Chapter 2
Cells and Circuits of the Suprachiasmatic Nucleus and the Control of Circadian Behaviour and Sleep

A. P. Patton, M. H. Hastings, and N. J. Smyllie

2.1 Introduction: Mammalian Circadian System Overview

Circadian clocks are self-sustaining biological timing mechanisms with an intrinsic period of approximately one day (hence, *circadian*). They are widely distributed across all forms of life (Edgar et al. 2012) because they confer adaptive value by facilitating the anticipation of, and thus preparation for, the alternating challenges and opportunities presented by daily and seasonal environmental cycles. Consequently, their influence impinges on virtually all aspects of metabolism, physiology and behaviour. In humans, the daily cycle of sleep and wakefulness is the most obvious output of the circadian system, but it is accompanied by equally dramatic cycles of autonomic function and endocrine status that maintain internal temporal coherence. In modern societies, this temporal coherence is compromised by factors such as rotational shift work, exposure to irregular lighting environments and increasingly prevalent age-related diseases, most notably neurodegenerative conditions. The principal organiser of our circadian life is the suprachiasmatic nucleus (SCN) of the hypothalamus, a cluster of ca. 20,000 cells sitting immediately above the optic chiasm, on either side of the midline third ventricle (Hastings et al. 2018) (Fig. 2.1a). It receives direct photic input via the retinal hypothalamic tract (RHT), which consists of the axonal projections of retinal ganglion cells (RGC). Many of these RGCs express melanopsin and are thus intrinsically photoreceptive (LeGates et al. 2014). Under normal circumstances, this input allows the SCN to align its internal representation of circadian time to external solar time. Importantly, however, the SCN will continue to maintain and generate a coherent representation of circadian time in the absence of environmental input, for example, when an animal is placed into constant darkness and even when the SCN is removed from a mouse and cultured as

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an explant. The discovery of circadian clock genes (see below) brought a totally unexpected level of circadian organisation into view. It is now clear that all major organ systems and many cell types, not least fibroblasts, have intrinsic circadian clocks (Reppert and Weaver 2002). This means that the role of the SCN is not as a driver of rhythms in a passive periphery, but rather that of a synchroniser of these innumerable, distributed local clocks (Fig. 2.1b). This dynamic interaction with the periphery highlights even further the power and sophistication of the SCN as a central timekeeper, and underscores the potential fragility of the overall system to genetic, physiological and environmental insults. The purpose of this review is, first, to consider the **molecular-genetic and cellular basis of circadian timekeeping** in mammals. Second, we discuss the **circadian properties of SCN neurons** followed by examination of the **circuit architecture of the SCN as a cellular network**. Fourth, we review the **role of astrocytes in the SCN**, before considering **SCN output pathways and their control over behaviour**, including the cycle of sleep and wakefulness. Finally, we note potential **future directions**.

2.2 Molecular-Genetic and Cellular Basis of Circadian Timekeeping in Mammals

2.2.1 The Core Feedback Loop—Genes and Molecules: Discoveries Through Mapping and Mutagenesis Screens

The possession of a cell-autonomous circadian clock is not unique to mammals. In the genetically tractable *Drosophila* and *Neurospora*, forward mutagenesis screens uncovered the first genetic components of eukaryotic circadian clocks: *period* (*per*) and *frequency* (*frq*), for each organism, respectively (Dunlap 1999). These were soon followed by the discoveries of *timeless* (*tim*), *clock* (*clk*) and *cycle* (*cyc*) in *Drosophila*. In these lower organisms, a common organisational feature emerged: transcriptional-translational feedback loops (TTFLs) were central to circadian timekeeping. Here, positive regulators transactivate transcription of negative regulator genes, whose protein products then, in turn, inhibit their own transcription. Importantly, these self-sustaining oscillations are entrained by light. In *Drosophila,* this is mediated by cryptochrome (CRY) proteins (Emery et al. 1998; Yuan et al. 2007), which are related to photolyase DNA repair enzymes, and in *Neurospora* by photosensitive white-collar complexes (WC-1, 2) (Froehlich et al. 2002). Although the *Tau* mutation, discovered in the Syrian hamster, was the first identified circadian clock mutant in mammals (Ralph and Menaker 1988), the turning point for assembling the mammalian molecular clock mechanism came with the discovery of the *CLOCK* gene, through a forward mutagenesis screen in mice (King and Takahashi 2000). A mutant allele that lengthened circadian period was positionally mapped (a heroic

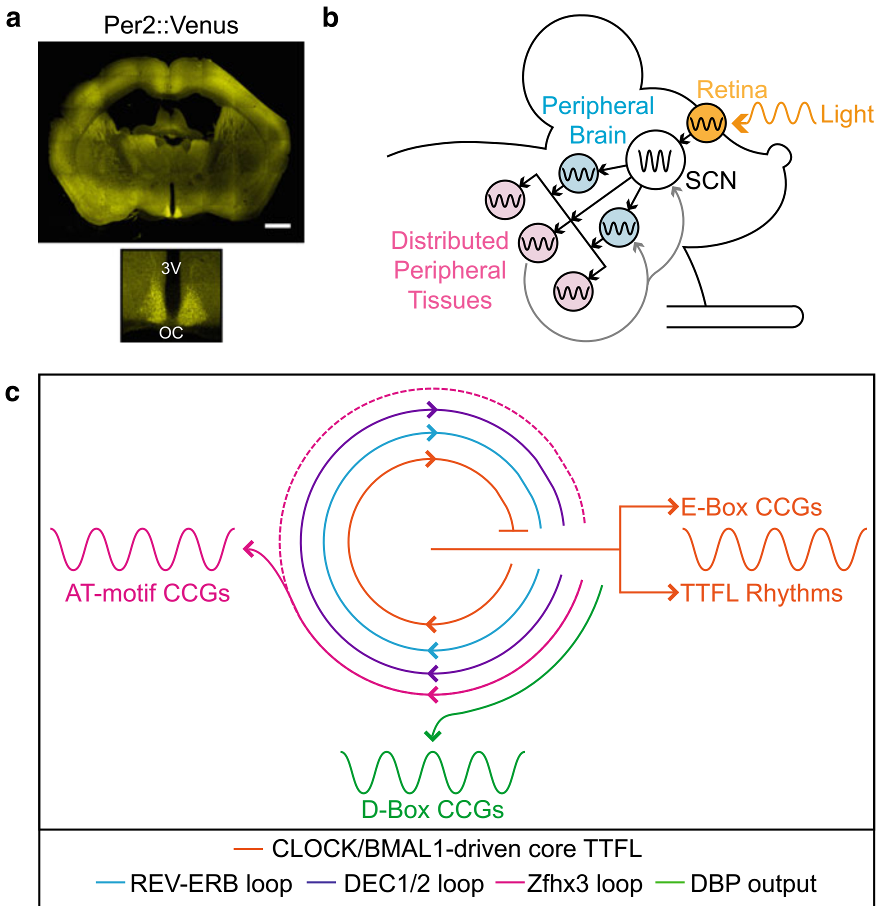

Fig. 2.1 The SCN and re-entrant motifs in the core clock and additional TTFLs. **a** Coronal section of mouse brain revealing location of the SCN by high expression level of endogenous PER2::Venus fluorescent protein. Scale 1 mm, inset higher power view to show nuclear localisation of PER2. 3 V: third ventricle, OC: optic chiasm. **b** Schematic view of the role of the SCN as the dominant, light-entrained circadian clock in mammals, orchestrating peripheral clocks in brain regions and major organs. **c** Schematic view of the inter-nested TTFL loops of the cell-autonomous clock. The core BMAL1:CLOCK-driven loop (centre) additionally drives rhythmic output from E-boxes in clock-controlled genes (CCGs) (orange). Robustness is conferred to this loop by the interwoven REV-ERB-driven (blue), DEC1/2-driven (purple) and ZFHX3-driven (magenta) additional loops. In addition to modulating the core clock TTFL, ZFHX3 also controls rhythmic transcriptional outputs via AT-motifs in CCGs. Finally, DBP-driven targets (green) are important for regulation of rhythmic outputs regulated by D-box regulatory elements in CCGs, but do not impinge on core clock function

undertaking in the pre-genome era) and then subsequently a series of rescue experiments showed that the mutation was mapped to exon 19 of the gene. The resulting exonal deletion (*CLOCK*^{delta19}) compromised the transcriptional activity of CLOCK protein as a core clock component. This was complemented by the discovery of mammalian homologues to *Drosophila per* and *cry* as negative transcriptional regulators (Tei et al. 1997; van der Horst et al. 1999). Interestingly, the mammalian *TIMELESS* gene is not closely related to the *Drosophila timeless* gene and is not a component of the mammalian clock (Gotter et al. 2000).

With the discovery of MOP3 (also known as BMAL1) (Bunger et al. 2000), the final piece was in place identifying the core factors in the mammalian TTFL clock: CLOCK and BMAL1 are basic helix-loop-helix (bHLH) transcriptional activators, which heterodimerise via PAS domains and activate the transcription of negative regulator genes *PERIOD* (*PER1, 2*) and *CRYPTOCHROME* (*CRY1, 2*). The production of PER and CRY proteins increases through the circadian day, during which they heterodimerise and translocate into the nucleus to inhibit their own transcription. PAS domains in PER likely facilitate interaction with CLOCK:BMAL1. The PER and CRY proteins are degraded through the subjective night, until the feedback inhibition is finally alleviated, after which the cycle can begin again. The dynamics of transcription, translation and protein stability provide a regulated delay within this feedback loop, which consequently takes approximately 24 h to complete (Fig. 2.1c). Importantly, there is redundancy inherent to the system. First, there are two PER and two CRY proteins, which have overlapping functionality and provide resilience to the negative limb of the clockwork, and second, NPAS2, a paralog to CLOCK, can also heterodimerise with BMAL1. This redundancy is clearly demonstrated by the fact that animals only become arrhythmic in their behaviour when both Clock and NPAS2 are deleted (DeBruyne et al. 2007). Interestingly, BMAL1 is the only component of the mammalian molecular clock that is irreplaceable. Global or SCN-restricted loss of the *Bmal1* gene alone results in arrhythmia in mice. The simple TTFL motif is that of a re-entrant loop whereby output becomes input and thus, by incorporating a delay, it establishes a self-sustained oscillation. This re-entrant loop motif lies at the heart of clock function at multiple levels (Fig. 2.1c). Finally, to be effective, the oscillatory TTFL must control cellular functions and the most direct way is for the periodic activation and repression of E-boxes across the genome to create circadian waves of transcription of "clock-controlled genes" (CCGs) (Akhtar et al. 2002; Koike et al. 2012). Surprisingly, across all tissues examined, the circadian clock controls the expression of 43% of genes (Zhang et al. 2014) and so any loss of circadian competence will inevitably disrupt physiology and thereby aggravate ageing and compromise longevity (Lowrey and Takahashi 2004).

