Title
Short Circuiting the Circadian System with a New Generation of Precision Tools

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genetic exaggeration to recapitulate the key features of AD.

Human clinical trials may provide a laboratory to test theories about the etiology of AD. Two large-scale prevention trials are currently underway to test the effects of anti-amyloid immunotherapy in people with FAD. One trial will enroll subjects with either APP or PS mutations, while the second trial will focus on a large Columbian kindred with a mutation in PS1. If the trials succeed, they will provide strong support for the Amyloid Cascade Hypothesis. However, if they fail, what can one conclude? Pharmacokinetic considerations aside, the most likely explanations are that: (1) the target (i.e., Aβ) was correct, but that the timing of intervention and/or the antibody were wrong, or (2) Aβ was the wrong target. If the trials fail to produce the expected results, the findings in Xia et al. (2015) may provide an early clue as to why.

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Short Circuiting the Circadian System with a New Generation of Precision Tools

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Circadian behavior in mammals is coordinated by neurons within the suprachiasmatic nucleus (SCN). In this issue, Lee et al. (2015) and Mieda et al. (2015) applied state-of-the-art genetic tools to dissect the microcircuits within the SCN generating circadian rhythmic behavior.

One of the fundamental goals of neuroscience is to link specific brain regions to specific functions. While in many cases this goal has proven elusive, an overwhelming body of evidence shows that the suprachiasmatic nuclei (SCN) of the anterior hypothalamus are the site of the master circadian pacemaker in mammals. The SCN functions to synchronize a network of circadian oscillations throughout the body; the resulting circadian rhythms have a profound impact on our health and wellbeing. In addition to the identification of the SCN as a key region regulating circadian activity, at the cellular level, we currently have a relatively firm understanding of the transcriptional/translational feedback loops that are responsible for generation of these molecular oscillations. However, major gaps remain in understanding circadian regulation at the intermediate level of analysis, including the roles of specific cell-types within the SCN. Two exciting back-to-back studies in this issue have applied state-of-the-art genetics tools to analyze the SCN and make headway in understanding its circuitry and its role in circadian rhythmic behavior (Lee et al., 2015; Mieda et al., 2015).

Some of the challenges in studying the function of the SCN and its subpopulations lie in its structure. Anatomical studies generally support the division of the SCN into at least two subdivisions including a dorsal (shell) region and a ventral (core) region (Figure 1; top). At the cellular/synaptic however, the SCN can be likened to a tightly packed ball, composed of GABAergic neurons whose synaptic connections form more of a plexus rather than an ordered structure like the hippocampus, cortex, or cerebellum. Furthermore, an influential study using fully isolated SCN neurons found

