Molecular and Genetic Analysis of Neuropeptide Signalling in Mammalian Circadian Timekeeping

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“Half of seeming clever is keeping your mouth shut at the right times.”
- Kvothe

“Ignorance is hardly unusual... The longer I live, the more I come to realize that it is the natural state of the human mind.”
- Jasnah Kholin
DECLARATION

All work presented in this thesis, unless otherwise stated, is the result of my own investigations during the years 2013-2017 under the supervision of Dr Michael Hastings at the MRC Laboratory of Molecular Biology, Cambridge, UK. The work presented here has not been submitted in whole or in part for any degree at the University of Cambridge or elsewhere, and does not exceed the limit of 60,000 words.
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ABSTRACT

The suprachiasmatic nucleus (SCN) of the hypothalamus is the master mammalian pacemaker, co-ordinating the multitude of cell-autonomous circadian oscillators across the body to ensure internal synchrony, as well as maintaining an adaptive phase relationship with the light-dark cycle via projections from the retina. Intercellular communication between SCN clock neurons synchronises their oscillations, resulting in coherent output signals to the periphery. Vasoactive intestinal peptide (VIP), a neuropeptide expressed in the retinorecipient ventrolateral region of the SCN, is vital to this circuit-level co-ordination by signalling to its cognate VPAC2 receptor. In addition, VIP is important for the integration of light input into the SCN oscillation. The aims of the work presented in this thesis were to determine the roles of the VIP and VPAC2 cells in controlling circadian rhythmicity, and to elucidate the mechanisms of VIP signalling that underpin these roles.

The first two experimental chapters utilise intersectional genetics and viral transduction to address separable roles for the VIP and VPAC2 cell populations. By diphtheria toxin-mediated cell ablation, or by adjusting cell-autonomous periodicity or rhythmicity specifically in these cell populations, I have identified that the VPAC2 cells are important for period setting and rhythmicity of both the SCN ex vivo and mouse behaviour in vivo, while the VIP cells play a vital role in behavioural rhythmicity and phase coherence across the SCN.

The next two chapters use application of VIP to SCN slices to address mechanisms of phase-resetting through pharmacological manipulation and microarray analysis. I find that VIP has long lasting effects on all major circadian parameters of the SCN slice oscillation at both the cellular and
circuit levels, and that it achieves this through a diversity of molecular pathways, in particular through cAMP/Ca\(^{2+}\) response elements within gene promoters.

The final chapter focuses primarily on DUSP4, a negative regulator of the MAP kinase pathway that I have demonstrated to be upregulated by VIP. Here I demonstrate that DUSP4 affects the steady-state period of SCN slices, as well as influences phase shifting characteristics of both slices and mice.

To conclude, the work presented here furthers our knowledge of neuropeptidergic communication in mammalian pacemaking. I have undertaken extensive characterisation of the molecular mechanisms through which the VIP neuropeptide influences SCN oscillators, and I have determined differential roles for the VIP and VPAC2 neurons in circadian timekeeping.
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<td>5-HT</td>
<td>5-hydroxytryptamine (serotonin)</td>
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<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
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<tr>
<td>AC</td>
<td>Adenylate cyclase</td>
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<td>ADAMTS</td>
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<td>AMPK</td>
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<td>Glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IEG</td>
<td>Immediate early gene</td>
</tr>
<tr>
<td>IGL</td>
<td>Intergeniculate leaflet</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>IP3</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>IVT</td>
<td>In vitro transcription</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LD</td>
<td>Light:dark</td>
</tr>
<tr>
<td>LHX1</td>
<td>Lim homeodomain transcription factor 1</td>
</tr>
<tr>
<td>LL</td>
<td>Constant light</td>
</tr>
<tr>
<td>MAB</td>
<td>Maleic Acid Buffer</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>MAPKK kinase</td>
</tr>
<tr>
<td>MAT2A</td>
<td>Methionine adenosyltransferase 2A</td>
</tr>
<tr>
<td>MECP2</td>
<td>Methyl CpG binding protein 2</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ERK Kinase</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>PACAP</td>
<td>Pituitary adenylate cyclase-activating polypeptide</td>
</tr>
<tr>
<td>PAM</td>
<td>Protoscaler adjacent motif</td>
</tr>
<tr>
<td>PAS</td>
<td>PER-ARNT-SIM</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
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<tr>
<td>PER</td>
<td>Period</td>
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<td>Paraformaldehyde</td>
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<td>PHI</td>
<td>Peptide histidine isoleucine</td>
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<td>Peptide histidine methionine</td>
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<td>Peptide histidine valine</td>
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<td>PKA</td>
<td>Protein kinase A</td>
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<td>Protein kinase C</td>
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<td>Phospholipase C</td>
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<td>PLD</td>
<td>Phospholipase D</td>
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<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>PTGS</td>
<td>Prostaglandin-endoperoxide synthase</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RA</td>
<td>Relative amplitude</td>
</tr>
<tr>
<td>RAE</td>
<td>Relative amplitude error</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
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<tr>
<td>RDB</td>
<td>RNA Dilution Buffer</td>
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<td>RHT</td>
<td>Retinohypothalamic tract</td>
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<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>ROR</td>
<td>RAR-related orphan receptor</td>
</tr>
<tr>
<td>RRE</td>
<td>ROR response element</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
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<tr>
<td>SCN</td>
<td>Suprachiasmatic nucleus</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SRE</td>
<td>Serum response element</td>
</tr>
<tr>
<td>TTFL</td>
<td>Transcriptional-translational feedback loop</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>VPAC2</td>
<td>VIP receptor</td>
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<tr>
<td>WT</td>
<td>Wild-type</td>
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<tr>
<td>β-TrCP</td>
<td>β-transducin repeat-containing protein</td>
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Publications and Presentations

Publications


Presentations


Hamnett, R., Hastings, M.H. Working Around the Clock: Circadian Modulation of Mammalian Sleep and Behaviour. Oral communication at Downing College MCR/SCR Seminar Night, Cambridge, UK, 20/05/15


CHAPTER 1

GENERAL INTRODUCTION

1.1 Circadian Rhythms

1.1.1 Rhythms on Earth

Cyclical events across a spectrum of timescales are present throughout the natural environment, ranging from the mechanics of a heartbeat to the seasonal migration of birds and insects. Pre-eminent among these are circadian rhythms, oscillations of approximately (circa-) one day (dies) that have evolved in response to the day-night cycle and encapsulate the timing of myriad biological phenomena (Pittendrigh, 1993). The presence of a circadian system thus constrains different aspects of physiology and behaviour to particular times of day or night, ensuring biological processes only occur during a suitable window, and allowing anticipation of predictable daily changes associated with light and dark (Aschoff, 1984). The near ubiquitous nature of rhythmicity, from single-celled eukaryotes to complex mammals, suggests it bestows a strong adaptive advantage. Direct evidence of this evolutionary value can be seen in studies of cyanobacteria and mammals, in which arrhythmic or non-resonant (with regards to period) organisms suffer more in competition assays or from predation compared to their rhythmic or resonant counterparts (DeCoursey et al., 2000; Ouyang et al., 1998).

In mammals, the most obvious manifestation of the circadian system is in the sleep-wake cycle, however numerous other processes are regulated temporally, including innate immunity (Curtis et al., 2014), body temperature (Aschoff, 1984), hormone release (Hastings, 1991) and cell division (Matsuo et al., 2003). Nevertheless, due to the universality of the circadian system,
significant pathologies can result if it is disrupted (Hastings et al., 2003). For example, shift work leads to dysregulation of the circadian system, and is associated with an increased prevalence of type II diabetes, cardiovascular conditions and cancer (Haus and Smolensky, 2013; Pan et al., 2011; Rararatnam and Arendt, 2001), while there is also some evidence to suggest that an aberrant circadian system is involved in the aetiology of psychiatric and neurodegenerative disorders (Kondratova and Kondratov, 2012; McClung, 2007).

1.1.2 Defining circadian rhythms

The first recorded observation of a circadian rhythm was noted by French scientist Jean-Jacques d’Ortous de Maira in the 18th century, who described 24 h leaf-opening behaviour of *Mimosa* plants that were not exposed to light. This suggested that the plants somehow “knew” the time of day and were responding accordingly. The persistence of an endogenous 24 h rhythm despite the lack of time-giving cues, or Zeitgebers, such as light or temperature, is one of the three hallmarks of a circadian oscillation, although these criteria were not established until some 300 years later by Colin Pittendrigh, a founder of the chronobiology field (Pittendrigh, 1960). The second criterion is that the clock must be able to entrain to environmental cues. Circadian rhythms are only approximately 24 h, therefore frequent resetting of the clock by external signals is required to ensure the endogenous cycle maintains synchronicity with the exogenous one. Finally, circadian rhythms must demonstrate temperature compensation, maintaining a 24 h rhythm even in the face of variable ambient temperature. The rates of biochemical reactions typically scale with temperature, occurring more quickly at higher temperatures, however a biological clock would have little value if it possessed these kinetics.
The main parameters that define an oscillation (Figure 1.1A) are period (distance between two equivalent points in the oscillation), amplitude (peak-trough distance) and phase (a defined point or interval within an oscillation, such as the peak). In mammals such as mice, these parameters are typically measured by recording wheel-running behaviour, which can then be plotted on an actogram. For example, mice can be exposed to a 12 h light, 12 h dark (12:12 LD) configuration, replicating a 24 h day, to determine entrainment, before being released into continuous darkness (DD) to allow the emergence of their free-running period (FRP; Figure 1.1B and C).

The phase of an oscillation is commonly described with reference to a phase-shift, whereby a defined point in an oscillation occurs later or earlier than expected based solely on its period, often due to an exogenous input (Figure 1.1D-H). By convention, this defined point occurring earlier would be a phase advance and would be denoted by a positive value (e.g. a 1 h phase shift), while the point occurring later would be referred to as a phase delay and would be negative (e.g. a -1 h shift). Furthermore, shifts can be described as either Type 0, in which the phase to which the oscillation is set is the same regardless of the phase at which the perturbation occurred, or Type 1, where the final phase following a perturbation is determined by the phase at which the resetting perturbation occurred. Thus by applying an input at different times throughout the day, a phase-response curve (PRC) can be generated (Figure 1.1I), which describes the response (phase shift) of an oscillation if it is perturbed in a given phase.

It is important to distinguish between the phase of the oscillation of an organism, and the phase of an external Zeitgeber. These can be separated through the use of circadian time (CT) and Zeitgeber time (ZT). CT refers to the endogenous time of the organism, and by convention CT12 denotes the start of the subjective night, coincident in nocturnal mammals, therefore, with
Figure 1.1 Measuring circadian oscillations

(A) Representation of two major circadian parameters: period, the distance between two equivalent points in an oscillation; and amplitude, the distance between the peak and trough. (B and C) Schematics of wild-type (WT) circadian activity, such as of gene expression (B) or wheel running behaviour, doubled plotted on an actogram in a 12:12 light:dark cycle (LD) followed by continuous darkness (DD) (C), compared to a short period (e.g. Ck1εTau/Tau) and long period (e.g. Fbxl3Af/Af). The short period mouse in (C) is showing no entrainment to the LD cycle, while the long period mouse is showing partial entrainment. Both free-run with their endogenous period in DD. Grey shading represents lights off. (D and E) Phase advance (D) or delay (E) in response to a stimulus (marked by *). Dashed line represents the continuation of an unperturbed rhythm, while the red line shows the shifted rhythm. (F-H) Schematics of wheel running activity of a WT mouse in LD and DD, followed by a stimulus applied in the middle of subjective day (F; no response), late subjective night (G; phase advance) or early subjective night (H; phase delay). Red lines represent the onsets of activity before and after the stimulus. (I) A schematic of a light phase response curve (PRC) generated from experiments such as those performed in (F-H), showing the magnitude of phase shift in response to a light stimulus presented at different times throughout the day, in DD.
activity onset. Furthermore, CT is always divided into 24 circadian hours, regardless of absolute period length. In contrast, ZT reflects solar time and is therefore relevant when an animal is in a light-dark cycle. ZT0 refers to dawn and, in 12:12 LD, ZT12 would refer to dusk, and again activity onset of nocturnal rodents.

1.1.3 Cell-autonomous oscillators

Despite circadian rhythms first being recognised at the organismal level, a molecular underpinning has now been well established, initially through chemical mutagenesis screens for circadian phenotypes. Konopka and Benzer were the first to identify circadian mutants using *Drosophila melanogaster*, observing flies that had a 19 h period, a 28 h period or were arrhythmic (Konopka and Benzer, 1971). Each of these mutants contained base substitutions that mapped to the same gene, named *period* (*per*), and suggested that the cellular basis of timekeeping might be protein-based and heritable.

The first mammalian circadian mutant, known as the Tau hamster, had a 20 h FRP in homozygotes, which struggle to entrain to a 24 h LD cycle (Ralph and Menaker, 1988), although it was only later that a single point mutation within the gene encoding casein kinase 1 epsilon (CK1ε) was found to be responsible (Lowrey et al., 2000). Further mammalian clock genes were discovered shortly thereafter. In 1994, an N-ethyl-N-nitrosourea (ENU) mutagenesis screen identified the circadian locomotor output cycles kaput (*Clock*) gene by mapping the locus of a mutation causing a 28 h FRP and unstable activity rhythms in homozygotes (Vitaterna et al., 1994). The CLOCK protein was identified as a transcription factor with a basic helix-loop helix (bHLH) DNA-binding domain, as well as a PER-ARNT-SIM (PAS) domain (Antoch et al.,
1997; King et al., 1997). PAS domains had been found to be important for PER clock protein interactions in *Drosophila* (Huang et al., 1993), suggesting that a binding partner for CLOCK may exist. BMAL1, also known as MOP3, was identified as such a binding partner, and was found to be necessary for activation of transcriptional targets (Gekakis et al., 1998; Hogenesch et al., 1998). Furthermore, genetic deletion of BMAL1 renders mice arrhythmic (Bunger et al., 2000), suggesting that it is a non-redundant component of the circadian system, in contrast to the other members of the core oscillator.

At a similar time, mammalian *Period (Per1 and Per2)* and *Cryptochrome (Cry1 and Cry 2)* homologues were discovered (van der Horst et al., 1999; Sun et al., 1997; Tei et al., 1997), with evidence for interactions between the components also emerging (Kume et al., 1999). Thus the core circadian transcriptional-translational feedback loop (TTFL), first posited by Hardin et al. in *Drosophila* (Hardin et al., 1990), was established in mammals, as shown in Figure 1.2A. During the day, CLOCK and BMAL1 heterodimerise to activate enhancer-box (E/E’-box) elements (sequence CACGTG) in the promoters of *Per* and *Cry* to induce their transcription. PER and CRY proteins then heterodimerise, translocate to the nucleus and feedback onto CLOCK:BMAL1 to inhibit transcription of their cognate genes. With time, progressive degradation of PER and CRY removes the inhibition on CLOCK:BMAL1, allowing the cycle to start again. The complete cycle of protein synthesis and degradation takes approximately 24 h, although the exact nature and kinetics of this cycle are not well understood. It is worth noting that this process of transcriptional activation followed by repression is well conserved across the cellular clocks of many types of organism, including plants (Nohales and Kay, 2016), fungi (Aronson et al., 1994) and *Drosophila* (Tataroglu and Emery, 2015), although the exact identities of the protein components differ considerably.
Figure 1.2 Molecular regulation of the mammalian circadian transcriptional-translational feedback loop (TTFL)

(A) The core loop begins with CLOCK:BMAL1 heterodimers stimulating the transcription of Per and Cry genes by activating E-box elements within their promoters. Period (PER) and Cryptochrome (CRY) are translated and heterodimerise before translocating to the nucleus to inhibit their own transcription. PER and CRY stability is regulated by post-translational modifications (PTMs; represented by yellow pentagons), including phosphorylation by CK1ε/δ and AMPK, and ubiquitination by β-TrCP and FBXL proteins, resulting in proteasomal degradation. An auxiliary loop is also present, whereby Rev-erb and Rora transcription are stimulated by CLOCK:BMAL1. REV-ERB and RORA proteins suppress and activate Bmal1 expression respectively through ROR response elements (RREs). Rhythmic transcription of clock-controlled genes (CCGs) results from these interlocking loops. Dashed lines represent transcription. AMPK, 5' adenosine monophosphate-activated protein kinase; β-TrCP, beta-transducin repeat containing protein; CK1, casein kinase 1; FBX, F-box protein; ROR, retinoic acid receptor-(RAR-) related orphan receptor. (B) Schematic representation of clock gene levels throughout the circadian cycle. Bmal1 expression is in an approximate antiphase with Per and Cry.
This core feedback loop is augmented by an auxiliary loop (Figure 1.2), providing an extra level of precision and robustness to the mammalian TTFL. Two orphan nuclear receptor families, REV-ERB and retinoic acid receptor-(RAR-) related orphan receptor (ROR), repress and activate Bmal1 expression, respectively, by binding ROR response elements (RREs; sequence [A/T][A/T]NT[A/G]GGTCA (Harding and Lazar, 1993)) within the Bmal1 promoter (Guillaumond et al., 2005). The actions of REV-ERB are delayed relative to ROR, resulting in rhythmic expression of Bmal1 (Preitner et al., 2002). The result is that components of the negative limb of the TTFL (PER and CRY) oscillate in approximate antiphase to BMAL1 in the positive limb (Figure 1.2B; Oishi et al., 1998; Shearman et al., 2000).

These two loops form the primary cell-autonomous oscillator, although new discoveries are frequently being made that add the complexity of the clock. These include translation elongation factor 4E-BP1, which regulates translation of Per mRNA (Cao et al., 2015), and CHRONO, which is regulated by BMAL1 and believed to form an independent negative feedback arm of the circadian clock (Goriki et al., 2014; Hatanaka et al., 2010). Furthermore, clock genes are not only controlled via E-box elements, but multiple other promoter elements including D-boxes (sequence TTA[T/C]GTAA; Falvey et al., 1996), cAMP response elements (CREs; sequence TGACGTCA (Shaywitz and Greenberg, 1999)) and serum response elements (SREs; sequence CC[A/T]6GG (Treisman, 1992)), forming a complex network of transcriptional regulation (Takahashi, 2017; Ueda et al., 2005). These elements also provide access to the TTFL for input pathways activated by exogenous stimuli to influence clock gene transcription, and as such serve as integration and entrainment points for the TTFL.
1.1.4 Non-transcriptional components of the TTFL

Despite the importance of the TTFL, transcriptional regulation is not the only important component of the cell-autonomous oscillator. Indeed, anucleate red blood cells maintain rhythmic oscillations in reduction and oxidation states of peroxiredoxin enzymes, despite the lack of transcription (O’Neill and Reddy, 2011), a mechanism that appears to be conserved across all domains of life (Edgar et al., 2012; O’Neill et al., 2011). Furthermore, the cellular clock of cyanobacteria is well established as not requiring transcriptional regulation, revolving around the rhythmic phosphorylation states of KaiA, KaiB and KaiC (Kageyama et al., 2006; Nishiwaki et al., 2000; Xu et al., 2003). This self-sustaining oscillator is then coupled to a slave TTFL to influence rhythmic gene expression (Qin et al., 2010).

Posttranslational modifications (PTMs) also play a large role in regulating the mammalian TTFL (Figure 1.2A). CK1ε and its homologue CK1δ both phosphorylate PER proteins to regulate stability as well as translocation to the nucleus, with the previously mentioned Tau mutation causing faster degradation of PER, resulting in a shorter period (Maywood et al., 2014; Meng et al., 2010, 2008). Phosphorylation of PER targets it for ubiquitination by β-transducin repeat-containing protein (β-TrCP), ultimately resulting in proteasomal degradation (Shirogane et al., 2005). CRY proteins are regulated in a similar fashion, whereby ubiquitination by F-box proteins FBXL3 and FBXL21 in a compartment-specific manner (Godinho et al., 2007; Siepka et al., 2007; Yoo et al., 2013) as a result of phosphorylation from several kinases (e.g. 5' adenosine monophosphate-activated protein kinase (AMPK; Lamia et al., 2009) and glycogen synthase kinase 3β (GSK-3β)/dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1A; Kurabayashi et al., 2010) also leads to degradation. As noted above, this degradation ultimately relieves inhibition on CLOCK:BMAL1, and thus forms a vital part of the
TTFL. This can be seen from their ability to affect TTFL period, as with the Tau mutation as well as the Afterhours mutation, which results in a loss of function of FBXL3 (Godinho et al., 2007). In Fbxl3^{Afh/Afh} mice, CRY is degraded more slowly, therefore a longer period is observed and this effect is independent of, and additive to, the acceleration caused by Tau-mediated PER instability.

A final non-transcriptional component of the mammalian cellular clock is that of cytosolic rhythms of 2nd messenger molecules, such as cAMP and Ca^{2+} (Hastings et al., 2008). Ca^{2+} rhythms in the cytosol were first thought of as an output of the TTFL, generated from intracellular stores and driven by the cell-autonomous oscillator (Colwell, 2000a; Ikeda et al., 2003). The ubiquity of Ca^{2+} in physiological processes would therefore likely result in the rhythmic expression of numerous downstream genes. These genes, however, include some core clock components, and the observation that manipulation of intracellular Ca^{2+} levels via activation of Gαq proteins induces changes in the circadian clock (Brancaccio et al., 2013) implies that Ca^{2+} is an input as well as an output of the cellular oscillator.

cAMP has also been shown to be both an input and output of the circadian clock (Nikaido and Takahashi, 1989; O’Neill et al., 2008), although stimulation of Gαs (which would increase cAMP levels) did not have a strong effect on circadian parameters compared to Gαq (Brancaccio et al., 2013), suggesting it serves a lesser role in the self-sustaining TTFL. Instead, cAMP acts as a mediator of extracellular signals by activating protein kinase A (PKA), which in turn phosphorylates cAMP response element-binding protein (CREB), activating transcription via CREs. Per1 and Per2 genes contain CREs within their promoters (Travnickova-Bendova et al., 2002), thus providing a clear pathway through which cAMP can influence the TTFL. Ca^{2+} levels can also influence the phosphorylation state of CREB, although this is primarily
through the mitogen-activated protein kinase (MAPK) pathway (Grewal et al., 2000).

1.1.5 Outputs of the molecular clock

A self-sustaining cellular oscillator is of little value if it has no means by which to influence cellular processes. The TTFL is able to exert effects over cellular activity, however, and indeed nearly half of all mammalian genes are rhythmically expressed in at least one tissue, although the identities of these genes are often highly tissue specific (Panda et al., 2002; Zhang et al., 2014). The influence of the clock on Ca\(^{2+}\) and cAMP levels, upstream of CRE activation, constitutes one mechanism by which the TTFL can alter gene regulation. Genes regulated directly by clock genes, known as clock-controlled genes (CCGs; Figure 1.2A), typically contain E-box elements in their promoters, much like Per and Cry, allowing CLOCK:BMAL1 to activate their transcription. In total, CLOCK and BMAL1 are thought to regulate ~3000 transcripts in the liver alone (Koike et al., 2012). A key example is D-element binding protein (DBP; Ripperger et al., 2000; Wuarin and Schibler, 1990), a transcription factor that binds to D-boxes in promoters to stimulate transcription. As well as providing a direct input into the TTFL (ROR family and Per gene promoters contain D-boxes; Takahashi, 2017), downstream genes are also regulated by DBP, particularly with regards to hepatic metabolic processes (Schrem et al., 2004).

The circadian repressors, particularly CRY1 and CRY2, also affect genomic regulation. In addition to repressing CLOCK:BMAL1 at their own promoters, CRY1 and CRY2 bind to several thousand sites, often independent of CLOCK:BMAL1, and frequently affecting the transcription of nuclear receptors (Koike et al., 2012). This includes the glucocorticoid receptor (GR),
with CRY deficiency greatly increasing the amount of GR-regulated transcripts induced in response to dexamethasone (Lamia et al., 2011). Transcriptional regulation by CRY, and indeed by other core clock components, frequently requires the recruitment of co-activators, such as other transcription factors and chromatin remodelling factors to either activate or repress transcription (Koike et al., 2012; Takahashi, 2017). Thus, the circadian TTFL results in a vast network of rhythmically regulated transcripts across diverse and major cellular processes (Panda et al., 2002), with a range of phases of expression, by which it can ultimately affect organismal physiology and behaviour.

1.2 The Suprachiasmatic Nucleus

1.2.1 The SCN is the master circadian pacemaker

The presence of circadian oscillators in almost every clock in the body suggests that a synchronising mechanism is required to prevent minor period differences or differential responsiveness to a variety of entraining cues from resulting in internal phase desynchrony (Figure 1.3). The role of master pacemaker falls to the suprachiasmatic nucleus (SCN) of the hypothalamus, a paired nucleus of approximately 20,000 cells situated immediately dorsal of the optic chiasm and either side of the 3rd ventricle (Welsh et al., 2010). Through dictating the rhythmicity of cellular clocks located throughout the body, the SCN thus determines rhythms in activity, sleep, metabolism and hormone release (e.g. from endocrine tissues such as the pituitary and pineal glands) among others (Figure 1.3). The SCN is robustly rhythmic in clock gene expression, metabolism, electrical activity and peptide release and is not easily perturbed by external influences, allowing it to maintain a consistent phase regardless of body temperature, stress and feeding (Weaver, 1998).
Nevertheless, light is capable of readily resetting it (Yamazaki et al., 2000), highlighting the prominence of light as the primary Zeitgeber in mammals. The SCN is ideally situated to respond to light information because it receives direct input from the retina via the retino-hypothalamic tract (RHT), therefore allowing it to match the phase of the organism to solar time.

The first strong evidence that the SCN was the location of the mammalian pacemaker came from two suprachiasmatic lesion studies in 1972, resulting in the abolition of rhythms of corticosterone (Moore and Eichler, 1972), drinking and locomotor activity (Stephan and Zucker, 1972). These studies had been prompted by an autoradiographical tract tracing study showing that the SCN
received direct retinal projections, separate from the pathway involved in vision forming (Moore and Lenn, 1972). Conclusive proof that the SCN was the site of the pacemaker came from later transplant studies. Transplantation of an intact foetal SCN into animals with an ablated SCN restored diurnal and circadian rhythmicity (Drucker-Colin et al., 1984; Lehman et al., 1987), while the behavioural period of the transplant recipient was determined by the period of the grafted SCN (Ralph et al., 1990). As well as *in vivo* work, the SCN can also be cultured *ex vivo* as an organotypic brain slice and maintain robust rhythmicity. This technique, combined with the development of numerous reporter strains, including fluorescent or bioluminescently tagged clock proteins and transcriptional reporters (e.g. Per1-GFP, Per1-Luciferase (Luc), Cry1-Luc and PER2::LUC), has greatly expanded study of how the SCN functions, offering the advantages of a reductionist system while still maintaining the complexity of the highly interconnected SCN circuit. Although individual SCN neurons do display rhythmicity when separated in dispersal cultures (Herzog et al., 1998; Honma et al., 1998; Welsh et al., 1995), the exact cytoarchitecture of the SCN appears to be important for its normal function.

