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REVIEW

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Effects of BMAL1 Manipulation on the Brain's Master Circadian Clock and Behavior

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Bmal1 is the only single circadian clock gene that is essential for rhythmic gene expression in the mammalian circadian timing system. Genetic approaches targeting *Bmal1* expression have been used to further assess its role in the circadian clock and to test for behavioral effects of clock disruption. In particular, disruptions in circadian clock function have been implicated in human mood disorders, and clock gene manipulation in mice may provide valuable models for studying depression-like behavior. In this review, we explore various approaches to manipulating *Bmal1* in mouse models and review their effects on the brain's master circadian pacemaker, on circadian rhythmicity in other brain regions, and on circadian and mood-related behavior.

INTRODUCTION

The suprachiasmatic nucleus (SCN†) of the hypothalamus is the master circadian pacemaker in mammals, driving ca. 24 hr oscillations in physiology and behavior and synchronizing clocks in peripheral tissues [1]. In SCN neurons and other cells, circadian rhythmicity is dependent on delayed negative feedback loops comprised of transcription factors and the clock genes they target. In cells, brain and muscle ARNT-like protein 1 (BMAL1) heterodimerizes with circadian locomotor output cycles kaput (CLOCK) and binds to DNA in promoter regions to activate the transcription of target gene families *Period (Per)* and *Cryptochrome (Cry)* [2]. As PER and CRY proteins build up in the cytoplasm over the day, they heterodimerize and form complexes with other proteins to enter the nucleus, where they bind to CLOCK:BMAL1 and repress their own transcription in a negative feedback loop. By dawn, this negative feedback is removed as PER and CRY degrade, and the circadian cycle repeats as CLOCK:BMAL1-mediated activation resumes. The CLOCK:BMAL1 complex is a pioneer-like transcription

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†Abbreviations: SCN, suprachiasmatic nucleus; BMAL1, brain and muscle ARNT-like protein 1; CLOCK, circadian locomotor output cycles kaput; *Per, Period; Cry, Cryptochrome;* ROR, RAR-related orphan nuclear receptor; BMAL1-KO, BMAL1 knockout; CRISPR, clustered regularly interspersed short palindromic repeats; SCNT, somatic cell nuclear transfer; WT, wild-type; *Bmal1*-cKO, conventional knock-out; *Bmal1*-iKO, inducible knock-out; FEO, food-entrainable oscillator; tTA, tetracycline transactivator; HA, hemagglutinin; BMAL1-HA, tagged BMAL1; *Acta1, alpha actin-1; Syt10, Synaptotagmin10;* BKO, forebrain/SCN-specific *Bmal1* knockout; DMH, dorsomedial hypothalamus; N*Bmal1*-KO, *Nestin-Cre+;Bmal1f/f*; AVP, arginine vasopressin; *Glast, glutamate aspartate transporter;* BMALcKO, *BMAL*$1^{flx/flx}$, *Glast*-CreER^{T2} +/−; Bmal1 GTΔC, C-terminal truncated *Bmal1* mutant mice; AAV, adeno-associated virus; SCN-BMAL1-KD, SCN-specific BMAL1-knockdown; NAc, nucleus accumbens.

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factor in this cycle, as rhythmic CLOCK and *Bmal1* binding promotes subsequent rhythmic chromatin opening [3].

In a parallel feedback loop, REV-ERBα contributes to the precision and stability of the clock by interacting with RAR-related orphan nuclear receptor (ROR) elements to auto-regulate *Bmal1* expression and influence downstream pathways [1,4]. In this loop (often referred to as the *Bmal1* loop), *Bmal1* expression is enhanced by ROR and repressed by REV-ERBα through ROR response elements [5]. Due to the strong repression of *Bmal1* with REV-ERBα accumulation, *Bmal1* transcription follows a high-amplitude circadian cycle [6]. Expression of both positive and negative regulatory elements is enhanced by CLOCK:BMAL1, resulting in an antiphasic phase relationship between the rhythms of BMAL1 and PER. Recent *in vivo* observation of *Per1* and *Bmal1* expression in freely moving mice carrying a bioluminescent reporter (*Per1-luc* or *Bmal1-luc*), as well as in cultured SCN slices from those mice, reveals that the BMAL1 loop has its own independent oscillatory nature [7].

