore, only in this group was there an increased expression of StAR and MRAP (a vital accessory for the MC2 receptor) mRNAs and StAR protein. This presumably explains the increased sensitivity to ACTH and the increased steroidogenesis at this time, which is quite

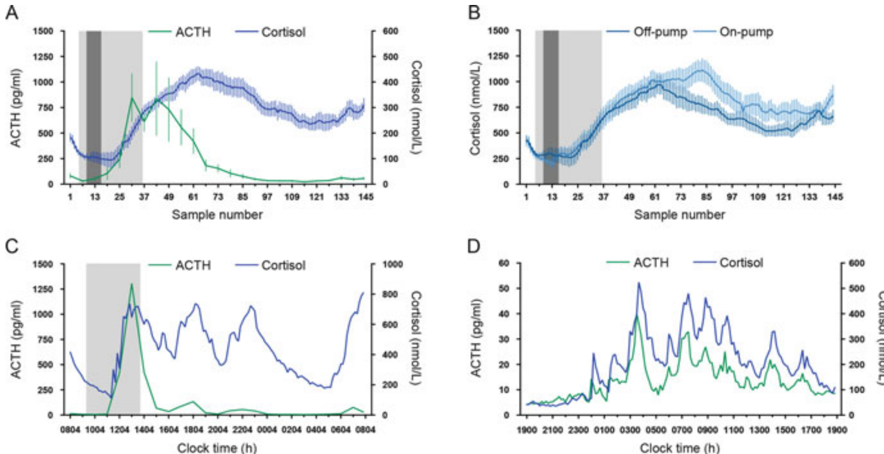

Fig. 2 Changes in cortisol and ACTH levels throughout the 24-h perioperative period of cardiac surgery. (**a**) Group mean ± SEM cortisol and ACTH. All sampling (i.e., the first sample in every case) started between 0800 and 0900 h. (**b**) Mean ± SEM 24-h cortisol profile from patients undergoing coronary artery bypass graft using the off-pump or the on-pump technique. All off-pump surgeries were performed between sample 5 and sample 35; all on-pump surgeries were performed between samples 5 and samples 36. (**a**) and (**b**), *light grey* area, period during which some patients were undergoing surgery. *Dark grey* area, period during which all patients were undergoing surgery. (**c**) Individual 24-h ACTH and cortisol profile of a patient undergoing off-pump CABG. *Light grey* area, period during which the patient was undergoing surgery (0919–1349-h). (**d**), Individual 24-h ACTH and cortisol profile of a healthy volunteer. Reproduced with permission from Gibbison et al. (2014)

different from the situation in patients who have had prolonged critical illness in whom steroidogenic genes appear to be depleted (Boonen et al. 2014).

Tissue Adaptation to Pulsatile CORT

The large oscillations of total CORT seen in blood are also reflected in similar large oscillations of the active free cortisol levels (unbound to cortisol binding globulin) in the brain and subcutaneous tissue (Droste et al. 2008; Qian et al. 2012; Bhake et al. 2013), indicating that both glucocorticoid (GR) and mineralocorticoid (MR) receptors will be exposed to oscillating levels of their ligand (Fig. 3). These receptors are latent transcription factors initially sequestered in the cytoplasm bound to chaperone molecules including HSP90 and p23. Upon binding of CORT, they undergo a conformational change dissociate from the chaperone complex and are actively transported into the nucleus where they rapidly cycle on and off glucocorticoid response elements (GREs) at the chromatin template (Fig. 4; Hager et al. 2006; Conway-Campbell et al. 2012). Each endogenous pulse of CORT results in a rapid increase in activated GR available for binding to GREs, with

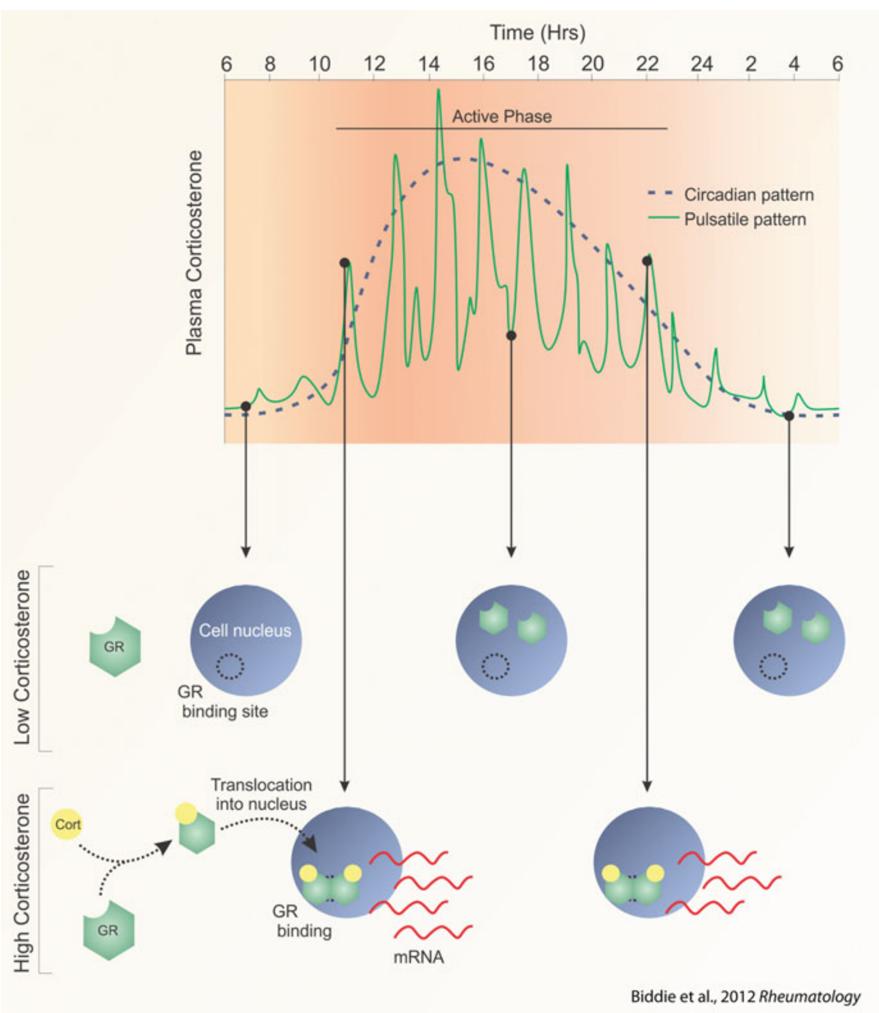

Fig. 3 Glucocorticoid pulsatility drives transient activation of GR-responsive genes. CORT levels rise in anticipation of the active phase. Hormone levels follow a circadian pattern, although the underlying pattern of hormone secretion is ultradian, where glucocorticoids are released approximately every hour. During a pulse, exposure to hormone drives GR translocation into the nucleus, where it binds to genomic elements to drive transcription. Hormone troughs result in GR dissociation from chromatin, releasing the receptor into the nucleoplasm ready to initiate transcription during further rises in hormone levels. The dynamics of the receptor and hormone secretion patterns allow rapid response to rapidly changing cellular and physiological conditions. Reproduced with permission from Biddie et al. (2011)

repeated pulses resulting in cyclical changes in GR chromatin association profiles on regulatory elements of endogenous CORT-regulated gene promoters (Fig. 4; Conway-Campbell et al. 2011). The interaction of GR with other accessory