### 1.2.2 The SCN as a network

What is special about the SCN that makes it so suited to pacemaking in mammals? Surprisingly, individual SCN neurons are actually considered to be “weak” clocks, with only ~60% demonstrating competent rhythmicity when dispersed at low density, and only ~30% when completely isolated (Webb et al., 2009), as well as no ability to maintain phase synchrony in the remaining rhythmic cells (Welsh et al., 1995). In contrast, fibroblasts are strong oscillators that require no input from surrounding cells to maintain rhythmicity (Nagoshi et al., 2004; Welsh et al., 2004), although they too exhibit
no capacity to maintain consistent phase relationships. Furthermore, fibroblasts are easily resettable by a number of factors, including temperature, glucocorticoids and serum shock (Balsalobre et al., 1998, 2000; Buhr et al., 2010). The key factor, therefore, in the ability of the SCN both in vivo and ex vivo to maintain robust rhythmicity is its highly interconnected circuitry of coupled oscillators (Aton and Herzog, 2005; Welsh et al., 2010). This can be directly demonstrated through the addition of tetrodotoxin (TTX), a potent neurotoxin that inhibits action potentials by blocking voltage-gated sodium channels. When neurons of the SCN are unable to communicate synaptically, their ability to maintain a coherent phase relationship is strongly attenuated (Patton et al., 2016; Schwartz et al., 1987; Yamaguchi et al., 2003).

When SCN circuit-level organisation is preserved, oscillations are highly robust and follow a defined phase relationship between its regions of specialised neuronal populations (discussed further in 1.2.3). This can be most easily observed through the use of bioluminescence reporters, such as the PER2::LUC fusion protein, which reports the sum of both transcriptional and translational activity of PER2 (Yoo et al., 2004). When visualised on camera, SCN slices show a defined spatiotemporal wave of bioluminescence, beginning in the dorsomedial region and progressing ventrolaterally (Welsh et al., 2010; Yan et al., 2007). This phase diversity allows output signals to the periphery to be differentially phased (Kalsbeek et al., 2006), and the spatiotemporal waveform itself may be a method of encoding photoperiod, as phase distribution is greater following exposure to long day photoperiods compared to short photoperiods (Evans et al., 2013; Meijer et al., 2010; Vanderleest et al., 2007).

Coupling also provides resistance to perturbation. For example, unlike peripheral tissues, the SCN is not affected by temperature cycles unless coupling is disrupted (Buhr et al., 2010). Remarkably, intercellular
communication is even able to overcome deficits in the TTFL, including mutations in *Clock* (Nakamura et al., 2002) as well deficiencies of *Bmal1, Per1, Cry1* or both *Cry1* and *Cry2* (CRY double KO or CryDKO) (Evans et al., 2012; Ko et al., 2010; Liu et al., 2007; Maywood et al., 2011). Thus although coupling mechanisms may exist to some extent in other tissues, particularly the retina and olfactory bulb (Abraham et al., 2005; Tosini and Menaker, 1996), intercellular communication appears to play a particularly vital role in the function of the SCN of maintaining phase synchrony with the external environment and ensuring organismal internal phase synchrony, a feat that is achieved and mediated primarily through its unique complement of neuropeptides.

1.2.3 SCN Anatomy and Neuropeptides

The vast majority of neurons within the SCN release γ-Aminobutyric acid (GABA) (Moore and Speh, 1993), however the SCN also expresses a diverse array of neuropeptides within specific regions, defining neuronal subclasses with distinct roles in SCN function (Figure 1.4A; Abrahamson and Moore, 2001; Antle and Silver, 2005; van den Pol and Tsujimoto, 1985). Recent peptidomic studies estimate there to be over 100 neuropeptides in the SCN, although many of these represent differentially regulated forms of common peptides (Lee et al., 2010b), and single-cell transcriptomic studies agree that the SCN network is more complex than previously anticipated (Park et al., 2016). Interestingly, many of the neuropeptides do not necessarily function at synapses, with evidence for paracrine or non-synaptic release (Castel et al., 1996; Maywood et al., 2011). This is further supported by the restoration of locomotor activity following an arrhythmic host receiving an SCN transplant that was situated inside a semipermeable capsule (Silver et al., 1996). This
Figure 1.4 Schematic representation of spatial organisation of cell populations, inputs and outputs of the mouse suprachiasmatic nucleus (SCN)

(A, Left) Major cell subpopulations in the SCN. The ventrolateral core is typically characterised by vasoactive intestinal peptide (VIP) and gastrin-releasing peptide (GRP) expression, while arginine vasopressin (AVP) is present in the dorsomedial shell near the 3rd ventricle (3V). The VIP receptor, VPAC2, is predominantly shell-localised. (A, Right) Terminal fields of input nerve fibres to the SCN and their predominant neurotransmitter or neuropeptide markers. Retinal input via the retinohypothalamic tract (RHT) is transmitted by glutamate (Glu) and pituitary adenylate cyclase-activating polypeptide (PACAP). Input from the raphe nucleus is mediated by serotonin (5-HT) and, along with retinal input, predominantly targets the core. Input from the intergeniculate leaflet (IGL) via the geniculohypothalamic tract (GHT), mediated by neuropeptide Y (NPY), also preferentially targets the core, with sparser projections further dorsal (represented by the colour gradient). Note that boundaries for all regions indicated are nebulous. OC, optic chiasm. (B) SCN output pathways. Most SCN outputs are relatively local, relying on nuclei such as the paraventricular nucleus (PVN) and subparaventricular zone (sPVZ), although direct connections to other areas have also been observed, as shown. Regions shown are not exhaustive. ARC, arcuate nucleus; BNST, bed nucleus of the stria terminalis; DMH, dorsomedial hypothalamus; POA, preoptic area.
capsule prevented neural outgrowth but allowed diffusion of humoral signals, although some functions of the SCN, such as reproduction-related rhythms, do appear to require “hard-wired” synaptic output (Lehman et al., 1987; Meyer-Bernstein et al., 1999).

The SCN is frequently described as having two main regions: the ventrolateral core and the dorsomedial shell (Moore et al., 2002). The core is typically characterised by retinal (as well as non-photic) innervation and presence of vasoactive intestinal peptide- (VIP-) and gastrin releasing peptide- (GRP-) expressing neurons, while the shell contains arginine vasopressin (AVP) and is largely responsible for the output of SCN signals (Abrahamson and Moore, 2001). The shell is also regarded as being more robustly rhythmic, such as in electrical firing rhythms, while the core is readily reset by light input (Antle and Silver, 2005; Hamada et al., 2001; Nakamura et al., 2001, 2005; Saeb-Parsy and Dyball, 2003). Despite this, the shell appears to require input from the core to maintain its rhythmicity, as surgical separation of the two results in desynchronisation within the shell, but little effect on the core (Yamaguchi et al., 2003). Further importance of the core is highlighted by selective microlesions, resulting in the abolition of multiple rhythmic processes (Kriegsfeld et al., 2004; LeSauter and Silver, 1999), although such surgical approaches lack anatomical precision.

This core/shell division is, however, a gross simplification (Morin, 2007). First, there are considerably more neuropeptides than just the three mentioned above, including met-enkephalin, angiotensin II and neurotensin, which adhere to the core/shell delineation to varying degrees (Abrahamson and Moore, 2001). Second, different divisions have also been found in the expression of receptors or other proteins which cross classical core/shell boundaries (Geoghegan and Carter, 2008; Lee et al., 2015; Morin et al., 2011; Smyllie et al., 2016). Furthermore, differences in neuropeptide expression
differ across the rostro-caudal axis as well as the dorso-ventral one, further blurring the core/shell distinction (Evans et al., 2011). Nevertheless, the terminology remains a useful shorthand to refer to regions of the SCN as described above, and as such is utilised throughout this thesis.

As well as separable roles for the core and shell, distinct functions of individual neuropeptides have also been elucidated. VIP is the best characterised in the SCN, and has roles in SCN synchrony as well as light input (Vosko et al., 2007), however this will be discussed extensively later on (section 1.3). AVP is thought to act as a major output signal from the SCN, in particular controlling the rhythmic activation of the hypothalamic-pituitary-adrenal (HPA) axis (Kalsbeek et al., 2010). Within the SCN, recent evidence has found a role for AVP in stabilising intercellular phases, thereby limiting entrainment to new lighting schedules: mice deficient in the AVP receptors V1a and V1b immediately re-entrain to 12:12 LD cycles phase advanced or delayed by 8 h, a finding replicated using V1a/1b antagonists (Yamaguchi et al., 2013). The SCN of these mice also showed poor phase organisation following treatment with translational inhibitor cycloheximide (CHX). This, combined with the wide expression of V1a in the SCN (Li et al., 2009; Zhu et al., 2012), suggests that AVP may feed back to the rest of the SCN to maintain a stable phase, particularly following lighting shifts, in order to prevent large shifts in overall SCN rhythmicity from occurring. AVP may also play a role in SCN synchrony, however this is only observable in the absence of VIP signalling, implying a strong hierarchy of coupling signals in the SCN (Maywood et al., 2011).

GRP, which acts through BB2 receptors, is also capable of compensating for VIP deficits (Brown et al., 2005; Maywood et al., 2006), although it is situated below AVP in the aforementioned hierarchy (Maywood et al., 2011). Deficiencies in GRP signalling tend to have minimal effects on rhythmicity
(Aida et al., 2002), however application of GRP to SCN slices, or injection into the SCN in vivo, results in a phase shift in a phase-dependent manner, which it achieves through induction of Per genes in the dorsal SCN (Aida et al., 2002; Gamble et al., 2007; McArthur et al., 2000; Piggins et al., 1995). BB2 knockout mice also phase shift significantly less to strong light, although a shift is still present (Aida et al., 2002). These data suggest that GRP can contribute to light responsiveness, but that it is not the only neuropeptide to do so, and is likely situated below VIP in the hierarchy for importance to both light input and general synchrony.

GABA has long been considered the primary neurotransmitter of the SCN, with extensive GABA and GABA receptor (GABA<sub>A</sub> and GABA<sub>B</sub>) expression throughout the SCN (Abrahamson and Moore, 2001; Belenky et al., 2003; Gao et al., 1995; Moore and Speh, 1993; O’Hara et al., 1995; van den Pol and Tsujimoto, 1985). Despite this ubiquity, the exact role of GABA in the SCN has been difficult to determine (Evans, 2016), not least because this canonically inhibitory neurotransmitter has been observed as being excitatory in the SCN under some conditions (Choi et al., 2008; Wagner et al., 2001). Furthermore, GABA has been shown to be capable of both synchronising (Liu and Reppert, 2000) and desynchronising (Aton et al., 2006) SCN neurons. Strong evidence for GABA playing a role in transmission of information from the retinorecipient cells to the dorsomedial cells is accumulating (Albus et al., 2005; Han et al., 2012), a role for which VIP is also known to be important. Interestingly, antagonism between GABA and VIP has been observed, whereby VIP acts as a potent synchronising agent whereas GABA desynchronises (Aton et al., 2006; Evans et al., 2013; Freeman et al., 2013). Thus, in weakly coupled VIP knockout SCN, GABA antagonism actually restores synchrony, suggesting that the desynchrony observed as a result of VIP deficiencies is not merely passive, but also active desynchronisation by
GABA (Evans, 2016; Evans et al., 2013). The fact that this occurs depending on the state of the network (e.g. based on previous photoperiod exposure (Evans et al., 2013)) may explain some of the seemingly contradictory results observed previously.

Prokineticin 2 (Prok2) is a clock- and light-controlled SCN output signal (Cheng et al., 2002; Zhang et al., 2009; Zhou and Cheng, 2005), however it seems to have no role in SCN rhythmicity per se (Li et al., 2006; Prosser et al., 2007). Little SAAS was discovered by neuropeptidomic studies as being present in the SCN (Hatcher et al., 2008; Lee et al., 2010b), and it appears to colocalise predominantly with GRP cells, serving a role in relaying light information, albeit independent of the actions of VIP and GRP (Atkins et al., 2010). Some additional peptides, such as calbindin (Kriegsfeld et al., 2004; LeSauter and Silver, 1999), neurotensin (Coogan et al., 2001; Meyer-Spasche et al., 2002) and angiotensin II (Brown et al., 2008; Mistlberger et al., 2001) do appear to influence SCN physiology, although less dramatically than the peptides already described, so have therefore received less attention. Nevertheless, considerable species differences can exist with regards to neuropeptide function, for example neurotensin appears to be particularly important in the human SCN (Goncharuk et al., 2001; Harper et al., 2008).

1.2.4 SCN inputs and outputs

While the SCN is vital to rhythmicity of the organism, to impose this it requires a downstream network of oscillators and effectors that will ultimately determine the behaviour of the organism. Moreover, despite its remarkable consistency, the period of the SCN is rarely exactly 24 h, thus to fulfil its role of maintaining an adaptive phase relationship with the external world, input is essential to reset the SCN each day. Therefore the mammalian
circadian system can be viewed as a multi-component system consisting of inputs, the SCN, and outputs (Figure 1.3). An array of techniques such as immunohistochemistry (Abrahamson et al., 2001) and both anterograde and retrograde tract tracing (Abrahamson and Moore, 2001; Lokshin et al., 2015; Moga and Moore, 1997; Watts and Swanson, 1987; Watts et al., 1987) have been employed to uncover the localisation of these input and output tissues and how they map onto the specific organisation of the SCN.

Inputs

Light-dependent retinal activation is the most important input to the SCN, transmitted via the RHT (Figure 1.4A). This retinal innervation maps primarily onto the ventrolateral region co-localising with VIP and GRP cells, with labelling becoming more sparse towards the dorsal region of the SCN (Aïoun et al., 1998; Lokshin et al., 2015; Moga and Moore, 1997). The circadian visual system is separate from the vision-forming system (involving the lateral geniculate thalamus and visual cortices), not just in the final destination but also in the photic receptors. Instead of the rods and cones required for vision, irradiance input to the circadian system is mediated by intrinsically photosensitive retinal ganglion cells (ipRGCs), which contain the light-receptive protein melanopsin, and the axons of which make up the RHT (Berson et al., 2002; Hattar et al., 2002; Lucas et al., 2001a, 2001b), although rods and cones do still contribute to circadian photic input (Hattar et al., 2003).

Activation of the RHT by light triggers the release of glutamate from terminals projecting to the SCN (reviewed in Ebling, 1996), which is modulated by pituitary adenylate cyclase-activating polypeptide (PACAP; Chen et al., 1999; Hannibal, 2002; Hannibal et al., 1997, 2000; Harrington et al.,...
Depolarisation of the retinorecipient cells follows, leading to Ca\textsuperscript{2+} influx (Meijer and Schwartz, 2003). This activates a web of kinases, including calcium/calmodulin-dependent protein kinase (CaMK) and MAPK pathways, ultimately resulting in the phosphorylation of CREB and subsequent expression of the Per genes (Wakamatsu et al., 2001). Phosphorylation of CREB occurs within minutes if a light pulse is applied during the subjective night, when light is readily capable of phase shifting rhythms (Gau et al., 2002; Ginty et al., 1993), and this gating is modulated at least in part by circadian glutamate receptor sensitivity (Colwell, 2001; Pennartz et al., 2001).

Regionality of terminal fields providing input to the SCN clock is not unique to the retina (Figure 1.4A). Similar targeting to the SCN core can also be observed in inputs from the thalamus, pretectum and median raphe nucleus (Morin, 2013; Morin and Allen, 2006), and these non-photic inputs can have differential effects on SCN phase and PER induction compared to photic (Maywood et al., 1999, 2002). Input from the intergeniculate leaflet of the thalamus (IGL) via the geniculohypothalamic tract (GHT) tends to be regarded as the second major input pathway to the SCN, with neuropeptide Y (NPY) in particular marking out GHT nerve terminals (Morin, 2013). Input from the IGL, as well as from the raphe nuclei using serotonin (5-hydroxytryptamine (5-HT)) as a neurotransmitter, may mediate non-photic input or non-linear photic input to the SCN (Hastings et al., 1997; Morin, 2013).

**Outputs**

The SCN communicates with the periphery and other regions of the brain using both humoral and electrical signals (Figure 1.3). Evidence of this can be found in the restoration of locomotor rhythms in SCN-ablated mice by foetal
SCN transplant, but not of endocrine rhythms (Lehman et al., 1987; Meyer-Bernstein et al., 1999; Silver et al., 1996). Diffusible signals maintaining peripheral rhythmicity have also been observed in culture, with fibroblasts capable of sustaining PER2::LUC rhythms for longer if co-cultured with the immortalised SCN2.2 cell line, implicating a humoral signal (Farnell et al., 2011). Within the brain, most SCN outputs are relatively local (e.g. in the hypothalamus; Figure 1.4B; Kalsbeek and Buijs, 2002; Watts et al., 1987), suggesting that much of the ability of the SCN to convey information is mediated by relay nuclei, such as the arcuate nucleus (Buijs et al., 2017). A key recipient of SCN projections is the paraventricular nucleus (PVN), and this is a key gateway to influencing peripheral tissues, such as the liver, through both the sympathetic and parasympathetic nervous systems (Buijs et al., 2003).

As mentioned previously, AVP and Prok2 represent two of the major output molecules from the SCN, with the shell having more output projections than the core (Kalsbeek et al., 2010; Leak and Moore, 2001; Zhang et al., 2009; Zhou and Cheng, 2005). Indeed, the phase of the SCN shell does seem the primary determinant of downstream tissue phase as established by desynchronising SCN regions with long photoperiod exposure (Evans et al., 2015), as well as REM sleep (Lee et al., 2009) and reproductive hormone phases (Smarr et al., 2012). However, the core does not merely project to the shell; it has numerous targets throughout the brain that it is capable of aligning in phase, and many SCN terminal fields have been found to contain both AVP- and VIP-positive fibres, although the recipient cells may constitute separate populations within a given region (Abrahamson and Moore, 2001; Kalsbeek and Buijs, 2002). Furthermore, the ventrolateral region has been found to project to the PVN (Yan et al., 2005) and gonadotropin-releasing hormone (GnRH) cells (van der Beek et al., 1993). Forced desynchrony protocols involving changing
photoperiod (e.g. to a 22 h day) can be used to investigate distinct outputs from the shell and core because the core typically stays entrained to the light:dark cycle while the shell free-runs (Mohawk and Takahashi, 2011). Under such protocols, multiple rhythms in locomotor activity, slow wave sleep and body temperature emerge, presumably as a result of different regional outputs from the SCN, while differential regulation of corticosterone rhythms can also be observed (Cambras et al., 2007; de la Iglesia et al., 2004; Wotus et al., 2013). Taken together, these results indicate that both the shell and the core are important for output signals from the SCN, although the core is typically more associated with environmental input that it then transmits to the shell via a number of neuropeptides, particularly VIP.

1.3 Vasoactive intestinal peptide in the SCN

1.3.1 VIP and its receptors

VIP is a 28 amino acid neuropeptide first isolated in 1970 from porcine duodenum (Said and Mutt, 1970), although its expression is widespread across both the central and peripheral nervous systems (Couvineau and Laburthe, 2012). VIP is involved in numerous physiological and pathological processes, and loss of VIP signalling causes disruptions in metabolism (Bechtold et al., 2008), cardiovascular function (Sheward et al., 2010) and reproduction (Loh et al., 2014), as well as circadian rhythms (Maywood et al., 2007). VIP is part of the secretin/VIP family, which includes peptides such as glucagon and PACAP that are structurally and functionally related (Couvineau and Laburthe, 2012). Similarly to PACAP, VIP is first translated as a larger precursor (prepro-VIP) that subsequently gets cleaved to produce VIP (Harmar et al., 2012) along with peptide histidine isoleucine (PHI; in non-human mammals (Tatemoto and Mutt, 1981)) or peptide histidine methionine
(PHM; in humans (Itoh et al., 1983)) and peptide histidine valine (PHV; a C-terminal extension of PHI/PHM (Yiangou et al., 1987)).

The activities of VIP are mediated through the receptors VPAC1 and VPAC2, which are class II G-protein-coupled receptors (GPCRs). This family of receptors includes receptors for secretin-family peptides as well as others, such as parathyroid hormone and calcitonin (Laburthe et al., 2007). In contrast to PAC1, which is a receptor selective for PACAP (although it will respond to VIP at higher concentrations), VPAC1 and VPAC2 have approximately equal affinity for PACAP and VIP (Harmar et al., 2012). Despite the wide expression of VPAC1 and VPAC2 across the nervous system and periphery, only VPAC2 is found in the SCN (Sheward et al., 1995; Usdin et al., 1994), along with PAC1, which mediates light input via the RHT (Hannibal, 2002).

Mechanistically, the VPAC2 receptor classically couples to the Gαs G-protein subunit (Figure 1.5; Couvineau et al., 1986; Kermode et al., 1992), stimulating adenylate cyclase (AC) to produce cAMP (Dickson and Finlayson, 2009). Notwithstanding its ubiquity, cAMP has relatively few direct targets, consisting primarily of PKA, exchange proteins activated by cAMP (Epac) and some cyclic nucleotide–gated ion channels (CNGCs). In contrast, PKA has a large number of substrates, the most important of which may be CREB, which then stimulates transcription via CREs. Additionally, VPAC2 has been reported as signalling via alternative pathways. One involves the G-proteins Gai and Gαq, which activate phospholipase C (PLC) to acutely increase Ca²⁺ levels (Figure 1.5; Dickson and Finlayson, 2009; Dickson et al., 2006; MacKenzie et al., 1996; Xia et al., 1996). Ca²⁺ has a large number of actions within the cell, prominent among which is the activation of protein kinase C (PKC) in concert with the second messengers inositol trisphosphate (IP₃) and diacylglycerol (DAG), two products of PLC activation. PKC can phosphorylate RAF-1, situated at the top of the MAPK cascade, resulting in
the phosphorylation of the MAPK extracellular signal-regulated kinases (ERK1/2) that can in turn phosphorylate CREB, amongst other transcription factors. A separate pathway activated by VPAC2 involves ADP-ribosylation factor- (ARF-) mediated induction of phospholipase D (PLD; Dickson and Finlayson, 2009; McCulloch et al., 2000), although the functional relevance of this is currently unclear.
1.3.2 VIP in the SCN

One of the most established roles for VIP in mammals is its critical function in circadian timekeeping (Vosko et al., 2007). Approximately 10% of SCN neurons express VIP and, as described in Figure 1.4, VIP neurons are found in the ventrolateral core of the SCN, receiving extensive input from RHT terminals (Abrahamson et al., 2001). VIP processes extend across much of the SCN (Card et al., 1981), and indeed many neurons in the SCN appear to express VPAC2, particularly in the dorsomedial SCN, although the exact proportion is still under debate (An et al., 2012; Fan et al., 2015; Kalamatianos et al., 2004; Kalló et al., 2004a; Park et al., 2016). Expression of VIP is strongly controlled by the transcription factor Lim homeodomain transcription factor 1 (LHX1), with relatively few LHX1-independent transcripts under VIP regulation (Bedont et al., 2014, 2017). Both VIP and VPAC2 are oscillatory within the SCN, with VIP peaking in the night, whereas VPAC2 expression appears somewhat biphasic (Cagampang et al., 1998a; Dardente et al., 2004; Glazer and Gozes, 1994; Shinohara et al., 1995). Rhythmic properties of the VIP and VPAC2 neurons themselves, such as rhythmic firing rates, are currently unclear (Fan et al., 2015; Hermanstyne et al., 2016), although VPAC2 cells in particular would be expected to be oscillatory due to being in the robustly rhythmic dorsomedial SCN.

As described above, VPAC2 receptor activation in the SCN stimulates an increase in cAMP and phosphorylation of CREB. The presence of CREs in the promoters of Per1 and Per2 genes thus provides a direct route for VIP to influence the circadian TTFL. As with other systems tested, VPAC2 in the SCN also appears to be able to transmit via the PLC pathway (Nielsen et al., 2002), with PLC-inhibitors (in addition to AC inhibitors) being required to block the effects of VIP (An et al., 2011). However, it is not entirely clear what the role of Ca\textsuperscript{2+} is in mediating the VIP response, as VIP has been shown to
decrease Ca\textsuperscript{2+} across the SCN slice (Irwin and Allen, 2010). This would appear to contrast with the traditional actions of PLC, but it is possible that VIP acutely increases Ca\textsuperscript{2+} in VIP-receptive cells, which in turn reduce Ca\textsuperscript{2+} in the rest of the SCN, such as through GABAergic communication. Therefore measures of Ca\textsuperscript{2+} in cells that are definitively VPAC2 cells are required to discern the molecular mechanisms downstream of VPAC2 activation. Nevertheless, kinases typically activated by these pathways do appear to be involved as expected, with PKA or MAPK kinase (MAPKK) inhibitors preventing phase-shifting effects of VIP (Meyer-Spasche and Piggins, 2004). While Per1 and Per2 are known to be induced (Nielsen et al., 2002), other transcripts directly and acutely stimulated by VIP have not yet been characterised, although transcriptome profiling in SCN chronically lacking VIP reveals dysregulation of numerous genes, including TTFL components and neuropeptides (Bedont et al., 2017).

VIP and VPAC2 also appear to be able to regulate electrical properties of SCN neurons. Vipr2\textsuperscript{-/-} cells tend to be more hyperpolarised, while acute application of VIP is capable of both increasing or suppressing firing of wild-type SCN, the latter of which may be mediated by GABA (Cutler et al., 2003; Itri and Colwell, 2003; Kudo et al., 2013; Pakhotin et al., 2006; Reed et al., 2002). VIP regulation of firing occurs through direct influences on both sodium and potassium channel activity, such as K\textsubscript{v}3.1 and K\textsubscript{v}3.2 (Kudo et al., 2013; Pakhotin et al., 2006). The voltage-gated sodium channel Na\textsubscript{v}1.1, known to be critical for transmission of light information from the core to the shell (Han et al., 2012), may be involved, given the role of VIP in this process, although direct evidence of that is currently lacking. Moreover, long-term effects of VIP on electrical firing appear to be dependent on Per1, with antisense oligonucleotides reported to block this response, however short-term effects
persist even in a *Per1* knockdown, suggesting multiple mechanisms (Kudo et al., 2013).

### 1.3.3 VIP is critical for SCN neuron synchrony

The vital role of VIP in circadian function came to be realised when mice lacking either VIP or VPAC2 had considerably compromised circadian rhythms. *Vip<sup>−/−</sup>* mice contain fewer rhythmic cells in the SCN, with the remaining cells displaying a broad range of phases and periods (Brown et al., 2007), a phenotype that can be rescued by daily application of a VPAC2 receptor agonist (Aton et al., 2005). This desynchrony has strong repercussions at the behavioural level, where either highly disrupted or arrhythmic behaviour is observed (Ciarleglio et al., 2009; Colwell et al., 2003). In *Vipr2<sup>−/−</sup>* mice, a similar loss of synchrony among SCN oscillators can be seen, and at the cellular level clock genes oscillate weakly (Harmar et al., 2002; Hughes et al., 2008; Maywood et al., 2006), implying VIP is important for both phase synchrony of cellular oscillators as well as sustaining the amplitude of cell-autonomous oscillation. At the behavioural level, VPAC2-null mice display either short periods or arrhythmicity (Brown et al., 2005; Sheward et al., 2007).

Intercellular coupling is capable of overcoming deficits in the TTFL (Evans et al., 2012; Ko et al., 2010; Liu et al., 2007), and VIP appears to have a key role in this. SCN slice grafting experiments demonstrate that paracrine signalling from an intact “graft” SCN slice is capable of restoring rhythmicity to a compromised *Vip<sup>−/−</sup>* or CryDKO “host” slice (Maywood et al., 2011). This inter-SCN communication is even capable of conferring period, with the period of the compromised host aligning to that of the graft. This observation that VIP is important for period determination was not replicated by a recent
intersectional study where the period of specifically the VIP cells was altered, appearing to have no effect on behavioural period (Lee et al., 2015).

1.3.4 VIP and its involvement in phase shifting

While the role of VIP in periodicity is still not clear, it has a well established role in photic responsiveness and phase shifting, which is unsurprising given the retinorecipience of VIP cells. Overexpression of VPAC2 in mice shows an enhanced ability to entrain to new lighting schedules (Shen et al., 2000), while $Vip^{+/−}$ mice struggle to entrain to light:dark cycles, show no adaptation to changing photoperiod and have aberrant phase shifting in response to presentation of light at night (Ciarleglio et al., 2009; Colwell et al., 2003; Dragich et al., 2010; Lucassen et al., 2012).