Interactions of circadian clock genes in the brain suggest that clock components including *Bmal1* can influence behaviors including locomotion, cognition, and mood. For instance, expression of the gene *monoamine oxidase A*, important for metabolizing dopamine, is rhythmic and dependent on clock genes, including *Bmal1* [8,9]. Also, with chronic unpredictable mild stress in mice, diurnal rhythms of *Bmal1* are delayed, which suggests a role of altered *Bmal1* rhythms in stress-induced mood dysfunction [10].

The crucial role of *Bmal1* as the only non-redundant gene in the core circadian clock has made it the focus of many studies investigating the effects of *Bmal1* manipulation on SCN tissue and neurons, on rhythmicity in other brain regions, and on behavior in mice models. We aim to highlight recent research that furthers our understanding of such manipulations.

FULL-BODY BMAL1 KNOCKOUT

A typical approach to investigating the circadian clock is to delete clock genes and investigate the resulting phenotypes, as depicted in Figure 1A. Whereas single gene knockout of most clock genes has revealed a compensatory mechanism for generating attenuated circadian rhythms [11], *Bmal1* knockout (*Bmal1*-KO) is the only single gene deletion that fully eliminates circadian clock function in the SCN and in peripheral tissues [12,13]. As a result, *Bmal1* deficient mice demonstrate arrhythmic circadian behavior and expression of clock target genes. *Bmal1*-KO mice also have reduced lifespans and display an early aging phenotype, a finding consistent with the increased levels of reactive oxygen species observed in some tissues of the *Bmal1*-KO mice [14]. Moreover, these mice display a host of other health issues, including decreased overall activity and decreased body weight [12,15,16].

Recent advances in gene editing technology have allowed for the generation of clonal macaque monkeys with full body knockout of *Bmal1*. Liu *et al*. accomplished this using clustered regularly interspersed short palindromic repeats (CRISPR)/Cas9 gene editing in combination with somatic cell nuclear transfer (SCNT) to transfer the nuclei of cultured *Bmal1*-KO fibroblasts into enucleated zygotes [17]. In the five monkeys that survived, knockout of *Bmal1* was confirmed both by DNA sequencing and by Western blot in several regions, including brain, kidneys, and liver. These monkeys were then used in a subsequent study in which they served as a model for psychiatric disorders involving disruption of circadian rhythms [18]. Actogram data showed clear circadian disruption, as the *Bmal1*-KO monkeys were active during the night while wild-type (WT) control monkeys were not. The *Bmal1*-KO monkeys also showed complete or near-complete loss of rhythmicity in several important endocrine signals such as melatonin, testosterone, and cortisol. In a standard activity tracking experiment, *Bmal1*-KO monkeys were nearly stationary for the entire 20-minute period while WT monkeys traveled roughly three times further on average. A low level of exploration and movement during the experiment was considered indicative of high stress. While promising, this research highlights a key potential issue of full-body knockouts for behavioral research. Considering the musculoskeletal degeneration and arthropathy found in *Bmal1*-KO mice by Bunger *et al*., it can be questioned to what degree these peripheral effects of *Bmal1*-KO may account for the inactivity assumed to reflect stress in the *Bmal1*-KO monkeys [15].