2.2.2 Additional Feedback Loops Support the TTFL

The discovery of the TTFL provided a range of new approaches, most notably real-time recording of circadian gene expression using bioluminescent and fluorescent reporters, to understand both cell-autonomous and tissue-based clock functions. Remarkably, not only the SCN but also peripheral tissues exhibits cell-autonomous TTFL cycles (Stokkan et al. 2001; Yoo et al. 2004). These discoveries revealed a completely unanticipated level to the sophistication of circadian co-ordination across the organism. This systemic complexity was complemented by the discovery at a molecular level of a series of additional feedback loops that stabilise and amplify the core TTFL. First, the transcription of nuclear receptors *ROR* α, *REV-ERB* α and *REV-ERB* β is activated by CLOCK:BMAL1, and these factors in turn act back on the TTFL by regulating the transcription of *BMAL1* itself, which carries ROR response elements (ROREs) (Preitner et al. 2002) (Fig. 2.1c). The *staggerer* mutation of *Ror* α results in the reduction of BMAL1 expression in mice and shortened circadian period (Akashi and Takumi 2005), whilst loss of both *Rev-Erb* α *and Rev-Erb* β is accompanied by arrhythmia (Cho et al. 2012). These factors are also important in sculpting circadian output because the circadian cycle of activation to ROREs across the genome drives further waves of CCG transcription, particularly metabolically relevant genes, thereby complementing control by E-boxes. An additional loop incorporates the circadian E-box and light-driven DEC1 and DEC2 bHLH transcriptional regulators. DEC1 in particular can repress CLOCK:BMAL1-mediated activation at E-boxes, including those of *PER1* (Honma et al. 2002), again closing a re-entrant loop around E-boxes. A third regulatory output of the TTFL pivots around the basic leucine zipper transcription factors DBP, TEF and HLF, which are expressed in a highly circadian manner in many tissues, including the SCN, where they further co-ordinate daily cycles of CCG expression, (Gachon et al. 2006), although their influence on the TTFL is minimal.

Forward mutagenesis screening in mice uncovered an additional loop centred on the transcription factor ZFHX3 which acts via "AT-box" regulatory elements to activate gene expression (Parsons et al. 2015). Mice carrying the dominant *Shortcircuit* autosomal mutation of *Zfhx3* ($Zfhx3^{Sci}$) have a significantly shorter circadian period (~ 0.6 h for each mutant allele), which accompanies a reduced transactivational potency of the protein at AT-boxes, whilst deletion of ZFHX3 in adult mice shortened behavioural period by ~ 1 h and in ~ 30% of mice caused arrhythmia (Wilcox et al. 2017). Not only does ZFHX3 therefore feed into the TTFL, but the AT-box axis is also under circadian regulation and so constitutes a downstream output of the TTFL (Parsons et al. 2015): again, a re-entrant loop motif (Fig. 2.1c). The contributions of cell-autonomous and network-level actions of ZFHX3 remain unclear, although the transactivational compromise in the mutant is reflected in lowered levels of expression of SCN neuropeptides (see below), several of which carry AT-boxes regulatory elements. The shortened period may therefore arise in part from changes in the interneuronal neuropeptidergic signalling network. More significantly, ZFHX3 regulates the expression of numerous genes in the SCN and so, alongside E-boxes,

and ROREs, it will be able to play a significant part in sculpting the circadian transcriptome that underpins the output signalling by the SCN. Finally, ZFHX3 plays a developmental role because SCN specification fails in mice carrying a conditional null allele from embryonic stages and so are behaviourally arrhythmic, even though circadian competence is retained outside the SCN (Wilcox et al. 2021). This argues further that the "re-entrant" actions of ZFHX3 are expressed at the level of the SCN circuit, whereas those of RORs and DEC are cell-autonomous (Fig. 2.1). In all cases, however, the net effect of these additional loops is to enhance robustness and amplitude of the core TTFL and to broadcast its timing cues via downstream transcriptional cascades.

2.2.3 Control of the Stability of Clock Proteins and Effect on Behaviour

It is implicit in the structure of the TTFL that changes to the rate of expression and/or stability of the mRNAs and proteins within it will alter its dynamics and therefore the period of overt measurable rhythms. Indeed, this relationship was foundational to the success of the forward genetic screens that identified clock genes, not least the *CLOCK*^{delta19} mutation that revealed CLOCK as a positive regulator in the TTFL of mammals (King and Takahashi 2000). The analysis of period mutants in rodents has revealed the post-translational modifications that determine the activity and stability of PER and CRY proteins. The *Tau* mutation provided the first evidence for the single-allele (as opposed to multigenic) control of circadian period in mammals, as well as identifying the SCN as the source of this control (Ralph et al. 1990). This mutation was later mapped to the *Casein Kinase 1 Epsilon* (*Ck1 ε*) gene (Lowrey et al. 2000) and re-engineered in mice to generate a comparable phenotype (Meng et al. 2008). Both in hamsters and mice, the mutant allele accelerates wheel-running rhythms by 2 h per copy. Biochemical studies in tissues and primary cells from *Tau* mutant mice indicated that this was a gain-of-function (GOF) mutation in CK1 ε that destabilised PER proteins, thereby accelerating their clearance and, therefore, the speed of the clock (Meng et al. 2008). Consistent with the gain-of-function mutation, genetic deletion of CK1 ε does not have a pronounced circadian phenotype: rather, CK1 δ is the principal endogenous regulator of PER stability and circadian period under normal conditions (Etchegaray et al. 2010), a conclusion confirmed by the contrasting effects of selective pharmacological inhibition of either CK1 δ or CK1 ε (Meng et al. 2010). It is now clear, however, that circadian period is tuned by the balance between kinase and phosphatase activity on PER proteins (Lee et al. 2011). Indeed, multiple CK1-dependent phosphorylation sites on PER can competitively stabilise or destabilise the protein (Philpott et al. 2020) by providing or denying access to the degron sequences that target it for ubiquitinylation by the ubiquitin ligase beta-TRCP and thus proteasomal degradation (D'Alessandro et al. 2017). Consequently, the *Early doors* mutation of PER2, which compromises packing of the PAS domain

and thus provides greater access to the degron sequences, destabilises the protein and shortens circadian period. In combination with the CK1ε Tau allele, it can accelerate SCN and behavioural rhythms to extremely short periods of below 19 h (Militi et al. 2016). Remarkably, these ultra-fast clocks remain stable and precise.

The period of the SCN and thus behavioural rhythms is also influenced by the activity of CRY proteins. Loss of CRY1 shortens period whilst loss of CRY2 lengthens it, and loss of both causes arrhythmia (van der Horst et al. 1999). Mutagenesis screens (Godinho et al. 2007; Siepka et al. 2007) revealed that stability of both proteins is regulated by the E3-ubiquitin ligase, FBXL3. Loss-of-function mutations (*Afterhours* and *Overtime*) of *Fbxl3* correspondingly increase the stability of CRY proteins and thereby lengthen SCN and behavioural periods in wild-type, *Cry1*-null and *Cry2*-null mice (Anand et al. 2013). Consequently, when combined with CRY2 deficiency, the *Afterhours* mutation lengthens SCN and behavioural periods to over 29 h (Anand et al. 2013). A second ubiquitin ligase, FBXL21, counterbalances the action of FBXL3: it is localised predominantly in the cytoplasm (in contrast to the nuclear FBXL3), stabilises CRY proteins (in contrast to the destabilising actions of FBXL3), and loss of FBXL21 attenuates the period lengthening caused by the absence of FBXL3 (Hirano et al. 2013). AMP kinase (AMPK) is an important upstream regulator of the ubiquitinylation of CRY proteins (Lamia et al. 2009), with loss of different catalytic isoforms shortening or lengthening TTFL period (Um et al. 2011). Given that AMPK is nutrient-responsive and the metabolic state of the cell is circadian, this provides an additional example of a cell-autonomous re-entrant loop that stabilises and tunes the TTFL. Finally, ubiquitinylation-dependent stability of REV-ERB proteins allows this additional loop to tune the levels of BMAL1 and therefore modulate the core TTFL (Stojkovic et al. 2014).

These discoveries in rodent models provide a satisfying confirmation of the elegant dynamics of the TTFL. They also offer insight into potential therapy and management of circadian disturbances, because the very same mechanisms direct the human TTFL and their compromise is at the heart of familial sleep disturbances. For example, mutations in human CK1 δ or at the kinase target sites of PER2 are associated with advanced sleep phase disorders (Chong et al. 2018). Equally, mutation of the human CRY1 that enhances its transcriptional repression leads to familial delayed sleep phase disorder (Patke et al. 2017). Agents that modify the molecular interactions within the TTFL therefore hold promise for therapeutic intervention. For example, small molecules that target the interaction between CRY and FBXL3 can lengthen period in cell assays (Hirota and Kay 2015), whilst RNAi screening in human cells has identified many potential circadian-regulatory targets (Zhang et al. 2009). Chronotherapy directed at the TTFL or at its innumerable outputs remains in its infancy, but its promise for therapeutic benefit is now evident (Zhang et al. 2014).

2.3 Circadian Properties of SCN Neurons

2.3.1 Observing Clock Proteins at the Cellular Level

The molecular clockwork does not operate in isolation—it oscillates within its cellular setting. Each component of the TTFL and auxiliary loops has its own distinct set of intracellular dynamics necessary for progression of the SCN clockwork. This is in part imposed by the compartmentalisation of the cell, where translocation from cytoplasm to nucleus is essential for PER and CRY proteins to inhibit their own transcription. Early over-expression studies in cell lines and immunostaining in mouse tissues made a link between PERs and CRYs and the regulation of their own nuclear translocation. More recently, RNAi screening in human cells revealed genes associated with nucleocytoplasmic translocation, including both canonical (beta importin-mediated) and novel (Transportin 1 (TNPO1)) pathways, knockdowns of which variously lengthened or shortened circadian period in line with altered protein localisation (Korge et al. 2018). Nevertheless, over-expression systems and "snap-shot" type single-timepoint imaging approaches carry limitations in terms of deriving quantitative, physiologically relevant measures. The recent creation of knock-in cell lines (Gabriel et al. 2021; Koch et al. 2022) and mice (Smyllie et al. 2016b; Yang et al. 2020) expressing fluorescently tagged versions of endogenous clock proteins has begun to give mechanistic insight and "put numbers" on to these intracellular dynamics. Real-time imaging of PER2::Venus revealed that although its abundance oscillates, when present, it was present in the nucleus throughout the circadian day and this nuclear retention is dependent on CRY proteins (Smyllie et al. 2022). Importantly, the mere presence of the CRY proteins is not sufficient: they themselves must also be able translocate into the nucleus for PER2 to also keep its nuclear localisation. This may be because CRY proteins reduce the mobility of PER2 molecules in nucleus and cytoplasm by a factor of ~ 2. Equally, PER2 slows down the mobility of CRY1.