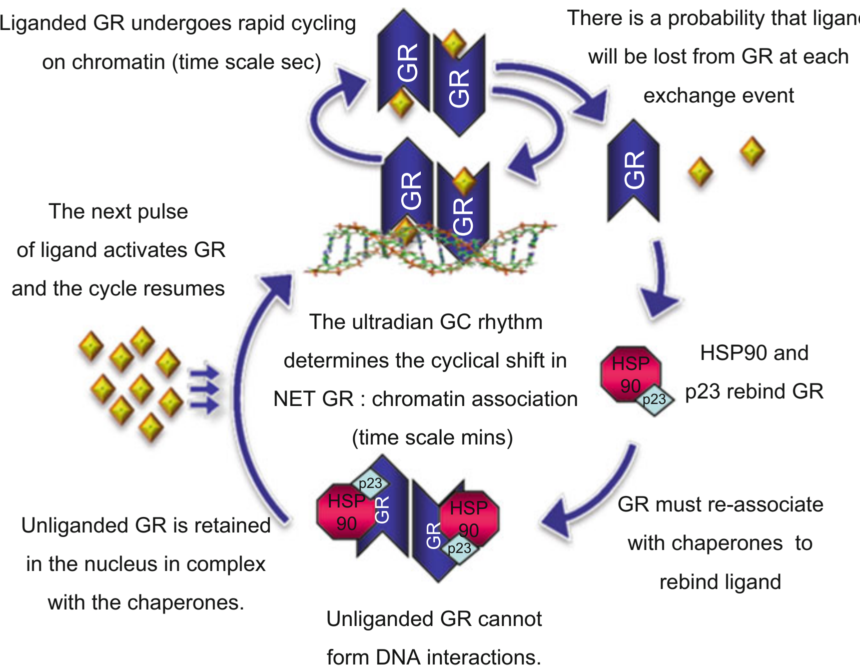

Fig. 4 GR ultradian cycling model. A schematic representation of how cyclical GR interactions with genomic response elements result in pulsatile transcriptional activity. The initial pulse of hormone causes nuclear translocation of GR, allowing GR to enter the chromatin binding cycle with rapid transient interactions with the chromatin template (stochastic action; time scale of seconds). The slower cyclical events related to the ultradian rhythm are not stochastic but are determined by pulses of ligand and the nuclear molecular chaperone cycle (time scale of minutes). Reproduced with permission from Conway-Campbell et al. (2012)

DNA-binding factors will clearly be cell and tissue specific, providing scope for differential responses to the same pattern of CORT pulses in different tissues.

There are now increasing data that ultradian pulsatility has considerable relevance for gene transcription. Ultradian oscillations of CORT induce cyclic GR-mediated pulses of gene transcription, both in vitro and in vivo, which differ from the response to equivalent constant levels of the ligand (Stavreva et al. 2009; Conway-Campbell et al. 2012; McMaster et al. 2011). Indeed, gene pulsing of the clock gene period 1 occurs in vivo in response to physiological pulses, both in the liver (Stavreva et al. 2009) and in the hippocampus (Conway-Campbell et al. 2010). In addition to these genomic effects, glucocorticoids have rapid non-genomic effects on neuronal activity in the brain (Karst et al. 2005; Evanson et al. 2010; Hill and Tasker 2012), with rapid effects on both excitatory and inhibitory inputs to the hippocampus (Karst et al. 2005), and evidence for a specific effect on the insertion of Ca^{2+}-permeable AMPA receptors into synapses (Whitehead et al. 2013). Glucocorticoid pulsatility has also been shown to have specific non-genomic effects on miniature excitatory postsynaptic current (mEPSC)

frequency in different brain areas, with differential effects being described for the amygdala and the hippocampus (Karst et al. 2010). Recently, using high-resolution imaging and electrophysiology, this group showed that, while a single pulse of CORT increases hippocampal AMPAR signalling and impairs the induction of LTP for several hours, a second pulse restores the potentiation range of the glutamate synapses (Sarabdjitsingh et al. 2014). This finding suggests that pulsatile exposure to CORT is necessary to maintain optimal glutamatergic neurotransmission.

It is also becoming clear that the pattern of glucocorticoid secretion has a considerable impact on behaviour. Adrenalectomised rats replaced with constant infusions of CORT show a blunted ACTH and behavioural response to a noise stress, whereas animals replaced with the same dose of CORT but in a physiological pulsatile pattern have normal responses (Sarabdjitsingh et al. 2010). Interestingly, these changes are associated with brain-specific differences in c-fos activation, most particularly in the amygdala, suggesting that different brain circuits respond differentially to different patterns of CORT pulsatility. This study also demonstrated a phase-dependent and region-specific response, revealing a different response during the ascending and descending phases of each CORT pulse. This finding is complementary to data from Haller et al. (2000a, b), who found that rats exposed to male intruders during a rising phase of an endogenous CORT pulse were more aggressive than rats exposed to the same stimulus on a falling phase.

The relevance of CORT pulsatility in man needs investigation. We do know that the use of non-pulsatile oral hydrocortisone replacement therapy in patients with Addisons's disease is associated with a doubling in mortality (Bergthorsdottir et al. 2006) as well as increased morbidity predominantly related to mental and physical fatigue (Løvås et al. 2002). We have now designed a technique to provide physiological CORT replacement (Russell et al. 2014) and will be using it to investigate the importance of pulsatility for optimal cognitive and metabolic function.

Conclusion

Oscillatory activity is widespread in both our physical and biological environment. At the biological level, it can occur in multiple time domains. Within the HPA there is a very rapid (seconds) interaction at the level of GR:chromatin interactions, a slower (minutes) interaction between GR and its associated chaperones, an hourly cycle that emerges as a natural consequence of subhypothalamic feedforward: feedback interactions, and a daily oscillation regulated by the SCN. The circadian variation in CORT is actually made up of changes in the amplitude of the underlying subhypothalamic ultradian rhythm. The ultradian rhythm provides digital signals for both ACTH signalling to the adrenal and CORT signalling to tissues across the whole body, including the nervous, cardiovascular, metabolic and immune systems. The body has adapted to read these signals in a tissue-specific manner, allowing one hormone to have many effects in different tissues. This

strategy of using a feedforward:feedback created digital signalling system is not unique to the HPA axis and is in fact commonly used across the endocrine system (Lightman and Terry 2014).

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The Genetics of Autism Spectrum Disorders

Guillaume Huguet, Marion Benabou, and Thomas Bourgeron

Abstract In the last 30 years, twin studies have indicated a strong genetic contribution to Autism Spectrum Disorders (ASD). The heritability of ASD is estimated to be 50 %, mostly captured by still unknown common variants. In approximately 10 % of patients with ASD, especially those with intellectual disability, *de novo* copy number or single nucleotide variants affecting clinically relevant genes for ASD can be identified. Given the function of these genes, it was hypothesized that abnormal synaptic plasticity and failure of neuronal/synaptic homeostasis could increase the risk of ASD. In parallel, abnormal levels of blood serotonin and melatonin were reported in a subset of patients with ASD. These biochemical imbalances could act as risk factors for the sleep/circadian disorders that are often observed in individuals with ASD. Here, we review the main pathways associated with ASD, with a focus on the roles of the synapse and the serotonin-NAS-melatonin pathway in the susceptibility of ASD.