In contrast to these studies using a conventional knock-out approach (*Bmal1*-cKO), Yang *et al*. (2016) studied mice with an *inducible* postnatal global deletion of *Bmal1* (*Bmal1*-iKO) [19]. *Bmal1*-iKO mice express *Bmal1* normally during embryogenesis, but not after birth. While in both approaches mice lose clock function in central and peripheral tissues, there are substantial phenotypic differences, as many of the pathologies observed in *Bmal1*-cKO mice are not seen with *Bmal1*-iKO. These mice do not have reduced life-spans, body weights, or fertility, demonstrating that these phenotypes may be due to developmental functions of BMAL1 that are not related to clock function. While locomotor activity was non-rhythmic in both cKOs and iKOs, a progressive reduction in overall locomotor activity was seen only in cKOs. These differences highlight the considerable advantage of the inducible knockout approach in providing a more selective way to manipulate the clock in adult animals without the confound of developmental effects

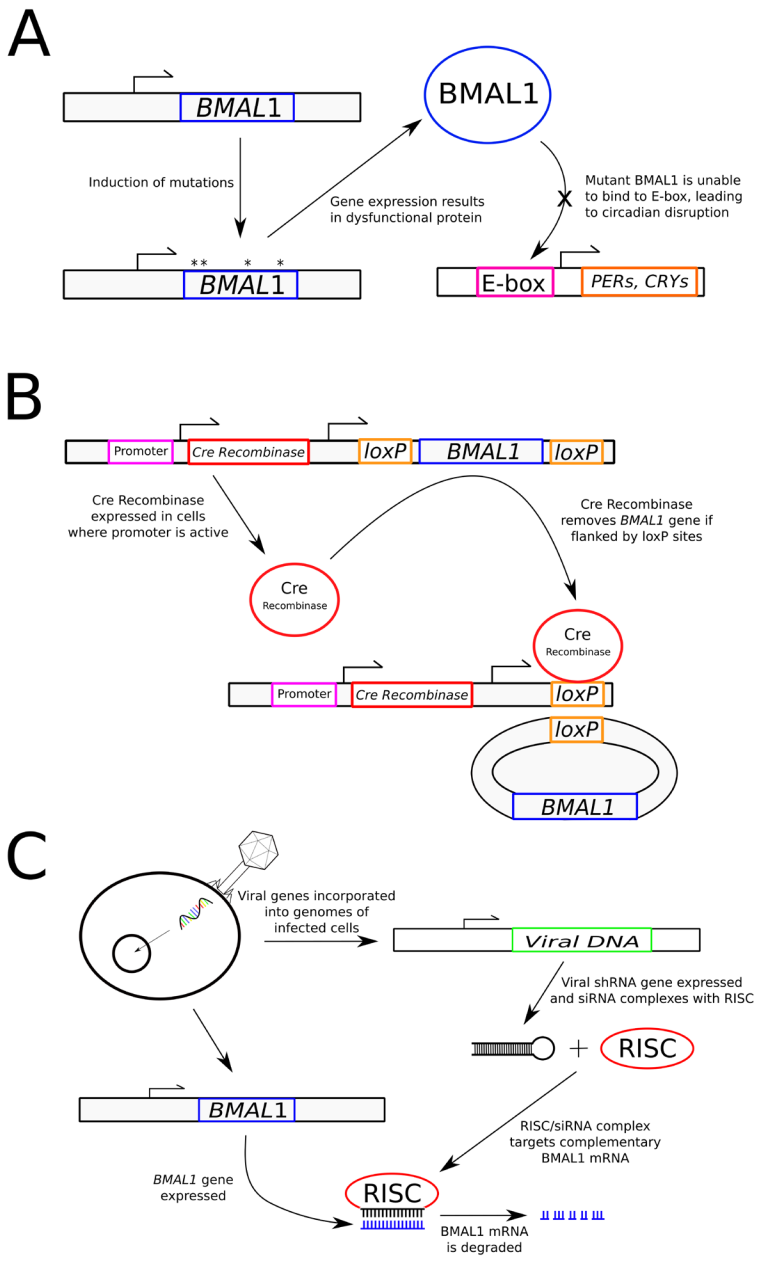

Figure 1. Summary of different manipulations used to modulate *Bmal1* expression in order to study the physiological/behavioral roles of circadian rhythms. (**A**) Full body knockouts of *Bmal1* are generated by a variety of gene editing techniques that induce loss of function mutations in the BMAL1 protein. Targeted mutations such as C-terminus deletion can be employed to study the structure and function of the BMAL1 protein in more detail. However, due to various roles of *Bmal1* throughout the body and in development, these mouse models suffer from many deficits that are not necessarily related to circadian clock function. (**B**) Cre-based knockouts avoid some of these problems by conferring region, tissue, or cell-type specificity of the *Bmal1* knockout by using a specific promoter linked to the *Cre recombinase* gene. (**C**) Adeno-associated virus (AAV) vectors allow for local knockdown of *Bmal1* in fully developed, wild type mice. Once the AAV vector is injected and its DNA is incorporated into the genomes of target cells, shRNA specific to *Bmal1* is expressed. The shRNA is processed into siRNA, which complexes with RISC and targets *Bmal1* mRNA for degradation.

that may not be related to circadian clock function.

TISSUE-SPECIFIC RESCUE OF BMAL1 IN FULL-BODY KNOCKOUT

Given the wide range of phenotypic characteristics resulting from *Bmal1* knockout, there is strong motivation to investigate the role of the protein in a more tissue-specific manner. One such approach is to use the full-body *Bmal1* knockout mouse, but to rescue expression of *Bmal1* in specific tissues. McDearmon *et al*. (2006) produced transgenic mice that express *Bmal1* in the brain (brain-rescued mice) or in the muscle (muscle-rescued mice) [20]. For brain-rescue, they used the tetracycline transactivator (tTA) system for the target gene *Bmal1* and the *Scg2* promoter