Fluorescence correlation spectroscopy (FCS) measurements in fibroblasts revealed that at the peak times of expression, between 3000 and 10,000 molecules of either PER2, BMAL1 or CRY1 are present in the nucleus. This relatively low molecular abundance may be an important feature of the TTFL because it sustains large-amplitude changes in TTFL phase in response to small changes in abundance. Importantly, the dynamics of endogenous PER2, BMAL1 and CRY1 proteins are very different: PER2::Venus has the highest rhythm amplitude of ~ tenfold over a relatively low baseline of fluorescence, whereas BMAL1 has a very low-amplitude rhythm of ~ 5%, with a very high baseline. Perhaps related to this, there is a negative correlation between rhythm amplitude and the half-lives of these proteins, whereas endogenous PER2 has a half-life of a few hours, BMAL1, which had the lowest amplitude oscillation, was found to be surprisingly stable and was not fully cleared after 3 days of cycloheximide treatment. Between PER2 and BMAL1, endogenous CRY1 oscillates with a high (~fivefold) amplitude but even at its nadir, there are sufficient molecules to effect nuclear translocation of PER2 (Smyllie et al. 2022). Surprisingly, in terms of molecular mobility, there is no evidence of circadian control

of these proteins: where measured in wild-type cells, the mobility of PER2 and BMAL1 did not vary between peak and trough of the cycle. Phases of expression do, however, vary markedly. Real-time imaging in SCN slices confirmed that endogenous PER2 peaks at CT12, but surprisingly, CRY1 peaks 6 h later at CT18 (Smyllie et al. 2022; Koch et al. 2022), followed by BMAL1 a few hours after that, at CT20 (Yang et al. 2020). This late expression peak of BMAL1 positions it temporally "poised" to be ready to transactivate E-box regulated *Per* and *Cry* genes to start a new cycle (Koike et al. 2012). The precise composition, and presumably the activity, of E-box-bound complexes containing CLOCK, BMAL1, CRY and PER proteins will therefore evolve through circadian time as the TTFL progresses. The temporal segregation of negative and positive regulators will enhance robustness and will also direct distinct, phase-appropriate waves of differential gene expression.

Finally, quantitative imaging in cells and associated modelling has indicated dual modes of action of PER2:CRY1 complexes. First, they conventionally repress transactivation via displacement of CLOCK:BMAL1 from target sites, but this in turn facilitates mobility of CLOCK:BMAL1 and binding to new target sites (Koch et al. 2022). Thus, PER2 acts both as part of a transcriptional repressor complex and as a facilitator of CLOCK:BMAL1 mobility to explore the genome. Given that CLOCK:BMAL1 will epigenetically mark its sites, such brief "visits" by relatively few molecules may nevertheless be sufficient to maintain genome-wide circadian co-ordination of the many CCGs (Koike et al. 2012).

2.3.2 SCN Neural Activity and Transcriptional Cycles

Beyond the TTFL, multi-channel and multi-modal recordings have enabled temporal "phase-mapping" to define the cell-autonomous programme of SCN neurons, mapping to the TTFL electrical activity and cytosolic signalling (Brancaccio et al. 2017). All cellular activities can be aligned to the well-characterised peak of PER2 expression, at circadian time 12 (CT12). Briefly, this programme begins in the subjective morning, when intracellular calcium levels $[Ca^{2+}]_i$ monitored with GCaMP fluorescent reporter, peak at CT7 (Brancaccio et al. 2013; Noguchi et al. 2017). This coincides with a circadian peak in levels of cAMP (O'Neill et al. 2008) and precedes gene transcription driven through cAMP/calcium response elements (CREs), monitored by lentiviral bioluminescent reporter, which peaks shortly afterwards at CT9 (Brancaccio et al. 2013). As *Per1* and *Per2* themselves carry CREs (Travnickova-Bendova et al. 2002), this provides a further example of rhythmic circadian outputs, here calcium and cAMP levels becoming inputs to the TTFL. Indeed, *Per1* and *Per2* expression peaks soon afterwards, followed by E-box-driven but CRE-independent CRY expression (Maywood et al. 2013). The outcome is a pronounced circadian cycle of metabolism and electrical activity in the SCN, with depolarisation and peak firing in the middle of circadian day, leading to the peak in $[Ca^{2+}]_i$. Electrical activity declines in circadian night as the neurons hyperpolarise and their metabolic redox state changes, as evidenced by increased super-oxidation of peroxiredoxin, a

highly conserved maker of circadian redox state (Edgar et al. 2012). These intracellular changes are accompanied by oscillations within the SCN extracellular milieu, including paracrine glutamate ($[Glu]_e$) and synaptic γ-aminobutyric acid (GABA) ($[GABA]_e$). Surprisingly, both of these neurochemical signals peak in circadian night, when neurons are electrically silent (see below). At a cell-autonomous level, a circadian output, in this case neural electrical activity, is again a re-entrant input to the TTFL. Consequently, compromise of the electrical state of the neurons, for example by pharmacological blockade of the firing of action potentials, in turns weakens the TTFL (Colwell 2011).

2.3.3 The Importance of Coupling Between SCN Neurons

The importance of electrical activity for stable progression of the TTFL is also evident at the level of the SCN circuit. Each SCN consists of ~ 10,000 neurons and ~ 3000 glial cells, the latter being principally astrocytes, but with single-cell RNA sequencing (scRNA-seq) revealing populations of ependymocytes, radial glia, oligodendrocytes and microglia (Wen et al. 2020; Morris et al. 2021). Conventionally, the SCN has been sub-divided into the retinorecipient "core", which is the termination zone of the RHT, and the surrounding "shell". Core and shell also have distinct patterns of efferent output and afferent input, as well as distinct neuropeptide expression (Abrahamson and Moore 2001). Vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP) are expressed in the core whilst arginine vasopressin (AVP) is in the shell and Prokineticin 2 (PROK2) and Neuromedin-S (NMS) straddle both domains (Lee et al. 2015; Cheng et al. 2002; Masumoto et al. 2006). The localised expression of their various cognate receptors sustains both intra- and inter-sub-divisional signalling (Wen et al. 2020; Morris et al. 2021). The importance of such intra-SCN communication is evident in several ways. First, both in vivo and in the SCN slice ex vivo, the expression of *Per* and *Cry* genes traces a spatiotemporal wave across the nucleus, starting in the dorsomedial lip of the shell, and progressing ventrally to the core and then dorsally and laterally before activity is curtailed, to be reinitiated on the next cycle (Hastings et al. 2018) (Fig. 2.2a). This spatiotemporal wave is additionally reflected in a wave of neuronal activity (represented by $[Ca^{2+}]_i$) that passes across the nucleus with the same trajectory as, but phase advanced to, gene expression. The spatiotemporal wave is therefore a clear demonstration of the flow of information through the circuit, and indicative of regional functionality. Its role is less clear, although one possibility is to segregate temporally distinct efferent signals in different neuroanatomical pathways, i.e. early and late timing cues to regulate appropriate output.

The second demonstration of the importance of coupling comes from the observation that in dispersed cultures, although SCN neurons can remain individually rhythmic in terms of gene expression and neural activity, these oscillations are less robust, less frequent and are poorly defined compared to those of intact SCN

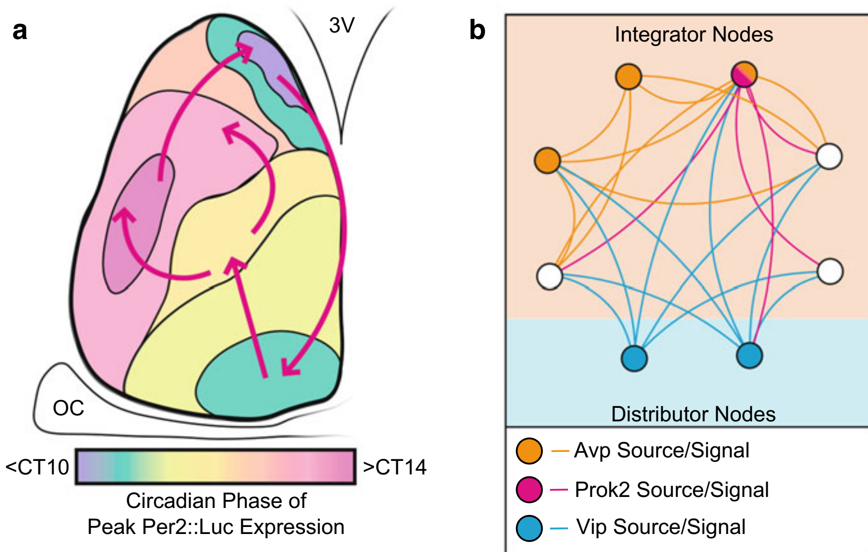

Fig. 2.2 Spatiotemporal dynamics of gene expression across the SCN indicate regional functionality and form a network-level re-entrant motif. **a** Schematic showing the spatiotemporal progression of peak PER2 gene expression passing across the coronal surface of the SCN indicated by differently phase-mapped regions. Arrows indicate the directional progression of the wave emanating from the dorsomedial lip of the SCN, adjacent to the 3rd ventricle and progressing sequentially: ventrally into the retinorecipient core of the SCN, dorsolaterally into the shell of the SCN, and finally into the dorsomedial region for the cycle to begin anew. **b** Neuropeptidergic topology of the SCN determined from scRNA-seq data (redrawn from Morris et al. 2021) and organised to represent the flow of information from and across distributor and integrator nodes in circadian day. Coloured circles indicate neuropeptide source nodes (cell populations) and coloured lines indicate inferred connectivity via that neuropeptide for VIP (blue), PROK2 (magenta), AVP (purple) and undetermined (white). Note that the PROK2 node is also a source node for AVP. This network is transcriptionally dismantled at night

slices (Noguchi et al. 2017). Moreover, mutations of the TTFL that do not compromise circadian behaviour of the animal nor timekeeping the SCN slice nevertheless disrupt further the rhythmicity of dispersed SCN neurons (Liu et al. 2007). Finally, compromise of electrical signalling across the circuit with reversible pharmacological (Yamaguchi et al. 2003) or genetic (Lee et al. 2015) means not only desynchronises the circadian cycles of the cells, but also causes them to lose amplitude and precision. The SCN circuit therefore creates a mutually reinforcing network, established by coupling between individual clock cells. In doing so, they establish additional emergent properties, including ensemble phase, ensemble period and phase-spread, as evidenced by the stereotypical spatiotemporal wave (Hastings et al. 2018). The re-entrant loop motif is therefore seen again, this time at the level of the SCN circuit.