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P. Sassone-Corsi, Y. Christen (eds.), *A Time for Metabolism and Hormones*,
Research and Perspectives in Endocrine Interactions,
DOI 10.1007/978-3-319-27069-2_11

Introduction

Autism Spectrum Disorders (ASD) are a group of neuropsychiatric disorders characterized by problems in social communication as well as the presence of restricted interests and stereotyped and repetitive behaviors (Kanner 1943; Asperger 1944; Coleman and Gillberg 2012). Epidemiological studies estimate that more than 1 % of the population could receive a diagnosis of ASD (Elsabbagh et al. 2012; Developmental Disabilities Monitoring Network Surveillance Year Principal 2014). Individuals with ASD can also suffer from other psychiatric and medical conditions, including intellectual disability (ID), epilepsy, motor control difficulties, Attention-Deficit Hyperactivity Disorder (ADHD), tics, anxiety, sleep disorders, epilepsy, depression or gastrointestinal problems (Gillberg 2010; Moreno-De-Luca et al. 2013). The term ESSENCE, for 'Early Symptomatic Syndromes Eliciting Neurodevelopmental Clinical Examinations,' was coined by Christopher Gillberg to take into account this clinical heterogeneity and syndrome overlap (Gillberg 2010). There are four to eight times more males than females with ASD (Elsabbagh et al. 2012), but the sex ratio is more balanced in patients with ID and/or dysmorphic features (Miles et al. 2005). Autism can be studied as a category (affected vs. unaffected) or as a quantitative trait using auto- or hetero-questionnaires such as the Social Responsiveness Scale (SRS) or the autism quotient (AQ) (Ronald et al. 2006; Skuse et al. 2009; Constantino 2011). Using these tools, autistic traits seem to be normally distributed in clinical cases as well as in the general population (Ronald et al. 2006; Skuse et al. 2009; Constantino 2011).

The causes of autism remain largely unknown, but twin studies have constantly shown a high genetic contribution to ASD. Molecular genetics studies have identified more than 100 ASD risk genes carrying rare and penetrant deleterious mutations in approximately 10–25 % of patients (Huguet et al. 2013; Gaugler et al. 2014; Bourgeron 2015). In addition, quantitative genetics studies have shown that common genetic variants could capture almost all the heritability of ASD (Huguet et al. 2013; Gaugler et al. 2014). The genetic landscape of ASD is shaped by a complex interplay between common and rare variants and is most likely different from one individual to another (Gardener et al. 2011; Hallmayer et al. 2011; Bourgeron 2015). Remarkably, the susceptibility genes seem (Huguet et al. 2013) to converge in a limited number of biological pathways, including chromatin remodeling, protein translation, actin dynamics and synaptic functions (Bourgeron 2009; Toro et al. 2010; Huguet et al. 2013; Bourgeron 2015). In addition, several studies have pointed to a dysfunction of the serotonin-NAS-melatonin pathway in patients with ASD. Abnormalities of this pathway might increase the risk of circadian/sleep disorders often observed in patients with ASD.

In this chapter, we will detail the advances in the genetics of ASD (Fig. 1) with a focus on the role of both synapses and biological rhythms in the susceptibility of ASD (Abrahams and Geschwind 2008; Bourgeron 2009; Toro et al. 2010; Devlin and Scherer 2012; Huguet et al. 2013).

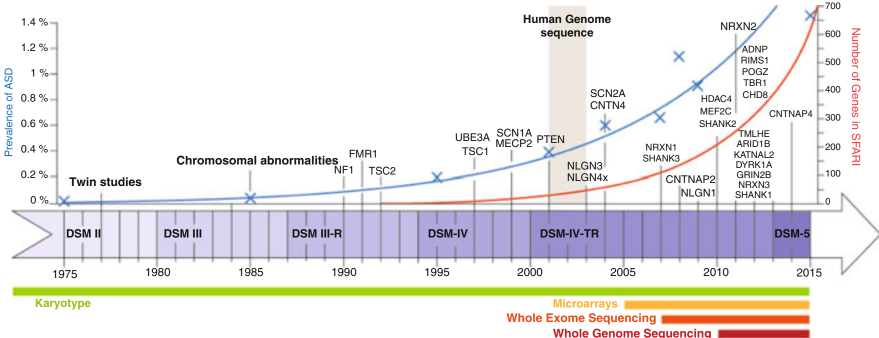

Fig. 1 The history of the genetics of autism from 1975 to 2015. The increase in the identified genes associated with ASD (SFARI—March 2015) is represented together with the prevalence of ASD (data taken from the Center for Disease Control and Prevention), the different versions of the Diagnostic Statistical Manual (from DSM II to DSM 5.0) and the advance in genetics technology (Adapted from Huguet and Bourgeron 2016)

Twin and Family Studies in ASD

Based on more than 13 twin studies published between 1977 and 2015, researchers have estimated the genetic and environmental contribution to ASD (Fig. 2). In 1977, the first twin study of autism by Folstein and Rutter (1977) reported on a cohort of 11 monozygotic (MZ) twins and 10 dizygotic (DZ) twins. This study showed that MZ twins were more concordant for autism—36 % (4/11)—compared with 0 % (0/10) for DZ twins. When a "broader autism phenotype" was used, the concordance increased to 92 % for MZ twins and to 10 % DZ twins (Bailey et al. 1995). Since this first small scale study, twin studies have constantly reported a higher concordance for ASD in MZ compared with DZ (Ritvo et al. 1985; Steffenburg et al. 1989; Bailey et al. 1995; Le Couteur et al. 1996). Between 2005 and 2009, three twin studies with relatively large groups of twins (285–3419) have reported high concordances for ASD in MZ twins (77–95 %) compared with DZ twins (31 %; Ronald et al. 2005; Taniai et al. 2008; Rosenberg et al. 2009). Notably, MZ concordances were similar to those reported in the previous studies, but DZ concordances were higher. In 2010, Lichtenstein et al. reported a relatively low concordance for ASD in 39 % of the MZ twins compared with other studies (the concordance for DZ twins in this study was 15 %). However, as previously indicated by studies using the "broader autism phenotype," all discordant MZ twins of this cohort had symptoms of ESSENCE (e.g., ID, ADHD, language delay, etc.). A significant proportion of the genetic contribution to ASD was shown to be shared with other neurodevelopmental disorders such as ADHD (>50 %) and learning disability (>40 %; Lichtenstein et al. 2010; Ronald et al. 2010; Lundstrom et al. 2011; Ronald and Hoekstra 2011). In summary, when all twin studies are taken into account, concordance for ASD is roughly 45 % for

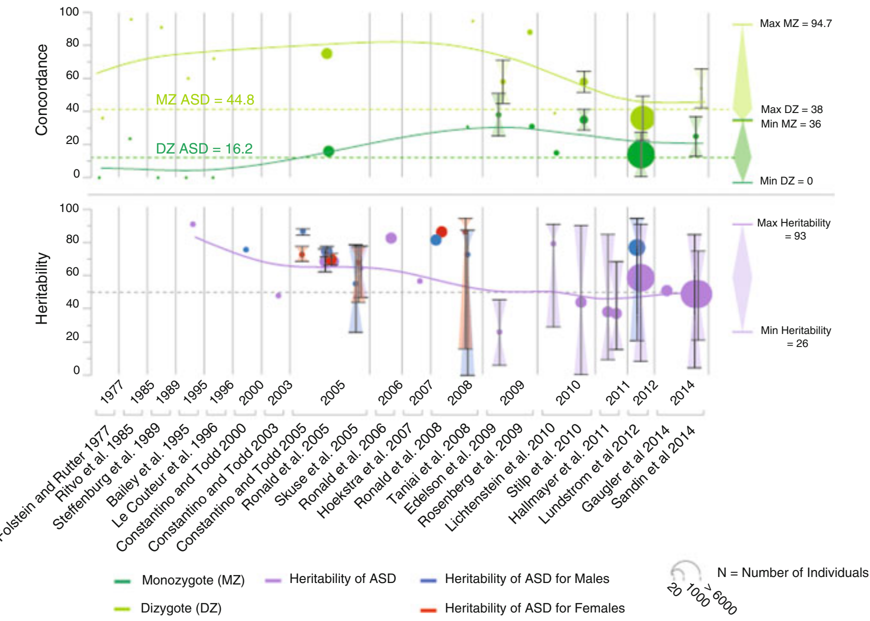

Fig. 2 The main twins studies in ASD. A total of 13 twins studies and 17 heritability studies are depicted. Means of concordance and heritability weighted by sample size are presented on the *right* of the figure (Adapted from Huguet and Bourgeron 2016)

MZ twins and 16 % for DZ twins (Ritvo et al. 1985; Steffenburg et al. 1989; Bailey et al. 1995; Le Couteur et al. 1996).