Mechanistically, cells in the core (including both VIP and GRP cells) respond directly to presentation of light at night through phosphorylation of kinases (e.g. ERK1/2) and induction of immediate early genes (IEGs), such as $c-Fos$, as well as $Per1/2$ (Gamble et al., 2007; Hughes et al., 2004; Karatsoreos et al., 2004; Kuhlman et al., 2003; Nielsen et al., 2002; Vosko et al., 2015). In WTs this response only occurs at night whereas in $Vipr2^{−/−}$ mice the gating of this induction is compromised such that light is stimulatory across all phases (Hughes et al., 2004).

Light information is then conveyed to the shell. While the core is capable of rapidly shifting in response to light, the shell does not, however the high degree of synchronisation between core neurons may explain their ability to entrain the shell (Davidson et al., 2009; Nagano et al., 2003; Rohling et al., 2011). Both VIP and GRP are implicated in this process, as both are capable of resetting slice or mouse rhythms (An et al., 2011; Chan et al., 2016; Gamble et al., 2007; Piggins et al., 1995), as well as rhythms in shell neuropeptide
expression (Watanabe et al., 2000). The exact mechanism for how the core entrains the shell is not known, although Per gene expression appears to be involved (Vosko et al., 2015). Moreover, high levels of VIP can desynchronise and reduce the amplitude of slice rhythmicity, which may make the shell amenable to novel entraining stimuli conveyed by the core (An et al., 2013).

1.4 Unanswered questions and aims

1.4.1 What are the differential roles of the VIP and VPAC2 cells?

Deficiencies in either VIP itself or in its receptor both have significant effects on the rhythmicity and photic responsiveness of mice, yet the cells that express these two proteins are likely to serve different functions given their largely differential localisation within the SCN. Thus the first two experimental chapters of this thesis (Chapters 3 and 4) aim to dissect out the roles of VIP and VPAC2 cells within the SCN slice and in the mouse in vivo. Are both necessary for SCN rhythmicity, and do they have distinct functions in controlling SCN coherence and periodicity?

1.4.2 Through what mechanisms does VIP entrain the SCN?

Much of the current work examining entrainment to light focuses on the immediate response of the retinorecipient region of the SCN, however conveying this information from the core to the shell is clearly a vital component of the entrainment process. Thus Chapters 5, 6 and 7 of this thesis aim to characterise mechanisms through which VIP can exert its effects at the transcriptional, cellular and circuit levels. How is intercellular communication converted into intracellular changes, and how is the balance between the cell-autonomous oscillator and cell-cell coupling determined?
CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Common Reagents

Common stock solutions were obtained from the media kitchen in the MRC Laboratory of Molecular Biology. These included LB (Luria-Bertani) media, 1 M MgCl₂, penicillin/streptomycin, Millipore water, Diethyl pyrocarbonate (DEPC)-treated water, 20x saline-sodium citrate (SSC) buffer, 5 M NaCl, 0.1 M ethylenediaminetetraacetic acid (EDTA; pH 7.4), trypsin, polyethylenimine (PEI), tris/glycine running buffer, 1 M tris-HCl (pH 7.4 or 8.0), 20x MOPS running buffer and TYE agar plates supplemented with antibiotics (ampicillin at 100 µg/ml or kanamycin at 30 µg/ml).

0.01M phosphate-buffered saline (PBS):

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄.2H₂O</td>
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<tr>
<td>NaH₂PO₄.2H₂O</td>
<td>1.49 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>137 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>2.68 mM</td>
</tr>
</tbody>
</table>

2.1.2 Antisera

Primary antisera used for immunohistochemistry (IHC), fluorescent in situ hybridisation (FISH) or western blot are detailed in Table 2.1.

All secondary antibodies used for IHC were highly cross-adsorbed IgGs purchased from Invitrogen and conjugated to Alexa Fluor fluorophores. For western blots the secondary antibody used was anti-rabbit IgG conjugated to horseradish peroxidase from CST (#7074).
2.1.3 Cell Lines

HEK293T (human embryonic kidney) cells for all experiments other than those used to generate adeno-associated virus (AAV) vectors in house were a gift from Yvonne Vallis, while those used to generate AAVs were a gift from Fabio Morgese. Neuro-2A neuroblastoma cells (N2As) were a gift from Ben Falcon.

<table>
<thead>
<tr>
<th>Name</th>
<th>Host</th>
<th>Supplier and Catalogue number</th>
<th>Application</th>
<th>Concentration used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-AVP</td>
<td>Rabbit</td>
<td>Penninsula T-4563</td>
<td>IHC</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-Bmal1</td>
<td>Rabbit</td>
<td>In-house</td>
<td>IHC</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti-Cre recombinase</td>
<td>Mouse (monoclonal)</td>
<td>Millipore MAB3120</td>
<td>IHC</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-GFP</td>
<td>Rabbit</td>
<td>Abcam ab6556</td>
<td>IHC</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-GRP</td>
<td>Rabbit</td>
<td>Immunostar 20073</td>
<td>IHC</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-VIP</td>
<td>Sheep</td>
<td>Millipore AB1581</td>
<td>IHC</td>
<td>1:1000</td>
</tr>
<tr>
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<td>Rabbit</td>
<td>Immunostar 20077</td>
<td>IHC</td>
<td>1:750</td>
</tr>
<tr>
<td>Anti-VIP</td>
<td>Guinea pig</td>
<td>Penninsula T-5030</td>
<td>IHC</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-VPAC2</td>
<td>Rabbit</td>
<td>Abcam ab28624</td>
<td>IHC</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-β-actin</td>
<td>Rabbit</td>
<td>Cell Signaling Technology 4967</td>
<td>Western Blot</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-myc tag</td>
<td>Rabbit</td>
<td>Abcam ab9106</td>
<td>Western Blot</td>
<td>1:2000</td>
</tr>
<tr>
<td>Anti-DIG-POD</td>
<td>Sheep</td>
<td>Roche 11 207 733 910</td>
<td>FISH</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-FLU-POD</td>
<td>Sheep</td>
<td>Roche 11 426 346 910</td>
<td>FISH</td>
<td>1:100</td>
</tr>
</tbody>
</table>

Table 2.1: Primary antisera used throughout this thesis for immunohistochemistry (IHC), western blotting or fluorescent in situ hybridisation (FISH). All antisera are polyclonal unless otherwise stated.
2.2 Animals

2.2.1 Welfare and Husbandry

All animal work carried out in these studies was licensed under the UK Animals (Scientific Procedures) Act 1986, with Local Ethical Review by the MRC. All mouse breeding and genetics were directed by Mrs Johanna Chesham and performed by members of the transgenic mouse facility Ares. All adult mice (older than P14) were culled by a Schedule 1 method: dislocation of the neck followed by exsanguination. All pups (P14 and younger) were culled by decapitation.

2.2.2 Genetically Modified Mice

Below is a list of transgenic and knock-in alleles used in the experiments described in this thesis:

- **PER2::LUC**: knock-in mouse generated by Dr. Joseph Takahashi (Yoo et al., 2004; University of Texas Southwestern Medical Center, Dallas). The luciferase gene has been inserted within the *Per2* gene downstream of exon 23, creating a fusion protein. This allows the detection of light that reflects PER2 protein dynamics when cultured with luciferin in the culture media.

- **Per1-Luc**: transgenic line generated by Hitoshi Okamura (Yamaguchi et al., 2000; Kobe University School of Medicine, Kobe) containing the 7.2 kb region upstream of the mPeriod1 gene translation start site driving a luciferase transgene. Unlike the above PER2::LUC mouse, this will act as a transcriptional reporter rather than a translational reporter, and is incorporated ectopically into the genome.

- **Cry1-Luc**: transgenic mouse generated in-house and validated by our laboratory (Maywood et al., 2013). The construct used to create the mouse is composed of a fragment of the *Cry1* promoter (-1504 to +107) driving a
luciferase transgene. Similarly to the Per1-Luc mouse, this is an ectopic transcriptional reporter.

- **Rosa-YFP**: This line contains an EYFP sequence inserted into the *Rosa26* locus, and is preceded by a floxed transcriptional stop sequence for Cre-mediated expression (Srinivas et al., 2001). These mice were originally acquired from Andrew McKenzie (LMB PNAC Division).

- **VIP-IRES-CreR (VipCre)**: This line contains an internal ribosome entry site (IRES) and the Cre recombinase coding sequence knocked in to the 3’ UTR of the *Vip* gene, resulting in Cre expression in VIP-expressing cells. Originally sourced from the JAX mouse database.

- **VPAC2-CreR (VpacCre)**: This line is the result of ectopic insertion of a BAC transgene containing Cre recombinase under the control of the VIP receptor (*Vipr2*) gene promoter. This mouse is also available from the JAX mouse database.

- **DarCre**: A mouse expressing transgenic Cre recombinase under the control of the dopamine receptor D1a promoter, originally obtained from GENSAT (Gene Expression in the Nervous System Atlas). The expression of Cre in this mouse has been shown to be high within the SCN but atypically low in other brain areas (Heintz, 2004; Smyllie et al., 2016).

- **Floxed Bmal1** – *Bmal1*lox/lox mice were a gift from Andrew Loudon, who in turn obtained them from Jackson Labs (stock no. 007668). These mice contain loxP sites flanking exon 8 of the *Bmal1* gene (Gibbs et al., 2012).

- **Floxed Ck1εTau/Tau**: Generated by Andrew Loudon (Meng et al., 2008; University of Manchester, UK), these mice have a 20h free-running period and contain loxP sites flanking exon 4 of the *Ck1ε* gene, which contains the catalytic domain.
Materials & Methods

• DUSP4\(^{+/−}\) and Floxed DUSP4: A mouse with a lacZ reporter inserted into the Dusp4 gene, along with several FRT and loxP sites creating a functional DUSP4 knockout (Figure 2.1), was originally purchased from European Mouse Mutant Archive. Crossing this mouse with a Flp recombinase mouse results in the removal of lacZ and neomycin cassettes, leaving loxP sites located either side of exon 3. This creates a “conditional ready” line, to be subsequently used in conjunction with Cre recombinase. Only the DUSP4\(^{+/−}\) mouse was used in this thesis. Future work will utilise the floxed DUSP4 mouse.

![Figure 2.1 Full allele map for the floxed Dusp4 mouse](image)

The lacZ reporter contains a stop codon, generating a Dusp4 knock-out. This mouse can be crossed with a strain that expresses the flippase recombinase to remove the lacZ-neomycin cassette and generate a “conditional ready” line by leaving two loxP sites either side of exon 3. Allele map from the European Mouse Mutant Archive.

• VPAC2-null: Vipr2\(^{+/−}\) mice were generated by Anthony J. Harmar (University of Edinburgh, Edinburgh; Harmar et al., 2002) as a targeted disruption of the endogenous Vipr2 gene.

• Cryptochrome-null mice: Cry1\(^{+/−}\) and Cry2\(^{+/−}\) mice were generated by Dr. G van der Horst through targeted disruption of the Cry1 and Cry2 alleles (van der Horst et al., 1999). These strains were then crossed in-house to generate Cry double knock out (CryDKO) mice.

2.2.3 Genotype Combinations

As well as utilising mouse strains in the forms described above, combinations of these strains were also utilised as shown in Table 2.2.
Name of mouse  Cre Recombinase  Floxed Allele(s)  Fluorescent Reporter  Bioluminescent Reporter
---  ---  ---  ---  ---
WT  --  --  --  PER2::LUC
VipCre-WT  VipCre  --  RosaYFP  PER2::LUC
VpacCre-WT  VpacCre  --  RosaYFP  PER2::LUC
Ck1εtau/tau  --  Ck1εtau/tau  --  PER2::LUC
VipCre-Ck1εtau/tau  VipCre  Ck1εtau/tau  --  PER2::LUC
VpacCre-Ck1εtau/tau  VpacCre  Ck1εtau/tau  --  PER2::LUC
Bmal1  --  Bmal1fx/fx  Bmal1fx/fx  --  PER2::LUC
DarCre-Bmal1  DarCre  Bmal1fx/fx  RosaYFP  PER2::LUC
VipCre-Bmal1  VipCre  Bmal1fx/fx  RosaYFP  PER2::LUC
VpacCre-Bmal1  VpacCre  Bmal1fx/fx  RosaYFP  PER2::LUC

Table 2.2 Mouse lines used in experiments described in this thesis
The table outlines the genotypes of the mouse strains used throughout this thesis and what they will be referred to. WT, wild-type (denotes the absence of a floxed allele).

2.2.4 Mouse Wheel-running Behaviour

Mice were individually housed and were kept in a light-controllable cabinet for the duration of behavioural monitoring. Their activity patterns were assessed using running wheels (Actimetrics) and passive infrared movement detectors. Mice were typically entrained to a 12:12 LD cycle (which mimicked that of their holding room) for at least 5 days before being exposed to different lighting schedules, including constant dim red light (DD) to investigate free-running period, and 10:10 LD cycles to determine range of entrainment, as well as 8 h phase advances or delays to determine phase shifting of different genotypes. Food and water were provided *ad libitum*.

2.2.5 Behavioural Analysis

Wheel running data were acquired and stored as wheel revolutions per six-minute bin. Data were analysed using ClockLab (ActiMetrix Inc.) to calculate behavioural
period in different lighting conditions (e.g. 12:12 LD vs. DD) by Chi-squared periodogram. Doubled-plotted actograms were produced to display behavioural data.

2.2.6 Stereotaxic Injections

Adult mice (20-25 weeks) were treated with 0.1 mg/kg Domitor (Orion Pharma) and 4 mg/kg Rimadyl (Pfizer) for sedation and analgesia respectively before receiving bilateral stereotaxic injections of AAV particles (0.3 µl per site of \(1.5 \times 10^{12}\) GC/ml) into the region of the SCN (±0.4 mm medio-lateral to Bregma, 5.5 mm deep to dural surface) under isoflurane anesthesia. 0.04% (w/v) Intra-epicaine (Dechra) was used topically throughout and 0.5 mg/kg Antisedan (Orion Pharma) was used as a reversing agent following completion of the surgery. Mice were culled and dissected for IHC two weeks after recovery to allow sufficient time for AAV expression.
2.3 Molecular Biology

2.3.1 General

All sequencing was performed by Source Bioscience, and all plasmid visualisation (e.g. for devising cloning strategies) was carried out using SnapGene Viewer software.

2.3.2 Restriction Enzyme Digest

All restriction endonuclease enzymes described in this thesis were purchased from New England Biolabs and used in their suggested buffer unless otherwise stated.

2.3.3 Transformation

Plasmids were transformed into either Top10 or Stbl3 chemically competent cells (Invitrogen, Paisley, UK), depending on the absence or presence respectively of terminal repeat sequences in the plasmid. 50 µl chemically competent cells were thawed on ice for 30 minutes before adding 1 µl of 5 ng/µl plasmid or 5 µl ligation product and gently mixing. Mixes were left on ice for 30 minutes, heat shocked for 30 s at 42°C and left on ice for 2 minutes, before adding 250 µl SOC outgrowth medium (Invitrogen) without antibiotic and incubated at 37°C for 45-60 minutes while shaking. Bacteria were then plated on warmed antibiotic-supplemented TYE agar plates and left overnight at 37°C.

2.3.4 Ligation

Ligations were performed using T4 DNA Ligase (NEB) incubated at room temperature for 20 minutes (Table 2.1). A 1:3 molar ratio of vector:insert DNA, as
calculated in Figure 2.2, was used in all ligations, keeping the amount of vector DNA constant at 50-100 ng.

\[
\frac{\text{ng of DNA (vector)}}{\text{Kbp (vector)}} : \frac{\text{ng of DNA (insert)}}{\text{Kbp (insert)}} = \frac{1}{n}
\]

**Figure 2.2** Formula for calculating molar ratios of vector and insert in ligation reactions. Ng refers to amount of DNA (in weight) while Kbp refers to length of DNA in kilobases. n refers to the molar ratio e.g. for the optimal vector:insert ratio of 1:3, n=3.

### 2.3.5 Miniprep of Plasmid DNA

Individual colonies were selected the day after transformation and were grown up in 8 ml LB overnight at 37°C with shaking. Plasmid DNA was extracted from these cultures using Qiaprep Spin Miniprep Kit (Qiagen Ltd., West Sussex, UK) according to the manufacturer’s instructions. 5 µl of each miniprep was sent to Source Bioscience (Cambridge, UK) for sequencing to confirm ligation success.

### 2.3.6 Maxiprep of Plasmid DNA

Larger preparations of plasmid DNA were required in order to generate AAV vectors. This was done using the Endotoxin-free Plasmid Maxi Kit from Qiagen, adapted from the manufacturer’s instructions. Following transformation into chemically competent bacteria, an individual colony was selected and incubated at 37°C overnight with shaking in 500 ml LB containing the appropriate antibiotic. This suspension was centrifuged at 6000 x g for 15 minutes at 4°C in the JLA 8.1000 rotor (Beckman Coulter). The pellet was then resuspended in Buffer P1 before the addition of P2, and incubated for 5 minutes at room temperature. Chilled Buffer P3 was then added, and the resulting solution (and precipitate) filtered through tissue paper into a beaker. Buffer ER was then added to the filtered lysate and incubated on ice for 30
Materials & Methods

minutes. The resulting solution was passed through an equilibrated Qiagen-tip 500. Following washing, the plasmid DNA bound to the column was eluted in Buffer QN before precipitation in isopropanol, centrifugation at 3124 x g for 30 minutes at 4°C and washing in endotoxin-free 70% ethanol. Once washed, the DNA was pelleted by centrifugation at 3124 x g for 10 minutes, allowed to air dry, and resuspended in 500 µl endotoxin-free Buffer TE.

2.3.7 Genomic DNA Extraction

Genomic DNA was extracted from SCN slices, non-SCN brain tissue and ear biopsies using DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer’s instructions. SCN slices were left in lysis buffer ATL for 1 h, while ear biopsies and 25 mg pieces of brain tissue were lysed for 4 h. Elution steps were carried out twice, with DNA from SCN slices being subsequently concentrated using an Eppendorf Concentrator Plus.

2.3.8 Polymerase Chain Reaction (PCR)

All endpoint PCR reactions using DNA as starting material described in this thesis were carried out using a TProfessional Basic Thermocycler (Biometra) and Q5 Hot Start High-Fidelity DNA Polymerase (NEB) according to manufacturer’s instructions. Primer concentrations were 500 nM for both the forward and reverse primers in each reaction. DNA was purified by a column centrifugation approach using a QIAquick PCR Purification kit (Qiagen) according to manufacturer’s instructions.
2.3.9 DNA Gel Electrophoresis

Following PCR or restriction digest, DNA was run through a gel containing 1% agarose (BioGene) dissolved in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM disodium EDTA dihydrate, pH 8.3) with 1X SYBR Safe DNA Gel Stain (Invitrogen). DNA was prepared in 4x sample running buffer containing 20% w/v glycerol, 1x TBE and 0.05% bromophenol blue, and was loaded in the gel alongside a lane containing TrackIt 1 Kb Plus DNA Ladder (Invitrogen) for accurate size determination. Following electrophoresis at 100 V for 15-30 minutes, DNA bands were visualised using a ChemiDoc MP Imaging System (Bio-Rad). If necessary, the appropriately sized band of interest was excised and the DNA purified using a QIAquick Gel Extraction Kit (Qiagen).

2.3.10 RNA Extraction from SCN Slices

RNA was extracted from organotypic SCN slices for downstream detection and quantification (qPCR and microarray) or as a source of RNA to generate RNA probes for fluorescent in situ hybridisation (FISH). SCN slices were washed in sterile PBS before detaching them from the membrane filter and storing them in RNALater (Qiagen) for 1-30 days at 4°C to preserve the RNA. RNA extraction was carried out with an RNeasy Micro Kit (Qiagen) using an adapted protocol from the manufacturer’s recommendation. RNALater surrounding the slice was removed by pipette and replaced with 350 µl Buffer RLT to disrupt and homogenise the tissue, assisted by 30 s of vortexing. One volume of 70% ethanol was added to the lysate and mixed, then transferred to an RNeasy MinElute spin column and centrifuged for 30 s at 12,000 x g. 350 µl Buffer RW1 was added to the column and spun through for 30 s at 12,000 x g, before carrying out a DNase I incubation to remove genomic DNA. 350 µl Buffer RW1 and 2x500 µl Buffer RPE washes were then performed, again for 30 s at 12,000 x g. A final wash with 80% ethanol was done, centrifuging for 2
minutes at 12,000 x g, with any remaining residue being removed by a subsequent 5 minute spin at full speed (~21,000 x g). RNA was eluted in 14 µl RNase-free water, and its purity and concentration were assessed via Nanodrop. Typical concentrations for individual cultured SCN slices ranged from 10-15 ng/µl (therefore typical yields of 140-210 ng) with 260/280 absorbance ratios of 1.8 and 260/230 ratios of 1.1.

2.3.11 cDNA Synthesis for qPCR Template Generation

To generate template cDNA for qPCR, RNA extracted from SCN slices was processed using an iScript cDNA synthesis kit (Bio-Rad). A consistent quantity (100 ng) of RNA was used per sample as a template to avoid bias during the qPCR stage. This was mixed with the provided reaction buffer, iScript reverse transcriptase and water then placed in a thermocycler where it underwent 5 min at 25°C, 20 min at 46°C and 1 min at 95°C. The resulting mixture was diluted 1:2 in nuclease-free water before further processing for qPCR (see section 2.5.12).

2.3.12 Riboprobe Generation for Fluorescent in situ Hybridisation (FISH)

Labelled riboprobes were generated via a one-step reverse transcriptase PCR (RT-PCR) protocol using gene-specific primers to generate cDNA, followed by an in vitro transcription (IVT) to create the riboprobe. First, primer sequences were sourced from the Allen Brain Atlas and checked for specificity using Primer BLAST (NCBI). Primers were ordered from Sigma containing either the bacterial promoter T7 sequence (TAATACGACTCACTATAGGGAGA; forward primer) or T3 sequence (AATTAACCCTCACTAAAGGGAGA; reverse primer) 5’ to the rest of the sequence. At the IVT stage, this would allow the respective polymerase to transcribe the RNA probe; T3 polymerase would produce the antisense probe and T7 polymerase would
produce the sense probe as a control. A full list of primers used in generating RNA probes can be found in Table 2.3.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer direction</th>
<th>Sequence</th>
<th>Probe Length (bp)</th>
<th>Probe Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vip</td>
<td>Foward</td>
<td>CCTGGCATTCTGACTCTTC</td>
<td>528</td>
<td>Allen Brain Atlas</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATTCTCTGATTTGCTCTGGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cre Recombinase</td>
<td>Foward</td>
<td>CCAATTTACTGACCGTACACCA</td>
<td>954</td>
<td>Allen Brain Atlas</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TATTTACATTGGTCCAGCACC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.3** Primers used to generate riboprobes for fluorescent in situ hybridisation against VIP and Cre recombinase transcripts.

Additionally, a plasmid containing a sequence corresponding to region 154-627 of the mouse arginine vasopressin (AVP) was kindly provided by Dr Seth Blackshaw for the purpose of generating an AVP riboprobe by in vitro transcription (IVT) as described below.

Using 10 µM stock solutions of the primers, a one-step RT-PCR was carried out to reverse transcribe RNA extracted from SCN slices and amplify the resultant cDNA. This was done using the SuperScript III One-Step RT-PCR System with Platinum Taq kit (Invitrogen) according to manufacturer’s instructions, using 50-100 ng RNA as template. Cycling conditions can be seen in Table 2.4.

<table>
<thead>
<tr>
<th>Number of cycles</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30 min</td>
<td>55°C</td>
</tr>
<tr>
<td>1</td>
<td>2 min</td>
<td>94°C</td>
</tr>
<tr>
<td>40x</td>
<td>15 s</td>
<td>94°C</td>
</tr>
<tr>
<td></td>
<td>30 s</td>
<td>55°C</td>
</tr>
<tr>
<td></td>
<td>1 min</td>
<td>68°C</td>
</tr>
<tr>
<td>1</td>
<td>5 min</td>
<td>68°C</td>
</tr>
</tbody>
</table>

**Table 2.4** Thermocycling conditions for one-step RT-PCR reaction on SCN RNA.
Once the resultant cDNA was purified using a QIAquick PCR Purification kit (Qiagen), 500 ng was \textit{in vitro} transcribed using either T3 or T7 polymerase (Roche), in a 20 µl reaction mix containing 1X transcription buffer (from the polymerase kit), 0.01M dithiothreitol (DTT; Melford), 2 µl digoxigenin (DIG) or fluorescein (FLU) labelling mix (Roche), 0.5 µl RNasin, 1 µl suitable polymerase and water. This was left at 37°C for 2 h in a thermocycler and was subsequently cleaned up using Micro Bio-Spin 6 columns (Bio-Rad) before diluting 1:5 in nuclease-free water and storing at -20°C.
2.4 Tissue and Cell Culture

2.4.1 Organotypic Slice Culture

*Media*

All media used in tissue and cell culture experiments were sterile-filtered prior to use.

### Culture Medium stock, pH 7.2, 315-320 mOsm

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eagle's Basal Medium (Sigma)</td>
<td>250 ml</td>
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<tr>
<td>EBSS (Gibco)</td>
<td>125 ml</td>
</tr>
<tr>
<td>Heat-inactivated horse serum (Invitrogen)</td>
<td>125 ml</td>
</tr>
<tr>
<td>D-glucose (Fisher)</td>
<td>5 mg/ml</td>
</tr>
<tr>
<td>Glutamax (Invitrogen)</td>
<td>5 ml</td>
</tr>
<tr>
<td>Penicillin</td>
<td>25 µg/ml</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>25 µg/ml</td>
</tr>
</tbody>
</table>

### Culture Medium Plus (CM+)

<table>
<thead>
<tr>
<th>Component</th>
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</tr>
</thead>
<tbody>
<tr>
<td>MgCl:</td>
<td>3 mM</td>
</tr>
<tr>
<td>MK-801 (Sigma)</td>
<td>100 nM</td>
</tr>
<tr>
<td>D-APV (DL-2-amino-5-phosphonovaleric acid; Sigma)</td>
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</table>

### Dissection Medium

<table>
<thead>
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</tr>
</thead>
<tbody>
<tr>
<td>GBSS (Sigma)</td>
<td>5 mg/ml</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>3 mM</td>
</tr>
<tr>
<td>MgCl:</td>
<td>3 mM</td>
</tr>
<tr>
<td>MK-801 (Sigma)</td>
<td>100 nM</td>
</tr>
<tr>
<td>D-APV (DL-2-amino-5-phosphonovaleric acid; Sigma)</td>
<td>50 µM</td>
</tr>
</tbody>
</table>

### Air Medium stock

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM (powder; Sigma)</td>
<td>8.3 mg/ml</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>5 mg/ml</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.35 mg/ml</td>
</tr>
<tr>
<td>HEPES (Sigma)</td>
<td>10 mM</td>
</tr>
<tr>
<td>Penicillin</td>
<td>25 µg/ml</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>25 µg/ml</td>
</tr>
</tbody>
</table>

### Air Medium Plus (AM+), pH 7.2, 315-320 mOsm

<table>
<thead>
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<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air Medium stock</td>
<td>93%</td>
</tr>
<tr>
<td>Foetal calf serum (FCS)</td>
<td>5%</td>
</tr>
<tr>
<td>B27 (Gibco)</td>
<td>1%</td>
</tr>
<tr>
<td>Glutamax</td>
<td>1%</td>
</tr>
<tr>
<td>Luciferin (Biosynth)</td>
<td>100 µM</td>
</tr>
</tbody>
</table>

### Peripheral Air Medium Plus (PAM+), pH 7.2, 350 mOsm

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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</thead>
<tbody>
<tr>
<td>Air Medium stock</td>
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</tr>
<tr>
<td>Foetal calf serum (FCS)</td>
<td>10%</td>
</tr>
<tr>
<td>B27</td>
<td>1%</td>
</tr>
<tr>
<td>Glutamax</td>
<td>1%</td>
</tr>
<tr>
<td>Luciferin (Biosynth)</td>
<td>100 µM</td>
</tr>
</tbody>
</table>
SCN organotypic slices were prepared as described in Hastings et al. (2005). Briefly, brains were dissected from P8-P11 pups and placed into ice-cold dissection medium. Extraneous tissue (e.g. cortex and tissue dorsal to the hypothalamus) was removed, and the remaining tissue (which contained the SCN) was cut into 300 µm slices using a McIlwain Tissue Chopper. These slices were microdissected to isolate the SCN, which was then transferred to a Millipore Millicell filter membrane in 1 ml CM+. SCN slices were allowed to acclimatise for 3-6 h at 37°C, 5% CO$_2$, before transferring membranes to 1.2 ml fresh CM. Slices were cultured for at least 7 days before any bioluminescence recording. For long-term slice maintenance, slices were kept at 37°C, 5% CO$_2$ with a medium change every 10 days.