to drive expression in the brain. tTA drives expression of hemagglutinin (HA)-tagged *Bmal1* (*Bmal1*-HA) cDNA, while doxycycline inhibits it. They then observed wheel-running patterns of brain-rescued *Bmal1*−/− mice and found that they exhibited a consistent circadian rhythm of behavior, but impaired locomotor activity. The researchers also noted that their free-running period was one hour shorter than that of WT mice, which was likely due to the lack of peripheral feedback to the SCN. Secondly, they produced transgenic mice expressing *Bmal1* selectively in muscle using the human *alpha actin-1 (Acta1)* promoter and observed no restoration of circadian rhythmic behavior, but much stronger locomotor activity. This tissue-specific rescue method led to the conclusion that *Bmal1* in the muscle is important for longevity, locomotor activity level, and body weight, but *Bmal1* in the brain is indispensable for circadian activity rhythms. Using the same transgenic model, a recent study by Ehlen *et al*. found that even though sleep timing is dependent on BMAL1 expression, most sleep amount phenotypes can be rescued by restoring BMAL1 selectively in skeletal muscle [21]. This surprising finding highlights the advantage of such tissue-specific approaches in understanding the relationship between central and peripheral clock mechanisms that depend on BMAL1.

TISSUE-SPECIFIC BMAL1 KNOCKOUT

Confirmation that *Bmal1* expression in the brain is important for circadian rhythmic behavior leads to questions about which brain regions are most important for various circadian functions. Several studies have explored the effects of tissue-specific knockout of *Bmal1* through Cre-lox recombination, where transgenic mice can be generated to express a Cre recombinase transgene under the control of promoters that are specific to the tissue of interest. This method, depicted in Figure 1B, allows for studies where *Bmal1* is knocked out in specific tissues, brain regions, or cell types.

The first target using this approach was naturally the SCN. One approach to targeting BMAL1 in the SCN was demonstrated by Husse *et al*. (2011), who generated a mouse expressing Cre recombinase driven by the promoter of *Synaptotagmin10 (Syt10),* a gene that is highly expressed within the SCN. Crossing this mouse to another mouse line in which the *Bmal1* gene is "floxed," or flanked by loxP sites, this group was able to delete *Bmal1* in the SCN but not in peripheral tissues, leading to behavioral arrhythmicity [22]. These mice also lacked circadian rhythms of gene expression in the SCN in either a light/dark cycle or constant darkness, but peripheral clocks maintained circadian rhythmicity and even reset to a shift of the light/dark cycle more quickly [23]. The investigators theorized that this may be due to phase advanced corticosterone from the adrenal clock, which could be tested by deleting *Bmal1* from both the SCN and adrenal glands.