Family studies also showed that the recurrence of having a child with ASD increases with the proportion of the genome that the individual shares with one affected sibling or parent (Constantino et al. 2010; Risch et al. 2014; Sandin et al. 2014). In a population-based sample of 14,516 children diagnosed with ASD (Sandin et al. 2014), the relative risk for ASD (compared to the general population) was estimated to be 153.0 [95 % confidence interval (CI): 56.7–412.8] for MZ twins, 8.2 (3.7–18.1) for DZ twins; 10.3 (9.4–11.3) for full siblings, 3.3 (95 % CI, 2.6–4.2) for maternal half siblings, 2.9 (95 % CI, 2.2–3.7) for paternal half siblings, and 2.0 (95 % CI, 1.8–2.2) for cousins.

Heritability is the proportion of the phenotypic variation in a trait of interest, measured in a given studied population and in a given environment, that is co-varying with genetic differences among individuals in the same population. In 1995, based on a twin study, Bailey et al. estimated the heritability of autism to be 91–93 %. Since then, the estimation of heritability has differed from one study to another, but the genetic variance has accounted for at least 38 % and up to 90 % of the phenotypic variance (Hallmayer et al. 2011; Ronald and Hoekstra 2011; Sandin et al. 2014). Using a large cohort of 14,516 children diagnosed with ASD Sandin et al. (2014), estimated the heritability to be 0.50 (95 % CI, 0.45–0.56) and the non-shared environmental influence was also 0.50 (95 % CI, 0.44–0.55).

Surprisingly, only the additive genetic component and the non-shared environment seemed to account for the risk of developing ASD (Sandin et al. 2014).

In summary, epidemiological studies provide crucial information about the heritability of ASD. However, they do not inform us about the genes involved or the number and frequency of their variants. In the last 15 years, candidate genes and whole-genome analyses have been performed to address these questions.

From Chromosomal Rearrangements to Copy Number Variants in ASD

The first genetic studies that associated genetic variants with ASD used observations from cytogenetic studies (Gillberg and Wahlstrom 1985). However, because of the low resolution of the karyotypes (several Mb), it was almost impossible to associate a specific gene to ASD using this approach. The prevalence of large chromosomal abnormalities is estimated to be less than 2 % (Vorstman et al. 2006). Thanks to progress in molecular technologies such as Comparative Genomic Hybridization (CGH) or SNP arrays, the resolution in the detection of genomic imbalances has dramatically increased. Depending on the platforms, Copy Number Variants (CNVs) of more than 50 kb are now robustly detected (Pinto et al. 2011). Since the first articles published in 2006, a very large number of studies have investigated the contribution of CNVs to ASD (Jacquemont et al. 2006; Sebat et al. 2007). Several studies using the Simons Simplex Collection could even provide an estimation of the frequency of the *de novo* CNVs in patients with ASD compared with their unaffected siblings (Sanders et al. 2011). All together, *de novo* CNVs are present in 4–7 % of the patients with ASD compared to 1–2 % in the unaffected siblings and controls (Glessner et al. 2009; Sanders et al. 2011; Pinto et al. 2014). The studies have also indicated that *de novo* CNVs identified in patients are most likely altering genes and most especially genes associated with synaptic functions and/or regulated by FMRP, the protein responsible for the fragile X syndrome (Pinto et al. 2010, 2014). Beyond ASD, large CNVs (>400 kb) affecting exons are present in 15 % of patients with Developmental Delay (DD) or ID (Cooper et al. 2011). Most of the CNVs are private to each individual, but some are recurrently observed in independent patients. For example, three loci on chromosomal regions 7q11, 15q11.2–13.3, and 16p11.2 have been strongly associated with ASD (Ballif et al. 2007; Kumar et al. 2008; Weiss et al. 2008; Szafranski et al. 2010; Sanders et al. 2011; Leblond et al. 2014).

In summary, large chromosomal rearrangements and CNVs increase the risk of having ASD in 5–10 % of the individuals (Vorstman et al. 2006; Pinto et al. 2010, 2014). To go further in the identification of the ASD risk genes, candidate genes and whole exome/genomes studies were performed.

From Candidate Genes to Whole Exome/Genome Sequencing Studies in ASD

The first approach to associate a gene with ASD was to select specific candidate genes based on data coming from functional or genetic studies or a combination of the two. This approach was successful in identifying several synaptic genes associated with ASD such as *NLGN3*, *NLGN4X*, *SHANK3* and *NRXN1* (Jamain et al. 2003; Durand et al. 2007; Szatmari et al. 2007). Thanks to the advance in Next Generation Sequencing (NGS), we can now interrogate all genes of the genome in an unbiased manner using Whole Exome/Genome Sequencing (WES, WGS).

To date, more than 18 WES studies of sporadic cases of ASD (O'Roak et al. 2011, 2012a; Chahrour et al. 2012; Iossifov et al. 2012; Neale et al. 2012; Sanders et al. 2012; He et al. 2013; Lim et al. 2013; Liu et al. 2013, 2014; Willsey et al. 2013; Yu et al. 2013; An et al. 2014; De Rubeis et al. 2014; Iossifov et al. 2014; Samocha et al. 2014; Chang et al. 2015; Krumm et al. 2015) have been performed, comprising altogether more than >4000 families (Table 1). In almost all these studies, the authors have especially focused their analysis on the contribution of *de novo* Single Nucleotide Variants (SNVs) in ASD. All together, the average number of *de novo* coding SNVs per individual (including missense, splicing, frameshift, and stop-gain variants) is estimated to be approximately 0.86 in female patients, 0.73 in male patients, and 0.60 in unaffected male and female siblings (Krumm et al. 2014; Ronemus et al. 2014). Interestingly, *de novo* SNVs were three times more likely to be on the paternal chromosome than on the maternal one (Kong et al. 2012; O'Roak et al. 2012a) with an increase of almost two *de novo* mutations per year and doubled every 16.5 years (Kong et al. 2012).

Based on these studies (Iossifov et al. 2012; Neale et al. 2012; O'Roak et al. 2012a; Sanders et al. 2012), 3.6–8.8 % of the patients were shown to carry a *de novo* causative mutation (Iossifov et al. 2012) with a twofold increase of deleterious mutations in the patients compared with their unaffected siblings. In a meta-analysis, using more than 2500 families, Iossifov et al. (2014) found that de novo Likely Gene Disrupting (LGD) mutations (frameshift, nonsense and splice site) were more frequent in patients with ASD compared with unaffected siblings ($P = 5 \times 10^{-7}$). The carriers of these *de novo* LGDs were more likely diagnosed with a low non-verbal IQ. The *de novo* LGDs are significantly enriched in genes involved in chromatin modeling factors ($P = 4 \times 10^{-6}$) and in genes regulated by the FMRP complex ($p = 4 \times 10^{-7}$). Following these whole exome studies, targeted re-sequencing studies of the most compelling candidate genes were performed (O'Roak et al. 2012b). All together, 10 genes carrying *de novo* mutations were significantly associated with ASD: *CHD8*, *DYRK1A*, *GRIN2B*, *KATNAL2*, *RIMS1*, *SCN2A*, *POGZ*, *ADNP*, *ARID1B* and *TBR1*.