Organotypic SCN and kidney slices from adults were prepared in the same way as above, but following acclimatisation in CM+ they were transferred into AM+ or PAM+ respectively for immediate bioluminescence recording.

2.4.2 Cell Culture

Media

Cell Culture Medium (CCM)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM Glutamax (Invitrogen)</td>
<td>90%</td>
</tr>
<tr>
<td>FCS</td>
<td>10%</td>
</tr>
</tbody>
</table>

HEK293 and N2A cells were used throughout these experiments for the validation of plasmid constructs prior to AAV generation. Both were cultured in CCM in a 37°C humidified incubator at 5% CO$_2$. The cells were not used above passage 40.

Passage of the cells was carried out every 3-4 days by removing the media, washing in warm PBS and treating with 0.05% trypsin for 2-3 minutes. Cells were then resuspended and triturated in warm CCM before splitting approximately 1 in 12 into a new culture flask.
HEK293 cells were used for the validation of shRNA constructs by western blot due to high transfection rates and expression levels. N2A cells were originally used for the validation of the diphtheria toxin receptor (DtR) construct due to being mouse-derived and thus being relatively unaffected by diphtheria toxin (Dtx) as compared to human cells (Dendy and Harris, 1973). However, given their ease of transfection and high expression levels, they were used for all subsequent construct validation experiments because they are closer in origin to SCN tissue (mouse neurons) than HEK293 cells. These experiments included all CRISPR validations.

2.4.3 Transient Transfection of Cells

For downstream western blot analysis as with shRNA, CRISPR and overexpression constructs, cells were seeded in 12-well plates at a density of $1 \times 10^5$ one day prior to transfection, ensuring confluence of 50-80% at the time of transfection. Typically 1 µg total plasmid DNA was used, divided between the number of plasmids being transfected (e.g. 0.5 µg each for 2 plasmids, 0.33 µg each for 3 plasmids). 1 µg DNA was incubated with 3 µl FuGene 6 Transfection Reagent (Promega) in 250 µl OptiMEM (GIBCO) per well for 20 minutes. This transfection solution was then gently added to each well. Cells were typically harvested 72 h after transfection.

For downstream fixation and imaging as with DtR constructs, cells were seeded in 24-well plates on poly-L-lysine-coated 13 mm coverslips to ensure cell attachment. Cells were seeded at a density of $5 \times 10^4$ per well one day prior to transfection and were transfected with FuGene 6, OptiMEM and 0.5 µg total DNA using the same ratios as stated above. Dtx treatment was administered 48 h after transfection and cells were fixed 48 h after treatment.
2.4.4 AAV Production

While many of the AAV viral vectors used in this thesis were purchased commercially (either from Penn Vector Core or from University of North Carolina), several were made in-house, particularly for pilot experiments. HEK293T cells were seeded in 15 cm plates at a density of 5 x 10^6 one day prior to transfection. Cells were cotransfected with 7µg AAV 2/1 (Rep/Cap proteins, for serotype 1), 20µg pHGTI-Adeno1 (adenoviral helper) and 7µg of the vector plasmid itself per plate, using polyethylenimine (PEI) in a 1:4 ratio DNA:PEI, mixed with 5 ml of DMEM. 6 h after transfection, the medium was changed, with the harvest beginning 72 h post-transfection.

The supernatant from each plate was harvested (and pooled if applicable) before centrifugation in a tabletop centrifuge at 3000 rpm for 30 minutes. The resultant supernatant was then filtered through a 0.45 µm Steriflip (Millipore) and transferred to polyallomer 30 ml ultracentrifuge tubes (Beckman Coulter). 2 ml of sterile 20% sucrose/PBS was gently pipetted to the bottom of these tubes before ultracentrifugation at 22,000 rpm at 4°C for 2 hours (Optima XPN-90 Ultracentrifuge, Beckman Coulter). The supernatant was subsequently removed and discarded, leaving the AAV pellet. The polyallomer tubes were allowed to air dry before the pellet was resuspended in 25 µl sterile, ice-cold PBS. This was left on ice for 1 h before combining resuspensions (if necessary), aliquotting and storing at -80°C.

2.4.5 Treatment of SCN slices with AAVs

SCN slices were given a medium change immediately prior to AAV treatment. 1 µl of AAV was placed directly on to the SCN slice and left for one week to allow expression. Another medium change was then performed before downstream experiments were undertaken. In experiments where two AAVs were utilised, AAVs were added sequentially over two weeks, with a medium change in between.
2.5 Detection and Visualisation

2.5.1 Bioluminescence Recordings of Organotypic Slices

Slices were transferred to 35 mm culture dishes containing 1.2 ml AM+ (or PAM+ for kidney slices), and were then air-sealed using glass coverslips secured with silicon grease. Firefly luciferase, fused to PER2 protein in the case of the PER2::LUC mouse or acting as a transcriptional reporter in Per1-Luc and Cry1-Luc strains, catalyses the conversion of luciferin to oxyluciferin resulting in the release of a photon. These photons can be amplified and recorded by photomultiplier tubes (PMTs), therefore acting as a real-time read out of protein or transcription levels.

PMTs (Hamamatsu) were housed above culture dish stages within a retractable light-tight sleeve in a light-tight atmospheric incubator kept at 37°C. They registered emitted photons every second (counts per second, cps) and integrated these counts over 6 minute bins.

2.5.2 Bioluminescence Imaging

SCN slices could also be imaged to study waveform or spatially localised alterations in the bioluminescence signal following drug treatments. This was achieved using a charge-coupled device (CCD) camera (Hamamatsu) and microscope with heated stage contained within a custom-built light-tight unit. Images were acquired at 1 frame per hour, which were then compiled in FIJI (Schindelin et al., 2012) post-acquisition. Brightfield images were also acquired at the start and end of the recording interval to determine any changes to morphology.

2.5.3 Widefield Multichannel Imaging

The LV200 Luminoview (Olympus) was used for long-term imaging of fluorescence and bioluminescence. The LV200 contains a heated sample stage and inverted
widefield microscope with fluorescence filter sets. All images were acquired at a rate of 2 frames per hour, with exposure times of 1 ms for brightfield, 200 ms for fluorescence and $1.75 \times 10^6$ ms (~30 minutes) for bioluminescence using a C9100-13 EM-CCD camera (Hamamatsu).

### 2.5.4 Bioluminescence Analysis

Bioluminescence recordings from adult and pup slice cultures were analysed to calculate circadian period length, amplitude and relative amplitude error (RAE; a measure of the robustness of a rhythm) using the Fast Fourier Transform – Non-Linear Least Squares (FFT-NLLS) function in the BioDare software (Zielinski et al., 2014). Changes in these values following drug treatment, rather than the absolute values themselves, were often used to account for intrinsic variability between SCN slices. Unless otherwise stated, values were calculated based on rhythms 4 days pre-treatment and 4 days post-treatment excepting the first 36 h immediately after treatment application.

**Phase Shift Analysis**

Bioluminescence recordings were also analysed to determine if a phase shift had occurred in response to a given treatment. First, the pre-treatment recording was used to predict when subsequent bioluminescence peaks would occur by extrapolating multiples of the measured period to the last identified peak before the treatment. These were compared with the actual observed peaks of bioluminescence following treatment and any differences were calculated in hours. Phase shifts at two different stages following a treatment are used in this thesis: the ‘immediate phase shift’, which shows the phase difference between the peak immediately after treatment with the predicted peak, and the ‘delayed phase shift’, which makes the
comparison between peaks three cycles after the treatment, taking into account any period effects of the treatment.

**PER2 Induction Analysis**

The induction of PER2::LUC was often calculated for the time interval immediately following a drug administration, such as VIP, to indicate the acute response of PER2. This was calculated by identifying the bioluminescence values for three peaks prior to drug treatment, and extrapolating what the next peak would have been using the ‘growth’ function in Microsoft Excel. The actual bioluminescence value of the peak following the drug treatment was compared to this predicted value, and a fold change could then be calculated.

**2.5.5 Time-lapse Image Analysis**

Once time-lapse recordings had been compiled into a single aligned TIFF file, basic analysis could be done by using the ‘plot z-axis profile’ function within FIJI to acquire bioluminescence intensity for export to, and subsequent analyse in, BioDare as described.

The Semi-automated route for image analysis (SARFIA; Dorostkar et al., 2010) package for Igor Pro software (v. 6.3; Wavemetrics) was used for time-lapse region of interest (ROI) and centre of mass (CoM) analysis. Two methods of region of interest (ROI) analysis were used: in one, ROIs within the slice were identified following despeckling and background subtraction in FIJI by thresholding the slice images and optimising pixel limits using SARFIA. The positions of these ROIs, their individual bioluminescence intensity profiles and raster plots generated from these profiles were then produced in Igor Pro. In the second method, which was used if there was noticeable movement of the slice (such as following diphtheria toxin
treatment where the slice gets smaller), following despeckling and background subtraction in FIJI, pixels were grouped as “superpixels”, which would represent 4x4-6x6 pixels and function as ROIs. Intensity profiles were then produced and normalised. Raster plots were then generated in Igor Pro. In both cases subsequent circadian analysis of ROIs using BioDare was performed. Rayleigh plots displaying phase coherence could be produced from this data by converting phase information of each ROI into circular data using Microsoft Excel, and plotted using the R ‘circular’ package.

Centre of mass (CoM) analysis can be used as an index of the wave of luminescence characteristic of PER2::LUC SCN slices (Brancaccio et al., 2013), to demonstrate if pharmacological treatments or genetic manipulations alter the phase relationship of cells within the slice. Time-lapse series were processed (despeckled, background subtracted and normalised) and thresholded in FIJI before using an in-house plug-in for Igor Pro. This plug-in identified the co-ordinates of the centre of mass (intensity of bioluminescence in each frame), which could then be superimposed over the original time-lapse images, or represented in graphical form in Prism to demonstrate any alterations in the trajectory of the two-dimensional wave moving across the SCN.

### 2.5.6 Immunohistochemistry (IHC) of Sections

#### Buffers

<table>
<thead>
<tr>
<th>Phosphate Buffer</th>
<th>Day 1 Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄·2H₂O 108 mM</td>
<td>PBS 0.01 M</td>
</tr>
<tr>
<td>NaH₂PO₄·2H₂O 25.3 mM</td>
<td>Bovine serum albumin (BSA) 1%</td>
</tr>
<tr>
<td></td>
<td>Triton-X 0.3%</td>
</tr>
</tbody>
</table>
Materials & Methods

Following careful dissection, brains were immediately post-fixed in 10 ml 4% paraformaldehyde (PFA; Alfa Aesar) made up in phosphate buffer and shaken for 4-5 h at room temperature before being cryopreserved in 20% sucrose (Fisher Chemical, UK) in PBS at 4°C overnight. The brains were then mounted on a freezing microtome using OCT embedding medium (Thermo Scientific) and 40 µm coronal sections were taken, rostral to caudal. Sections of interest (containing the SCN) were selected based on the presence and structure of the optic chiasm. Sections were placed in wells of WHO dimple trays containing 0.01M PBS.

Sections were washed twice in PBS and then blocked for 1 h at room temperature with shaking in 2% normal serum in Day 1 Buffer to reduce non-specific binding (serum donor was the same as that of the secondary antibody, for example goat serum was used with secondary antibodies raised in goat). Sections were then incubated in primary antisera (in Day 1 Buffer) overnight at 4°C with shaking.

Sections were next washed in Day 2 Buffer (1:3 dilution of Day 1 Buffer in 0.01 M PBS) twice and incubated with appropriate secondary antibodies, diluted 1:500 in Day 2 Buffer, for 1 h with shaking at room temperature. Sections were washed twice more in Day 2 Buffer, then in 0.01 M PBS before mounting onto Superfrost Plus slides (Thermo Fisher), rinsing in water and coverslipping using Vectashield Hardset mounting medium with DAPI to preserve fluorescence and assist in morphological recognition of cells.
**Materials & Methods**

**X-gal Staining of Sections**

**Buffers**

<table>
<thead>
<tr>
<th>Solution A</th>
<th>Solution B</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-gal</td>
<td>Potassium</td>
</tr>
<tr>
<td>N,N-dimethylformamide</td>
<td>hexacyanoferrate (II) trihydrate</td>
</tr>
<tr>
<td>1 M MgCl₂</td>
<td>Potassium</td>
</tr>
<tr>
<td>PBS</td>
<td>hexacyanoferrate (III)</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
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</tr>
</tbody>
</table>

_X-gal staining solution_ is made by mixing Solution A:B in a ratio of 9:1.

To visualise the expression of β-galactosidase in adult mice, brains were fixed for 2 hours at 4°C, then cryopreserved and sectioned as described above. Following washes in PBS, the sections were then incubated in X-gal staining solution at 37°C in a humidified chamber for 12-48 h. The sections were washed in PBS and then mounted onto slides before being allowed to air dry. The slides were briefly rinsed in water to remove residual salts and then dehydrated in 95% ethanol and 100% ethanol for 2 minutes each. Slides were placed in Clear-Rite 3 (Thermo Scientific) for 2 minutes and finally coverslipped using Pertex Mounting Medium (HistoLab).

**2.5.7 Immunohistochemistry of Slices**

Organotypic SCN slices were washed in PBS, fixed in 4% PFA for 1 h at 4°C with shaking and washed twice more in PBS. A 1x1 cm² region of membrane filter containing the slice was cut out from the insert using a scalpel and transferred to a WHO tray well containing PBS. From this point they were processed and mounted on slides in the same way as previously described for sections, although wash steps following secondary antibody incubation were longer (30-60 minutes) to reduce the greater background fluorescence that tended to be observed when imaging slices.
If only endogenous fluorescence (e.g. genetically encoded or virally encoded fluorescence) was to be observed, then slices were mounted immediately following excision from the inserts.

2.5.8 Dot Blot

Buffers

<table>
<thead>
<tr>
<th>RNA Dilution Buffer (RDB; 10 ml)</th>
<th>Maleic Acid Buffer (MAB) pH 7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>20x SSC</td>
<td>Maleic Acid</td>
</tr>
<tr>
<td>3 ml</td>
<td>(Sigma)</td>
</tr>
<tr>
<td>37% formaldehyde (VWR)</td>
<td>NaCl (Fisher)</td>
</tr>
<tr>
<td>2 ml</td>
<td>8.7 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>Tween-20</td>
</tr>
<tr>
<td>5 ml</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Dot blots were carried out to confirm the presence of labelled nucleotides in the RNA probes. An appropriate area of positively charged nylon membrane was excised, with dilutions of the stock of each probe being ‘dotted’ onto the membrane, allowing for 1 cm² per dot. Following an initial dilution of 1:50, 6 1:3 serial dilutions were carried out, along with a negative control of just RDB.

The RNA probes were fixed to the membrane using a UV Stratalinker, and then rinsed in MAB. The membrane was then incubated in blocking solution composed of 1% Blocking Reagent (Roche) in MAB for 30 minutes followed by antisera solutions containing either anti-fluorescein or anti-digoxigenin conjugated to horseradish peroxidase (anti-Flu-POD and anti-DIG-POD respectively) at a 1:1000 dilution in the blocking solution. Membranes were washed twice in MAB before signal development using a DAB Peroxidase (HRP) Substrate Kit (Vector Labs) according to manufacturer’s instructions, incubating membranes for at least 20 minutes to see a precipitate.
2.5.9 Fluorescent in situ Hybridisation (FISH)

Buffers

All reagents used prior to or during probe hybridisation were DEPC-treated where possible to remove RNase contamination.

**Hybridisation Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionised formamide</td>
<td>62.5%</td>
</tr>
<tr>
<td>NaCl</td>
<td>375 mM</td>
</tr>
<tr>
<td>Denhardt’s Solution</td>
<td>1.25x</td>
</tr>
<tr>
<td>Tris pH 8</td>
<td>12.5 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.25 mM</td>
</tr>
<tr>
<td>Dextran sulphate (in DEPC-H₂O)</td>
<td>12.5%</td>
</tr>
</tbody>
</table>

**RNase A Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 M NaCl</td>
<td>5 ml</td>
</tr>
<tr>
<td>1 M Tris pH 8</td>
<td>500 µl</td>
</tr>
<tr>
<td>0.1 M EDTA</td>
<td>500 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>44 ml</td>
</tr>
<tr>
<td>RNase A</td>
<td>20 µg/ml</td>
</tr>
</tbody>
</table>

**Working Hyb Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybridisation Buffer</td>
<td></td>
</tr>
<tr>
<td>Torula yeast RNA (Sigma)</td>
<td>250 µg/ml</td>
</tr>
<tr>
<td>DTT</td>
<td>12.5 mM</td>
</tr>
</tbody>
</table>

**Tris-NaCl Buffer (TNB)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 M NaCl</td>
<td>30 ml</td>
</tr>
<tr>
<td>1 M Tris pH 8</td>
<td>100 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>870 ml</td>
</tr>
</tbody>
</table>

Dissected brains were immediately placed on to aluminium foil on top of dry ice, SCN facing upwards, to freeze them. These could then be wrapped in foil and stored at -80°C until sectioning. 12 µm sections were taken using a cryostat at -18°C by mounting the brains on to the chuck with a small amount of OCT embedding medium, cutting a section, collecting onto a SuperFrost Plus slide and allowing to air dry. Sections were usually taken in series across 6 separate slides, and were stored at -80°C until further processing.

When ready for processing, slides containing the sections were allowed to equilibrate to room temperature and then post-fixed in 4% PFA made up in DEPC-PBS for 20 minutes at 4°C. Following three washes in DEPC-PBS, sections were incubated in triethanolamine (TEA; Sigma) solution (1.4 M TEA in DEPC-H₂O) for three minutes at room temperature before being acetylated in 0.25% acetic anhydride
in TEA solution for 10 minutes at room temperature. Sections were subsequently washed in DEPC-PBS before dehydration by ascending concentrations of ethanol (50%, 70%, 95% and finally 100%) for three minutes each. Sections were then left to air dry before addition of hybridisation buffer.

RNA probes were diluted 1:100 in working hybridisation buffer, heated at 60°C for 10 minutes to unravel any secondary structure and then immediately chilled on ice for 5 minutes. 80 µl of probe solution was then added to each slide and a coverslip was carefully placed on top of the solution to ensure uniform spreading and reduce evaporation. Slides were placed in a hybridisation chamber (saturated with 50% formamide and 50% PBS to prevent evaporation) and incubated overnight in a ventilated oven at 58°C.

Following hybridisation, coverslips were removed by washing in 4x SSC before treating with RNase A Buffer for 30 minutes at 37°C. The sections were subsequently washed in decreasing SSC concentrations as follows:

- 2x SSC: 10 minutes, Room temperature
- 1x SSC: 10 minutes, Room temperature
- 0.5x SSC: 10 minutes, Room temperature
- 0.1x SSC: 30 minutes, 60°C
- 0.1x SSC: Rinse, Room temperature

Sections were then washed and equilibrated in TNB before blocking in 5% normal sheep serum (in TNB) for 30 minutes. The primary antibody (anti-DIG-POD) was diluted in this blocking buffer at a concentration of 1:100 before adding 80 µl of this antibody solution to each slide, coverslipping and incubating at 4°C overnight.

Sections were washed three times in TNB to remove the antibody solution and were then treated with TSA working solution (TSA Plus Cy3 diluted in TSA Diluent 1:50; Perkin Elmer): 80 µl was added to each slide, which were then coverslipped and incubated in the dark at room temperature for 1 h to produce a fluorescent precipitate. Three washes in TNB followed before incubating the slides in 3% H₂O₂.
(in TNB) for 30 minutes to block the activity of the existing horseradish peroxidase and thus allow a second round of antibody and TSA treatment. Thus sections were again blocked and treated with the remaining antibody (anti-FLU-POD) and incubated overnight. The washes and TSA treatment were also repeated, this time using TSA Plus Fluorescein. Three washes in TNB and a final rinse in H₂O were carried out before immediately coverslipping with Vectashield Hardset mounting medium with DAPI and imaging on a confocal microscope.

2.5.10 Fluorescent Image Acquisition and Analysis

Confocal imaging was conducted using Zeiss 710 and 780 confocal microscopes. Whole SCN sections or slices on slides were imaged using a 20x air objective (NA 0.5 or 0.8 on the 710 and 780 microscopes respectively) while more detailed images (required for cell counting analysis) were acquired with a 63x oil objective, NA 1.4 before subsequent automated tile stitching performed by the Zeiss software (Zen 9 or 10).

Automated counts of cells in SCN sections were performed in FIJI using an in-house macro, kindly assisted by Jerome Boulanger (Light Microscopy, MRC LMB). Multi-channel images were separated into individual files, blurred and thresholded, with a watershed function applied if necessary. The ‘Analyze particles’ function was then applied to the SCN region of the thresholded DAPI image, resulting in the number of nuclei within the SCN being counted and a drawing of those nuclei, which could be further used as a mask for counting other signals. Using the Image Calculator in FIJI, this drawing (showing only the nuclei of the SCN) could be multiplied with another thresholded images, such as EYFP or Bmal1. The result of this was an intersection between DAPI and the second signal, which could be counted using ‘Analyze particles’ again. If necessary, for example if colocalisation between EYFP
and Bmal1 was being analysed, further intersections between other signals could be computed.

Cell culture experiments were imaged on the Nikon high content analysis (HCA) inverted fluorescence microscope using a 20x air objective (NA 0.75). Analysis was performed on Nikon NIS Elements software (Nikon).

Brain sections expressing beta-galactosidase were imaged on an Olympus BX41 microscope with a Nikon DS2mv camera attachment.

2.5.11 SDS-Polyacrylamide Gel Electrophoresis and Western Blotting

Buffers

<table>
<thead>
<tr>
<th>RIPA Lysis Buffer</th>
<th>Transfer Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris pH 7.4</td>
<td>5x Tris/Glycine</td>
</tr>
<tr>
<td>NaCl</td>
<td>running buffer</td>
</tr>
<tr>
<td>Sodium dodecyl</td>
<td>Methanol</td>
</tr>
<tr>
<td>sulphate (SDS)</td>
<td>200 ml</td>
</tr>
<tr>
<td>Sodium-deoxycholate</td>
<td>100 ml (200 ml for low kDa protein blots)</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>SDS</td>
</tr>
<tr>
<td></td>
<td>1g</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
</tr>
<tr>
<td></td>
<td>700 ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TBS-(T) (10x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris, pH 7.4</td>
</tr>
<tr>
<td>5M NaCl</td>
</tr>
<tr>
<td>H₂O</td>
</tr>
<tr>
<td>(Tween-20 5 ml)</td>
</tr>
</tbody>
</table>

72 hours after transfection (Section 2.4.3), HEK293 or N2A cells were washed in warm PBS and harvested in 100µl RIPA buffer supplemented with one cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche) tablet per 10 ml. Samples were subsequently sonicated to ensure cell lysis and to shear DNA before centrifugation at 21,000 x g at 4°C for 10 minutes to obtain the soluble protein fraction.
**SDS-PAGE**

Equal amounts of protein (typically 10 µg; concentration of each sample determined by bicinchoninic acid (BCA) assay using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific)) was denatured for 5 minutes at 90°C in the presence of 4x NuPAGE LDS Sample Buffer (Invitrogen) and 25 mM DTT. Samples were loaded onto 4-12% Bis-Tris NuPage gels (Invitrogen) alongside a Novex Sharp Pre-Stained Protein Standard (Invitrogen) and run using the MOPS buffer system at 200 V for 45 minutes.

**Western Blot**

Proteins were transferred using a wet tank system at 30 V for 1 h at 4°C (30 minutes for Cartpt blotting) onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P Membrane, 0.45 µm, Millipore) activated in methanol. Membranes were subsequently blocked for 1 h at room temperature in 5% (w/v) non-fat dried milk (Marvel) in TBS-T and incubated with primary antisera (Section 2.1.2) overnight at 4°C. Membranes were then washed three times in TBS-T for 15 minutes each and incubated in anti-rabbit IgG conjugated to horseradish peroxidase. After three more 20 minute washes in TBS-T, the ECL Prime (GE Healthcare) chemiluminescence system was used for detection, imaged using a GelDoc system (Bio-Rad) and analysed in Image Lab software (Bio-Rad), typically using rabbit anti-β-actin as a loading control.

2.5.12 Quantitative PCR (qPCR) for SCN mRNA

cDNA was generated from SCN slices as described previously (Section 2.3.11) and 15µl of each sample was pooled to generate the highest concentration standard. Serial dilutions were then performed to produce the remaining standards. Samples
were diluted 1:10 to ensure they fell within this standard curve, and to provide sufficient sample volume for all genes investigated.

qPCR was carried out using a Prime Pro 48 machine (Techne) and KAPA SYBR Fast qPCR reagents (KAPA Biosystems). Each sample well consisted of 6.5 µl KAPA SYBR master mix, 0.5 µl of each primer at 10 µM (see Table 2.5 below) and 2.5 µl nuclease-free water prepared as a master mix, plus 2.5 µl cDNA. Plates were sealed, vortexed and centrifuged briefly, and were then run in the Prime Pro 48 with the following conditions: 95°C for 2 minutes, then 40 cycles of 5 seconds at 95°C and 30 seconds at an optimised annealing temperature. At the end of the cycling, a melt curve programme was run which involved heating to 95°C for 15 s, then dropping to 55°C for 15 s and gradually increasing in temperature to 95°C. Analysis was carried out using Prime Pro Study software, excluding wells with unexpected melt curves (suggesting a different PCR product). Samples were compared to the standard curve to determine relative quantity of a given transcript, which was in turn normalised to RNS18 quantity to control for variable total RNA levels.