2.4 Circuit Architecture of the SCN as a Neuronal Network

2.4.1 *Entrainment of the SCN Network: Photic and Non-photic Cues*

The powerful role of intercellular coupling within the SCN immediately begs the question of how SCN cells communicate within the circuit and how the circuit as a whole is entrained by inputs conveying cues regarding external time (i.e. retinal) and internal state. Entrainment to solar time is mediated by glutamatergic signals from the RHT, which act on the SCN via AMPA- and NMDA-type glutamate receptors expressed on retinorecipient neurons, including those that express VIP and GRP (Colwell 2011). The NMDA receptors all contain NR1, in combination with NR2A or NR2B sub-units that confer specific properties, such as calcium channel kinetics. These in turn determine the responses of retinorecipient neurons, including enhanced firing rates that ultimately lead to changes in the TTFL and thus entrainment to light (Mazuski et al. 2018). This control of the TTFL is achieved principally via induction of *Per* gene expression as it spontaneously declines or increases in early and late circadian night, respectively. Although it occurs initially in the core neurons, induction of *Per* expression spreads rapidly to the SCN shell, mediated by non-glutamatergic signals. Consequently, the spontaneous oscillation of the entire SCN is delayed by light after dusk (when induction opposes the spontaneous decline) or advanced by light before dawn (when induction accelerates the spontaneous increase) (Shigeyoshi et al. 1997). In nature, such shifts are small, of the order of minutes, as the SCN tracks solar dusk and dawn. Experimentally, however, the presentation of artificial light in the middle of night can shift the SCN and circadian behaviour by several hours. This perturbation also transiently uncouples the TTFL oscillations in the core, which responds rapidly, and the shell, which lags behind the core because of its indirect regulation by the RHT (Nagano et al. 2003). In modern society, nocturnal exposure to bright artificial lighting for recreational or work-related reasons is a cause of circadian and sleep disruptions that compromise well-being (Chang et al. 2015). Conversely, progressive decline in these entraining pathways at all and any level can lead to poor circadian coherence and sleep disturbance during ageing that can be associated with cognitive and other health-related difficulties (Duffy et al. 2015; Robbins et al. 2021).

Complementing photic entrainment by the induction of *Per* gene expression, the suppression of *Per* gene expression during circadian day, when it is high, can also reset the TTFL by advancing the spontaneous decline. This is best understood in experimental rodents, in which acute behavioural arousal is signalled to the core SCN by thalamic, brain stem and basal forebrain centres via cholinergic, neuropeptide Y- and serotonin-mediated cues (Mistlberger and Antle 2011; Yamakawa et al. 2016). Furthermore, photic and arousing cues can interact at the SCN, modulating and even cancelling out the entraining effect of the other, depending on the balance of *Per* expression (Maywood et al. 2002). The basic sequence of information flow is therefore core to shell and then to targets outside the SCN, with recurrent feedback

from those targets that reflects activity levels. This is important because it allows the stereotypical control of behaviour by the SCN to be over-ridden, and modified to match circumstances. A striking example of this is the recent finding that in mice fed a high-calorie diet, dopamine receptors expressed in the SCN mediate a decrease in neuronal excitability in response to extra-SCN dopamine. This reduction in SCN neuronal electrical activity promotes food intake at inappropriate phases, leading to severe metabolic dysfunction (Grippo et al. 2020). Similar neurochemical pathways exist to the human SCN and so may mediate the entraining effect of altered schedules of activity (e.g. shift work, meal timing) on the circadian system. In addition, from a therapeutic perspective, non-photic behaviourally mediated cues may sustain circadian entrainment when retinal signalling is compromised, most obviously following damage to the eye (Lockley et al. 2007), but also with progressive ageing.

2.4.2 SCN Network Synchrony: GABA and Neuropeptides

In the context of within-circuit coupling, SCN neurons constitute a homogeneous population that ubiquitously synthesises and utilises the inhibitory neurotransmitter GABA, and both ionotropic $GABA_A$ receptors and Gi-coupled metabotropic $GABA_B$ receptors are expressed in the SCN alongside ancillary proteins involved in GABA metabolism and transport (Albers et al. 2017). It is therefore reasonable to infer that GABA plays a role in determining network synchrony. Indeed, exposure of dissociated SCN neurons to exogenous GABA suppresses their electrical firing, elicits phase delays when applied in late circadian day/early circadian night and daily treatment with GABA synchronously entrains their firing rhythms (Evans et al. 2013; Liu and Reppert 2000; Rohr et al. 2019). These effects are dependent on the $GABA_A$ receptors, as the $GABA_A$-specific agonist muscimol recapitulates them, whereas the $GABA_B$-specific agonist baclofen cannot (Liu and Reppert 2000). Notwithstanding these observations, pharmacological or genetic loss of GABAergic signalling in intact SCN explants does not disrupt aggregate SCN circadian timekeeping: a counter-intuitive observation (Aton et al. 2006; Freeman et al. 2013a, b; Ono et al. 2019; Patton et al. 2016). SCN explants from mice deficient in the vesicular GABA transporter (vGAT) do have elevated electrical activity, associated with synchronous burst firing across the network, which reveals a reduced GABAergic tone, but ensemble PER2::LUC rhythms are unaffected (Ono et al. 2019). Pharmacological GABA antagonism does have a subtle network-level effect, increasing the amplitude of cellular *Per1*-LUC oscillations and reducing the period distribution of individual SCN neurons (Aton et al. 2006; Freeman et al. 2013a). This indicates that within the SCN network, GABAergic transmission appears to “repulsively couple” neurons. A consequent decrease of network precision could potentially make the network more susceptible to phase-shifting stimuli, and thereby responsive to seasonal changes in photoperiod, a response thought to be mediated by GABAergic signals (Meijer and Michel 2015). Thus, although implicated in coupling of SCN neurons and the encoding of photoperiodic information, the definitive role(s) of GABA within

the SCN remains unclear (Albers et al. 2017). Indeed, GABA signalling may be more important for the time-dependent inhibitory control by SCN neurons of their extra-SCN targets (Paul et al. 2020; Ono et al. 2021).

SCN factors beyond GABA must, therefore, sustain its potent ensemble time-keeping. Dense core vesicles (DCV) for neuropeptide release are found in the terminals of SCN GABAergic neurons (Albers et al. 2017) and to some extent, therefore, GABAergic and neuropeptidergic signalling presumably work in concert in the SCN. One model is that the repulsive coupling mediated by GABA is counterbalanced by the attractive coupling of neuropeptides such as VIP. Certainly, $GABA_A$ antagonism can induce synchronous rhythmicity within previously asynchronous VIP-null SCN explants (Freeman et al. 2013a). In such a scenario, where neither repulsive nor attractive cues are active, other signalling axes are able to sustain circuit function. Under normal circumstances, by balancing destabilising GABA against neuropeptidergic synchronisation, the network is able to generate correctly timed spatiotemporal dynamics. It is likely that GABA and neuropeptides interact together at the network level to help SCN neurons (alongside their cell-autonomous electrical programme) reach a synchronous happy point of depolarisation that sustains firing and quiescence by clamping resting membrane potential within a permissive range.

Although most attention has focused on VIP, a range of neuropeptides and their cognate receptors is expressed in the SCN, including AVP, GRP, PROK2, cholecystokinin (CCK), Neuromedin-S (NMS) and –U (NMU), Neurotensin (NT), Angiotensin II (AII), methionine enkephalin (mENK), somatostatin (SST) and substance P (SP) (Abrahamson and Moore 2001; Karatsoreos and Silver 2007; van den Pol and Tsujimoto 1985). Notwithstanding variation between species, in unbiased transcriptomic analysis of single cells from the mouse SCN, these genes have been used to define neuronal clusters across the population (Wen et al. 2020; Morris et al. 2021) (Fig. 2.2b). Thus, it is important to consider the SCN network organisation on three levels: the active neuropeptide, its cognate receptor and the distinct cellular populations expressing these factors.

2.4.3 VIP Axis: Mediator of SCN Photic Entrainment and Neuronal Synchrony

VIP is the best characterised neuropeptide in the SCN. Expressed in the SCN core in ~ 10% of SCN neurons, it forms a paracrine signalling axis with VPAC2 receptor-expressing neurons in the SCN shell (~ 35% of SCN neurons) (Morris et al. 2021; Patton et al. 2020). SCN VIP expression is rhythmic, regulated by clock-driven upstream E-box elements (Silver et al. 1999), and AT-motifs, driven by ZFHX3 (Parsons et al. 2015). VPAC2, a Gs-coupled GPCR is also rhythmic, peaking in circadian day at ~ CT4 (An et al. 2012; Doi et al. 2016). Consistent with the retinorecipient character of VIP cells, VIP is a potent regulator, phase-shifting SCN slices ex vivo and animals in vivo, in a phase-dependent mimic of light pulses, with delays

and advances in early and late circadian night, respectively (An et al. 2011; Hamnett et al. 2019; Piggins et al. 1995). It can also accelerate the speed of re-entrainment of mice to a new lighting schedule (An et al. 2013). In steady-state oscillation, VIP synchronises and maintains SCN neuronal and behavioural rhythms, which are disorganised in VIP- or VPAC2-null mice (Harmar et al. 2002; Colwell et al. 2003; Hughes et al. 2004; Aton et al. 2005). This is caused by a loss of network synchrony and reduced amplitude of SCN neuronal rhythms (Atkinson et al. 2011; Aton et al. 2005; Maywood et al. 2011, 2006) and can be restored acutely in VIP-null SCN by treatment with VIP or a VPAC2 agonist (Atkinson et al. 2011; Aton et al. 2005; Maywood et al. 2006) or chronically by co-culture with a VIP-proficient SCN. This is effected by paracrine release of VIP (Maywood et al. 2011; Ono et al. 2016). Activation of the VPAC2 receptor by VIP stimulates intracellular cAMP- (An et al. 2011; Hamnett et al. 2019) and kinase-dependent signalling cascades, acting through the extracellular-signal regulated kinase 1/2 (ERK1/2) pathway and dual specificity phosphatase 4 (DUSP4) (Hamnett et al. 2019) to tune TTFL phase. It also increases electrical activity of SCN neurons by activation of a fast-delayed rectifier (FDR) current (Kudo et al. 2013).