Only a few studies have analyzed the contribution of inherited SNVs in ASD. In 2013, Lim et al. analyzed whole exome sequencing of 933 cases (ASD) and 869 controls for the presence of rare complete human knockouts (KO) with homozygous or compound heterozygous loss-of-function (LoF) variants (≤5 %

Table 1 Summary of the main whole exome/genome sequencing studies in ASD

Studies	Tech	#ASD analyzed in the study	# ASD specific to this study	#ASD coming from other studies	#Controls	#Unaffected sibling	#Parents	Analysis of *de novo* variants	Analysis of inherited variants
O'Roak et al. (2011)	WES	20	20	–	–	20	38	X	–
O'Roak et al. (2012a)	WES	229	209	20 (O'Roak et al. 2011)	–	50	418	X	–
Neale et al. (2012)	WES	175	175	–	–	–	350	X	–
Sanders et al. (2012)	WES	238	238	–	–	200	476	X	–
Iossifov et al. (2012)	WES	343	343	–	–	343	686	X	X
Chahrour et al. (2012)	WES	16	16	–	–	–	–	–	X
Yu et al. (2013)	WES	401	163	238 (Iossifov et al. 2012; Neale et al. 2012; Sanders et al. 2012)	–	114	326	X	X
Lim et al. (2013)	WES	1496	1004	492 (Sanders et al. 2012)	5474	–	–	–	X
Liu et al. (2013)	WES	1039	–	1039 (O'Roak et al. 2011, 2012a; Iossifov et al. 2012; Neale et al. 2012; Sanders et al. 2012; Liu et al. 2013)	869	–	–	–	X
He et al. (2013)	WES	1867	–	1867 (O'Roak et al. 2011, 2012a; Iossifov et al. 2012; Neale et al. 2012; Sanders et al. 2012; Liu et al. 2013)	870	593	1870	X	X
Willsey et al. (2013)	WES	1099	56	1043 (O'Roak et al. 2011, 2012a; Iossifov et al. 2012; Neale et al. 2012; Sanders et al. 2012; Liu et al. 2013)	–	56	112	X	X

(continued)

Table 1 (continued)

Studies	Tech	#ASD analyzed in the study	# ASD specific to this study	#ASD coming from other studies	#Controls	#Unaffected sibling	#Parents	Analysis of *de novo* variants	Analysis of inherited variants
Liu et al. (2014)	WES	1967	–	1967 (O'Roak et al. 2011, 2012a; Iossifov et al. 2012; Kong et al. 2012; Neale et al. 2012; Sanders et al. 2012; Liu et al. 2013; Willsey et al. 2013)	870	593	2070	X	X
Samocha et al. (2014)	WES	1078	–	1078 (Asperger 1944; Coleman and Gillberg 2012; Developmental Disabilities Monitoring Network Surveillance Year Principal 2014; Elsabbagh et al. 2012; Gillberg 2010; Constantino 2011)	–	343	2156	X	–
Iossifov et al. (2014)	WES	2	1576	932 (O'Roak et al. 2011, 2012a; Iossifov et al. 2012; Neale et al. 2012; Sanders et al. 2012; Liu et al. 2013)	–	1911	5016	X	–
An et al. (2014)	WES	40	40	–	–	8	80	X	X
De Rubeis et al. (2014a)	WES	2270	–	2270 (O'Roak et al. 2011, 2012a; Iossifov et al. 2012, 2014; Neale et al. 2012; Sanders et al. 2012)	5397	–	4540	X	X
Chang et al. (2015)	WES	932	–	932 (Levy et al. 2011; O'Roak et al. 2011, 2012a; Iossifov et al. 2012; Sanders et al. 2012)	–	593	1580	X	X
Krumm et al. (2015)	WES	2377	–	2377 (O'Roak et al. 2011, 2012a; Iossifov et al. 2012, 2014; Neale et al. 2012; Sanders et al. 2012)	–	1786	4754	X	X

Kong et al. (2012)	WGS	40	44	–	–	7	136	X	–
Michaelson et al. (2012)	WGS	20	20	–	–	–	–	X	–
Shi et al. (2013)	WGS	1	1	–	–	6	2	X	X
Jiang et al. (2013)	WGS	32	32	–	–	–	64	X	X
Yuen et al. (2015)	WGS	85	85	32 (Jiang et al. 2013)	–	–	170	X	X
Nemirovsky et al. (2015)	WGS	1		–	–	–	–	–	X

frequency). They observed a significant twofold increase in complete KOs in patients with ASD compared to controls. They estimated that such complete KO mutations could account for 3 % of the patients with ASD. For the X chromosome, there was a significant 1.5-fold increase in complete KO in affected males compared to unaffected males that could account for 2 % of males with ASD (Lim et al. 2013). The same year, Yu et al. (2013) analyzed 104 consanguineous families including 79 families with a single child with ASD (simplex families) and 25 families with more than one affected individual (multiplex families) collected by the Homozygosity Mapping Collaborative for Autism (HMCA). They identified biallelic mutations in *AMT*, *PEX7*, *SYNE1*, *VPS13B*, *PAH*, and *POMGNT1*. Finally, a very recent study by Krumm et al. (2015) ascertained the relative impact of inherited and *de novo* variants (CNVs or SNVs) on ASD risk in 2377 families. Inherited truncating variants were enriched in probands (for SNV odds ratio = 1.14, P = 0.0002; for CNV odds ratio = 1.23, P = 0.001) in comparison to unaffected siblings (Krumm et al. 2015). Interestingly, they also observed a significant maternal transmission bias of inherited LGD to sons. New ASD-risk genes were also identified such as *RIMS1*, *CUL7* and *LZTR1*.

To date, few whole genome sequencing studies have been published for ASD (Table 1). Michaelson et al. (2012) analyzed 40 WGS of monozygotic twins concordant for ASD and their parents. They proposed that ASD-risk genes could be hot spots of mutation in the genome and confirmed the association between ASD and *de novo* mutations in *GPR98*, *KIRREL3* and *TCF4*. Shi et al. (2013) analyzed a large pedigree with two sons affected with ASD and six unaffected siblings, focusing on inherited mutations. They identified *ANK3* as the most likely candidate gene. In 2015, Yuen et al. analyzed 85 families with two children affected with ASD. This study represents the largest published WGS data set in ASD. They identified 46 ASD-relevant mutations present in 36 of 85 (42.4 %) families. Only 16 ASD-relevant mutations of 46 (35 %) identified were *de novo*. Very interestingly, for more than half of the families (69.4 %; 25 of 36), the two affected siblings did not share the same rare penetrant ASD risk variant(s).

Whole genome sequencing is also very efficient to identify mutations in regions of the human genome that are wrongly annotated and in regions that are highly GC rich. For example, mutations on the *SHANK3* gene were rarely identified using whole exome sequencing, given its high GC content (Leblond et al. 2014). In contrast, whole genome sequencing could successfully identify *SHANK3* mutations (Nemirovsky et al. 2015; Yuen et al. 2015).

The Common Variants in ASD

In the general population, one individual carries on average three million genetic variants in comparison to the reference human genome sequence (Xue et al. 2012; Fu et al. 2013; Genome of the Netherlands and Genome of the Netherlands 2014). The vast majority of the variants (>95 %) are the so-called common variants shared

with more than 5 % of the human population (Xue et al. 2012; Fu et al. 2013; Genome of the Netherlands and Genome of the Netherlands 2014). While there is not a clear border between common and rare variants, it is nevertheless interesting to estimate the role of the genetic variants found in the general population in the susceptibility to ASD.