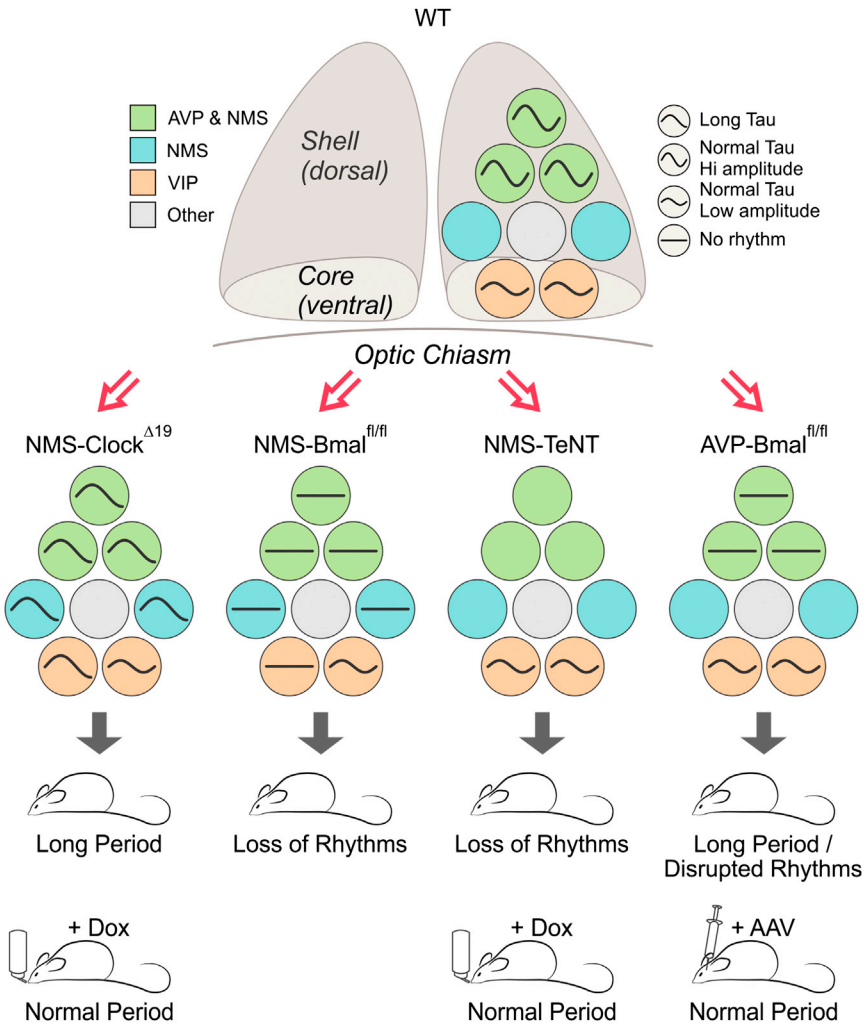

Figure 1. Schematic Illustrating the Key Findings

In this highly stylized representation, AVP-expressing neurons are localized in the shell region of the SCN while NMS-expressing neurons are localized throughout the nucleus. NMS (blue) appear to be co-expressed with the majority of AVP (green) and VIP (orange) expressing neurons. Disrupting the molecular clock in NMS or AVP neurons produced abnormal circadian rhythms. Lengthening the intracellular circadian period of NMS neurons lengthens behavioral circadian period. Blocking synaptic transmission from NMS neurons similarly leads to the loss of coherent circadian rhythms. These phenotypes were reversible with appropriate genetic manipulations.

no evidence for a specialized or anatomically localized class of cell-autonomous oscillators (Webb et al., 2009). Instead, Webb et al. proposed that different SCN cell types have intrinsic circadian oscillation mechanisms, but that these oscillators are unstable and rely on network interactions for stability. Without a clear wiring diagram, it is hard to even pick your target for cellular analysis. In addition, the SCN network is extremely resilient in the face of perturbations. At a genetic level, the clock genes can compensate for the loss of one gene by increased expression of another. As an example, *Clock* knockout (KO) mice that lack the transcription factor *CLOCK* hardly display any circadian phenotype, likely because of compensation by another transcription factor, *NPAS2* (DeBruyne et al., 2007). To provide another example, the SCN network will continue to generate circadian oscillations at a population level even when most of the cell-autonomous oscillations are compromised due to the loss of the *Cry* gene (Liu et al., 2007). Like those old Timex watches, the SCN can "take a licking and keep on ticking." The potential biological benefit of this redundancy in the circadian mechanisms is clear, but scientifically it is challenging to tease apart the components of this circuit. To delineate the circadian circuits, one needs a new combination of approaches ranging from computational analysis to new genetic tools. The difficulties outlined above in the analysis of this dynamic systems are by no means limited to the specific microcircuits of the SCN, and it seems likely that the lessons learned in this "simpler" brain region can be applied elsewhere in more complicated circuits.