VIP cells peak in electrical activity during mid-circadian day at ~ CT6.5 (Enoki et al. 2017; Hermanstyne et al. 2016; Mazuski et al. 2018; Patton et al. 2020; Paul et al. 2020), while VPAC2 cells peak ~ 1.5 h later (~ CT8) (Patton et al. 2020). This serial activation from VIP to VPAC2 cells within the network-level spatiotemporal wave is also evident in their TTFL and cytosolic calcium rhythms (Patton et al. 2020). Both cell types are electrically quiescent in circadian night, when acute optogenetic stimulation of VIP cells (mimicking their activation via the RHT by nocturnal light) can shift the phase of circadian behaviour or ensemble molecular rhythms (Jones et al. 2018; Mazuski et al. 2018; Patton et al. 2020). VPAC2 receptor antagonism blocks such optogenetically induced shifts (Jones et al. 2015), whilst sustained chemogenetic activation of VIP cells phase-shifts the SCN and irreversibly re-programmes the spatiotemporal wave of gene expression in the SCN (Brancaccio et al. 2013). In contrast, optogenetic stimulation of VPAC2 cells is not sufficient to shift SCN phase (Patton et al. 2020) suggesting that VIP cells entrain the TTFL of VPAC2 cells by controlling activity-independent signalling pathways (which likely include ERK1/2 and DUSP4) (Hamnett et al. 2019). Ablation of VIP cells in adult mice has behavioural effects ranging from minor loss of precision and shortening of free-running period (Mazuski et al. 2020) to behavioural arrhythmia (Todd et al. 2020). Ablation of VIP or VPAC2 cells in SCN slices severely attenuated the amplitude of molecular rhythms (Mazuski et al. 2020; Patton et al. 2020), with less severe effects following VPAC2 cell ablation (Patton et al. 2020). Thus, loss of the cells that express the neuropeptide source or receptor is not as detrimental to circadian time-keeping as the loss of the neuropeptide or receptor itself. This indicates that the VIP or VPAC2 cells release other factors, and focussed loss of VIP-VPAC2 signalling causes a neurochemical imbalance that desynchronises the SCN network. As noted above, unopposed synaptic GABAergic communication may cause this (Fan et al. 2015; Paul et al. 2020; Freeman et al. 2013a).

Finally, beyond acute phase-shifting and sustained cellular synchrony, the VIP-VPAC2 cellular axis acts as pacemaker to the SCN circuit. AAV-mediated genetic complementation in CRY-deficient mice to alter the properties of the cell-autonomous TTFLs of VIP and/or VPAC2 cells can change the ensemble period of the SCN to match that of the VIP-VPAC2 cellular axis. It can also establish robust TTFL rhythms across otherwise arrhythmic SCN when only VIP and VPAC2 cells, together, have competent TTFLs. The initiation of circadian competence in the VIP-VPAC2 cellular axis also initiates behavioural rhythms in previously arrhythmic CRY-null mice (Patton et al. 2020). Indeed, the cell-autonomous TTFL of VPAC2 cells, the cellular output of this neurochemical axis, determines the period and circadian competence of behavioural rhythms in mice (Hamnett et al. 2021).

2.4.4 GRP Axis: An Accessory Entrainment and Synchronisation Pathway

GRP is expressed in the SCN core, in retinorecipient cells that constitute in ~ 5% of SCN neurons, with some overlap in a subset of the VIP cells (Morris et al. 2021; Wen et al. 2020). Its expression is circadian (Dardente et al. 2004), driven at least in part by AT-motifs activated by ZFHX3 (Parsons et al. 2015). The GRP receptor, GRPR (also known as BB_2) is a Gq-coupled GPCR expressed in the SCN shell (Karatsoreos et al. 2006). Both in mice in vivo and in SCN slices ex vivo, exogenous GRP can trigger phase-shifts and acute gene expression comparable to the effect of light pulses (Piggins et al. 1995; Gamble et al. 2007). GRP therefore acts in parallel to, and converges with, the VIP-VPAC2 axis by activating the TTFL of shell AVP neurons. Correspondingly, mice lacking GRPR have reduced responses to photic cues (Aida et al. 2002), whilst GRPR antagonists can suppress peak firing rate (Brown et al. 2005). Moreover, in the absence of effective VIP-mediated signalling, GRP can restore cellular synchrony to SCN (Maywood et al. 2006, 2011).

2.4.5 AVP Axis: Within-Shell Coupling and Circadian Output

AVP is enriched in 15–20% of SCN neurons located in the shell. Its transcription peaks in circadian day, controlled by TTFL E-box elements (Jin et al. 1999), cAMP-response elements (CREs) (Arima et al. 2002) and ZFHX3-driven AT-motifs (Parsons et al. 2015). AVP receptors (AVPR) V1a and V1b are Gq-coupled GPCRs that are expressed rhythmically in the SCN shell (Bedont et al. 2018; Morris et al. 2021) (Morris et al. 2021) peaking during circadian night. Consistent with this, V1a/1b agonists induce phase delays when administered to SCN explants during circadian night (Rohr et al. 2021). Genetic deletion of the V1a and V1b receptors creates a more loosely synchronised circuit, which allows both the SCN and animal to entrain more

rapidly to external perturbation (Yamaguchi et al. 2013), and in the absence of VIP-signalling, AVP can act as a synchronising factor (Maywood et al. 2011; Ono et al. 2016). Additionally, treatment with V1a/V1b antagonists lengthened the period of SCN explants and decreased spatiotemporal phase dispersion (Bedont et al. 2018). It also prevented initiation of de novo SCN oscillation following CRY1 complementation, suggesting that AVP is required to couple previously desynchronised oscillatory cells (Edwards et al. 2016).

AVP neurons are highly rhythmic, with calcium peaking in advance of voltage (Enoki et al. 2017) and both leading PER2::LUC (Shan et al. 2020). Rhythmicity requires the BMAL1-dependent cell-autonomous TTFL, although loss of rhythmicity in AVP cells does not compromise the rest of the SCN, which remains synchronously rhythmic (Mieda et al. 2015). Interestingly, in contrast to the VPAC2 cells (Hamnett et al. 2021), which encompass 85% of the AVP cells (Patton et al. 2020), conditional ablation of BMAL1 in AVP neurons lengthened the period of locomotor activity, but did not induce arrhythmia (Mieda et al. 2015; Shan et al. 2020), indicating that AVP neurons only play a role in coordinating some SCN outputs, while additional VPAC2-expressing cells also function to sustain network rhythmicity (Hamnett et al. 2021). AVP cells can, however, act as behavioural pacemakers: conditional deletion or over-expression of CK1 δ into AVP cells lengthens or shortens behavioural period, respectively, but again without altering intrinsic SCN explant period (Mieda et al. 2016). Thus, AVP neurons form at least part of the output from the SCN and when GABAergic transmission from AVP neurons is compromised, activity time is lengthened independent of behavioural period (Maejima et al. 2021). This is consistent with the phenotype of the AVP BMAL1 knockout where in addition to a lengthening of period, circadian activity time also lengthens (Mieda et al. 2015).

2.4.6 Prokineticin-2 Axis: More Than an SCN Output?

PROK2 is enriched in ~ 12% of SCN neurons, straddling the core–shell division and overlapping to varying degrees other neuropeptidergic populations as well as cells that express its cognate receptor, PROKR2 (Cheng et al. 2002; Masumoto et al. 2006). PROK2 transcription is highly rhythmic, driven by TTFL E-box elements (Cheng et al. 2005) and AT-motifs controlled by ZFHX3 (Parsons et al. 2015). It peaks during circadian day and is correspondingly low during circadian night, when it can be induced by light. PROKR2 is a Gs-coupled GPCR enriched in ~ 16% of SCN neurons, 10% of which co-expresses PROK2 (Morris et al. 2021). Within the SCN, PROKR2 expression is also rhythmic at the transcript level following the pattern of PROK2 expression: peaking during the early circadian day and reaching its lowest abundance during the early circadian night.

Knockout of PROK2 or its receptor disrupts clock-controlled behaviour and physiology (Li et al. 2006; Prosser et al. 2007; Jethwa et al. 2008) without apparent compromise of SCN function and so PROK2 has been ascribed a role in SCN output. It can, however, suppress GABAergic signalling in SCN slices (Ren et al. 2011) and through

this disinhibition increase the baseline of PER2::LUC bioluminescence without altering SCN ensemble phase (Morris et al. 2021). Conversely, PROKR2 antagonists acutely suppress the amplitude of the subsequent PER2::LUC peak (Morris et al. 2021), presumably via a disruption of signalling to CREs in the promoters of TTFL components (Colwell 2011). Pharmacological inhibition of PROKR2 also reversibly lengthens SCN period (Morris et al. 2021) with a similar small lengthening of behavioural period observed in mice lacking PROK2 or PROKR2 (Li et al. 2006; Prosser et al. 2007). Furthermore, ectopic expression of PROK2 under the control of the PROKR2 promoter disrupts the SCN TTFL and circadian behaviour (Li et al. 2018). Coherent SCN function therefore depends on where PROK2 is expressed and how it is signalling to target cells. Consistent with this, intersectional manipulations of the PROK2-PROKR2 cellular axis have revealed a pacemaking function parallel to that of the VIP-VPAC2 axis. Both PROK2 and PROKR2 populations express TTFL rhythms, with PROK2 cells in advance of the PROKR2 cells by ~ 0.5 h (Morris et al. 2021). As with VIP-VPAC2 (Patton et al. 2020), this difference between ligand- and receptor-expressing populations may contribute to the circuit-wide spatiotemporal waves of activity. Moreover, intersectional manipulations of the cell-autonomous TTFLs of PROK2 and PROKR2 alter the ensemble period of the SCN and initiate rhythms in an otherwise arrhythmic circuit (Morris et al. 2021). In contrast to the VIP-VPAC2 axis, where ensemble period is only controlled when both cell populations are targeted (Patton et al. 2020), both PROK2 cells or PROKR2 cells can individually control circuit period. To initiate rhythms, however, both PROK2 and PROKR2 populations need to have activated TTFLs, as is the case for the VIP-VPAC2 axis.

2.4.7 An Emerging View of the Functional Topology of the SCN Network

The time-base over which the SCN operates, 24 h, is very different from that of other neural circuits, which process information over much shorter intervals. Various features of the SCN network architecture appear especially well adapted to this role. The first is the paracrine nature of neuropeptidergic signalling which sustains a slow but progressive flow of information through the circuit. This is reflected in the spatiotemporal waves of TTFL and neural activity, which loop across the SCN in a stereotypical pattern. The wave may comprise distinct stages, each consisting of the serial activation of ligand-releasing and receptor-expressing populations within distinct neuropeptidergic axes, themselves linked in a chain (Fig. 2.2b). Available evidence from the VIP-VPAC2 and PROK2-PROKR2 axes supports this view, and future studies may expand the network to include AVP-, GRP- and other neuropeptide-mediated signalling. The ability of distinct neuropeptidergic populations to control ensemble period, driving it at their own cell-autonomous period, further supports this view of the network as one built around serial activation through

a chain of cellular hubs. Indeed, the de novo establishment of network-wide oscillations by circadian-competent VIP-VPAC2 or PROK2-PROKR2 axes emphasises the ability of cell-derived (neuropeptidergic) circadian signals to propagate through and organise an otherwise circadian-incompetent circuit. For the purposes of entrainment to external time and to internal cues, the VIP and GRP cells are the entry point to the network. This may explain why the circadian-competent VIP-VPAC2 axis is able to establish spatiotemporal order across the SCN, whereas the PROK2-PROKR2 axis is unable to achieve this order, even though it can initiate basic rhythmicity (Patton et al. 2020; Morris et al. 2021). These results support a model whereby VIP and VPAC2 cells work together as a distributor node in the SCN network to transfer circadian timing information from the retinorecipient cells to the rest of the network, while the PROK2 and PROKR2 cells work together to integrate temporal information across the network (Fig. 2.2b).