Using quantitative genetics, Klei et al. (2012) estimated that common variants were contributing to a high proportion of the liability of ASD: 40 % in simplex families and 60 % in multiplex families. In 2014, a study by Gaugler et al. used the same methodology (Yang et al. 2011) and provided an estimation of the heritability (52.4 %) that is almost exclusively due to common variation, leaving only 2.6 % of the liability to the rare variants. The contribution of common variants is therefore important, but unfortunately the causative SNPs still remain unknown since they are numerous (>1000) and each is associated with a low risk. To date, the largest genome wide association studies (GWAS) performed on <5000 families with ASD were underpowered to identify a single SNP with genome wide significance (Anney et al. 2012; Cross-Disorder Group of the Psychiatric Genomics 2013).

The recruitment of larger cohorts of patients with dimensional phenotypes is therefore warranted to better ascertain the heritability of ASD and to identify the genetic variants, which explain most of the genetic variance.

The Genetic Architecture of ASD

Based on the results obtained from epidemiological and molecular studies, it is now accepted that the genetic susceptibility to ASD can be different from one individual to another with a combination of rare deleterious variants (R) and a myriad of low-risk alleles [also defined as the genetic background (B)]. Most of the inherited part of ASD seems to be due to common variants observed in the general population, with only a small contribution from rare variants (Fig. 3). Importantly, while the *de novo* mutations are considered per se as genetic factors, they do not contribute to the heritability since they are only present in the patient (with the relatively rare exception of germinal mosaicisms present in one of the parental germlines and transmitted to multiple children). These *de novo* events could therefore be considered as “environmental causes” of ASD but acting on the DNA molecule. It is currently estimated that more than 500–1000 genes could account for these “monogenic forms” of ASD (Iossifov et al. 2012; Sanders et al. 2012), confirming the high degree of genetic heterogeneity.

The interplay between the rare or *de novo* variants R and the background B will also influence the phenotypic diversity observed in the patients carrying rare deleterious mutations. In some individuals, a genetic background B will be able to buffer or compensate the impact of the rare genetic variations R. In contrast, in some individuals, the buffering capacity of B will not be sufficient to compensate the impact of *R* and they will develop ASD (Rutherford 2000; Hartman et al. 2001). In the R‘n’B model, ASD can be regarded as a collection of many genetic forms of

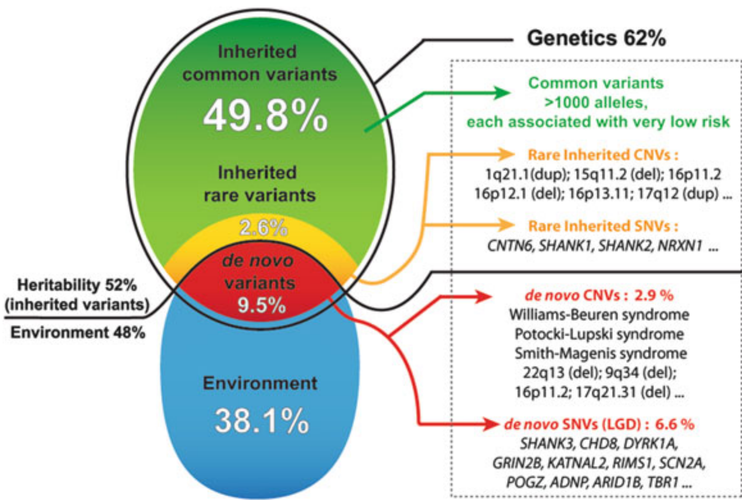

Fig. 3 Relative contribution of the genetics and environment in ASD. Based on twin and familial studies, it is estimated that the genetic and environmental contributions to ASD are approximately 50/50 %. Most of the heritable part seems to be due to common variants observed in the general population, with a small contribution of rare variants. Importantly, the *de novo* mutations are genetic causes of ASD but do not contribute to the heritability since there are only present in the patient. These *de novo* events are therefore considered to be "environmental causes" of ASD, but acting on the DNA molecule (Adapted from Huguet and Bourgeron 2016)

"autisms," each with a different etiology ranging from monogenic to polygenic models.

The presence of multiple hits of rare CNVs, SNVs or indels in a single individual also illustrates the complexity of the genetic landscape of ASD (Girirajan et al. 2010, 2012; Leblond et al. 2012). In addition, the analysis of the whole genome sequence of multiplex families also indicates that clinically relevant mutations can be different from one affected sib to another even in a single family (Yuen et al. 2015). It is therefore still difficult to ascertain robust genotype-phenotype relationships based on our current knowledge.

Fortunately, although the ASD-risk genes are numerous, they seem to converge in a limited number of biological pathways that are currently scrutinized by many researchers.

The Biological Pathways Associated with ASD

Unbiased pathway analyses indicated that ASD-risk genes seem to be enriched in groups of proteins with specific functions (Voineagu et al. 2011; De Rubeis et al. 2014; Ronemus et al. 2014; Uddin et al. 2014; Hormozdiari et al. 2015). Pinto et al. (2014) analyzed the burden of CNVs in 2446 individuals with ASD and

2640 controls and found enrichment in genes coding post-synaptic density proteins and FMRP targets. Ronemus et al. (2014) reviewed the results of four whole exome sequencing studies and showed an enrichment of mutated genes in chromatin modifier genes ($P = 4 \times 10^{-6}$) and FMRP targets ($P = 7 \times 10^{-6}$). Protein-protein interactions (PPI) analyses of the genes carrying LGD mutations also showed enrichment in proteins involved in neuronal development and axon guidance, signaling pathways and chromatin and transcription regulation. De Rubeis et al. (2014) also used the PPI network and showed enrichment in clusters of proteins involved in the cell junction TGF beta pathway, cell communication and synaptic transmission, neurodegeneration and transcriptional regulation.

In parallel to the genetic studies, several transcriptomic analyses were performed using post-mortem brain of individuals with ASD (Voineagu et al. 2011; Gupta et al. 2014). Several genes were differentially expressed or correlated between brain regions. Two network modules were identified. The first module was related to interneurons and to genes involved in synaptic function (downregulated in brains from ASD patients compared to controls). The second module was related to immunity and microglia activation (upregulated in brains from ASD patients compared to controls).

Based on these results, neurobiological studies using cellular and animal models have been performed to identify the main mechanisms leading to ASD. Remarkably, several studies showed that neuronal activity seems to regulate the function of many of the ASD-risk genes, leading to the hypothesis that abnormal synaptic plasticity and failure of neuronal/synaptic homeostasis could play a key role in the susceptibility to ASD (Belmonte and Bourgeron 2006; Auerbach et al. 2011; Toro et al. 2010). Here, we will only depict four main biological pathways associated with ASD: chromatin remodeling, protein synthesis, protein degradation, and synaptic function (Fig. 4). In parallel, several biochemical studies have indicated a dysfunction in the serotonin-NAS-melatonin pathway.

Chromatin Remodeling Mutations in genes encoding key regulators of chromatin remodeling and gene transcription (e.g., *MECP2*, *MEF2C*, *HDAC4*, *CHD8* and *CTNNB1*) have been reported in individuals with ASD (Fig. 4). Remarkably, a subset of these genes is regulated by neuronal activity and influences neuronal connectivity and synaptic plasticity (Cohen et al. 2011; Sando et al. 2012; Ebert et al. 2013).