The primers in Table 2.5 were made by Sigma and validated by either members of the O’Neill laboratory (LMB Cell Biology Division) or myself. In addition to these, pre-validated PrimePCR SYBR Green Assay primers were purchased from Bio-Rad for mouse Mat2a and Cxcl10 transcripts. For Mat2a, a 147bp amplicon was amplified from the 115-291bp region of the Mat2a transcript. For Cxcl10, a 61bp amplicon was amplified from the 608-698bp region of the Cxcl10 transcript. Both primers used 60°C as their annealing temperature.
<table>
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<th>Gene name</th>
<th>Primer direction</th>
<th>Sequence</th>
<th>Amplicon Length (bp)</th>
<th>Annealing Temperature (°C)</th>
<th>Primer pair source</th>
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<td><strong>Adams1</strong></td>
<td>Forward</td>
<td>AGTGGTGTGTCAGTG GCAAG</td>
<td>166</td>
<td>65</td>
<td>(Xu et al., 2006)</td>
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<td></td>
<td>Reverse</td>
<td>TTCTTTGGGACTGGGT TGTC</td>
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<td></td>
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<tr>
<td><strong>Cartpt</strong></td>
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<td>Reverse</td>
<td>TCTGGGATCCATCTC CTC</td>
<td></td>
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</tbody>
</table>

**Table 2.5** Primers used for qPCR of genes to validate microarray data.
qPCR Primer Design and Annealing Temperature Optimisation

Primers were either sourced from the literature or designed using the NCBI Basic Local Alignment Search Tool (BLAST) Primer Blast programme. Primers were designed to amplify a <200 bp region of the gene of interest, with a preference for no off-target amplicons and primer pairs in which one spanned an exon-exon junction. Oligonucleotides were subsequently synthesised by Sigma Aldrich in a desalted form.

Annealing temperatures were found for all primer pairs by running multiple identical PCR reactions using KAPA SYBR Fast qPCR reagents in a standard thermocycler, but utilising a temperature gradient during the annealing step centred on the predicted annealing temperature. Products were then visualised on a 1% agarose gel, and optimal annealing temperatures were identified as the highest temperature that produced the expected band and had no off-target products. This temperature was then confirmed as being applicable to the Prime Pro 48 qPCR machine by running qPCR reactions with the optimised annealing temperature and examining the resulting melt curves.
2.6 General Analysis

Statistical tests and graphical representation of data (typically mean ± SEM) was performed using Prism 6 or 7 software (Graphpad). Throughout this thesis, significance between the measured means of experimental groups is denoted as follows: \( *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 \).

The following analyses were typically used throughout this thesis unless specified otherwise. Two-tailed, unpaired Student’s T-tests were performed in experiments containing two groups separated by one independent variable. More than two groups separated by one independent variable were tested using an Ordinary one-way ANOVA, with Tukey correction for multiple comparisons where every group is compared to every other group, or Dunnett correction where groups are compared to a control group. Two-way ANOVAs with Tukey correction for multiple comparisons between all groups were performed on experiments involving multiple independent factors (e.g. time and drug treatment). If no significant differences are found between individual groups, then any significant differences between factors are reported. Pearson’s correlation coefficient was calculated to determine if two variables were correlated and, if applicable, linear regression was used to see if one variable could be predicted from another.
CHAPTER 3

CELL TYPE-SPECIFIC ABLATION OF SCN
SUBPOPULATIONS

3.1 Introduction

The SCN is the central mammalian pacemaker, signalling distal regions of the organism to ensure internal phase synchrony. These output signals are typically thought to emanate from the shell region of the SCN, characterised by AVP, PK2 and VPAC2 expression (Kalsbeek et al., 2010; Yamaguchi et al., 2003; Zhou and Cheng, 2005), while the core region of the SCN, characterised by VIP and GRP, receives inputs, such as from the retina, regarding the outside world (Abrahamson and Moore, 2001; Morin and Allen, 2006). Intercellular communication within the SCN is therefore essential for a coherent and accurate output signal that is truly reflective of the external environment.

VIP signalling within the SCN has previously been shown to be vital to this circuit-level communication. In VIP-null mice, fewer electrically rhythmic cells within the SCN are seen, while those that are rhythmic have a broader range of phases and periods (Brown et al., 2007). This phase dispersal is reflected at the behavioural level, where VIP-null mice show disrupted or arrhythmic behaviour patterns (Ciarleglio et al., 2009; Colwell et al., 2003). In SCN lacking the VPAC2 receptor, individual neurons demonstrate weak oscillations in core clock genes and phase synchrony is lost compared to wild-type SCN slices, resulting in a lack of robustness to perturbations (Harmar et al., 2002; Hughes et al., 2008; Maywood et al., 2006). Behaviourally, VPAC2-null mice are either arrhythmic or display a short period (Brown et al., 2005;
Harmar et al., 2002; Sheward et al., 2007). Responses to light in both knockout strains are attenuated, with responses to light pulses and the gating of those responses being disrupted in VIP-nulls (Dragich et al., 2010), while VPAC2-nulls are able to shift to new lighting regimes more rapidly than WT mice (Harmar et al., 2002) and actually demonstrate improved rhythmicity under constant light conditions (Hughes et al., 2015).

Despite the significant advances these null strains have allowed, they suffer from the same issues as all global knockout mice, such as confounding developmental factors and off-target effects (especially given the wide expression of VIP and VPAC2 (Dickson and Finlayson, 2009)). For example, Hughes et al. (2008) found that the VPAC2 antagonist PG 99-465 was only partially able to recapitulate the effects of a VPAC2 knock out, but did not reduce the percentage of electrically rhythmic cells, suggesting that VIP signalling during development may have a role in the generation of rhythmicity. Other techniques for elucidating the role that VIP and VPAC2 cells play in circadian timekeeping, such as partial lesions of the core (Kriegsfeld et al., 2004; LeSauter and Silver, 1999) or surgical separation of the dorsal SCN from the ventral (Yamaguchi et al., 2003) suffer from a lack of specificity given the imperfect spatial segregation of different cell types in the SCN.

A combination of flexed, virally transduced vectors and cell-type specific Cre recombinase expression can overcome many of the aforementioned issues, bypassing developmental factors by injecting the virus in adulthood, and achieving specificity through localised expression of Cre. These approaches can be used in concert with recent cell ablation techniques to further understand the role of specific cell types. One such method involves the use of diphtheria toxin (Dtx). Dtx enters cells through the heparin-binding epidermal growth factor (Naglich et al., 1992), also known as the diphtheria
toxin receptor (DtR), which is then endocytosed into the cell. Acidification of the endosome results in the separation of the two Dtx subunits, A and B, after which the A subunit exits the endosome into the cytosol through the pore formed by the B subunit. The A subunit subsequently ADP-ribosylates eukaryotic elongation factor 2 (eEF2), an essential factor for protein synthesis, blocking protein synthesis in the process, and resulting in apoptotic cell death (Bennett and Clausen, 2007; Collier, 1975; Naglich et al., 1992). Mice are naturally more resistant to Dtx than humans and other primates due to three amino acid changes in the DtR that are critical for binding of the B subunit (Mitamura et al., 1997). Thus expression of the human or simian DtR in specific mouse cells should render them susceptible to Dtx-mediated cell death, leaving other cells intact (Bennett and Clausen, 2007; Buch et al., 2005; Cha et al., 2003; Hatori et al., 2008). This approach has been used in neuroscience to ablate retinal ganglion cells (Hatori et al., 2008) and oligodendrocytes (Buch et al., 2005) by delivering Dtx by intraperitoneal injection, demonstrating that the toxin is capable of crossing the blood-brain barrier and reaching targets in the central nervous system. Thus far, however, the DtR has been expressed genomically, which is unsuitable for studies in which the role of a common cell type is to be investigated within a specific region of the brain.

The experiments presented in this chapter describe a novel approach to investigating the role of the VIP and VPAC2 cells in circadian timekeeping by specifically ablating one or other cell population through the use of Cre-mediated expression of the DtR transduced by AAV. It is important to note that this approach differs from VIP- and VPAC2-null strains in that not only is VIPergic signalling abolished, but all functions carried out by these cell types, including receiving inputs to the SCN (particularly for VIP cells) and projecting outputs to the rest of the body (particularly for VPAC2 cells).
Furthermore, while VIP and VPAC2 knockout SCN may be expected to display similar phenotypes, ablation of the VPAC2 cells may be seen as more severe due to not only eradicating VIP signalling, but also canonical “shell” signalling neuropeptides, such as AVP.

Here I present validation of the VipCre and VpacCre mouse strains as well as the flexed diphtheria toxin AAV. While this work is preliminary and as such only focuses on the *ex vivo* role of these two cell populations in SCN slices, it appears that VIP cell ablation has a more detrimental effect on SCN rhythmicity than VPAC2 cell ablation, despite its smaller cell number and ostensible lack of effect on other canonical SCN neuropeptides. Moreover, ablation of either cell population has the ability to affect all single-cell oscillators within the SCN, but, in these preliminary studies, only VIP cell ablation drastically affected circuit-level communication.
3.2 Methods

3.2.1 Intersectional genetic scheme

The provenance of the mice used throughout this chapter is described in General Methods (Chapter 2). To visualise the expression of VipCre in mouse SCN, VipCre mice were crossed with mice containing a YFP reporter inserted at the Rosa26 locus (RYFP) for ubiquitous expression, preceded by a loxP-flanked transcriptional “stop” sequence.

3.2.2 Gibson Cloning of the Diptheria toxin receptor (DtR)

For packaging into AAV vectors, the diphtheria toxin receptor-containing transgenic cassette was cloned into pAAV-EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA (Addgene #20297), an AAV plasmid containing: AAV inverted terminal repeats required for AAV packaging, a WPRE sequence (WHP Posttranscriptional Regulatory Element) to enhance expression (Donello et al., 1998), the Ef-1a promoter for strong, ubiquitous expression and four loxP sites to allow transgenes to be flexed (leads to expression only in the presence of Cre recombinase). The Gibson method of cloning (Gibson et al., 2009) was used to insert an mCherry fluorescent protein and the simian diptheria toxin receptor (DtR) separated by a P2A peptide (to generate separate proteins in equimolar amounts) between the four loxP sites.

hChR2-mCherry was excised from pAAV-EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA using BsrGI and NheI, linearising it in the process. mCherry-P2A was amplified from HIV.CMV.mCherry-P2A-Cre, kindly donated by Mat Edwards, using the forward primer 5’-TAACTTCGTA TAGGATACTTTATACGAAGTTATGCTAGCCACCatggtagcaagggcgagg-3’ and the reverse primer 5’-GCTTCATaggccggattctctccagtc-3’, where the capitalised, red lettering represents regions of the primers complementary to...
the vector backbone and the DtR sequence respectively. The DtR sequence was amplified from $pAAV.pCAG.DIO.DtR.WPRE$, kindly donated by Fabio Morgese, using the forward primer 5'-GTGGAGGAGAATCCCGGCCCTatatgaagctgctgccgtcgg-3' and the reverse primer 5'-CATTATACGAAGTTATGGCGCGCCTTACTTGTACAtcagtgggaattagtcatgccccacttc-3', where the capitalised, red lettering represents regions of the primers complementary to the P2A sequence and the vector backbone respectively.

The resulting PCR fragments were purified and incubated with the linearised vector backbone and Gibson mix (NEB) for 30 minutes at 50°C. The reaction was chilled on ice before adding 5 µl of the mix to chemically competent cells, kindly donated by Ernesto Ciabatti. This was left on ice for 2 minutes and then plated on to LB plates containing ampicillin. Colonies were selected, mini-prepped and confirmed by sequencing. Following validation in cells, the $Ef1a.DiO.mCherry-P2A-DtR$ plasmid was packaged into AAV vectors as described in Chapter 2.

3.2.3 N2A cell and SCN slice treatment with diphtheria toxin (Dtx)

Diphtheria toxin (D0564, Sigma) was dissolved in sterile water and stored in 1 mg/ml aliquots at -80°C. N2A cells were treated with either 100 or 200 ng/ml by diluting the toxin in cell culture media and performing a full media change. Following pilot studies using the sensitive PER2::LUC reporter to detect non-specific (receptor-independent) effects of Dtx, SCN slices were treated with 20 ng/ml Dtx by diluting it in air medium (AM) to 10 µg/ml before adding 2.4 µl to 1.2 ml AM in the culture dish. Wash off was carried out after 5-8 days post-Dtx application.
3.3 Results

3.3.1 Effective Cre expression in targeted VIP and VPAC2 neuronal populations

Functional Cre expression was visualised in sections from VipCre and VpacCre mice using YFP fluorescence as a reporter and additionally in VpacCre sections using an anti-Cre antibody. Native VIP and VPAC2 respectively were co-stained alongside these signals to determine if Cre expression had the expected core or shell localisation (Figure 3.1 and 3.2). VipCre-YFP cells were observed in the core region of the SCN as expected (Figure 3.1A), demonstrating clear colocalisation with the native VIP staining in both cell bodies and neural projections (Figure 3.1B). Furthermore, VipCre-YFP cells were found to occupy approximately 15% of the SCN as marked by DAPI, which is consistent with previous literature (Abrahamson and Moore, 2001). Cre immunostaining in VpacCre SCN sections revealed a shell distribution, particularly in the rostro-caudal mid-point of the SCN, while it could be seen across the whole section in more rostral or caudal regions, as has been shown previously (King et al., 2003). This pattern was mirrored by VPAC2 co-staining (Figure 3.2A). Although the nuclear Cre signal was difficult to definitively colocalise with the membrane-localised VPAC2 receptor, many Cre-positive cells demonstrated a VPAC2 “halo” (Figure 3.2B). When the Cre signal was compared to DAPI across the whole SCN, it was found that almost 40% of all SCN cells express VpacCre.

VipCre-YFP was found to colocalise very little with AVP immunostaining (Figure 3.3A), as has been reported previously (Lee et al., 2015), whereas, as expected, VipCre expression colocalised with almost 100% of VIP cells as determined by fluorescent in situ hybridisation (FISH; Figure 3.3B), which was utilised to limit fluorescent signals to the cell body. 85% of AVP cells were VpacCre-YFP-positive, but this occupied only ~30% of all VpacCre-YFP
cells (Figure 3.3C), suggesting that while VIP cells directly communicate with most AVP cells, there is extensive VPAC2-mediated communication throughout the SCN from VIP-positive to AVP-negative cells as well. Finally, limited evidence of VpacCre-YFP colocalising with VIP immunostaining was observed: approximately 13% of VIP cells appeared to express VpacCre, but this accounted for less than 2% of all VpacCre cells (Figure 3.3D). Thus the
Cell type-specific ablation of SCN subpopulations

VipCre and VpacCre cell populations appear to share limited overlap in their expression within the SCN (Figure 3.3E).

3.3.2 VipCre and VpacCre are active throughout development in the SCN

The use of flexed reporters packaged in AAV vectors and delivered acutely allows visualisation of current Cre expression, as opposed to genomically encoded reporters, which may additionally reflect some developmental expression. AAVs containing a flexed TdTomato reporter under the
Figure 3.3 VIP and VPAC2 cell populations are largely separate in the SCN
(A) Representative VipCre-YFP fluorescence alongside immunostaining for AVP in SCN
(legend continued on next page)
constitutive CAG promoter (Penn Vector Core) were stereotactically injected into adult mouse SCN. Sectioning of the SCN and fluorescence microscopy revealed clear TdTomato localisation in similar regions to those demonstrated in Figures 3.1-3.3 through the genomic YFP reporter (Figure 3.4A), and cell counts of TdTomato-positive cells compared to DAPI were not significantly different to those of YFP-positive cells (Figure 3.4B). This demonstrates that AAVs are an effective tool for delivering expression constructs to the SCN with high transduction efficiencies, expression can be dependent on Cre-mediated recombination, and that neither the VipCre nor VpacCre line demonstrates significant developmental Cre expression within the SCN. Finally, neonatal pup slices were also treated with the flexed TdTomato AAV, again displaying the expected localisation in VipCre and VpacCre SCN (Figure 3.4C), demonstrating that Cre is reliably active in both juvenile and adult mice.

3.3.3 Cre-mediated diphtheria toxin receptor (DtR) expression makes cells susceptible to the diphtheria toxin (Dtx)

The human or simian diphtheria toxin receptor (DtR) has previously been shown to render mouse cells susceptible to ablation by the diphtheria toxin

Figure 3.3 continued
sections, with higher magnification images showing minimal colocalisation. (B) Representative images of double fluorescent in situ hybridisation to VIP and Cre recombinase in VipCre SCN sections compared to single-probe controls shows almost 100% colocalisation in higher magnification images. (C and D) Representative VpacCre-YFP fluorescence alongside immunostaining for AVP (C) and VIP (D) in SCN sections, with higher magnification images showing that most AVP cells are VpacCre-positive, while few VIP cells are (cyan arrows show colocalisation). Scale bar in lower magnification images represents 50 µm. Location of all higher magnification images marked by white box on lower magnification images; scale bar represents 10 µm. All colocalisation percentages given represent mean ± SEM. (E) Proportional Venn diagram representing different cell populations of the SCN. DAPI represents all SCN cells.
Cell type-specific ablation of SCN subpopulations

Figure 3.4 Cre recombinase is active throughout life in VipCre and VpacCre SCN

(A) Representative VipCre and VpacCre SCN sections expressing a stereotaxically injected flexed TdTomato AAV demonstrating expected localisation. (B) Cell counts for TdTomato expression and genomically encoded YFP expression (mean ± SEM) in VipCre and VpacCre mice (n = 4-6 sections from 4-6 mice per group). No significant differences were observed between TdTomato and YFP counts for either strain. ns, P > 0.05, one-way ANOVA with Tukey’s multiple comparisons test. (C) Neonatal VipCre and VpacCre SCN slices expressing the flexed TdTomato. Scale bars represent 50 µm.
(Dtx) (Buch et al., 2005; Cha et al., 2003). To utilise DtR as an effective tool for understanding the roles of VIP and VPAC2 cells in the SCN, a flexed DtR was cloned into an AAV backbone along with an mCherry reporter to make EF1a.DiO.mCherry-P2A-DtR (section 3.2.2; Figure 3.5A). Validation of this plasmid was performed in neuron-like N2A cells that were co-transfected with a nuclear-localised Cre::GFP plasmid (kindly donated by Ernesto Ciabatti). Addition of either 100 or 200 ng/ml Dtx effectively ablated all mCherry-positive cells (Figure 3.5B-D). While these concentrations of toxin were additionally causing non-specific cell death, as seen by the reduced number of DAPI-positive cells counted in cultures lacking the receptor, the toxin nevertheless preferentially ablated cells expressing DtR because when cell numbers were normalised to the remaining Cre::GFP cells, no mCherry cells could be seen (Figure 3.5C). If it was not selective, similar proportions to the vehicle-treated cells would have been observed. Finally, no leakiness was observed with the plasmid, with mCherry expression only ever being seen in Cre::GFP-positive cells (Figure 3.5D).

3.3.4 Dtx-mediated VIP cell ablation has severe effects on the SCN network

Once the flexed mCherry-DtR construct was packaged into AAV particles, EF1a.DiO.mCherry-P2A-DtR (mCherry-DtR) was used to target and subsequently ablate VIP and VPAC2 neurons in VipCre (VipCre-DtR; Figures 3.6-3.8) and VpacCre (VpacCre-DtR; Figures 3.9-3.11) SCN slices. Control (no DtR) slices were treated with the flexed TdTomato AAV as a marker for VIP and VPAC2 cell populations. Addition of 20 ng/ml Dtx to VipCre-DtR slices immediately reduced the amplitude of PER2::LUC bioluminescence rhythms, presumably through translational inhibition before ultimately killing the cells. This effect was not reversed by wash-off, although the remaining cells of the slice remained weakly rhythmic (Figure 3.6A and B). Ablation of the VIP cells
Cell type-specific ablation of SCN subpopulations

Figure 3.5 Validation of flexed diphtheria toxin receptor (DtR) in N2A cells

(A) Plasmid map of EF1a.DiO.mCherry-P2A-DtR (mCherry-DtR). mCherry and DtR will only be expressed in the presence of Cre. (B) N2A counts of DAPI-, Cre::GFP- and mCherry-DtR-positive cells with and without Dtx treatment (mean ± SEM; n = 4 wells of cells per group, with each well value representing an average of 3 different field counts. Range of 513-2138 cells counted per well, mean of 1078 cells per well, total of 24788 cells counted). When transfected with mCherry-DtR and treated with either concentration of Dtx, 4 cells were counted as mCherry-positive, compared to a total of 1058 in the vehicle-treated condition. (C) Percentage of cells expressing either Cre::GFP or mCherry-DtR normalised to the average Cre::GFP cell count value for each group (mean ± SEM). (D) Representative images of N2A cells transfected with Cre::GFP or Cre::GFP and mCherry-DtR, treated with vehicle or Dtx. Scale bar represents 50 µm.
Figure 3.6 Ablation of VIP cells in SCN slice has severe effects on PER2::LUC rhythms. 

(A and B) Representative PER2::LUC bioluminescence rhythms of Dtx-treated VipCre-DtR and VipCre-TdTom slices. Dtx was added after 4 days and washed off after a further 8 days. Bioluminescence has been normalised to the first peak. (B) A close-up view of the last 4 days of recording from the same slices. (C) Period change (mean ± SEM) in VipCre-DtR Dtx-treated and control slices. Dtx application significantly increased the period in VipCre-DtR slices (n = 6) compared to vehicle treated (n = 6) and VipCre-TdTom slices (Dtx: n = 5; vehicle: n = 3). (D-E) Percentage amplitude (D) and RAE (E) changes (mean ± SEM) compared to pre-treatment in VipCre-DtR Dtx-treated and control slices. Amplitude was significantly reduced in VipCre-DtR Dtx-treated slices compared to all controls, while RAE

(legend continued on next page)
affected a number of circadian parameters: a significantly increased period, reduced amplitude and decreased robustness of ensemble oscillation (Figure 3.6C-E). After wash-off, slices were treated with a flexed YFP AAV (EF1a.DiO.EYFP; Penn Vector Core) before fixation and microscopy (Figure 3.6F). The flexed YFP was used to demonstrate that the Dtx had genuinely resulted in apoptosis of the cells (rather than merely affecting mCherry expression), and to highlight the number of Cre-positive cells not originally transduced by mCherry-DtR. No mCherry-positive and few YFP-positive (if any) were observed across the VipCre-DtR slices treated with Dtx compared to controls, and immunostaining for VIP itself showed a strong signal in control slices and none in VipCre-DtR slices, demonstrating an effective toxin-mediated ablation of VIP cells. Furthermore, remaining cells in VipCre-DtR slices treated with Dtx appeared to have normal morphology when comparing DAPI staining to control slices (Figure 3.6G).

The effect of ablating the VIP cells on the SCN network was next examined with time-lapse CCD imaging (Figures 3.7 and 3.8). This revealed that regions of interest (ROIs; each ROI = 5x5 pixels), which were highly synchronous before Dtx application, lost phase coherence, particularly after wash-off (Figure 3.7A-C). Furthermore, the immediate reduction in amplitude was not merely due to the loss of bioluminescent signal from the VIP cells; all ROIs appeared to subsequently oscillate with reduced amplitude (Figure 3.7A). The period increase was also observed at the level of single oscillators, with most

**Figure 3.6 continued**

significantly increased compared to Dtx-treated VipCre-TdTom but not vehicle-treated slices (n numbers as in (C)). All tests were two-way ANOVAs with Tukey’s multiple comparisons test, *P < 0.05; **P < 0.01, ****P < 0.0001. (F) Representative images of VipCre SCN slices transduced with AAV-flexed EYFP and immunostained for VIP alongside either flexed TdTomato or mCherry-DtR following Dtx or vehicle treatment. No YFP-, mCherry or VIP-positive cells can be seen in Dtx-treated VipCre-DtR slices, although some mCherry cell debris is visible. Scale bars represent 50 µm. (G) High magnification images of white-boxed regions in (F) demonstrating normal cell morphology in Dtx treated slices. Scale bars represent 10 µm.
ROIs displaying a lengthened period, although the period dispersal was also greater, and this average increase in period did not persist after wash off (Figure 3.7D-F).

Figure 3.7 Ablation of VIP cells affects all SCN cell-autonomous oscillators
(A and B) Representative raster plots (A) and associated Rayleigh plot (B) of PER2::LUC bioluminescence rhythms of one of two Dtx-treated VipCre-DtR slices imaged on CCD camera. The two raster plots represent the same recording: left has had brightness and contrast adjusted following Dtx treatment (marked by +) to better show lack of phase coherence among oscillators following Dtx; right is the unadjusted plot to demonstrate amplitude reduction in all ROIs. * represents wash off. (C) Length of Rayleigh plot vector (mean ± SEM, example in (B)) before and after Dtx treatment and after wash-off to demonstrate loss of phase coherence. (D) Relative cumulative frequency (mean ± SEM) of ROIs with a given period before and after Dtx treatment and after wash-off. (E) Periods for all oscillators across two slices (mean ± SEM). (F) Standard deviation of periods (mean ± SEM) to demonstrate period dispersal amongst oscillators following Dtx treatment and its maintenance after wash-off.
Given the role of VIP in synchronising SCN neurons, as well as a reduced amplitude of oscillation being observed in all neurons across the slice, the network dynamics of PER2::LUC expression were next examined using centre of mass (CoM) analysis (Brancaccio et al., 2013). Wild-type SCN slices exhibit a stereotypical wave of bioluminescence as a result of variations in peak PER2 expression within different regions of the SCN. This wave progresses ventrolaterally from the dorsomedial region over the course of the day, and the CoM dynamically tracks the area of strongest bioluminescence. Thus, changes to the CoM can be seen as changes in the phase relationship between regions of the SCN, resulting either from differential responses of each region to a given treatment, or from changes to intercellular communication. VipCre-DtR slices showed the characteristic wave of bioluminescence before Dtx treatment, and this could still be seen 24 h and 96 h after Dtx application, although this did gradually worsen as time went on (Figure 3.8A-C). However, upon wash off, the CoM showed very little displacement, suggesting that the spatiotemporal wave had largely been abolished (Figure 3.8B and C). What is also clear from the time-lapse imaging is that cell ablation results in a reduction in size for the slice, presumably due to the death of some cells and migration of others (Figure 3.8A).

3.3.5 Dtx-mediated VPAC2 cell ablation reduces amplitude, but not synchrony of SCN slices

Interestingly, ablation of the VPAC2 cells did not exactly mirror VIP cell ablation, which might have been expected, as both manipulations result in the absence of VIPergic signalling. One common feature, however, was a reduction in amplitude, due to translational inhibition and then cell apoptosis, that could not be reversed by toxin wash-off (Figure 3.9A and C). There was also a trend for increased period in VpacCre-DtR Dtx-treated slices.
Figure 3.8 Ablation of VIP cells affects SCN circuit-level function

(A) Representative images of PER2::LUC bioluminescence rhythms of one of two Dtx-treated VipCre-DtR slices visualised on CCD camera before and after Dtx and after wash-off. Each set of images has been separately adjusted for maximum contrast. (B) Mean centre of mass (CoM) path vectors for both SCN slices visualised (one SCN per slice); means were calculated over 5 days in the given time window. (C) Representative CoM path vectors over time for one slice before and after Dtx, and after wash-off. Note that following wash-off, the stereotypical spatiotemporal wave of bioluminescence is completely abolished.
Figure 3.9 Ablation of VPAC2 cells has strong effects on SCN slice rhythmicity

(A) Representative PER2::LUC bioluminescence rhythms of Dtx-treated VpacCre-DtIR and VpacCre-TdTom slices. Dtx was added after 6 days and washed off after a further 7 days. Bioluminescence has been normalised to the first peak. (B) Period change in VpacCre-DtIR Dtx-treated and control slices (mean ± SEM). No significant differences were observed in period change, although a trend in interaction between AAV and treatment was observed (P = 0.07). (C-D) Percentage amplitude (C) and RAE (D) change (mean ± SEM) compared to pre-treatment in VpacCre-DtIR Dtx-treated and control

(legend continued on next page)
Cell type-specific ablation of SCN subpopulations

(Figure 3.9B and D), but these were not significant ($P = 0.07$ for interaction between Dtx and AAV treatments for period change, two-way ANOVA with Tukey’s multiple comparisons test). Effective cell ablation was confirmed at the end of the experiment by addition of flexed EYFP AAV, followed by fixation and microscopy as with the VipCre-DtR slices. Once again, complete ablation of mCherry-expressing cells was observed in VpacCre-DtR slices treated with Dtx, while controls appeared unaffected (Figure 3.9E). Immunostaining was performed for AVP as an approximate marker for VPAC2 cells (due to inherent difficulties of VPAC2 staining in SCN slices), which showed a dramatic reduction in VpacCre-DtR slices after Dtx treatment. The morphology of remaining cells in VpacCre-DtR/Dtx slices appeared healthy (Figure 3.9F), suggesting non-VPAC2 cells were not adversely affected by the death of DtR-positive cells. Moreover, VIP cells appeared to remain intact and in a separate location to any surviving YFP-positive VpacCre cells (Figure 3.9G), demonstrating that death of VPAC2 cells does not result in non-specific death of other cell types, and providing further evidence that the VPAC2 and VIP cell populations are separate within the SCN.