Orthogonal to the anatomical structure of the SCN circuit, analysis of single-cell RNA sequencing data has made it possible to construct formal network topologies based on the inferred relationships between neuropeptide ligand- and receptor-expressing populations (Wen et al. 2020; Morris et al. 2021). These support both linear, recurrent and circular topologies, the latter two likely facilitating self-sustained oscillation as (again) output becomes input. Importantly, all of the neuropeptidergic axes are under circadian regulation, and from a transcriptional perspective, they are assembled during circadian day when neural electrical activity is maximal and dissolved in circadian night, when neural firing rate is minimal. The expression of VIP, AVP, GRP and PROK2, as well as their cognate receptors, all exhibit this temporal plasticity, with the PROK2-PROKR2 axis being the most dramatic as, transcriptionally speaking, it disappears at night. Assuming this is carried through at the levels of ligand release and subsequent receptor activation, it means that neuropeptidergic signals are effective during circadian daytime but exert little effect on the network at night. This implies that other, non-neuropeptidergic cues are important for nocturnal advancement and co-ordination of cell-autonomous and network-level oscillations.

2.5 The Role of Astrocytes in the SCN

Alongside the neurons in the SCN network sit the glial cells, of which astrocytes are the most numerous (Guldner 1983; Morris et al. 2021; Wen et al. 2020). In common with most mammalian cell types, astrocytes display clear circadian TTFL rhythms (Prolo et al. 2005; Marpegan et al. 2011; Brancaccio et al. 2017; Tso et al. 2017) alongside cytosolic circadian cycles of $[Ca^{2+}]_i$ (Brancaccio et al. 2017; Patton et al. 2022). Strikingly, however, these rhythms in the intact SCN network sit in antiphase to neuronal rhythms, i.e. they peak in circadian night when neurons are inactive (Fig. 2.3a). The overriding question, therefore, has been whether, despite being outnumbered by neurons by a factor of 3–1, astrocytes actively participate in network timekeeping?

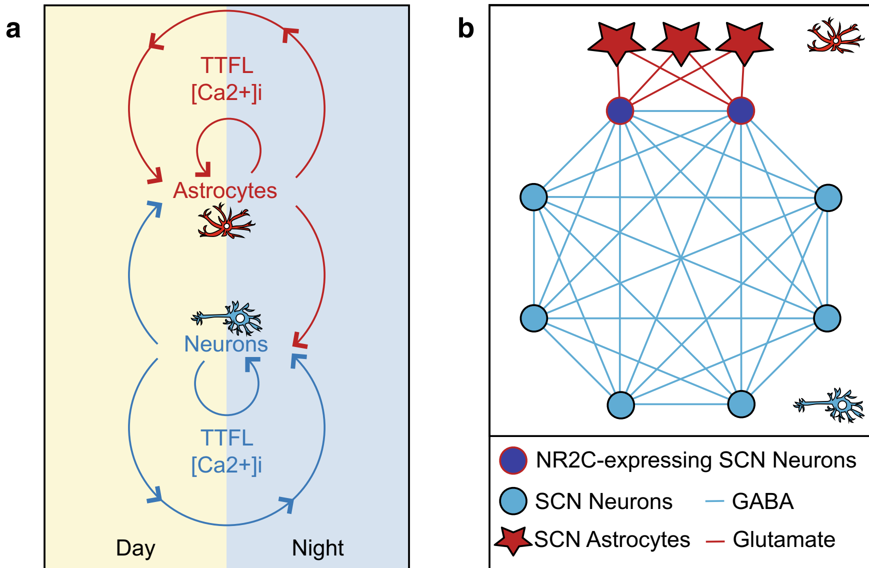

Fig. 2.3 Neurons and astrocytes communicate within the SCN network and form a network-level re-entrant motif. **a** Astrocytes and neurons communicate with one another to correctly phase their respective TTFLs. Importantly, neurons are active during circadian day, consistent with the timing of the transcriptional presence of neuropeptidergic signalling hubs, while astrocytes are active during the night, consistent with the timing of astrocyte-derived glutamate signals. Thus, while neurons direct daytime network functions, astrocytes appear to direct night-time functions. **b** A network schematic for astrocytic control of the SCN neuronal network via nocturnal release of glutamate. This signal is sensed by a subset of SCN neurons expressing NR2C-containing NMDA receptors, which in turn utilise synaptic GABA to suppress the activity of the rest of the neuronal network

2.5.1 Astrocytic Control of Circadian Rhythms

Astrocytic metabolism can be disrupted by treatment with the glial-specific metabolic toxin fluorocitrate, a suicide inhibitor of aconitase, which arrests carbon flux through the astrocytic citric acid cycle (Fonnum et al. 1997). Treatment of acute SCN explants from rats with fluorocitrate at CT6 disrupts ensemble neuronal electrical activity rhythms on the following cycles, indicating that on some level SCN astrocytes are able to direct activity within the SCN network (Prosser et al. 1994). Similarly, chronic treatment of free-running SCN explants with fluorocitrate disrupts astrocytic $[Ca^{2+}]_i$ oscillation, supresses the amplitude of PER2::LUC oscillations and compromises network synchrony (Patton et al. 2022). Furthermore, disruption of astrocytic communication through pharmacological or genetic manipulation of gap junctions or hemichannels (Shinohara et al. 2000; Brancaccio et al. 2019) and disruption of glial proliferation through antimitotic treatments (Shinohara et al. 1995) also disrupts SCN neuronal rhythms. Astrocytic competence is necessary, therefore, to maintain network-level SCN rhythmicity (Fig. 2.3a).

The sufficiency of astrocytic clocks to control the SCN circuit is demonstrated in two ways. First, if the period of the cell-autonomous TTFL specifically of astrocytes is lengthened by intersectional genetic means, then the ensemble period of the SCN is also lengthened (Brancaccio et al. 2017; Tso et al. 2017; Patton et al. 2022). In addition, deletion of BMAL1 from astrocytes lengthens ensemble period, which suggests that a BMAL1-dependent signal from astrocytes is required for timely progression of the network cycle. In contrast, shortening of the cell-autonomous period of astrocytes does not shorten ensemble period to the degree seen when the period of neurons is shortened, indicative of some differential potency between astrocytic and neuronal pacemaking (Patton et al. 2022). Second, if astrocytes are the only cells in the SCN with a functional clock, they are nevertheless able to impose rhythmicity, monitored as both TTFL and neuronal $[Ca^{2+}]_i$ oscillations, on the rest of a previously arrhythmic circuit (Brancaccio et al. 2019; Patton et al. 2022). Albeit the time-course is slower than that of neuronal initiation. SCN astrocytes are therefore not passive components within the SCN network: rather, they are effective pacemakers, and this competence is emphasised further by in vivo studies. In mice where only the cell-autonomous period of SCN astrocytes is lengthened, locomotor activity rhythms are also lengthened (Tso et al. 2017; Brancaccio et al. 2017). Furthermore, disruption of the TTFL by conditional deletion of BMAL1 only in astrocytes results in a lengthening of behavioural period (Barca-Mayo et al. 2017). Finally, CRY1-complementation into SCN astrocytes of CRY-null mice initiates behavioural rhythmicity, indicating that astrocytes are able to adequately organise neurons within the SCN network to coherently control their output (Brancaccio et al. 2019). These results therefore elevate astrocytes to active participants in SCN network timekeeping.

2.5.2 *Astrocyte-To-Neuron-To-Astrocyte Communication Within the SCN Network*

How, then, are SCN astrocytes able to control circadian rhythmicity? The first clue to this emerged from imaging approaches that revealed rhythmic release of glutamate within isolated free-running SCN explants. This rhythmic release is striking on two fronts: first, SCN neurons are exclusively GABAergic and, second, glutamate rhythms within the SCN peak during the night, in antiphase to neuronal activity (Brancaccio et al. 2017). Glutamate release in the extracellular space is therefore co-phasic with astrocytic metabolic activity rhythms, suggesting that they are the source of this transmitter and, indeed, blockade of astrocytic glutamine synthetase, which synthesises glutamine from glutamate, increased extracellular glutamate in SCN slices. Astrocytic control was confirmed by a significant disruption in extracellular glutamate rhythms following either caspase-3 driven ablation of SCN astrocytes (but not neurons) (Brancaccio et al. 2017) or pharmacological blockade of astrocytic connexin-43 (Cx-43) hemichannels (Brancaccio et al. 2019). These manipulations in turn disrupted SCN TTFL rhythms, confirming the relevance of astrocytic control

of glutamate for clock function. So how might glutamate act on the SCN neural circuitry? Whereas NMDA receptors with NR2A and NR2B sub-units mediate retinal entrainment in the core SCN (Colwell 2011), the dorsal SCN expresses NR2C sub-units which confer different dynamics and agonist-sensitive properties to the oligomeric NMDA receptor (Fig. 2.3b). Pharmacological blockade of NR2C-containing NMDARs reduced the amplitude of SCN molecular oscillations and depolarised cells during the night (Brancaccio et al. 2017). Importantly, it also eliminated astrocyte-initiated rhythms (Brancaccio et al. 2019), indicating that signalling via the NR2C subunit is vital for linking astrocytic and neuronal clock function (Fig. 2.3b). Curiously, however, glutamate release from astrocytes appears to be inhibitory to the neuronal network, i.e. blockade of NR2C depolarised neurons, even though glutamate is conventionally an excitatory neurotransmitter. This can be reconciled by the observation that the subset of neurons that express NR2C display pre-synaptic calcium elevations during the night, and this nocturnal rise presumably facilitates the synaptic release of GABA. This will in turn suppress neuronal activity across the network, even though the action potential firing of GABAergic cells is not altered (Brancaccio et al. 2017). The effect seen in the SCN may be more general, in so far as astrocytes can manipulate extracellular levels of several neurotransmitters to mediate astrocyte-to-neuron function. The first is active release of glutamate from astrocytes, which is sensed by neuronal NR2C-containing NMDARs to control SCN GABAergic transmission (as described above) (Brancaccio et al. 2017). Second, the uptake of GABA may rebalance VIP/GABAergic signalling (Barca-Mayo et al. 2017) (and see Sect. 4.2), and, third, the active release of adenosine from astrocytes may alter neuronal GABAergic signalling (Hablitz et al. 2020). While these mechanisms are qualitatively different, they converge on a common theme: the control of GABAergic signalling.