To study the effect of SCN *Bmal1* expression on peripheral clocks, Izumo and colleagues (2014) reduced BMAL1 by > 90 percent using floxed *Bmal1* and pan-neuronal Cre lines to generate forebrain/SCN-specific *Bmal1* knockout (BKO) mice [24]. As a result of this deletion, BKO mice demonstrate a total loss of circadian rhythmic behavior in constant conditions, but do not have shortened life-spans or other serious health defects as observed in global knockout mice. The SCNs of BKOs have suppressed but observable rhythms (likely due to glial cells and residual unfloxed neurons still expressing *Bmal1*), and rhythms from the dorsomedial hypothalamus (DMH) were attenuated as well, but circadian rhythmicity was retained in peripheral tissues. BKO mice still displayed food entrainment behavior, and food restriction allowed synchronization of peripheral clocks, indicating that external cues can compensate for the loss of the SCN as a synchronizer of peripheral clocks in BKOs. Interestingly, BKO mice demonstrated significantly more locomotor activity than controls in constant conditions, indicating that the reduced activity previously observed in *Bmal1* global knock-outs was due to *Bmal1* deficiency in peripheral tissues rather than in forebrain or SCN.

To investigate the role of *Bmal1* in circadian clocks within the brain, but outside the SCN, Mieda and colleagues (2017) generated Nkx2.1-Bmal1−/− mice in which Cre recombinase was limited such that *Bmal1* was deleted specifically in the ventral forebrain, including much of the hypothalamus but excluding the SCN [25]. As expected, PER2::LUC oscillations in the mediobasal hypothalamus were attenuated while those in the SCN were sustained. While the mutant mice were still rhythmic behaviorally, Nkx2.1-Bmal1−/− mice differed from controls in that they were less active during the first half of subjective night and more active in the second half, the inverse of the activity pattern for controls. This difference

had implications for sleep-wake cycles and for feeding patterns and indicated that *Bmal1*-dependent clocks in the ventral forebrain are important for the precise timing of circadian behavioral patterns. Using a similar method, Snider and colleagues deleted *Bmal1* from forebrain circuits, but left expression in the SCN intact, in order to observe the role of forebrain *Bmal1* in learning, memory, behavioral despair, and anxiety. While these mice had impaired ability in certain cognitive tasks, their performance in other, more affective measures such as the elevated plus maze, open field assay, and tail suspension test was not affected [26]. This indicates that *Bmal1* in the forebrain is important for cognition and memory, but not for mood-related behaviors.

Tissue-specific *Bmal1* knockout through the Cre-lox system has allowed researchers to investigate the role of *Bmal1* in many more specific physiological functions. For example, to investigate the role of *Bmal1* specifically in astrocytes, Lananna *et al*. compared the brains of *Nestin-Cre+;Bmal1f/f* (N*Bmal1*-KO) mice (in which *Bmal1* is deleted in both neurons and astrocytes), with neuron-specific *Bmal1* knockout (KO) mice (Camk2a-iCre;*Bmal1*f/f) [27]. Mereness *et al*. conditionally deleted *Bmal1* specifically in ovarian theca cells and observed that, although ovulation was disrupted, behavioral rhythms were not affected [28]. Another study used a nervous system specific deletion and found that the SCN-independent food-entrainable oscillator (FEO) in the nervous system requires *Bmal1* in order to adapt circadian locomotor activity to timed feeding [29].