Protein Synthesis The level of synaptic proteins can be influenced by neuronal activity through global and local synaptic mRNA translation (Ma and Blenis 2009). Several genes involved in such activity-driven regulation of synaptic proteins have been found to be mutated in individuals with ASD (Kelleher and Bear 2008). For example, the mTOR pathway controls global mRNA translation and its deregulation causes diseases associated with increased cell proliferation and loss of autophagy, including cancer (Ma and Blenis 2009), but also increases the risk for ASD. Remarkably, mutations in the repressor of the mTOR pathway such as *NF1*, *PTEN* and *SynGAP1* cause an increase of translation in neurons and at the synapse (Auerbach et al. 2011). Mutations of the FMRP–EIF4E–CYFIP1 complex cause the

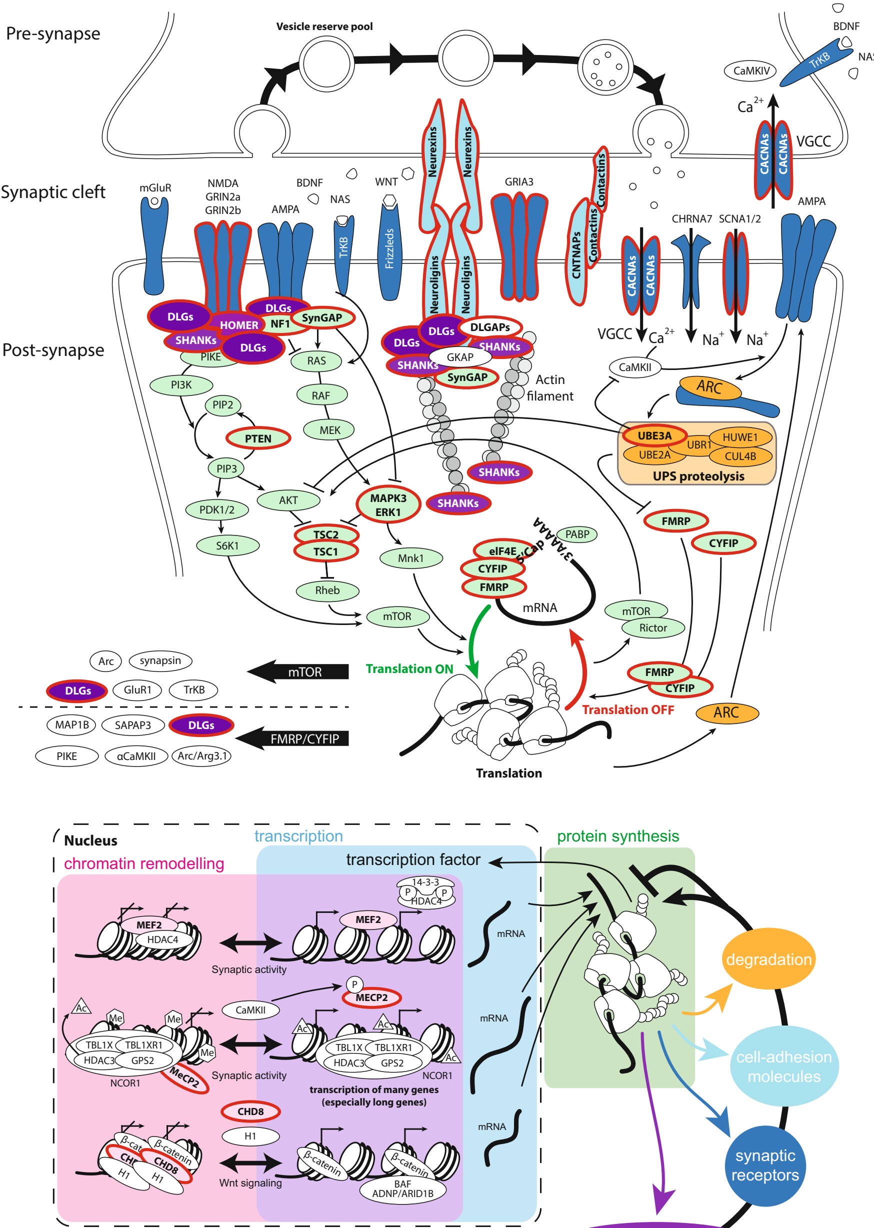

Fig. 4 Examples of the biological pathways associated with ASD. The ASD-risk genes code for proteins involved in chromatin remodeling, transcription, protein synthesis and degradation, cytoskeleton dynamics, and synaptic functions. Proteins associated with ASD are *circled* in *red* (Adapted from Huguet and Bourgeron 2016)

fragile X syndrome and increase the risk of ASD (Budimirovic and Kaufmann 2011). This protein complex controls local translation of mRNA at the synapse and acts downstream of the Ras-ERK signaling pathway. This complex regulates the translation of more than 1000 specific genes, many of which are ASD risk genes (De Rubeis et al. 2013; Fernandez et al. 2013; Gkogkas et al. 2013; Santini et al. 2013). An alteration of this FMRP–EIF4E–CYFIP1 complex should therefore create an imbalance in the level of many synaptic proteins that are associated with ASD.

Protein Degradation The Ubiquitin-Proteasome System (UPS) is central for the degradation of the proteins and, consequently, for the regulation of synapse composition, assembly and elimination (Mabb and Ehlers 2010). The *UBE3A* gene encodes a ubiquitin ligase, is mutated in patients with Angelman syndrome and is duplicated on the maternal chromosome 15q11 in individuals with ASD. Neuronal activity increases *UBE3A* transcription through the MEF2 complex and regulates excitatory synapse development by controlling the degradation of ARC, a synaptic protein that decreases long-term potentiation by promoting the internalization of AMPA receptors (Greer et al. 2010).

Synaptic Functions Many proteins encoded by ASD-risk genes participate in different aspects of neuronal connectivity, such as glutamatergic (e.g., GRIN2B), GABAergic (e.g., GABRA3 and GABRB3) and glycinergic (e.g., GLRA2) neurotransmission, neuritogenesis (e.g., CNTN), the establishment of synaptic identity (e.g., cadherins and protocadherins), neuronal conduction (CNTNAP2) and permeability to ions (CACNA1, CACNA2D3 and SCN1A). Some of these proteins are directly involved in activity-driven synapse formation, such as the neurexins (NRXNs) and neuroligins (NLGNs). Some are scaffolding proteins involved in the positioning of cell-adhesion molecules and neurotransmitter receptors at the synapse (Sheng and Kim 2011; Choquet and Triller 2013). For example, deletions, duplications and coding mutations in the three *SHANK* genes (*SHANK1*, *SHANK2* and *SHANK3*) have been recurrently reported in individuals with ASD (Leblond et al. 2014). SHANK proteins assemble into large molecular platforms in interaction with glutamate receptors and actin-associated proteins (Grabrucker et al. 2011). In vitro, *SHANK3* mutations identified in individuals with ASD reduce actin accumulation in spines affecting the development and morphology of dendrites as well as the axonal growth cone motility (Durand et al. 2012). In mice, mutations in *SHANK3* decrease spine density in the hippocampus but also increase dendritic arborizations in striatal neurons (Peca et al. 2011). Mice mutated in *SHANK* present with behavior resembling autistic features in humans. *Shank1* knockout mice display increased anxiety, decreased vocal communication, decreased locomotion and, remarkably, enhanced working memory, but decreased long-term memory (Hung et al. 2008; Silverman et al. 2011; Wohr et al. 2011). *Shank2* knockout mice present hyperactivity, increased anxiety, repetitive grooming, and abnormalities in vocal and social behaviors (Schmeisser et al. 2012; Won et al. 2012). *Shank3* knockout mice show self-injurious repetitive

grooming, and deficits in social interaction and communication (Bozdagi et al. 2010; Peca et al. 2011; Wang et al. 2011; Yang et al. 2012).