If astrocytes are signalling to neurons to control neural activity, the neurons must also be signalling back to modulate astrocytic function. Consistent with this, endocannabinoids released by neurons induce calcium activity in SCN astrocytes following activation of astrocytic cannabinoid 1 receptors (CB1R) and thereby facilitate adenosine release (Hablitz et al. 2020). In addition, cultured cortical astrocytes can be entrained to daily VIP exposure (Marpegan et al. 2009). To understand the contributions of astrocytes to circadian timekeeping, we therefore need to understand how SCN astrocytes and neurons communicate time of day information to one another (Fig. 2.3). Such signalling adds to the emerging network model, being consistent with a necessarily slow and progressive information flow on a long timebase. This further level of paracrine cues (paracrine glutamate controlling synaptic GABA) and its nocturnally specific cellular activity will allow SCN timekeeping to "bridge" the gap when the diurnally active neuropeptidergic network is dismantled. Moreover, this adds yet another re-entrant loop motif to the SCN timekeeper.

2.6 SCN Outputs and Control of Circadian Behaviour and Sleep

2.6.1 SCN Outputs and Control of Circadian Behaviour

The neural pathways that mediate SCN-dependent control over circadian behaviour including sleep are not well defined. This may in part reflect the diverse and highly distributed nature of circadian control. Neuropeptidergic axons from both core (VIP, GRP) and shell (AVP, PROK2) SCN neurons project out of the nucleus to the surrounding hypothalamus, where direct inputs to neuroendocrine nuclei such as the paraventricular nucleus and arcuate nucleus will mediate circadian regulation of hormonal and metabolic status (Paul et al. 2020; Mendez-Hernandez et al. 2020). SCN projections to the dorsolateral hypothalamus will impinge on wake-regulatory centres containing orexinergic and MCH-expressing neurons. A broader distribution of circadian signals arises from the sub-paraventricular zone (SPZ), which is adjacent to, and receives input from, the SCN. It projects to the medial forebrain, thalamus, hypothalamus and brainstem to provide several parallel and pathways for segregated circadian regulation of behaviour and physiology (Vujovic et al. 2015). This includes GABAergic efferents from the SPZ to the ventromedial hypothalamus that confer circadian control of aggression (Todd et al. 2018), as well as extensive input to the periaqueductal central grey, an area strongly associated with behavioural arousal. The midline paraventricular nucleus (PVT) is also interconnected, reciprocally, with the SCN and SPZ. It receives direct and indirect photic input and is densely innervated by orexinergic neurons which direct arousal-state transitions. It is, therefore, a likely route for circadian modification of affective behaviours, such as mood and motivation (Colavito et al. 2015). Notwithstanding this growing neuroanatomical knowledge, the means by which specific SCN neuronal populations control behaviour by specific pathways remains to be determined. AAV-mediated delivery of Cre-recombinase in vivo into the SCN of floxed VGAT mice decreased the quality and amplitude of circadian locomotor activity rhythms without altering period (Ono et al. 2019). Furthermore, this loss of GABAergic signalling did not disrupt timekeeping within SCN explants, suggesting that synaptically released GABA is potentially more important in the regulation of SCN outputs rather than SCN timekeeping itself (see above). Consistent with this, the same manipulation within just the AVP cells, similarly disrupted circadian behaviour without altering SCN rhythmicity (Maejima et al. 2021).

2.6.2 SCN in the Circadian Regulation of Sleep

The principal overriding output of the circadian system is the control of the sleep–wake cycle. Although the exact function of sleep is not yet fully understood, its importance in relation to health is evident. Even acute sleep deprivation has known

effects on cognition and alertness (Lo et al. 2012). This is particularly relevant to the large numbers of shift workers in the population, where night shift work is associated with loss of concentration and increased workplace accidents (Budnick et al. 1994; Ryu et al. 2017). Furthermore, a breakdown of daily sleep–wake patterns is associated with many neurological and psychiatric illnesses (Leng et al. 2019; Sato and Sassone-Corsi 2021), although the extent to which these reflect cause or effect awaits clarification.

The current accepted model for sleep control involves two processes: a sleep homeostat, or Process "S" that measures sleep pressure as a function of length of time spent awake and a circadian Process "C" that allows sleep to be timed relative to the light–dark cycle (Borbely et al. 2016). In this model, the circadian clock could either behave as a passive gate on the homeostatic process, or it could actively promote sleep and/or wake at the appropriate circadian time. It is only by separating process S and C that this can be tested, and forced desynchrony protocols, which uncouple rest/activity rhythms from the internal circadian clock, have enabled this to some degree in human subjects. This has revealed that the circadian clock can modulate sleep propensity and structure and that ageing affects sleep regulation (Dijk et al. 1999). Equally, there is evidence that sleep pressure can influence circadian control of sleep [reviewed in (Deboer 2018)].

The identity of the sleep homeostat is unknown but through advanced techniques including opto- and pharmaco-genetics, viral tracing, fluorescent reporters of neuronal activity and human brain imaging, it has been possible to map individual sleep- and wake-promoting neuronal circuits within and between known sleep/wake-controlling regions in forebrain, hypothalamus and brain stem (Weber and Dan 2016; Boes et al. 2018). Within these networks, neural hubs control sleep state (REM and NREM), transitions between sleep and wakefulness and also presumably are sensitive to time spent awake. Whether homeostat(s) and wake- and sleep-promoting centres are co-located is not clear.

For Process C, early ablation studies across nocturnal and diurnal mammals demonstrated the necessity of the SCN for circadian timing of sleep, and most suggest that there is little or no effect on sleep homeostasis [reviewed in (Mistlberger 2005)]. However, SCN-ablated mice are reported to have reduced NREM compared to control animals during recovery from sleep deprivation (Easton et al. 2004), which suggests that the SCN may play a greater role in the control of sleep, beyond sleep timing. An additional level of complexity to the localisation of Process C came with the discovery of local TTFL-based clocks in various brain regions, including hippocampus, cerebellum and cerebral cortex, which can maintain autonomous rhythms in ex vivo culture for several cycles (Abe et al. 2002). These local clocks may confer circadian modulation of local functions, such as cognition (Kyriacou and Hastings 2010; Wright et al. 2012) that "chime" with central SCN-determined phases of sleep and wakefulness. As noted above, projections via the SPZ will mediate internal synchrony between SCN and local brain clocks. But what are their relative contributions to circadian control of sleep?

Genetic approaches confirm a role for the TTFL in Process C. Double deletions of either *Cry1, 2* or *Per1, 2*, or single deletion of *Bmal1* in mice abolish sleep/wake

rhythms under constant darkness (Wisor et al. 2002; Shiromani et al. 2004; Laposky et al. 2005). In contrast to the SCN neural ablations, genetic ablations variously affected total sleep time, sleep fragmentation and NREM recovery after sleep deprivation. These may arise from loss of local TTFL competence and/or the non-circadian pleiotropic effects of disrupted transcriptional programmes. Moreover, such universal genetic deletions cannot discriminate between effects originating from the SCN and those from other brain clocks. More specific conditional deletion can be achieved by intersectional means. For example, deletion of *Bmal1* in the histaminergic cells of the tuberomammillary nucleus (TMN) disrupted sleep architecture and recovery from sleep deprivation, without affecting overall circadian timing of sleep–wake cycles of mice (Yu et al. 2014). This suggests that the TTFL within local brain clocks can contribute to circadian sleep control, in this case, likely through appropriate circadian control of histamine synthesis. An alternative to such loss-of-function is genetic complementation to achieve gain-of-function. In CRY-null mice, local AAV-mediated expression of CRY1 in the SCN not only restored circadian behavioural rhythms but it also organised the previously arrhythmic sleep/wake cycle (Maywood et al. 2021a) (Fig. 2.4a–c). Moreover, deficits in NREM recovery sleep and sleep-dependent memory were reversed, as was novel object memory, a measure of sleep-dependent cognition. Thus, molecular circadian competence solely in the SCN is sufficient to effect Process C function in an otherwise "clockless" mouse. Clock competence in other brain regions is, therefore, not necessary. This does not mean, however that local clocks have no auxiliary role to play in circadian control of sleep and wakefulness. In the CRY-null mouse, local clocks are disabled, but what if time-keeping in SCN and local clocks is mismatched? Temporally chimeric mice can be created by intersectional means, such that SCN (and other) cells expressing the dopamine 1a receptor (*Drd1a*) have a cell-autonomous period of ~ 24 h, whilst all other cells and tissues have a period of ~ 20 h. This provides a suitable system where the brain has its own internal forced desynchrony, or misalignment: mutant local brain clocks running at a 20 h period, whereas the SCN provides a circadian output at ~ 24 h. The dominant pacemaking effect of the SCN *Drd1a* cells imposes a stable ~ 24 h rhythm to the SCN TTFL and also to rest/activity cycles (Smyllie et al. 2016a). In such mice, the sleep-wakefulness cycle also has a ~ 24 h period, but sleep is more fragmented compared to 24 h and 20 h, non-chimeric, control groups. Moreover, sleep-dependent novel object recognition memory is severely impaired in the temporally chimeric mice, but not in the controls, indicative of compromised sleep function (Maywood et al. 2021b) (Fig. 2.4d). This indicates that extra-SCN local brain clocks do likely play a role in circadian regulation of sleep, insofar as sleep and sleep-dependent memory are optimal when local clocks are "in tune" with the dominant circadian signal of the SCN.