In tissue-specific knockout of *Bmal1*, understanding the role of *Bmal1* in the target tissue can be furthered by comparing phenotypes with the global knockout. Musiek *et al*. (2013) studied brain pathology in global knockouts at 4 to 6 months (before peripheral pathologies become severe) and compared it to that of *Nestin-Cre+:Bmal1f/f* mice, in which *Bmal1* is deleted in most neurons, astrocytes, and oligodendrocytes, with residual *Bmal1* expression in microglia [30]. These mice had intact behavioral circadian rhythms and rhythmic sleep-wake, but showed the same severe age-dependent astrogliosis observed in global *Bmal1*-KO mice. They then investigated behavioral abnormalities and found novelty-induced hyperactivity in the *NestinCre+;Bmal1f/f* mice, but found that these mice had less impaired habituation to novelty than global KOs. They were thus able to conclude that the brain phenotype observed due to *Bmal1*-KO is due to local loss of BMAL1 function within neurons and glia, and not due to peripheral pathologies, changes in the sleep-wake cycle, or loss of peripheral circadian rhythms.

CELL-SPECIFIC DOWNREGULATION

As arginine vasopressin (AVP) neurons are predominantly found in the dorsal SCN, they are an interesting target for *Bmal1* manipulation within the context of the circadian network, and specifically to investigate whether they have a substantial role in entraining the whole SCN. These neurons are GABAergic and often contain co-localized neuropeptides. Mieda *et al*. (2015) crossed mice expressing Cre recombinase under the control of the AVP promoter (AVP-Cre mice) with mice harboring floxed *Bmal1* alleles, generating AVP-*Bmal1*−/− mice, in which *Bmal1* was deleted selectively in AVP neurons [31]. These mice had lengthened free-running periods and increased duration of circadian locomotor activity time. They also showed reduced photoperiodic responses in the long-day condition and temporary arrhythmicity under constant conditions. Finally, AVP-*Bmal1*−/− mice had reduced SCN *Per1* mRNA expression after a light pulse, indicating that *Bmal1* in AVP neurons may be important for the SCN response to light.

A similar cell-type-specific deletion experiment was conducted in astrocytes by Barca-Mayo and colleagues (2017) [32]. They generated a mouse model where Cre-recombinase is expressed under the control of an astrocyte-specific promoter for the gene *glutamate aspartate transporter (Glast)*, allowing *Bmal1* knockout specifically in astrocytes. These *Bmal1*$^{flx/flx}$, *Glast*-CreER^{T2} +/− (*Bmal1*cKO) mice exhibited no loss of behavioral rhythms. They had entrained locomotor activity rhythms very similar to controls, with around the same level of activity, and the same length of free-running period in constant conditions. However, in constant conditions, *Bmal1*cKO mice had a delay in the timing of activity onset, and they took longer to entrain to a new light-dark cycle after constant conditions. They also displayed cognitive deficits. Upon further investigation, the team found that GABA signaling was an important mechanism at play, as modulation of GABA-A-receptor signaling rescued the behavioral phenotypes of *Bmal1*cKO mice. These results indicate that astrocytes have an important role in coordinating neuronal circadian clocks, and that this requires *Bmal1*.

BMAL1 KNOCKDOWN

Knockdown of *Bmal1* levels is another approach to studying the role of *Bmal1* without completely eliminating circadian function. Landgraf and colleagues, for example, observed the effects of SCN-specific *Bmal1*-knockdown (SCN-*Bmal1*-KD) on circadian rhythms and depression-like behavior in mice. As in Figure 1C, adeno-associated virus (AAV) vectors encoding shRNAs were designed to target and downregulate *Bmal1* expression, achieving a > 60 percent reduction of *Bmal1* protein levels in the SCN [33]. In SCN slices from these mice, PER2::LUC rhythms had amplitudes

decreased by ~80 percent and lengthened periods when compared to slices from control mice, indicative of a substantially attenuated circadian clock. In tests of mood-related behavior, these mice exhibited more depression and anxiety-like characteristics, taking longer to escape in the learned helplessness paradigm, displaying more immobility in the tail suspension test, and spending less time in the lighted section of a light/dark box. In addition, these mice had greater weight gain and decreased corticosterone release in response to stress. This knockdown model is advantageous for investigating the effects of *Bmal1* in mood-regulating brain areas. By maintaining normal brain development and anatomy and restricting clock effects to the SCN in SCN-*Bmal1*-KD mice, the group was able to offer a new animal model for depression that more closely resembles mood dysregulation in humans.