The Serotonin-NAS-Melatonin Pathway

In parallel to the genetic investigations pointing to the biological pathways described above, several biological abnormalities have been reported in individuals with ASD, including neurochemical, immunological, endocrine or metabolic traits (Lam et al. 2006; Rossignol and Frye 2012), which may provide insights into the pathophysiology of ASD. Among these, elevated blood serotonin is one of the most replicated findings (Pagan et al. 2012) (Fig. 5). A deficit in melatonin (which chemically derives from serotonin) in the blood or urine of individuals with ASD has also been described in several studies (Tordjman et al. 2005; Melke et al. 2008) and is associated with increased peripheral *N*-acetylserotonin (NAS), the intermediate metabolite between serotonin and melatonin. Several mutations affecting this pathway were identified but the mechanisms of these biochemical impairments remain mostly unexplained. Melatonin is a neurohormone mainly synthesized in

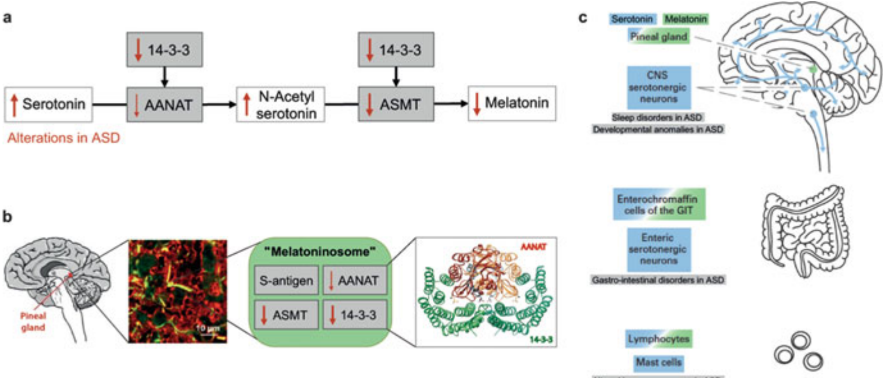

Fig. 5 The serotonin-NAS-melatonin pathway in ASD. (**a**) The serotonin-NAS-melatonin synthesis pathway consists of two enzymatic steps involving first the *N*-acetylation of serotonin to *N*-acetylserotonin (NAS) by the rate-limiting enzyme AANAT and the methylation of NAS by the ASMT (also known as HIOMT). Different alterations such as higher serotonin or low melatonin levels were observed in the blood of patients with ASD. The enzymes are represented in *gray* and metabolites are in *white*. Alterations of the biochemical parameters are shown with *red arrows*. (**b**) A schematic view of the pineal gland with the pinealocytes that contain the "melatoninosome." This complex includes at least four proteins: AANAT, ASMT, 14-3-3 and S-antigen. The immunofluorescence confocal image of AANAT (*green*) and 14-3-3δ/ε (*red*) in the pinealocytes is adapted from Maronde et al. (2011). The structure of the 14-3-3ζ homodimer binding to AANAT is adapted from Obsil et al. (2001). (**c**) Main sources of serotonin (*blue*) and melatonin (*green*) and the symptoms or comorbidities of ASD relevant to alterations in serotonin and melatonin levels observed in ASD (Adapted from Pagan et al. 2012)

the pineal gland during the night. It is a biological signal of light/dark cycles and is considered to be a major circadian synchronizer. It is also a modulator of metabolism, immunity, reproduction and digestive function. Furthermore, it displays antioxidant and neuroprotective properties and can directly modulate neuronal networks (Bourgeron 2007). Melatonin appears as a therapeutic target of the frequently reported sleep disorders associated with ASD (Andersen et al. 2008; Wright et al. 2011; Malow et al. 2012). NAS displays intrinsic biological properties: it is an agonist of the TrkB receptor and may thus share the neurotrophic properties of brain-derived neurotrophic factor (BDNF), the canonical TrkB ligand (Jang et al. 2010; Sompol et al. 2011). Serotonin conversion into melatonin involves two sequential enzymatic steps: *N*-acetylation of serotonin into NAS by arylalkylamine *N*-acetyltranferase (AANAT, EC: 2.3.1.87) followed by methylation by acetylserotonin *O*-methyltransferase (ASMT, also called hydroxyindole O-methyltransferase HIOMT, EC: 2.1.1.4) (Fig. 5a). We previously showed that deleterious mutations of the *ASMT* gene could disrupt melatonin synthesis in a subset of patients with ASD. Nevertheless, the frequency of such a deleterious mutation is too low (2 % of the cases) to explain the relatively high frequency of melatonin deficit in ASD (>50 % of the patients, taking as a threshold the fifth percentile of the controls). More recently, we observed a low level of the 14-3-3 proteins both in the blood platelet and pineal gland of patients with ASD (Pagan et al. 2014). These ubiquitous chaperone proteins are known to form a protein complex, the 'melatoninosome,' involving AANAT and ASMT in pinealocytes (Obsil et al. 2001; Maronde et al. 2011). This interaction between 14-3-3 and AANAT and/or ASMT might be essential for the production of melatonin and an adequate balance of the serotonin-NAS-melatonin pathway. Indeed, a low level of 14-3-3 could eventually lead to a deficit in enzyme activity, contributing to the global disruption of the serotonin-NAS-melatonin pathway observed in ASD. Studies investigating the regulation of the complex 14-3-3/ASMT/AANAT in ASD and controls are in progress.

Perspectives

In the last 30 years, very significant progress has been made in the genetics of ASD. We now have a better knowledge on the genetic architecture of this heterogeneous syndrome and some of the biological pathways have been investigated using different approaches such as cellular and animal models. There are, however, many aspects of the genetics of ASD that remain largely unknown.

The first challenge concerns the role of the common variants. These variants are most likely playing a key role in the susceptibility to ASD and in the severity of the symptoms. But, because the impact of each single SNP is very low, it is currently impossible to identify the risk alleles using conventional GWAS. In human quantitative traits such as height, neuroanatomical diversity or intellectual quotient, very

large cohorts of many thousands of individuals are necessary to identify the main causative SNPs (Toro et al. 2014; Yang et al. 2010; Deary et al. 2012).

The second challenge concerns the stratification of the patients and the role of the ASD-risk genes during brain development/function. Based on our current knowledge, the genetic architecture of ASD seems to be different from one individual to another, with possibly contrasting impact on when and where neuronal connectivity could be atypical compared to the general population. For example, in animal models, several mutations lead to higher connectivity whereas other mutations alter synaptic density. It is therefore crucial to increase our knowledge from a basic research perspective about the biological roles of the ASD-risk genes and their partners.

Finally, while we all agree that biological research is necessary to improve the quality of life of the patients and their families (for example, to alleviate the comorbidities associated with ASD like sleep and gastrointestinal problems), progress should also be made toward better recognition and inclusion of people with neuropsychiatric conditions in our societies (no mind left behind). Hopefully, increasing knowledge in genetics, neurology and psychology should allow for better diagnosis, care for and integration of individuals with autism.

Acknowledgments We thank Roberto Toro for critical reading of the manuscript. This work was funded by the Institut Pasteur, the Bettencourt-Schueller foundation, Centre National de la Recherche Scientifique, University Paris Diderot, Agence Nationale de la Recherche (SynDiv-ASD), the Conny-Maeva Charitable Foundation, the Cognacq Jay Foundation, the Orange Foundation, and the Fondamental Foundation.

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P. Sassone-Corsi, Y. Christen (eds.), *A Time for Metabolism and Hormones*,
Research and Perspectives in Endocrine Interactions,
DOI 10.1007/978-3-319-27069-2

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