Time-lapse CCD imaging was again used to investigate the effect of cell

**Figure 3.9 continued**

slices. Amplitude was significantly reduced in VpacCre-DtR Dtx-treated slices ($n = 6$) compared to VipCre-TdTom ($n = 4$) and vehicle-treated controls (DtR: $n = 5$; TdTom: $n = 3$). No significant differences were observed for RAE change. All tests were two-way ANOVAs with Tukey’s multiple comparisons test, $**P < 0.01$, $***P < 0.001$. (E) Representative images of VpacCre SCN slices transduced with flexed EYFP and immunostained for AVP alongside either flexed TdTomato or mCherry-DtR following Dtx or vehicle treatment. No YFP- or mCherry-positive cells can be seen in Dtx-treated VipCre-DtR slices, although some mCherry cell debris is visible. Scale bars represent 50 µm. (F) High magnification images of white-boxed regions in (E) demonstrating normal cell morphology in Dtx treated slices. Scale bars represent 10 µm. (G) Representative images of VpacCre SCN slices transduced with flexed EYFP and immunostained for VIP alongside either flexed TdTomato or mCherry-DtR following Dtx treatment. Some YFP-positive cells can be seen but no mCherry cells are present. Note that VIP staining (cyan arrows) is intact and separate from the YFP cells (yellow arrow). Top: 20x magnification, bottom: 63x magnification. Scale bars represent 50 µm (top) or 10 µm (bottom).
ablation on SCN oscillators and the network. ROI analysis showed that the amplitude drop was again due to an amplitude reduction at the single cell level, rather than just the loss of VPAC2 cells (Figure 3.10A). Interestingly however, unlike when the VIP cells were ablated, synchronicity was not strongly affected after Dtx administration, although wash-off again did result in reduced synchronicity, presumably due to differential resetting effects on different regions of the SCN (Figure 3.10A-C). Period effects were also seen for all oscillators (Figure 3.10D-F), although again the effect was not as pronounced as in VipCre-DtR slices.

Figure 3.10 Ablation of VPAC2 cells affects all SCN cell-autonomous oscillators
(A and B) Representative raster plot (A) and associated Rayleigh plot (B) of PER2::LUC bioluminescence rhythms of one of two Dtx-treated VpacCre-DtR slices visualised on CCD camera. + marks Dtx treatment, * marks wash off. (C) Length of Rayleigh plot vector (mean ± SEM; example in (B)) before and after Dtx treatment to demonstrate phase coherence. (D) Periods (mean ± SEM) for all oscillators across two slices before and after Dtx, and after wash-off. (E) Standard deviation of periods (mean ± SEM) to demonstrate period dispersal amongst oscillators following Dtx treatment. (F) Relative cumulative frequency (mean ± SEM, %) of ROIs with a given period before and after Dtx treatment and after wash-off.
Remarkably, the spatiotemporal wave of PER2::LUC bioluminescence was mostly preserved after VPAC2 cell ablation (Figure 3.11). The stereotypical dorsomedial-ventrolateral movement of the CoM was still intact 96 h after Dtx treatment, and even after wash-off there remained considerable displacement of the CoM, which resembled the untreated CoM in both slices observed, although it was clearly less robust than the untreated CoM (Figure 3.11A-C). When compared to VipCre-DtR slices, the relative perimeter of the CoM (a quantification for CoM displacement) does not seem to be as large, particularly after wash-off (Figure 3.11D), implying killing the VPAC2 cells had a weaker effect on the spatiotemporal wave than VIP cell death. As with VipCre-DtR, a reduction in slice size was also seen in these slices (Figure 3.11A).
Figure 3.11 Ablation of VPAC2 cells affects SCN circuit-level function
(A) Representative images of PER2::LUC bioluminescence rhythms of one of two Dtx-treated VpacCre-DtR slices visualised on CCD camera before and after Dtx and after wash-off. Each set of images has been separately adjusted for maximum contrast. (B) Mean centre of mass (CoM) path vectors for both SCN slices visualised; averages were taken over 5 days in the given time window. (C) Representative CoM path vectors over time for one slice before and after Dtx, and after wash-off. Note that the CoM is still largely intact even after wash-off. (D) Relative CoM perimeter measurements (mean ± SEM) post-Dtx and post-wash compared to pre-Dtx perimeter lengths for both VpacCre-DtR and VipCre-DtR slices.
3.4 Discussion

3.4.1 VIP and VPAC2 cell populations are largely distinct in the SCN

Two important questions that had to be addressed regarding the VipCre and VpacCre mouse lines before any subsequent experiments were performed were, first, does Cre expression reflect native VIP or VPAC2 expression? Second, are the VIP and VPAC2 cell populations distinct or do they overlap? With regards to the first question, comparisons with VIP or VPAC2 immunostaining or FISH would indeed suggest that Cre expression mimics core or shell regionality. Two-colour FISH on the knock-in VipCre strain convincingly demonstrated that Cre is limited to, and present in all, VIP cells. FISH in the VpacCre line presented some difficulty (data not shown), presumably due to the low expression of the VPAC2 receptor mRNA in contrast to the typically high levels of pre-pro-neuropeptide mRNA expression. Comparisons between nuclear-localised Cre and membrane-localised VPAC2 immunostaining demonstrated that the two signals qualitatively displayed the same patterning within the SCN, as did the clear existence of VPAC2 “halos” around Cre-positive nuclei. If any doubt remain, future work could combine VPAC2 immunostaining with expression of a flexed membrane-bound marker (such as fluorescently tagged channelrhodopsin), which would be expected to overlap. For the purposes of this study, however, the fidelity of the cell type-specific expression of Cre was very high.

The question of whether VIP and VPAC2 are co-expressed, and thus if VIP signalling can act in an autocrine manner, has previously received conflicting answers. One paper utilised a β-galactosidase reporter of VPAC2 expression and found that approximately one third of VIP cells expressed VPAC2, although it did not report what proportion of VPAC2 cells this represented (Kalló et al., 2004a). More recently, immunostaining for VPAC2 in SCN
sections and SCN dispersed cell cultures suggested that VPAC2 was expressed in almost all SCN cells (An et al., 2012). However, an approach relying entirely on immunostaining suffers from the problems of staining for VPAC2 described above: that it is difficult to determine colocalisation due to its membrane-bound localisation, including in neural processes (An et al., 2012). Furthermore, the images of SCN sections presented in this particular article are limited to a low magnification, making single cell (and single plane) resolution difficult, while images of cell culture show the receptor being expressed across the cell body, in contrast to its localisation in SCN neurons in situ. This may suggest that there is some non-specific binding of the antisera when used in cell cultures, or that expression of VPAC2 itself changes during as a consequence of dissociation.

Use of the VpacCre line in concert with Cre immunostaining or a fluorescent reporter allow a more reliable cell count due to colocalisation with the nuclear marker DAPI, and counts using this method suggest that VPAC2 is expressed in slightly less than 40% of SCN cells. Furthermore, only 13% of VIP cells (identified by immunostaining) were positive for VpacCre-YFP. While this is likely to be a slight underestimate due to the VIP neuropeptide often residing in neural processes rather than the cell body, certainly only a small minority of VIP cells appear to have the capacity for autocrine signalling, an observation supported by single-cell transcriptomics (Park et al., 2016). Another possibility is that VpacCre expression in VIP-positive cells only occurred during development, releasing the EYFP reporter, but is no longer active during adulthood. The extent of co-expression between VIP and VPAC2 is also in stark contrast to AVP-VPAC2 colocalisation, where the vast majority of AVP cells are receptive to VIP signalling. The significance of putative VPAC2-expressing VIP-positive cells is not clear, but one suggestion previously put forward was that this might facilitate communication between
the two nuclei of the SCN, which has previously been observed to be VIPergic in nature and to primarily involve connections between the two core regions (Abrahamson and Moore, 2001; Kalló et al., 2004a).

3.4.2 Flexed diphtheria toxin receptor expression allows selective ablation of cells

As well as validation of mouse strains, the DtR construct also required validation to demonstrate that its use resulted only in ablation of the targeted cell population. Work in N2A cells, used because they are derived from mice (rather than humans which would express a native DtR) and are neuron-like, demonstrated that the plasmid was indeed only expressed in Cre-expressing cells, and that application of Dtx selectively ablated DtR-expressing (mCherry-positive) cells. It was also observed that Dtx was having non-specific effects on DtR-negative cells. However the Dtx concentration was not optimised in these experiments once the construct had been satisfactorily validated due to the expectation that different concentrations would be required in different experimental systems (such as SCN slices vs. N2A cells), and the N2A cells themselves were not going to be used experimentally. The optimal Dtx concentration to use in SCN slices was determined through pilot experiments, looking to establish the highest concentration that could be used which had no detrimental receptor-independent effect on the sensitive PER2::LUC translational reporter in DtR-negative slices. This was determined to be 20 ng/ml toxin. Given that it has previously been shown that a single molecule of the A subunit of Dtx is sufficient to kill a cell (Yamaizumi et al., 1978), it is likely that no Dtx is entering DtR-negative cells in either WT slices or in DtR-expressing slices, attesting to the specificity of the approach. This was further demonstrated by clear VIP immunostaining in VpacCre-DtR slices that had been treated with Dtx.
To my knowledge, the experiments presented in this chapter represent the first use of virally transduced DtR as a method to ablate cells. This approach offers a number of advantages over other cell ablation methods, such as caspase-mediated apoptosis (pro-taCasp3; Yang et al., 2013). First, DtR itself does not affect cell function (Hatori et al., 2008), requiring the presence of the toxin to be effective. This therefore allows a great deal of temporal control, particularly given the rapid effects of Dtx. Conversely, the single-component system of pro-taCasp3-mediated cell death would potentially take time to express to sufficient levels if delivered virally, which in turn may vary between cells. Secondly, Dtx can be delivered by intraperitoneal injection because it crosses the blood-brain barrier (Buch et al., 2005; Hatori et al., 2008). This is particularly useful for long-term circadian experiments, in which cells of interest can be ablated in vivo without significant disruption to the normal behaviour of a mouse often seen with, for example, a surgical approach. Finally, the virally mediated delivery, rather than genomic nature of the system means that it can be targeted to a small region such as the SCN to be studied in isolation. It also allows it to be easily transferred to any Cre recombinase strain to study other cell populations of interest. The primary drawback of using this approach appears to be potential non-specific effects, requiring careful optimisation of effective toxin concentration in a given system before undertaking experiments.

3.4.3 Ablating VIP vs. VPAC2 cells

At the outset of this project, ablation of VPAC2 cells was hypothesised to be more detrimental to SCN rhythmicity than VIP cell ablation due to their greater number across the SCN, allied to loss of not only VIP signalling but also the majority of AVP cells. Furthermore, previous work has shown that AVP and GRP are capable of at least partially compensating VIPergic
deficiencies (Brown et al., 2005; Maywood et al., 2011), thus ablation of the VIP cells might only have had a minor effect. In reality, deletion of the VIP cells appeared to be far more pronounced. Not only did it cause a more rapid reduction in amplitude of the molecular rhythm in the SCN slice than in VpacCre-DtR slices, it also had a greater impact on both cellular synchrony and the spatiotemporal wave of bioluminescence when examined with time-lapse imaging.

Alone, these effects are not surprising, as deficiencies in VIP signalling have previously been shown to result in reduced synchrony of neurons and weak oscillations of core clock genes (Brown et al., 2007; Harmar et al., 2002; Hughes et al., 2008; Maywood et al., 2006). Moreover, this lack of synchrony could be explained by a lack of balance in VIPergic and GABAergic tone within the SCN, as these two neural signals have previously been demonstrated to be antagonistic: VIP synchronises while GABA actively desynchronises (Aton et al., 2006; Evans et al., 2013). Loss of the cells would therefore remove both VIP and the associated GABAergic cues from the VIP cells, which could potentially have left the remnant SCN circuit unperturbed (except for a proportional (~15%) loss of bioluminescent amplitude). Expression of GABA is SCN-wide (Abrahamson and Moore, 2001), however, so the remaining cells may still be expressing GABA and causing desynchronisation. If this were the case, GABA receptor antagonists would be expected to improve synchrony in VIP-ablated slices.

Similarly, it was expected that ablation of VPAC2 cells would also cause severe effects on SCN rhythmicity, for example disrupting the spatiotemporal wave, given the resultant VIP-signalling deficit, and given that the “leading” AVP cells were likely ablated. Although the wave of PER2::LUC expression does begin in the dorsomedial region of the SCN, this does not necessarily mean that they are essential to nor initiators of the wave, as one study has
shown that surgical separation of the core from the shell led to disrupted phase relationships between cells in the shell, but not in the core (Yamaguchi et al., 2003). The current study would therefore support this idea that it is the core that is more important for appropriate phasing across the circuit. One intriguing possibility for the surprisingly weak effects of VPAC2 cell ablation is if more antagonistic signals exist within the SCN than just VIP and GABA. Some antagonism between the core and shell regions is expected given that the SCN as a whole is largely resistant to perturbation (such as light pulses or lighting shifts), taking several days to align to a new lighting schedule, despite the SCN core shifting rapidly (Albus et al., 2005). GABA has previously been implicated in allowing the core to have a greater degree of phase shifting influence over the shell (Albus et al., 2005), however the shell is clearly partly resistant to this, otherwise it would also be shifted immediately. Thus it is possible that in ablating the core, only an antagonistic shell signal of an unknown, non-AVP nature remains and this results in desynchrony, which is not present when the VPAC2 cells are ablated. Antagonists of canonical shell neuropeptides, or indeed GABA itself which is expressed across the SCN (Abrahamson and Moore, 2001), may therefore be expected to improve synchrony in VIP-ablated slices.

A potential example of such antagonism could be the expression of recently discovered Gpr176 in VPAC2 cells (Doi et al., 2016). This G-protein coupled receptor was found to be expressed in antiphase to VPAC2, supressing cAMP production counter to the action of VPAC2. However, Gpr176 is unlikely to be the location of such antagonism, as VPAC2 and Gpr176 double knockout mice do not show a reversion to rhythmicity relative to the single VPAC2 knockout, and indeed it exacerbates their short period (Doi et al., 2016). Nevertheless, this antagonistic pair of receptors (at least with regards to
3.4.4 Future work and conclusions

The work involving the DtR presented in this chapter represents a proof-of-principle, having thus far only characterised the effects of ablating the VIP or VPAC2 cells on core circadian parameters in \textit{ex vivo} SCN slices. Larger sample sizes are still required to ensure the reliability and reproducibility of the results, particularly for observations made using time-lapse CCD imaging. Following on from that, there are a number of obvious areas into which this work could expand to further elucidate the roles of the VIP and VPAC2 cells in SCN timekeeping and intercellular communication in addition to those already discussed (such as the use of receptor antagonists).

First, characterisation of canonical SCN neuropeptides following region ablation would be important to identify any potential compensatory mechanisms. For example, following VIP or VPAC2 ablation, are AVP or GRP responsible for the residual level of cell synchrony as has previously been suggested (Maywood et al., 2011)? Simple immunohistochemical staining of these neuropeptides or their receptors may reveal compensatory increases or decreases, while antagonists against their receptors may expand existing knowledge on the hierarchy of synchronising signals in the SCN (Brown et al., 2005; Maywood et al., 2011).

Establishing the effects of cell ablation \textit{in vivo} is likely to demonstrate the true value of this approach, where both inputs into and outputs from these two regions will also be affected. The core region of the SCN is known to mediate photic input from the retinohypothalamic tract (RHT), with both VIP and GRP signalling playing a role, although they are regarded as separate cell
populations (Abrahamson and Moore, 2001; Aioun et al., 1998; Karatsoreos et al., 2004; Piggins et al., 1995; Reed et al., 2001). Thus the ability of cells utilising GRP and other traditional core neuropeptides to convey photic information, and indeed non-photic information such as from the geniculohypothalamic tract or the raphe nuclei (Card and Moore, 1982; Hastings et al., 1997; Meyer-Bernstein and Morin, 1999; Ribak and Peters, 1975; Sumova et al., 1996), in the absence of VIP cells could be investigated.

With regards to SCN outputs, while the shell is typically thought of as being important in signalling to distal regions (Evans et al., 2015), it is also known that the core outputs to areas both distinct from (van der Beek et al., 1993; Yan et al., 2005) and common to (Abrahamson et al., 2001; Kalsbeek and Buijs, 2002; Watts and Swanson, 1987; Watts et al., 1987) the shell. Different types of signal have even been suggested to be responsible for different circadian rhythms within an organism (Meyer-Bernstein et al., 1999). Ablation of different SCN cellular regions, and thus their outputs, might therefore render mice rhythmic in some rhythms but not others, such as locomotor activity but not corticosterone oscillations.

To conclude, I have developed a flexible system to allow highly selective cell ablation. Although the work is currently in its preliminary stages, the potential uses for this AAV both in the circadian field and elsewhere are extensive, and offer a number of advantages over traditional genetic knockout and surgical lesioning approaches. I have found that ablation of both the VIP and VPAC2 cell populations within the SCN significantly reduces the amplitude of all SCN oscillators, beyond that expected on a pro-rata basis for cell loss, while ablation of the VIP cells (which occupy ~15% of the SCN) had more extensive effects on SCN rhythmicity than that of VPAC2 cells (~40% of the SCN), including significant period lengthening and considerable attenuation of the spatiotemporal wave of core clock gene expression.
CHAPTER 4

GENETIC MANIPULATION OF VIP AND VPAC2 CELL POPULATIONS

4.1 Introduction

Circadian timekeeping in mammals is organised hierarchically: while most cells in the body have the ability to maintain rhythmicity, they require coordination from the central pacemaker, the suprachiasmatic nucleus (SCN; Reppert and Weaver, 2002). The SCN synchronises downstream oscillators via a variety of output signals, which are powerful enough to drive even transplanted tissue to its own period, although this stops short of inducing rhythmicity in arrhythmic cells (Pando et al., 2002).

In addition to serving as distal synchronising cues, SCN signalling molecules are also essential for robust and coherent rhythmicity amongst SCN cells. It has been proposed that individual SCN neurons are actually weak clocks and that intercellular communication is responsible for the precise, high amplitude rhythmicity typically seen in vivo or in SCN explants (Herzog et al., 2004). Evidence for this can be found when SCN are plated as dispersal cultures: at low density, ~60% of SCN neurons are rhythmic, while only ~30% remain so in complete isolation (Webb et al., 2009). Coupled SCN neurons are also less easily perturbed by external factors, such as temperature oscillations (Abraham et al., 2010; Buhr et al., 2010), although light input via the retinohypothalamic tract can readily reset the SCN (Yamazaki et al., 2000). Intercellular communication is even able to compensate for cell-autonomous oscillator defects, including in CLOCK (Nakamura et al., 2002), BMAL1 (Ko et al., 2010) and PER/CRY deficiencies (Liu et al., 2007; Maywood et al., 2011).
The SCN comprises a diverse range of cell subpopulations, often characterised by their expression of neurotransmitters, neuropeptides and receptors or their anatomical location within the nuclei (Abrahamson and Moore, 2001; Antle and Silver, 2005). It is frequently described as containing “core” and “shell” regions, whereby the ventral core, characterised by VIP and GRP neuropeptides, receives input from the retina as well as non-visual regions of the brain (Morin and Allen, 2006) while the dorsal shell, characterised by AVP, PK2 and VPAC2, is more intrinsically rhythmic, receives information from the core and outputs rhythmic timing cues to the rest of the body (Kalsbeek et al., 2010; Yamaguchi et al., 2003; Zhou and Cheng, 2005).

The highly heterogeneous and complex nature of the SCN thus asks an obvious question: does the master pacemaker contain its own pacemaking cells? Both in vivo and in single-cell dispersal cultures, not all SCN neurons are rhythmic (Hamada et al., 2001; Webb et al., 2009), which may suggest that the remaining rhythmic cells are the pacemaking cells of the SCN, dictating period, phase and amplitude. However, Webb et al. (2009) found that the isolated rhythmic cells contained both VIP- and AVP-expressing neurons. While this could argue against a pacemaking population, it may also simply mean that the pacemaking cells are not found along traditional SCN divisions defined by neuropeptides and location, the accuracy of which have previously been questioned (Morin, 2007), or that in vivo those rhythmic cells do not have equal power, with the responsiveness of recipient cells also playing a role.

An alternative scenario is that all SCN neurons contribute equally to periodicity, with the emergent period simply representing an average of integrated periods. However, in a study by Low-Zeddies and Takahashi (2001) in which chimeric mice containing SCN with a random proportion of
WT or Clock/Clock cells were generated, wheel-running behaviour typically either had a 24 h or a 28 h period, reflective of complete WT or Clock/Clock mice, with few intermediates observed. If periodicity truly is an average of all SCN cell periods, it would be expected that periods would linearly reflect the ratio of WT to Clock/Clock cells. This study also found that period and rhythm amplitude shared little common variance, raising the possibility that not all circadian parameters are controlled by the same cell population.

Further evidence of a pacemaking cell population arises from co-culture experiments (Maywood et al., 2011), in which SCN slices of different intrinsic periods were grafted to weakly rhythmic VIP-null slices. Not only could the grafts restore robust rhythmicity, they could also drive the VIP-null slice to their own period (20 h for Tau grafts, 24 h for WT or 28 h for Afterhours), presumably by effectively providing a phase-resetting stimulus each cycle. Importantly, however, when the host slice was VPAC2-null (and therefore could not receive VIPergic signalling from the graft), although rhythmicity could be restored, periodicity could not, for example a 28 h graft was only capable of driving 24 h rhythms in the host. This provides strong evidence that VIPergic signalling is key to period determination, although the role that host VPAC2 cells play in transmitting this information to the rest of the slice is not known.

Intriguingly, however, the finding that VIP, but not AVP, determines period has not been borne out by more recent intersectional approaches. One such study generated mice with chimeric SCN, whereby Cre recombinase was expressed under the control of the VIP gene, which in turn activated the tetO promoter transactivator, otherwise silenced by the presence of a floxed STOP cassette (Lee et al., 2015). These mice were crossed with tetO-ClockΔ19 mice, resulting in VIP cells with longer cell autonomous periods in the absence of doxycycline (dox). This had no effect on overall behavioural period. In
Genetic manipulation of VIP and VPAC2 cell populations

contrast, when Cre was expressed in neuromedin S (NMS) cells, which make up 40% of the SCN and comprise both VIP and AVP cell populations, a clear lengthening of behavioural period was observed. However, NMS itself was shown to be dispensable for time-keeping, thus whether the period lengthening was due to the AVP cells or to as-yet uncharacterised AVP-negative, NMS-positive cells is not clear.

Manipulation of period in both core and shell regions of the SCN was also investigated using Cre expressed in dopamine 1a receptor cells (DarCre) to remove the Tau mutant form of casein kinase 1 epsilon (CK1ε\(^{\text{Tau/Tau}}\); Smyllie et al., 2016), a mutation which if expressed globally results in a free-running period of 20 h (Meng et al., 2008). Thus, DarCre cells would be reverted to 24 h while the rest of the organism remained at 20 h. DarCre is expressed in ~80% of VIP cells and ~60% of AVP cells, and was able to revert behavioural period to 24 h in some mice, while others remained at 20 h, and a third group displayed unstable periodicity. This incomplete reversion in all mice compared to the complete alteration seen in NMS-Clock mice may be due to the non-DarCre AVP and VIP cells, in which NMS is expressed, although both DarCre and NMS populations contain large numbers of unidentified cells with regards to neuropeptidergic identity.

AVP cells have also been investigated in isolation by expressing Cre in AVP cells and deleting casein kinase 1 delta (CK1δ), which lengthens the cell autonomous clock (Mieda et al., 2016). Behaviourally, this resulted in a lengthened period of 24.7 h, which could be reversed by virally overexpressing a flexed CK1δ. Interestingly, this result was not replicated at the SCN slice level, where the dorsal region was phase delayed relative to the core region in AVP-CK1δ SCN, but period persisted at 24 h. Nevertheless, at the whole organism level, AVP cells are clearly able to exert an effect on period, while that of VIP cells appears minimal.
The observation that period and amplitude are not covariate (Low-Zeddies and Takahashi, 2001) and that SCN outputs cannot induce rhythmicity in arrhythmic peripheral cells (Pando et al., 2002) suggest that different circadian characteristics may depend on different populations of cells, and driving rhythmic cells with a different period is easier than inducing rhythmicity from nothing. Thus, SCN cell subpopulations capable of altering the overall rhythmicity of the animal may differ to those capable of dictating period. AVP and NMS cells have been investigated in this regard by deleting BMAL1 in these cell types (Lee et al., 2015; Mieda et al., 2015), rendering the cells arrhythmic (Bunger et al., 2000; Gibbs et al., 2012). In NMS-Bmal1 mice, arrhythmicity is observed in constant conditions (DD), whereas in AVP-Bmal1 mice, period is lengthened, possibly suggesting that NMS cells are important for behavioural rhythmicity, whereas arrhythmic AVP cells may just destabilise rhythm robustness and coherence, but are not sufficient to control the rhythmicity of other cell types. This provides an interesting contrast to period manipulation, where AVP cells were capable of dictating period at the behavioural level (Mieda et al., 2016). If the behavioural arrhythmicity in NMS-Bmal1 mice is not, therefore, due to AVP cells, the possibility of VIP cells being important for rhythmicity in the SCN remains.

The experiments presented in this chapter aim to further explore the existence and nature of pacemaker cells within the SCN. Utilising intersectional genetics approaches in which Cre recombinase is expressed in either VIP neuropeptide or VPAC2 receptor cells, the ability of these two predominantly separate cell populations (see Chapter 3) to dictate periodicity and affect rhythmicity through the removal of Tau and BMAL1 respectively are investigated. I demonstrate that VPAC2, not VIP, cells are capable of affecting period, while rhythmicity is necessary in both populations for completely
reliable circadian timekeeping at the behavioural level, although this is not always reflected in the isolated SCN.
4.2 Methods

4.2.1 Intersectional genetic scheme

The provenance of the mice used throughout this chapter is described in General Methods (chapter 2). VipCre and VpacCre mice containing no floxed alleles were crossed with mice containing the Tau mutation and a floxed exon within the CK1ε gene (Ck1ε^{Tau/Tau} mice). The Ck1ε^{Tau/Tau} mouse free-runs with a period of 20 h (Meng et al., 2008), and Cre-mediated removal of the floxed exon results in a phenocopy of the Ck1ε^{-/-} mouse, which free-runs with a period of 24 h (Meng et al., 2008). Thus the resultant mice will be temporal chimaeras, with 24 h VIP (VipCre-Ck1ε^{Tau/Tau}; Figure 4.1A) or VPAC2 (VpacCre-Ck1ε^{Tau/Tau}, Figure 4.1B) cells, while cells expressing no Cre will remain at 20 h.