To date, the heterogeneity of the neurons that make up the SCN has made it difficult to specifically ablate or modify gene expression function in the SCN. Prior work (Husse et al., 2011) came closest to SCN-specific genetic manipulation with the use of a *Synaptotagmin10 (Syt10)* driver, which showed enriched expression in the SCN, but with possible off-target effects in other brain regions. The ablation of a gene critical for circadian rhythms, *Bmal1,* in *Syt10*+ cells resulted in a greatly shortened free-running period in locomotor activity, but interpretation of the work was marred by incomplete Cre recombinase excision at the $Bmal1^{flx/flx}$ locus. In addition to using a more effective Cre recombinase, the present studies (Lee et al., 2015; Mieda et al., 2015) make use of genetic manipulations to address the relative importance of an intact circadian oscillator in SCN neuronal sub-types as defined by neuropeptidergic content. A caveat to bear in mind is that the genetic drivers used in both studies also show some expression outside of the SCN. In fact, Mieda and colleagues (2015) acknowledge and deal with this issue by specifically restoring gene expression using focal injection of a rescue viral construct in the SCN. Both studies combine their specific genetic drivers with *Bmal1* ablation, the only available mutation that fully disrupts the molecular clock. The study by Lee and colleagues (2015) goes even further in their use of genetic tools in the SCN, specifically altering the speed of the molecular oscillator using the $Clock^{\Delta 19}$ period-lengthening mutation; holding one component of the circadian oscillator at a permanent high using *Period2*

overexpression; and, notably, blocking synaptic transmission with a modified tetanus light chain molecule (TeNT) (Figure 1; middle/bottom, columns 1–3). The tetanus-like molecule targets and cleaves synaptobrevin, preventing vesicular docking/fusion and hence synaptic transmission, which is critical for synchronization of SCN neurons. They also elegantly employ the doxycycline-induced *tet-On* system to reverse the resulting phenotypes, thus demonstrating that the effects of the genetic mutations are functional and not a result of aberrant development of the SCN circuitry.

Mieda and colleagues (2015) provide new insights into the role of vasopressin (AVP)-producing neurons of the SCN. These neurons appear to generate the most robust circadian oscillations, at least at the level of gene expression. The neurons in the SCN shell express AVP as well as GABA. Although the peptide AVP defines one of the SCN cell populations, these neurons received so far less attention partly because the AVP-deficient Brattleboro rats display little abnormality in circadian pacemaking other than a reduced amplitude in their behavioral rhythms (Kalsbeek et al., 2010). More recent work, however, has implicated AVP in the coupling of SCN neurons (Li et al., 2009; Yamaguchi et al., 2013), and together the data is consistent with the hypothesis that cellular oscillators in AVP neurons are required for high-amplitude circadian output of the SCN. Mieda et al. (2015) tested this hypothesis by ablating *Bmal1* from *Avp* producing neurons (AVP+) (Figure 1; middle/bottom, right column). Behavioral analysis indicated that these mice exhibited a longer free-running period of locomotor activity rhythms. As expected with a reduced amplitude of the circadian oscillator, the mutant mice showed a faster re-entrainment to changes in the light/dark cycle. Somewhat unexpectedly for a mouse with a disrupted circadian clock in the dorsal SCN, the mice also showed a reduced phase shift induced by light and reduced clock gene induction by light in ventral SCN regions. While most photic information flows from the ventral to the dorsal SCN, this finding illustrates that the communication is bi-directional. Importantly, the expression of crucial SCN output genes *Avp*, *Prok2,* and *Rgs16* were drastically reduced in the dorsal SCN in the mutants. Finally, PERIOD2 bioluminescence rhythms in the SCN had unstable and lengthened periods. At the behavioral levels, *Avp-Bmal1*$^{fl/fl}$ showed abnormal circadian rhythms. Critically, the abnormal circadian rhythms in locomotor activity were rescued by injections of an adeno-associated virus expressing *Bmal1* into the SCN of adult *Avp-Bmal1*$^{fl/fl}$ mice, which emphasizes the anatomical specificity of the mutation's impact. Future studies will need to parse the impact of disrupting the clock alone or intercellular communication alone in a defined set of cells. As noted by Mieda et al., the phenotype of the *Avp-Bmal1*$^{fl/fl}$ mutants cannot be solely due to the loss of AVP expression, as mice without AVP receptors still express fairly normal rhythms. The study by Lee and colleagues (2015) shows one possible strategy for parsing the impact of disruption of cell-autonomous clocks versus disruption of cellular communication.