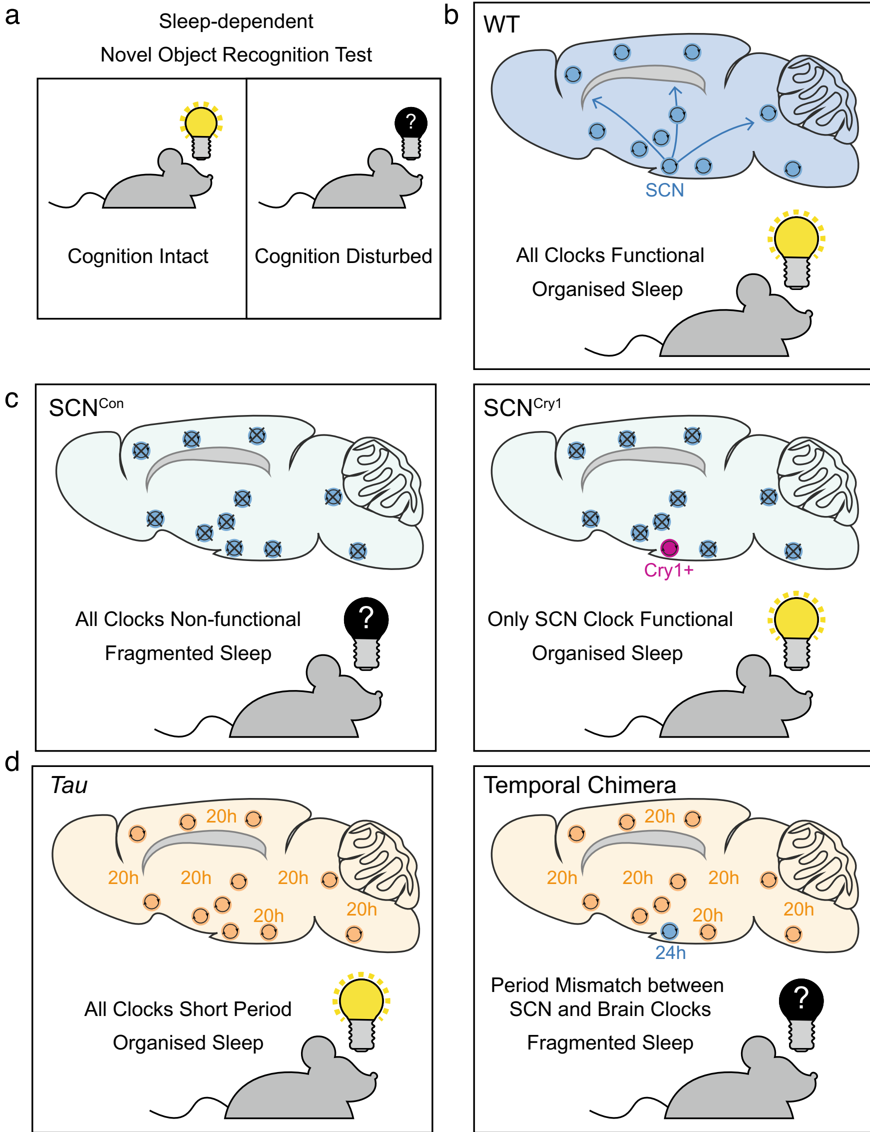

Fig. 2.4 Circadian disruption of sleep leads to cognitive deficits. **a** Novel object recognition is a task to study sleep-dependent cognition. **b** In a wild-type mouse where all of the cell-autonomous clocks in the animal are coherent, sleep is well organised and the mouse is cognitively competent. **c** In a CRY-null mouse (left, SCN^{Con}) where all cell-autonomous clocks are arrhythmic, sleep is fragmented and the mouse displays cognitive deficits. If CRY1 is expressed via AAV into just the SCN of a CRY-null mouse (right, SCN^{Cry1}), sleep becomes organised and the mouse is cognitively competent. **d** In a short period (20 h), $CK1\varepsilon^{Tau}$ mouse (left, *Tau*) where all cell-autonomous clocks share a common, coherent period (left), sleep is well organised and the mouse is cognitively competent. In a mouse where excision of the short period $CK1\varepsilon^{Tau}$ allele is excised specifically in the SCN (right, Temporal Chimera), the SCN expresses a long period cell-autonomous oscillation (24 h) which is mismatched relative to the (20 h) cell-autonomous clocks in the rest of the mouse. This results in fragmented sleep and cognitive deficits in these mice

2.7 Conclusion and Future Perspectives

At all levels of organisation, the mammalian circadian timing system, focussed on the SCN, features re-entrant feedback loops that confer high-amplitude oscillation, precision and robustness (Fig. 2.5). The delineation of the cell-autonomous TTFL of mammals is a major achievement in chronobiology and neuroscience. Nevertheless, the current model is very qualitative and we have limited understanding of the exact and quantitative functions of the TTFL. This is an unmet opportunity because the operations of the SCN are ideal for formal analysis, more so than any other behaviourally relevant circuits, because its outputs are so "crystalline" and precise and the system spans seamlessly from molecules through cells and circuits to behaviour (Fig. 2.5). It is vital, therefore, to understand molecular abundances of the TTFL components through circadian time, and their affinities in the formation of complexes and how this directs their intracellular behaviour: mobility, stability and localisation. The smooth transitions between states of neural activity and quiescence reflect progressive changes in the expression of genes controlling excitability and metabolism, but how this is orchestrated at the level of the genome remains unclear.

The emerging model of the SCN circuit, based around slow and progressive paracrine signalling of time, looping around the network, highlights several critical features, such as the inter-dependence of the TTFL and neural activity, the serial activation of ligand- and receptor-expressing cell groups, and the capacity of some of these groups to impose their cell-autonomous properties on other SCN circuits. The requirement now is to understand how the assembly of these elements creates a greater whole, conferring emergent properties that are lacking from the individual cells. We also lack a clear understanding of both the topography (beyond core and shell) and topology of the circuit that could delineate the exact contribution of individual nodes (pacemaker, distributor and integrator nodes) and how they are related. Furthermore, what circuit elements actually close the loop of the spatiotemporal wave to take it full circle, and what does that wave represent for SCN output? The discovery of the central role of astrocytes in maintaining SCN timekeeping has raised a series of questions regarding neuron-to-astrocyte-to-neuron signalling, another circular motif that confers amplitude and stability. Clearly, SCN output cues are delivered by neurons but the cell-autonomous clocks of their astrocytic partners work through that neural circuitry. The transfer of information from astrocytes to neurons by paracrine means (possibly by astrocytic regulation of extracellular glutamate and thereby GABAergic tone) may be more general across the brain, and influence local changes in neural activity, such as during sleep stages and vigilance states that similarly occur over a longer time-base. The SCN is so powerful a pacemaker that if it is the only competent circadian clock in the animal, it can nevertheless impose appropriate cycles of sleep and wakefulness and in doing so maintain sleep-dependent cognitive function. The neural and neurochemical pathways by which the SCN achieves this regulation are unclear, and so we do not understand how the SCN affects the sleep–wake cycle. Is it primarily a promoter of wakefulness, and if so, does it activate wake-promoting centres and inhibit the complementary sleep-promoting circuits?

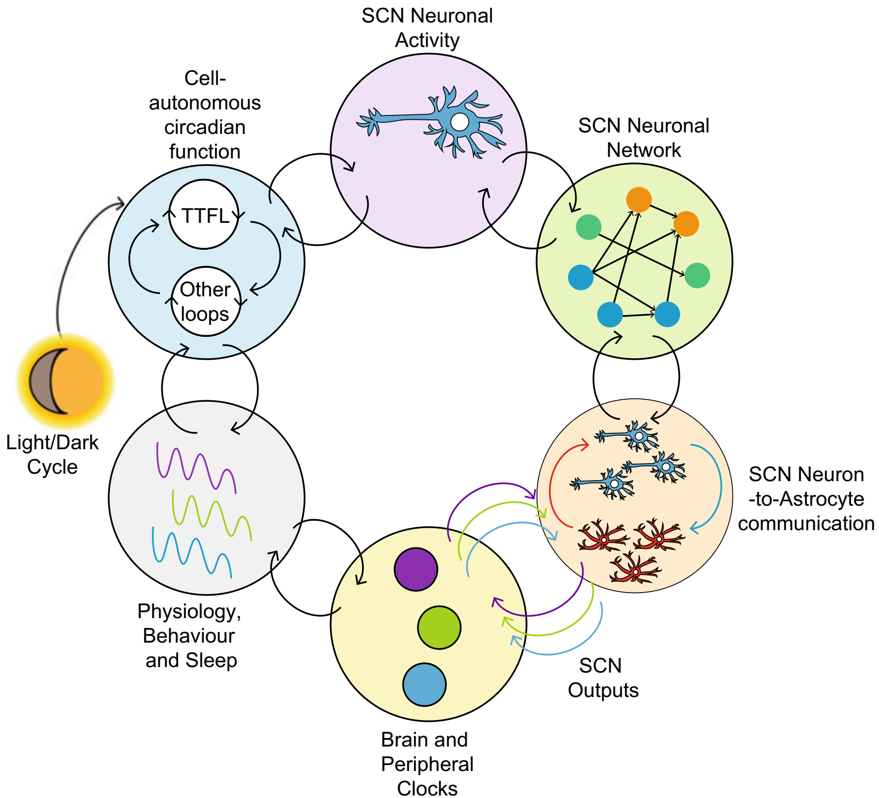

Fig. 2.5 Different levels of circadian organisation form a re-entrant motif. Circadian organisation of physiology and behaviour extends with exquisite precision from the molecular level to the SCN neuronal and network level, to bidirectionally coupled SCN neurons and astrocytes and ultimately to SCN outputs that control behaviour and physiology on a daily timescale. Remarkably, temporal information flows seamlessly from one level to the next, with feedback between molecular and cellular elements increasing the overall precision, amplitude and robustness of the various rhythmic processes. Thus, at every level of circadian timekeeping, a re-entrant motif can be observed

Although the cell-autonomous TTFL clock is active across tissues and brain regions, the SCN is *primus inter pares*. Nevertheless, the deleterious effects of circadian chimerism, in which SCN and local brain clocks are mismatched, show that they operate in concert. Cognitive decline during ageing may therefore have some circadian origin, arising from progressive dysfunction in the SCN, in local brain clocks, and/or in their abilities to interact. This may arise in neurons and/or astrocytes in SCN and brain regions, either within their cell-autonomous clock or in cellular pathways regulated by the clock. In the context of neurodegeneration, this trajectory of decline is accelerated, particularly in diseases associated with the accumulation of toxic aggregates of misfolded proteins. Given the central role of the TTFL and its outputs in cellular homeostasis, age- or disease-dependent compromise of local TTFLs may

facilitate disease progression by reducing the capacity of the cell to prevent, process and neutralise such aggregates. These physiological defences are present in both neurons and astrocytes, the capacities of which are both compromised in neurodegenerative conditions. At the level of the whole brain, it is also clear that sleep, a dominant output of the circadian system, favours restorative functions, including cellular metabolism and brain-wide clearance of metabolites via the extracellular space and cerebrospinal fluid. Consequently, perturbed cell-autonomous clocks and sleep/wake cycles may well exacerbate the progression of aggregate-based neurodegeneration. Even though clock dysfunction may not be the primary cause of such diseases, by identifying the casual links in these processes, it should be possible to develop novel avenues to management, and possibly therapy. These translational applications of newly found circadian knowledge are in their infancy, but the pervasive roles of circadian clocks to cellular and brain health highlight the enormous range and scope of opportunities in this area.

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