Figure 4.1 Intersectional genetics strategy used to generate VipCre-Tau, VipCre-Bmal1, VpacCre-Tau and VpacCre-Bmal1 mice
(A and B) Mice containing Cre recombinase knocked in to the VIP locus following an internal ribosome entry site (IRES) sequence (A) or as a transgene controlled by the VPAC2 promoter (B) were crossed with either Ck1ε^{Tau/Tau} or Bmal1^{Flx/Flx} mice. The result was mice containing chimeric SCN, with either VIP or VPAC2 cells lacking the Tau allele or Bmal1, reverting them to a 24 h period as Ck1ε^{-/-} or rendering them arrhythmic as Bmal1^{-/-} respectively.
The strains will be referred to as VipCre-Tau and VpacCre-Tau respectively, which additionally encompasses controls that are WT for one or both alleles. This strategy was previously utilised in Smyllie et al. (2016), which utilised Cre expressed in dopamine receptor 1a cells (DarCre).

Separately, VipCre and VpacCre mice were also crossed with mice containing a floxed exon within the Bmal1 gene (Bmal1^{flx/flx} mice, herein Bmal1 mice) to generate VipCre-Bmal1 (Figure 4.1A) and VpacCre-Bmal1 (Figure 4.1B) mice respectively. Removal of this exon renders cells arrhythmic as assessed by a PER2::LUC reporter (Gibbs et al., 2012), thus the resulting chimeric mice should contain arrhythmic VIP (VipCre-Bmal1) or VPAC2 (VpacCre-Bmal1) cells on a rhythmic background. The mouse strains will be referred to as VipCre-Bmal1 and VpacCre-Bmal1 strains respectively, encompassing controls WT for one or both alleles.

VipCre- and VpacCre-Bmal1 strains were also crossed with mice containing a YFP reporter inserted at the Rosa26 locus (RYFP) for ubiquitous expression, preceded by a loxP-flanked transcriptional “stop” sequence, allowing visualisation of Cre expression. This was subsequently used to establish the efficiency of Cre-mediated recombination at the Bmal1 locus.

Bmal1 mice were also crossed with mice expressing DarCre, a Cre line previously shown to have extensive expression in the SCN, covering 63% of SCN cells and colocalising with 62% of AVP cells and 81% of VIP cells (Smyllie et al., 2016). The DarCre population has also been shown as being capable of dictating period by crossing with Ck1ε \textsuperscript{Tau/Tau} mice as described above, however it is not known whether dictating rhythmicity (through the removal of Bmal1) will show similar results. Furthermore, given the unusual distribution of DarCre across both the shell and the core of the SCN, it may serve as a useful control for the VipCre and VpacCre lines.
4.2.2 Wheel running analysis

Emergence of phenotype at a certain day in Bmal1 experiments was assessed by eye, and subsequent survival (of rhythmicity) curves were generated in Prism. Robustness of rhythms in Bmal1 experiments was assessed using the relative amplitude (RA) non-parametric measure available in Clocklab v6, which compares intervals of highest and lowest activity according to the equation:

$$\text{RA} = \frac{(M \, \text{Avg} - L \, \text{Avg})}{(M \, \text{Avg} + L \, \text{Avg})}$$

where $M \, \text{Avg}$ refers to the average activity during the most active phase (10 hours) of the activity profile, and $L \, \text{Avg}$ is the average activity during the least active phase (5 hours).

4.2.3 Fluorescent image analysis

Fluorescent confocal images were obtained as described in section 2.5.10. Fluorescence intensity analysis of SCN neuropeptides was carried out in FIJI using the in-built Measure function in areas of high expression within a given section.

4.2.4 SCN slice bioluminescence analysis

Slices obtained from mice involved in the Ck1ε experiments were analysed as described in section 2.5.4, as well as by peak-to-peak analysis. Peaks were identified by eye and differences across the recording window were calculated. This allows a more dynamic assessment of period change throughout the experiment.
4.2.5 Statistics

Throughout this chapter, two-way ANOVAs were typically carried out to determine statistical differences between groups or interactions between factors, using Cre genotype (VipCre or VpacCre) and floxed genotype (Ck1ε or Bmal1) as the two factors, with Tukey’s correction for multiple comparisons. One-way ANOVAs were performed in experiments involving VpacCre-Bmal1 mice, due to the inclusion of DarCre-Bmal1 mice as an extra group, with Tukey’s correction for multiple comparisons. Kruskal-Wallis tests were performed when comparing RA scores between genotypes due to the non-parametric nature of the measure, followed by Dunn’s correction for multiple comparisons.
4.3 Results

4.3.1 VpacCre-Tau Genotyping

In light of experimental experience, genotyping of the Ck1ε gene was redesigned to allow the identification of a null allele (that is, the removal of the floxed exon) within the line (Figure 4.2). Two PCR reactions were utilised, as described in Smyllie et al. (2016). The ‘Ck1ε intact reaction’ (Figure 4.2A) identified the presence of at least one copy of exon 4 of the Ck1ε gene, while the ‘Ck1ε null reaction’ (Figure 4.2B) identified the presence of at least one copy of the null allele. The combination of these two reactions revealed that all VpacCre-Ck1ε\textsuperscript{Tau/Tau} mice were actually heterozygous for the null allele,

![PCR genotyping strategies](image)

**Figure 4.2** The VpacCre-Tau line carries a Ck1ε null allele across all genotypes

(A and B) PCR genotyping strategies devised to identify the intact Ck1ε allele in which exon 4 is present (A) and the null Ck1ε allele in which exon 4 has been removed by Cre-mediated recombination, thus bringing the two primer binding sites closer together (B). (C) Representative genomic DNA samples of each genotype run on 1% agarose gels following PCR amplification as described in A and B. Prior to this new strategy, all mice were thought to be Ck1ε\textsuperscript{Tau/Tau}, however the prevalence of the null allele across all genotypes can be seen, suggesting a genotype of Ck1ε\textsuperscript{Tau/-}. Note that two ostensibly Ck1ε\textsuperscript{Tau/Tau} samples have been run due to free-running period differences; this can be explained by the presence of the null allele in one (22.1 h) but not the other (20.4 h). Positive and negative control samples were kindly given by Dr. Qing-Jun Meng (University of Manchester).
and that the null allele was prevalent in some mice across all genotypes within the same strain (Figure 4.2C). It has previously been shown that mice heterozygous for the Tau mutation have a 22 h free-running period, thus the expected period for cells that were not expressing Cre within the VpacCre-Tau line was 22 h. Subsequently, all mice used in experiments involving the VpacCre-Ck1εTau/ mouse were heterozygous for the Ck1ε null allele to serve as appropriate controls. The VipCre-Tau line was unaffected, thus is as described in Figure 4.1.

4.3.2 Removal of Tau has a limited effect on SCN neuropeptide expression

VIP, AVP and GRP were visualised using immunohistochemistry (IHC) in VipCre-Ck1εTau/Tau and VpacCre-Ck1εTau/- adult mouse brain sections (Figure 4.3). The presence of the Tau allele significantly reduced AVP levels in both VipCre- and VpacCre-Tau strains (Ck1εTau/Tau, VipCre-Ck1εTau/Tau and VpacCre-Ck1εTau/-) compared to mice carrying the Ck1ε wild-type allele (WT, VipCre-WT or VpacCre-WT), however the presence of Cre appeared to have no significant effect (Figure 4.3A and D), indicating that the removal of Tau from VIP or VPAC2 cells does not reduce AVP levels. Mice containing Cre knocked in to the VIP locus had significantly reduced levels of VIP, although the presence or absence of Tau had no effect (Figure 4.3B and E). In contrast, no significant effect was observed in the VpacCre-Tau strain on VIP levels due to the presence of Cre or Tau (Figure 4.3B). In all conditions, GRP levels appeared to be unchanged by Cre or Tau genotype (Figure 4.3C).
Figure 4.3 SCN neuropeptides are unaffected by the removal of Tau from VIP or VPAC2 cells

(A-C) Fluorescence intensities (mean ± SEM) for immunohistochemical staining of AVP (A), VIP (B) and GRP (C) in SCN sections of VipCre-Ck1εTau/Tau (top) or VpacCre-Ck1εTau/− (bottom) mice with their respective controls (n = 3-5 per genotype). Significant differences were observed for AVP (A) for the Ck1ε genotype but not for Cre genotype nor for any interaction in both VipCre-Tau and VpacCre-Tau strains, and for VIP (B) for the Cre genotype but not Ck1ε genotype or interaction in the VipCre-Tau strain. All tests two-way ANOVA with Tukey’s multiple comparisons, **P < 0.01, ****P < 0.0001.

(D-E) Representative images of SCN in VipCre-Ck1εTau/Tau mice and controls for AVP (D) and VIP (E) IHC. Scale bar represents 50 µm.
4.3.3 Removal of Tau from VPAC2, but not VIP, cells alters behavioural period

Behavioural circadian phenotypes of adult mice were assessed using wheel-running activity in 12:12 light:dark (LD) cycles, 10:10 LD or constant conditions (constant dim red light, DD). In 12:12 LD, there was no significant effect on wheel running period by expression of VipCre (Figure 4.4A and C). However, as expected in the VipCre-Tau line, the homozygous Ck1ε\textsuperscript{Tau/Tau} mice did not fully entrain to the 12:12 LD cycle, with most of their activity being masked during the light phase. The VipCre-Ck1ε\textsuperscript{Tau/Tau} mice phenocopied this, demonstrating a lack of entrainment to the 12:12 cycle. On entry into DD, both the Ck1ε\textsuperscript{Tau/Tau} and VipCre-Ck1ε\textsuperscript{Tau/Tau} mice free-ran with a period of approximately 20 h (Figure 4.4E), while the WT and VipCre-WT free-ran at 24 h, suggesting that the reversion of the VIP cells to 24 h was not sufficient to alter the behaviour of the whole organism.

In contrast to the VipCre-Tau strain, there was no significant difference between any groups in the VpacCre-Tau strain in 12:12 LD (Figure 4.4B and D). The difference between the Tau groups with no Cre expression in the two strains was anticipated given that the Ck1ε\textsuperscript{Tau/-} mice within the VpacCre-Tau line are heterozygous for Tau due to the null allele (section 4.3.1), and should therefore have a longer free-running period than their homozygous counterparts, allowing them to entrain more easily to a 24 h day. This longer period was observed in DD, presenting as a 22 h free-running period (Figure 4.4F). The removal of this Tau allele from the VPAC2 cells significantly lengthened the period, with VpacCre-Ck1ε\textsuperscript{Tau/-} mice free-running with a 23.5 h period, demonstrating an almost complete reversion towards the WT 24 h period seen in the WT and VpacCre-WT groups. Nevertheless, a statistically significant difference still remained, thus the VPAC2 cells are not able to completely dictate periodicity.
Figure 4.4 VPAC2 cells, but not VIP cells, can partially dictate behavioural periodicity (A and B) Representative double-plotted actograms of wheel-running activity of VipCre-Ck1ε^WT/WT (A) and VpacCre-Ck1ε^WT/WT (B) mice and their respective controls exposed to a 12:12 light:dark (LD) cycle followed by continuous dim red light (DD) conditions. Grey shading represents lights off. (C and D) Periods (mean ± SEM) observed in 12:12 LD for VipCre-Ck1ε^Tau/Tau (C) and VpacCre-Ck1ε^Tau/Tau (D) mice. Significant differences were observed in the VipCre-Tau strain (C) for the Ck1ε genotype but not Cre genotype or interaction (n = 4-9 per group), and no differences were seen in the VpacCre-Tau strain (D; n = 3-7 per group). Note that Ck1ε^Tau/Tau and VipCre-Ck1ε^Tau/Tau mice struggle to entrain to a 12:12 LD cycle (A), hence the seeming spread of periods in (C). (E and F) Periods (mean ± SEM) observed in DD after 12:12 LD for VipCre-Ck1ε^Tau/Tau (E) and VpacCre-Ck1ε^Tau/Tau (F) mice. VipCre-Ck1ε^Tau/Tau mice (n = 6) did not significantly differ from Ck1ε^Tau/Tau mice (n = 10) (E), while VpacCre-Ck1ε^Tau/Tau mice (n = 17) differed significantly from Ck1ε^Tau/Tau mice (n = 9) as well as WT and VpacCre-WT controls (WT: n = 9; VpacCre-WT: n = 8) (F), the latter of which suggests an incomplete reversion to WT period. All tests were two-way ANOVAs with Tukey’s multiple comparisons test, **P < 0.01, ****P < 0.0001.
While a reversion to a 24 h period is seen in most DarCre-Ck1ε\textsuperscript{Tau/Tau} mice, the relative dominance of the 24 h DarCre and 20 h non-Cre-expressing cells can be altered by previous lighting conditions; for example 20 h cells would dominate in DD immediately following 10:10 LD cycles (Smyllie et al., 2016). Thus a similar paradigm was adopted here to determine if the dominance of, in particular, the VPAC2 cells could be diminished.

During the 10:10 LD cycles, mice containing the Ck1ε WT allele struggle to entrain to the 20 h day, often only showing relative co-ordination (Figure 4.5A and B). In contrast, the homozygote Ck1ε\textsuperscript{Tau/Tau} and VipCre-Ck1ε\textsuperscript{Tau/Tau} mice were able to entrain to the 10:10 LD cycle. There was a trend for the heterozygote Ck1ε\textsuperscript{Tau/-} and VpacCre-Ck1ε\textsuperscript{Tau/-} mice to entrain somewhat more easily to the 10:10 LD cycle, although no significant differences were observed. The free-running periods in DD following the 10:10 LD cycles showed no obvious differences from that which followed 12:12 LD; this is of particular interest in the VpacCre-Ck1ε\textsuperscript{Tau/-} mice, which had an average period of 23.4 h, suggesting that previous lighting conditions did not alter the relative dominance of different cell populations within the chimeric VpacCre-Ck1ε\textsuperscript{Tau/-} SCN.

4.3.4 SCN slices from VpacCre-Ck1ε\textsuperscript{Tau/-} mice do not necessarily reflect behavioural phenotypes

Following the recording of wheel-running, adult mouse SCN slices were dissected and circadian rhythmicity was measured using a PER2::LUC bioluminescence reporter for mice within the VpacCre-Tau strain (Figure 4.6 and Table 4.1; VipCre-Tau strain mice were not investigated due to the absence of any obvious behavioural phenotype). As with behavioural periods, the presence of VpacCre itself did not affect period, while the presence of a
Figure 4.5 10:10 lighting schedule does not modulate the dominant pacemaking populations

(A and B) Representative double-plotted actograms of wheel running activity of VipCre-Ck1ε\textsuperscript{Tau/Tau} (A) and VpacCre-Ck1ε\textsuperscript{Tau/\textminus} (B) mice and their respective controls exposed to a 10:10 light:dark (LD) cycle followed by continuous dim red light (DD) conditions. Grey shading represents lights off. (C and D) Periods (mean ± SEM) observed in 10:10 LD for VipCre-Ck1ε\textsuperscript{Tau/Tau} (C) and VpacCre-Ck1ε\textsuperscript{Tau/\textminus} (D) mice. No significant differences were observed between any genotypes tested in the VipCre-Tau strain (C; n = 4-7 per group) or the VpacCre-Tau strain (D; n = 3-7 per group). (E and F) Periods (mean ± SEM) observed in DD after 10:10 LD for VipCre-Ck1ε\textsuperscript{Tau/Tau} (E) and VpacCre-Ck1ε\textsuperscript{Tau/\textminus} (F) mice. VipCre-Ck1ε\textsuperscript{Tau/Tau} mice (n = 6) did not significantly differ from Ck1ε\textsuperscript{Tau/Tau} mice (n = 6) (E), but did from other controls (WT: n = 4; VipCre-WT: n = 7). VpacCre-Ck1ε\textsuperscript{Tau/\textminus} mice (n = 6) differed significantly from Ck1ε\textsuperscript{Tau/\textminus} mice (n = 5) as well as WT controls (WT: n = 6; VpacCre-WT: n = 3) (F) similarly to DD following 12:12 LD. All tests were two-way ANOVAs with...
single Tau allele shortened the period by approximately 2 h as expected (Figure 4.6A and B). However, in contrast to the behaviour, while VpacCre-Ck1ε\textsuperscript{Tau/} mice showed rhythmicity similar to controls (Figure 4.6A), there was no significant difference between the periods of VpacCre-Ck1ε\textsuperscript{Tau/} adult SCN slices and Ck1ε\textsuperscript{Tau/Tau} slices, with both showing a period of approximately 22 h (Figure 4.6B). Thus, period lengthening observed \textit{in vivo} was not evident from the VpacCre-Ck1ε\textsuperscript{Tau/} slice in culture. Despite this seeming lack of difference in periodicity, however, the variability in VpacCre-Ck1ε\textsuperscript{Tau/} slice periods was far more pronounced, with a range of 5.56 h between the longest and shortest slice periods in the first 4 days of recording (Table 4.1).

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Table 4.1 Column statistics for adult SCN slices taken from mice within the VpacCre-Tau strain. Note the large variability in VpacCre-Ck1ε\textsuperscript{Tau/} slice period.

Furthermore, a difference in the stability of slices was observed between VpacCre-Ck1ε\textsuperscript{Tau/} and control groups, whereby the period of VpacCre-Ck1ε\textsuperscript{Tau/} slices tended to increase over time (Figure 4.6C-E); this can clearly be seen in Figure 4.6D where rhythms from a Ck1ε\textsuperscript{Tau/} slice and a VpacCre-Ck1ε\textsuperscript{Tau/} slice are aligned for the first 6 cycles before beginning to diverge. This increase in period over time is not statistically significant when mean periods are compared due to the variability of VpacCre-Ck1ε\textsuperscript{Tau/} slice periodicity. Nevertheless, if the average period increase per day is compared, VpacCre-Ck1ε\textsuperscript{Tau/} slices appear to increase their period by almost 30 minutes.
Figure 4.6 Adult SCN slices from VpacCre-Ck1ετau− show no reversion in period
(A) Representative base-line corrected PER2::LUC bioluminescence rhythms from WT, VpacCre-WT, Ck1ετau− and VpacCre-Ck1ετau−. SCN dissected following wheel-running recordings. (B) Periods (mean ± SEM) of the first 4 bioluminescent cycles of adult SCN slices in which VpacCre-Ck1ετau− (n = 10) did not significantly differ from Ck1ετau− slices (n = 4). WT: n = 5; VpacCre-WT: n = 7. (C) Periods (mean ± SEM) of the first 3 and last 3 cycles of adult SCN slices suggesting a slight period increase in VpacCre-Ck1ετau− slices over time. (D) Representative base-line corrected PER2::LUC bioluminescence rhythms from Ck1ετau− and VpacCre-Ck1ετau−. SCN slices displaying phase alignment for the first 5 cycles before a period lengthening in the VpacCre-Ck1ετau− slice. (E) Peak-to-peak differences (mean ± SEM) of PER2::LUC bioluminescence rhythms, demonstrating an increase in period over time in VpacCre-Ck1ετau− slices. (F) Period change per day (mean ± SEM) in adult SCN slices. VpacCre-Ck1ετau− slice periods increased significantly more than WT and Ck1ετau− slices and show a trend against VpacCre-WT slices (P = 0.08). (G) Bioluminescence amplitude (mean ± SEM) in the last 3 cycles as a percentage of the amplitude in the first 3 cycles, demonstrating no significant differences. All tests were two-way ANOVAs with Tukey’s multiple comparisons test, *P < 0.05, ****P < 0.0001.
per day compared to control slices, which show greater stability (Figure 4.6F). Importantly, there was no significant difference in the amplitude reduction observed over time in all genotypes (Figure 4.6G), suggesting that the period lengthening is not merely the result of cells within the slice desynchronising.

The periods observed in SCN slices (from the last 4 cycles of each recording) were directly compared to the behavioural phenotypes of the mice from which they were derived (Figure 4.7). The large variation in SCN slice period of VpacCre-Ck1ε\textsuperscript{Tau/-} mice as described in Figure 4.6 is striking when contrasted with the behavioural periods, which cluster tightly around a period of 23.5 h (Figure 4.7A). In control groups, a strong positive correlation can be seen between SCN and behavioural periods, and a linear regression fit suggests that the SCN period is predictive of the behavioural period (Figure 4.7B). In contrast, no significant correlation was observed among VpacCre-Ck1ε\textsuperscript{Tau/-} mice, and the SCN period showed no predictive ability for behavioural period.

**Figure 4.7** Adult SCN slices from VpacCre-Ck1ε\textsuperscript{Tau/-} mice do not correlate with behaviour
(A) Free-running behaviour and slice periods from WT, VpacCre-WT, Ck1ε\textsuperscript{Tau/-} and VpacCre-Ck1ε\textsuperscript{Tau/-} mice (n = 4-10 per group). (B) Scatter plot of slice periods vs. behavioural periods. VpacCre-Ck1ε\textsuperscript{Tau/-} mice show no significant correlation (P = 0.72, Pearson’s correlation) but grouped control slices do (****P < 0.0001, Pearson’s correlation). Furthermore, linear regressions were computed for the two sets of data: VpacCre-Ck1ε\textsuperscript{Tau/-}, \( r^2 = 0.02, Y = 0.02196X + 23.01 \); Controls \( r^2 = 0.85, Y = 0.5321X + 10.64 \).
4.3.5 SCN slices from neonatal Cre-Tau mice show no phenotype

All SCN slice data described thus far have been taken from adult (>P60) mice. To determine if the effects of Tau removal from SCN cell subpopulations can also be seen prenatally or neonatally, SCN slices were dissected from mouse pups (P9-P11) and rhythms were visualised using a PER2::LUC (Table 4.2 and Figure 4.8). Removal of Tau from VIP cell populations showed no phenotype in pups, consistent with the behavioural phenotypes of these mice (Figure 4.8A), with both Ck1ε\textsuperscript{Tau/Tau} and VipCre-Ck1ε\textsuperscript{Tau/Tau} pup SCN slices displaying periods of approximately 20 h.

Interestingly, VpacCre-Ck1ε\textsuperscript{Tau/-} slices did not show the same phenotype as their adult counterparts: while the period is approximately 22 h as in the adults, they displayed none of the variability or range of periods associated with adult VpacCre-Ck1ε\textsuperscript{Tau/-} slices (Table 4.2 and Figure 4.8B), nor did they show a change in period over time (Figure 4.8C).

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Table 4.2 Column statistics for pup SCN slices taken from mice within the VipCre-Tau and VpacCre-Tau strains. VpacCre-Ck1ε\textsuperscript{Tau/-} pup slices lack the variability of their adult counterparts.
One possibility for such a discrepancy between neonatal and adult slices in VpacCre-Ck1εTaul/− mice is that the regulation of the VPAC2 promoter, and therefore of Cre expression, is altered throughout development into adulthood. Therefore IHC of SCN sections for the VPAC2 receptor was carried out on a range of ages from P2 through to adulthood in WT mice (Figure 4.9). No obvious qualitative differences were observed between ages, with clear VPAC2 expression visible at all developmental stages investigated. This raises the intriguing possibility that the relative dominance of cell populations within the SCN can vary with age, or that circuit reorganisation occurs throughout development.
Figure 4.9 VPAC2 and VIP expression do not appear to change with age
Representative SCN sections stained for DAPI and immunostained for VIP (red) and VPAC2 (green) at different ages. Mice were culled at approximately the same time during the light phase. If possible, a section at approximately the same point along the rostro-caudal axis of the SCN was chosen. P, postnatal day.
4.3.6 VpacCre-dependent period manipulation does not occur in the adult mouse at sites lacking the VPAC2 receptor

Cre-mediated recombination within the Tau gene should only occur in parts of the body where VPAC2 is naturally expressed, while other cells throughout the body should remain unaffected. To confirm that cells that do not normally express VPAC2 maintained a short period in Ck1ε\textsuperscript{Tau\texttext{-}} mice, regardless of extraneous Cre expression, slices were taken from the kidneys of the same adult mice from which SCN were dissected following wheel running experiments (Figure 4.10). The kidney was chosen because VPAC2 expression is not thought to occur in the kidney, excepting the blood vessels of the renal cortex (Harmar et al., 2004). In contrast to VpacCre-Ck1ε\textsuperscript{Tau\texttext{-}} wheel running behaviour and SCN rhythmicity where the presence of VpacCre resulted, respectively, in reversion to a near wild-type period and period instability, no phenotype was observed in kidney slices, which appeared to maintain a stable rhythm not significantly different in period from Ck1ε\textsuperscript{Tau\text{-}} mouse kidneys (Figure 4.10A and B).

Figure 4.10 Kidney slices are unaffected by Cre-mediated removal of Tau in VPAC2 cells

(A) Representative base-line corrected PER2::LUC bioluminescence rhythms from WT, VpacCre-WT, Ck1ε\textsuperscript{Tau\text{-}} and VpacCre-Ck1ε\textsuperscript{Tau\text{-}} kidney slices dissected following wheel-running recordings. (B) Periods (mean ± SEM) of kidney slices showing a significant effect of Ck1ε genotype but not Cre genotype or interaction (n = 3-4 per group). Two-way ANOVA with Tukey’s post-hoc test, *P < 0.05.
4.3.7 Bmal1 can be partially removed from VIP and VPAC2 cell populations

The efficiency with which Cre-mediated recombination occurred at the *Bmal1* locus in VipCre-*Bmal1* and VpacCre-*Bmal1* mice was determined using IHC for BMAL1 on SCN sections, and comparing this to the expression of a YFP reporter for Cre expression (Figure 4.11). Within VipCre-*Bmal1* mice, BMAL1 expression was reduced by approximately 10% across all SCN cells (as marked by DAPI) compared to VipCre-WT mice, while having two floxed Bmal1 alleles in the absence of VipCre did not change Bmal1 expression significantly (Figure 4.11A and B). Further, Bmal1 expression was abolished in approximately 45% of VIP cells in VipCre mice (Figure 4.11C).