BMAL1 C-TERMINAL TRUNCATION

A possible approach to understanding the function of *Bmal1* at a detailed molecular level is to mutate *Bmal1*. A circadian mutant mouse was studied by Park and colleagues, who developed C-terminal truncated *Bmal1* mutant mice (*Bmal1* GTΔC) by injecting ES cells harboring the truncated gene into blastocysts and investigated the effects on circadian rhythms in the resulting transgenic mice [34]. Examining wheel-running patterns showed that homozygous mutant mice were immediately arrhythmic. Unlike homozygous mutant mice, heterozygous mice showed gradual loss of rhythmicity under constant conditions, which could indicate a semi-dominant negative allele. These mice had a decreased period length but no reduction in amplitude of behavioral rhythms. The phenotypes of these mice support previous suggestions that the C-terminus of *Bmal1* is important for maintaining the balance between circadian transcriptional activation and suppression [35]. Specifically, the functional switch between activation and suppression of CLOCK:BMAL1 is thought to be established by CRY1 competing with coactivators to bind to the C-terminal transactivation domain of BMAL1 [36].

TARGETING THE *BMAL1* LOOP BY MANIPULATING REV-ERBα

An alternative to direct knockdown of *Bmal1* is to manipulate REV-ERBα to indirectly down-regulate *Bmal1*, and this can be done in a tissue-specific and drug-dependent fashion. To isolate the influence of the SCN as opposed to local regulators such as temperature in the synchronization of the hepatic clock, Kornmann *et al*. engineered a mouse in which REV-ERBα was constitutively expressed in liver cells unless doxycycline was administered orally. As REV-ERBα is a potent inhibitor of *Bmal1* expression, this allowed the researchers to control the expression of *Bmal1* in the liver through administration of doxycycline [5]. This was confirmed by Western Blot analysis, which showed only trace levels of BMAL1 from hepatic tissue extract in the absence of doxycycline, and much stronger expression of *Bmal1* in its presence [37]. This type of model has not been used in the SCN, but it has been applied to the nucleus accumbens (NAc), a brain region strongly implicated in regulation of mood. In the NAc, knockdown of REV-ERBα using shRNA was shown to reduce anxiety-like behavior in female mice and to upregulate the expression of the circadian proteins PER1 and PER2 [38]. This illuminates a potential disadvantage of this indirect approach to regulating *Bmal1*, as it is unclear whether the effects of REV-ERBα in the liver or in the NAc were due to changes in *Bmal1* itself or a *Bmal1*-independent pathway controlled by REV-ERBα. However, like *Bmal1* knockdown, this approach to manipulating *Bmal1* does avoid the health and developmental issues of a total knockout of *Bmal1*, as *Bmal1* can be both spatially and temporally manipulated through REV-ERBα.

CONCLUSIONS AND OUTLOOK

The crucial role of *Bmal1* in circadian behavior has made it a point of great interest in circadian research, but the absolute dependence of rhythmicity on the presence of *Bmal1* also makes *Bmal1* mechanisms challenging to study. By increasing the specificity of methods for *Bmal1* manipulation, researchers have made progress in understanding the role of *Bmal1* in particular brain regions, peripheral tissues, and cell types. *Bmal1* manipulation in mouse models also helps to create a framework for *Bmal1* research in human cells. For example, an siRNA screen of circadian clock modifiers in human cells revealed low amplitude and arrhythmic clock gene expression patterns as a result of *Bmal1* knockdown, a phenotype consistent with those observed in animal models [39]. Manipulations of *Bmal1* have also illuminated the role of clock gene expression in circadian clock function and rhythmicity in the brain, and in turn, on mood regulation and depression-like behavior. Mood disorders such as bipolar disorder and major depressive disorder are associated with disrupted cellular circadian clocks, but the SCN is not known to be directly involved in mood regulation [40]. Manipulating *Bmal1* and studying downstream effects in other brain regions will shed light on circadian clocks in brain regions that play a more direct role in mood regulation, as well as in other cognitive functions.

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