While AVP is a well-studied anatomical marker within the SCN, very little is known about neuromedin S (NMS). This peptide has highly enriched expression within the SCN and is the ligand for neuromedin U receptors (Mori et al., 2005). Due to its SCN-enriched expression, NMS represents an attractive genetic target for exploiting and understanding the organization of the central circadian clock. In their study, Lee and colleagues (2015) demonstrate that a subpopulation of SCN neurons expressing NMS play a critical role in regulating circadian behavior. NMS+ neurons make up approximately 40% of SCN neurons, and NMS is co-expressed in AVP+ and VIP+ neurons (Figure 1). When the period-lengthening *Clock*$^{\Delta 19}$ mutation was expressed in NMS neurons, the behavioral rhythm and PERIOD2 expression in the SCN exhibited a longer circadian period characteristic of this mutation in a reversible manner (Figure 1). In addition, knocking out of *Bmal1* in these neurons caused disruption of the behavioral rhythm and of PERIOD2 expression in the SCN. Overexpression of PERIOD2 selectively in NMS neurons also disrupted behavioral rhythmicity and SCN synchronization. Finally, inhibition of synaptic transmission from NMS neurons resulted in arrhythmic behavioral rhythms and desynchronized PERIOD2 expression in the SCN. This final clincher of an experiment demonstrates the power of the new genetic tools in mammals, previously only available to *Drosophila* geneticists. The necessity of synaptic transmission in the SCN for behavioral rhythmicity could previously only be tested in ex vivo or in vitro systems, which hence lacked the critical link of brain-to-behavior effects. In fact, from our perspective, this study goes a long way in bringing the investigation of the circadian system in mammals closer to the sophisticated analysis that could previously only be carried out in the fly.

Importantly, it is neither the neuropeptide NMS itself nor its relative NMU that are functionally essential, as double knockouts of the neuropeptides remain rhythmic (Lee et al., 2015). This raises some intriguing questions: Does NMS truly label a critical population of pacemaker neurons? Of note, NMS-Bmal1 knockout mice continue to show rhythmicity for 12 days on average under constant dark conditions. Could it be that the main effect of the NMS genetic manipulation is in fact due to the NMS driver also mutating AVP neurons, which mostly overlap with NMS expression? Or could there be some type of mass action effect, where regardless of the specific neuronal sub-type being manipulated, altering 40% of SCN neurons, as done in both studies discussed here, disrupts just enough of this synchronized plexus of neurons to impair the circadian circuit? The VIP-Bmal1 knockout mouse showed what looked like a normal circadian rhythm, but the VIP cell population is sparser than the AVP or NMS expressing neurons. A prior study floxing out Bmal1 in up to 65% of the SCN using the pan-neuronal *Syt10* driver showed minimal effects on behavioral rhythms (Husse et al., 2011). The larger *Syt10*-driven ablation, along with older studies of partial SCN lesions and SCN transplantation rescue studies, suggests that even a small percentage of intact SCN neurons is sufficient to drive circadian behavior, and argues against the possibility that mass action underlies the effects observed using the NMS driver. It is also worth pointing out that previous work has demonstrated that micro-lesions of a specific region in the center of the hamster SCN leads to behavioral and neuroendocrine

arrhythmicity (Kriegsfeld et al., 2004). So it is not as though all neurons in the SCN have an equal, functional weight.

We believe these are early days in a new era of understanding the microcircuits that comprise the circadian pacemaker in the SCN. Future work will need to firmly link the molecular oscillator with neuropeptide secretion and the neural membrane events that form the backbone of SCN pacemaking. The next generation of "circuit breaking" tools such as optogenetics (Fan et al., 2015; Jones et al., 2015) and DREADDs (Brancaccio et al., 2013), along with more sophisticated computational analysis (e.g., Evans et al. [2013]), are starting to be applied to better understand the SCN circuit that times our daily lives.

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