It is important to note that, as with the removal of the Tau allele (section 4.3.1), it was observed that mice carrying VpacCre were found to contain only one floxed Bmal1 allele alongside a null allele that had already undergone Cre-mediated recombination. It has previously been shown that mice containing only one copy of the *Bmal1* gene are no different from WT littermates in a number of circadian parameters (Bunger et al., 2000), however *Bmal1*^{flx/} and DarCre-*Bmal1*^{flx/} have been included in experiments involving VpacCre-*Bmal1* mice herein as controls (as opposed to containing two floxed alleles). The deletion of the remaining Bmal1 allele in VPAC2 cells had a more profound effect on overall SCN Bmal1 expression, as would be expected due to its more abundant expression compared to VipCre, reducing BMAL1 expression by almost 20% across the SCN compared to VpacCre-WT mice (Figure 4.11A and B). Within the VPAC2 cells themselves, only 31% of YFP-positive cells were found to express BMAL1 in VpacCre-*Bmal1* SCN (Figure 4.11C), suggesting a stronger recombination than that which occurred in VipCre-*Bmal1* SCN. Finally, in DarCre-*Bmal1* mice, BMAL1 was greatly reduced, remaining in only 31% of all SCN cells, and 17% of DarCre-expressing cells (Figure 4.11B and C).
Genetic manipulation of VIP and VPAC2 cell populations

**Figure 4.11 Bmal1 can be partially removed from Cre-expressing populations**

(A) Representative 63x tiled confocal micrographs of Cre expression as reported by a genetically encoded YFP reporter (green) and Bmal1 immunohistochemistry (red) in VipCre-WT, VipCre-Bmal1, VpacCre-WT and VpacCre-Bmal1 SCN sections. Locations of magnified images are indicated by white rectangles. White arrows indicate colocalisation between YFP and Bmal1 signal. Scale bars represent 50 µm in stitched images, 10 µm in magnified images. (B) Percentage of SCN cells (marked by DAPI; mean ± SEM) expressing Bmal1 across genotypes. Significantly fewer cells were found to be expressing Bmal1 in DarCre-Bmal1 (n = 3 mice), VipCre-Bmal1 (n = 8 mice) and VpacCre-Bmal1 mice (n = 8) compared to WT mice (n = 7; one-way ANOVA with Dunnett’s multiple comparisons test). (C) Percentage of Cre-positive cells (marked by YFP; mean ± SEM) expressing Bmal1 across genotypes. Significantly fewer cells were found in VipCre-Bmal1 sections (n = 4) compared to VipCre-WT (n = 3) and in VpacCre-Bmal1 sections (n = 4) compared to VpacCre-WT (n = 2). The efficiency of removal was significantly better in VpacCre-Bmal1 and DarCre-Bmal1 (n = 3) than in VipCre-Bmal1 mice. One-way ANOVA with Tukey’s multiple comparisons test. (D and E) Number of Cre-negative, Bmal1-positive cells did not differ between VipCre-WT (n = 3) and VipCre-Bmal1 (n = 5), nor VpacCre-WT (n = 3) and VpacCre-Bmal1 (n = 4) mice; unpaired t-test. (F and G) Number of Cre-positive cells did not differ between VipCre-WT and VipCre-Bmal1, nor VpacCre-WT and VpacCre-Bmal1 mice; unpaired t-test. *P < 0.05, **P < 0.001, ***P < 0.0001.
Importantly, the number of Cre-negative, BMAL1-positive cells did not differ between any genotypes compared (Figure 4.11D and E), suggesting that recombination only occurred in Cre-expressing cells. Moreover, the number of Cre-positive cells did not differ between those containing a floxed Bmal1 allele and those without (Figure 4.11F and G), thus Bmal1 deletion did not appear to affect the viability of cells.

4.3.8 Bmal1 deletion has a minor effect on SCN neuropeptide expression

As with neuropeptidergic expression in VipCre-Ck1εTau/Tau mice (section 4.3.2), no interaction was found between Cre and Bmal1 genotype in VipCre-Bmal1 mice on the expression of AVP, VIP and GRP (Figure 4.12A-C), although VIP was again found to be reduced due to the presence of VipCre alone, regardless of Bmal1 genotype (Figure 4.12B and D). In contrast, removal of Bmal1 from VPAC2 cells had a small effect on AVP expression in the SCN, reducing it to approximately 70% of control levels (Figure 4.12A and E), while the expression of VIP and GRP appeared unaffected.

4.3.9 VipCre-Bmal1 and VpacCre-Bmal1 mice display variable phenotypes in DD

In 12:12 LD, no phenotype was observed for mice of any genotype (Figure 4.13A-D), suggesting that Bmal1 in the VIP or VPAC2 cells is not necessary for entrainment to an LD schedule.

Previous studies utilising chimeric mice involving the removal of Bmal1 from SCN subpopulations have found that phenotypes do not always emerge immediately when mice are allowed to free-run in DD, sometimes taking several weeks to emerge (Lee et al., 2015). Thus, following exposure to a 12:12
Figure 4.12 AVP is slightly reduced by removal of Bmal1 from VPAC2 cells

(A-C) Fluorescence intensities (mean ± SEM) for immunohistochemical staining of AVP (A), VIP (B) and GRP (C) in SCN sections of VipCre-Bmal1 (top) or VpacCre-Bmal1 (bottom) mice with their respective controls (n = 3-4 per genotype). AVP was significantly reduced (A) in VpacCre-Bmal1 mice compared to controls. Significant differences in VIP levels were found (B) for the Cre genotype and Bmal1 genotype but no interaction was seen in VipCre-Bmal1 mice; two-way ANOVA with Tukey’s post-hoc test, **P < 0.01, ****P < 0.0001. (D-E) Representative images of SCN in VipCre-Bmal1 mice and controls for VIP (D) and in VpacCre-Bmal1 mice and controls for AVP (E) IHC. Scale bar represents 50 µm.
LD cycle to identify entrainment phenotypes, mice were placed into DD for up to 7 weeks to allow the emergence of a phenotype.

When placed into DD, VipCre-Bmal1 mice showed variable phenotypes, several of which did not emerge immediately (Figure 4.14). Some mice (7/16) showed no phenotype which differed from controls for the duration of the 7 week DD time-frame, while others (6/16) showed arrhythmic behaviour (Figure 4.14A and B). Of the remaining 3 mice, 2 showed highly fragmented behaviour (Figure 4.14A), while one displayed a short period (Figure 4.14A).
Figure 4.14 Removal of Bmal1 from VIP cells has variable effects on activity in DD

(A) Representative double-plotted actograms of wheel-running activity of control mice, and all double-plotted actograms of VipCre-Bmal1 mice (n = 16) in DD, ranked in order of relative amplitude (RA) from highest to lowest. (B) Chi-squared periodograms of VipCre-Bmal1 mice mice shown in (A). (C) Relative amplitude scores (mean ± SEM) of the last 14 days of wheel

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and D), although given that another mouse did display a short period prior to becoming arrhythmic, it is possible that this subsequent arrhythmicity was not observed due to the experimental time window. A significant difference was also found when comparing the relative amplitude (RA) of wheel running activity between VipCre-Bmal1 mice and controls (Figure 4.14C), suggesting that even when a rhythm is present in these mice, it is less robust than that of controls. Interestingly, if a phenotype was present, the time at which it emerged was highly variable (Figure 4.14E), with several mice displaying a phenotype immediately, whereas others did not emerge until several weeks into the DD recording.

As with VipCre-Bmal1 mice, VpacCre-Bmal1 mice also displayed variable phenotypes at variable times throughout the experiment, although removal of Bmal1 from VPAC2 cells only resulted in arrhythmicity in 4/14 mice (Figure 4.15A and B). Fragmented or “split” behaviour was the more common result (7/14), with several mice displaying multiple significant periods, while 3/14 did not show an obvious phenotype (Figure 4.15A and B). Interestingly, none of the DarCre-Bmal1 mice showed a severe phenotype (Figure 4.15A), with only some mild disruption of the wheel running being evident in two actograms of 2/4 mice investigated; these two mice also had slightly lower RA scores (Figure 4.15C). The RA of VpacCre-Bmal1 mice was significantly more compromised, although a spectrum of scores was evident, and scores were

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**Figure 4.14 continued**

running activity. VipCre-Bmal1 mice (n = 16) show significantly less robust rhythms than WT (n = 12) and Bmal1<sup>flx/flx</sup> (n = 12) but not VipCre-WT (n = 4) mice as assessed by Kruskal-Wallis test with Dunn’s multiple comparisons. (D) Periods (mean ± SEM) of VipCre-Bmal1 mice were not significantly different from controls, although arrhythmic VipCre-Bmal1 mice (star symbols on graph, n = 6) were not included in the comparison; two-way ANOVA with Tukey’s multiple comparisons. (E) Percentage of VipCre-Bmal1 mice displaying any behavioural phenotype (green) or specifically arrhythmic behaviour (red) over time. ***P < 0.001.
Figure 4.15 Removal of Bmal1 from VPAC2 cells has variable effects on activity in constant conditions

(A) Representative double-plotted actograms of wheel running activity of control mice, and all (legend continued on next page)
generally less severe than in VipCre-Bmal1 mice due to some form of rhythmicity (or multiple periodicities) being present. Clearly the periods of VpacCre-Bmal1 mice were highly variable, thus no significant difference was found overall when compared to controls (Figure 4.15D). As with VipCre-Bmal1 mice, phenotypes appeared at a diverse range of time points throughout the experiment (Figure 4.15E); some mice immediately showed split or arrhythmic behaviour, while others took several weeks and appeared similar to WTs until that point. Given the lack of variability in deletion efficiency across both VipCre-Bmal1 and VpacCre-Bmal1 mice (Figure 4.11), the extent of deletion would appear unlikely to be the cause of the variability observed, raising the possibilities of either subpopulations within the VIP or VPAC2 cell populations, or of a circuit-level effect being responsible instead.

4.3.10 Phenotypes of Bmal1-deleted mice are not permanent and can be reset by light

To determine if the phenotypes observed in DD are a permanent feature of mouse behaviour once emerged, mice were placed back into 12:12 LD after time in DD, and then placed into DD again (Figure 4.16). All mice were able...
to immediately re-entrain to the LD cycle, showing none of their previous arrhythmic or split behaviour (Figure 4.16), suggesting that light input can compensate for a compromised chimeric circuit. Strikingly, when returned to DD (2nd DD), none of the VipCre-Bmal1 mice exposed to this paradigm (4/4) displayed a disrupted behavioural phenotype, regardless of their activity in the 1st DD interval (Figure 4.16A), instead showing WT-like activity patterns. Improvements in RA scores to those comparable with controls were also observed (Figure 4.16B). In contrast, the 2 VpacCre-Bmal1 mice exposed to this schedule that showed a phenotype in the 1st DD also showed a disrupted

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**Figure 4.16 Phenotypes of mice with Cre-mediated Bmal1 removal can be reset by light and are inconsistent within individual mice**

(A) Representative double-plotted actograms of wheel-running activity of VipCre-Bmal1 and VpacCre-Bmal1 exposed to, sequentially, 12:12 LD, DD, 12:12 LD and DD light schedules. Grey shading indicates lights off. Note the absence of a phenotype in the 2nd DD interval in the VipCre-Bmal1 mouse, and the different nature of the phenotype, as well as time in DD until its emergence, in the VpacCre-Bmal1 mouse. (B) Relative amplitude scores compared between 1st and 2nd DD exposures. All VipCre-Bmal1 scores increase (n = 4), while VpacCre-Bmal1 scores stay approximately the same or worsen (n = 3).
phenotype in the 2nd DD, maintaining or attaining lower RA scores, while the 3rd mouse showed WT-like activity in both DD windows (Figure 4.16A and B). Extraordinarily, the disrupted behaviour of these 2 VpacCre-Bmal1 mice did not emerge in a similar time frame, nor did it necessarily present itself as the same phenotype: the example presented in Figure 4.16A demonstrates a short, though unstable, period to start with, while in the 2nd DD it shows true splitting behaviour consisting of two separate activity bouts per day.

4.3.11 Adult SCN slices of VpacCre-Bmal1, but not VipCre-Bmal1, mice are highly disrupted

As with experiments involving Cre-mediated removal of the Tau allele (section 4.3.4), SCN slices were prepared from adult mice following wheel running recording to establish if behavioural phenotypes were reflected by, and thus likely resulted from, SCN rhythmicity. When dissected from mice displaying disrupted behaviour, VpacCre-Bmal1 SCN slices were also highly disrupted, showing jagged and unstable bioluminescence recordings, and more variable periods (Figure 4.17A and B), although period measurements are somewhat unreliable in these slices, given the significantly worse relative amplitude error (RAE) scores compared to control slices (Figure 4.17C). DarCre-Bmal1 SCN slices also showed stable rhythmicity as anticipated based on wheel running behaviour, although RAE scores tended to be higher, which was not significant, possibly due to insufficient sample size (Figure 4.17A-C). Contrary to expectations, VipCre-Bmal1 slices from arrhythmic mice showed rhythmic PER2::LUC expression, demonstrating sinusoidal wave forms and periods which did not significantly differ from controls (Figure 4.17A and B), although there was a slight increase in RAE scores, suggesting less robust rhythmicity (Figure 4.17C). No significant amplitude differences were observed for any genotype investigated (Figure 4.17D).
Figure 4.17 VpacCre-Bmal1, but not VipCre- or DarCre-Bmal1, slices are significantly disrupted

(A) Representative base-line corrected PER2::LUC bioluminescence rhythms from control, DarCre-Bmal1, VipCre-Bmal1 and VpacCre-Bmal1 SCN dissected in dim red light following wheel running recordings in DD. (B and C) Period (mean ± SEM) of the first 4 bioluminescent

(legend continued on next page)
SCN slices were next directly compared to the mice from which they were obtained (Figure 4.18). In VipCre-Bmal1 mice, there did not appear to be any obvious relationship between the behaviour of the mouse and the rhythmicity of the SCN; almost all slices appeared to have robust rhythmicity, regardless of rhythmic or arrhythmic behaviour (Figure 4.18A). This is even more evident when comparing relative amplitude scores of behavioural robustness with relative amplitude error scores of slice reliability, where no clear correlation emerges (Figure 4.18B). If period measurement was possible in VipCre-Bmal1 mice (i.e. if they were not arrhythmic), then the SCN slices tended to reflect their 24 h periodicity, although interestingly the one mouse displaying a 22 h period had an almost 24 h slice rhythm (Figure 4.18C).

In contrast, VpacCre-Bmal1 slices appeared to reflect behavioural phenotype qualitatively, suggesting that disrupted behaviour was a result of disrupted SCN (Figure 4.18A). A slight negative correlation between RA and RAE scores was observed as would be expected for SCN slices to reflect behaviour, although this was not significant, possibly because the RA scores of VpacCre-Bmal1 mice were only slightly reduced compared to controls (Figure 4.15C) due to a level of robustness in the split rhythms observed. Periods in slices did not reflect behavioural periods in VpacCre-Bmal1 mice compared to controls, but this is unsurprising given that period determination was unreliable at both behavioural and slice levels in these mice (Figure 4.18C).

**Figure 4.17 continued**
cycles of adult SCN slices in which VipCre-Bmal1 (B; n = 11), DarCre-Bmal1 (C; n = 3) and VpacCre-Bmal1 (C; n = 7) slice periods did not significantly differ from control slices (n = 3-8 per group), although VpacCre-Bmal1 slices show considerable spread. (D and E) Amplitude of bioluminescent recordings (mean ± SEM) from SCN slices. No significant differences were observed between any genotypes. (F and G) Relative amplitude error (RAE) values (mean ± SEM) for bioluminescent recordings from SCN slices. VipCre-Bmal1 (F; n = 11) slices were found to be significantly different from VipCre-WT (n = 5) and Bmal1flx/flx (n = 4) but not WT slices (n = 8). VpacCre-Bmal1 slices (G; n = 7) were significantly different from WT (n = 8), VpacCre-WT (n = 3) and Bmal1 slices (n = 7). No significant differences were observed for DarCre-Bmal1 slices (n = 3). All tests two-way ANOVA for VipCre-Bmal1 slices, one-way ANOVA for DarCre- or VpacCre-Bmal1 slices, with Tukey’s test for multiple comparisons. *P < 0.05, **P < 0.01. WT values re-plotted in B-C, D-E and F-G.
Figure 4.18 Disrupted VpacCre-Bmal1, but not VipCre-Bmal1, slices correlate with disrupted wheel-running behaviour

(A and B) Representative PER2::LUC bioluminescence rhythms from VipCre-Bmal1 (A) and VpacCre-Bmal1 (B) adult slices prepared in dim red light after wheel running recordings in DD, alongside respective actograms and periodograms. (C) Scatter plot of individual relative amplitude scores for behaviour against RAE scores for slices, with linear regression lines for each group. Note in particular how poor RA scores for VipCre-Bmal1 mice are not reflected at all by RAE slice scores. (D) Free-running behavioural periods plotted against slice periods for each genotype. Note that period measurements for VpacCre-Bmal1 behaviour and slices are inherently unreliable.
4.3.12 Neonatal slices differ considerably from adult slices

BMAL1 has a role in development (Yang et al., 2016), thus it might be expected that the effects of removing Bmal1 from selected subpopulations could occur prenatally or neonatally. To determine if neonatal slices were compromised in VipCre- and VpacCre-Bmal1 mice, SCN slices were prepared from P9-P11 pups and PER2::LUC bioluminescence was used as a measure of rhythmicity (Figure 4.19). As in adult slices, all VipCre-Bmal1 slices appeared to be robust oscillators, although 3/7 slices did have a short period compared to controls, which correlated with low amplitudes (Figure 4.19A, C, E and I). This is an interesting contrast with the adult behavioural and SCN observations, where two mice showed at least transient short periods at the behavioural level, but not at the slice level. No significant effect was seen on RAE (Figure 4.19G).

Much like VpacCre-Ck1εTaul/– pup slices (Figure 4.8), no significant difference was observed between VpacCre-Bmal1 pup slices and control slices (Figure 4.19B, D, F and H), although an ordinary one-way ANOVA reveals that DarCre-Bmal1 slices have a slightly shorter period than two control groups. Importantly, however, this is not significantly different from Bmal1 or VpacCre-Bmal1 slice periods and, if DarCre-Bmal1 slices are excluded and a two-way ANOVA performed with VpacCre and the Bmal1flx/– genotype as the two factors, then Bmal1flx/– emerges as resulting in a significantly shorter period. Nevertheless, it is clear that the highly disrupted PER2::LUC expression exhibited by adult VpacCre-Bmal1 slices is absent, suggesting that this occurs at a later developmental stage.
Figure 4.19 Regional Bmal1 deletion in pup SCN shows minimal effects on slice rhythmicity

(A and B) Representative PER2::LUC bioluminescence rhythms from VipCre-Bmal1 (A) and VpacCre-Bmal1 (B) pup slices. (C and D) Period (mean ± SEM) of VipCre-Bmal1 (C), DarCre-Bmal1 and VpacCre-Bmal1 (D) pup slice bioluminescence with respective controls (n = 3-9 per group). VipCre-Bmal1 slices (n = 7) were not significantly different from controls, although a separation can be seen within the group, which contains WT-like slices (n = 4) and short period

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4.3.13 Kidney slices are unaffected by the removal of Bmal1 from VIP or VPAC2 cells

Bmal1 removal was only expected to occur in cells where native VIP and VPAC2 expression occurs. This was confirmed by preparing kidney slices from adult mice following wheel running experiments (Figure 4.20), coincident with the dissection of SCN slices (section 4.3.11). Slices from all genotypes produced robust PER2::LUC rhythms (Figure 4.20A), with no obvious effects on period (Figure 4.20B) or RAE (4.20C), although a significant difference between VipCre-positive and non-Cre-expressing slices was found for RAE scores.

Figure 4.19 continued
slices \( n = 3 \). DarCre-Bmal1 \( n = 3 \) were significantly shorter than WT slices \( n = 8 \) but not from Bmal1\(^{flx/}\) \( n = 4 \) or VpacCre-Bmal1 \( n = 6 \) slices. \( \text{E and F} \) Amplitude of bioluminescence rhythms (mean ± SEM) of VipCre-Bmal1 \( \text{E} \), DarCre-Bmal1 and VpacCre-Bmal1 \( \text{F} \) pup slices. No significant differences were found between any groups, however the separation observed in \( \text{C} \) was also seen in \( \text{E} \). \( \text{G and H} \) RAE scores (mean ± SEM) of VipCre-Bmal1 \( \text{G} \), DarCre-Bmal1 and VpacCre-Bmal1 \( \text{H} \) pup slice bioluminescence. No significant differences were observed for any genotypes. \( \text{I} \) Scatter plot of slice periods vs. bioluminescence amplitude in VipCre-Bmal1 slices. A significant correlation is observed for VipCre-Bmal1 slices, but not for grouped controls (Pearson’s correlation). All tests two-way ANOVA for VipCre-Bmal1 slices, one-way ANOVA for DarCre- or VpacCre-Bmal1 slices, with Tukey’s test for multiple comparisons unless otherwise specified. \( *P < 0.05 \), \( **P < 0.01 \), \( ****P < 0.0001 \).
Figure 4.20 Kidney slices are unaffected by Cre-mediated removal of Bmal1 in VIP or VPAC2 cells

(A) Representative base-line subtracted PER2::LUC bioluminescence rhythms from WT, VipCre-Bmal1 and VpacCre-Bmal1 kidney slices dissected following wheel-running recordings. (B and C) Periods (mean ± SEM) of kidney slices showed no significant effect of genotype. (D and E) RAE scores (mean ± SEM) of kidney slices showed a significant effect of Cre genotype (***P < 0.01) but not Bmal1 genotype nor any interaction in VipCre-Bmal1 slices. No significant effects were observed for DarCre- or VpacCre-Bmal1 slices. All tests two-way ANOVA for VipCre-Bmal1 slices, one-way ANOVA for DarCre- or VpacCre-Bmal1 slices, with Tukey’s test for multiple comparisons.
4.4 Discussion

The SCN offers a unique insight into the workings of neuronal circuits, due to the existence of a clear link between this conserved region of the brain and its control on behaviour, as well as the ability to study the SCN in culture for many weeks or months (Brancaccio et al., 2014). Due to the complex heterogeneity present within the SCN, it also offers an opportunity to study how different cell types interact within a circuit. Early transplant approaches demonstrated the importance of the SCN for dictating behavioural period (King et al., 2003; Ralph et al., 1990), but could not address the relevance of potential cellular hierarchies within the SCN. Thus, a long-standing question within the field remains: do pacemaker cells exist, or is SCN timekeeping the result of the entire circuit? Intersectional genetics offers a multitude of benefits over the transplantation technique, including the preservation of cellular connections, and the ability to investigate specific cellular subtypes. Here, using an intersectional genetics approach, I have demonstrated that VPAC2 cells, not VIP cells, are able to control mouse free-running behavioural period. I have also explored the difference between period determination and rhythmicity generation, in which it appears that both populations in the VIP signalling axis are capable of affecting the rhythmicity of the entire organism.

4.4.1 Do pacemaker cells exist?

The experiments presented in the first half of this chapter indicate that VPAC2 cells are at least partial pacemakers, reverting a mouse with predominantly 22 h cells to a 23.5 h free-running period through deletion of a Tau allele in these cells in the dorsal region of the SCN. In contrast, deletion of Tau from the retinorecipient VIP cells had no effect on period. A similar
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approach was previously used by Smyllie et al (2016). In all cases, no effect on SCN neuropeptide expression was observed, demonstrating the usefulness of deleting a kinase rather than manipulating a transcription factor, which may lead to confounding factors such as reduced neuropeptide expression (Mieda et al., 2015).

These results complement those seen in recent papers using similar approaches, but targeting different alleles or cell populations. Lengthening the period specifically in VIP cells on a WT background (Vip-ClockΔ19) had no effect on free-running period, while using the same approach in NMS cells (NMS-ClockΔ19), which contain both AVP and VIP cells, did result in a behavioural period lengthening (Lee et al., 2015). Similarly, period-lengthening in DarCre cells (DarCre-Ck1εTau/Tau) was able to completely control the periodicity of at least some mice (Smyllie et al., 2016), while altering the period of AVP cells alone was also capable of manipulating free-running period (Mieda et al., 2016). Given that the VPAC2 cell population encompasses ~85% of AVP cells, it is possible that the effects seen in my experiments are a direct result of simply altering AVP cell-autonomous oscillators. However, it is unlikely that this is the case. First, DarCre expression includes approximately 60% of AVP cells; this is considerably less than VPAC2 cells cover, and yet many DarCre-Ck1εTau/Tau mice show a complete reversion to a 24 h period from 20 h. Furthermore, a global AVP knockout has only a minor effect on period (Groblewski et al., 1981). Thus, the AVP cell population may be important in period determination, but the AVP neuropeptide itself is unlikely to be able to dictate period, suggesting that AVP cells may be acting as a marker for another period-determining neuropeptide or signalling molecule.

Interestingly, the results presented above and those of my own work do not appear to agree with co-culture experiments, which have demonstrated that
VIP is sufficient to drive VIP-deficient host slices to the period of a WT graft, yet AVP and GRP cannot (Maywood et al., 2011). In the experiments presented here, 24 h VipCre-Ck1ε<sup>Tau/Tau</sup> cells cannot drive other cells within the circuit to be 24 h, while in VpacCre- Ck1ε<sup>Tau−/−</sup> mice, 24 h VPAC2 cells can override timing cues emanating from 22 h VIP cells. Differences in these results may arise from the fact that in intersectional approaches, cell types of different intrinsic periods are present together throughout development, whereas in co-culture experiments, exogenous signals appear post-natally. Thus, it may be that co-culture experiments are more analogous to entrainment to LD cycles (in which rhythmic VIP release (Shinohara et al., 1995) acts as the “light”, given that VIP cells are stimulated by light) entraining a VIP-null oscillator, the weakened intercellular coupling of which may result in an increased range of entrainment. Graft slices are also only able to signal through diffusible factors, whereas in my experiments electrical and non-synaptic connections will also exist. Given that circadian electrical communication is not just an output, but also an input to the clock (Jones et al., 2015), differences may arise through this as well.

### 4.4.2 Rhythmicity vs. Periodicity

There appear to be inherent differences between periodicity and rhythmicity in circadian timekeeping, and while certain cell populations within the SCN may be sufficient to affect period, this does not necessarily indicate that they are sufficient or essential for rhythmicity. For example, when NMS cells have a longer cell-autonomous period, so too does the whole organism, and when NMS cells are arrhythmic, wheel running behaviour reflects this in DD (Lee et al., 2015). In contrast, AVP cells appear to be capable of affecting period, yet when they are arrhythmic, whole organism arrhythmicity does not follow (Mieda et al., 2015, 2016). I have shown evidence that suggests that both
VPAC2 and VIP cell populations are able to alter the rhythmicity of whole animal behaviour, demonstrating a clear contrast to their influence on period, where VIP had no effect.

It is important to note that mice containing arrhythmic VIP or VPAC2 cells are not merely phenocopies of VIP- or VPAC2-null mice. The most obvious difference between them is that VIP signalling remains present in VipCre- and VpacCre-Bmal1 mice, albeit with presumably aberrant timing. At the behavioural level, VIP- and VPAC2-null mice both struggle to completely entrain to LD cycles, and immediately show either arrhythmicity or significantly shorter periods upon entry into DD (Aton et al., 2005; Ciarleglio et al., 2009; Colwell et al., 2003; Harmar et al., 2002). In contrast, the ability to entrain to a light-dark cycle was not significantly compromised in VipCre- or VpacCre-Bmal1 mice. Moreover, while arrhythmicity did occur in a subset of VipCre-Bmal1 mice, this did not necessarily occur immediately, while in VpacCre-Bmal1 mice, residual rhythmicity of multiple periods could often be seen in DD. The lack of an LD phenotype may be unsurprising: it has been suggested that many cells within the retinorecipient region are not intrinsically rhythmic (Hamada et al., 2001), thus responses to an LD schedule may not require an intact cell-autonomous oscillator. Future work could investigate whether VIP signalling in VipCre-Bmal1 is indeed arrhythmic and thus the cause of the lack of rhythmicity seen in DD, such as through the use of a graft/co-culture system.

As with identifying cell subpopulations important for pacemaking, it appears likely that certain defined subclasses of cells are important for rhythmicity, rather than merely a certain number of cells being required to reach a certain threshold, or a linear change in rhythmicity being observed as more and more cells are rendered arrhythmic. For example, using mice heterozygous for Synaptotagmin10-Cre (Syt10Cre/+)) and containing one floxed and one null
Bmal1 allele (Bmal1<sup>flx/</sup>-), one study reduced Bmal1 expression in the SCN by 65%, yet no behavioural phenotype was observed (Husse et al., 2011). A second copy of Cre was required (Syt10<sup>Cre/Cre</sup>), leaving just 17% of the SCN expressing Bmal1, in order to see arrhythmic behaviour in DD. Furthermore, loss of Bmal1 from ~30% of neurons using a Nestin-Cre line did not have any effect on behavioural rhythms in DD (Mieda and Sakurai, 2011), whereas deletion of Bmal1 from NMS neurons, occupying 40% of the SCN, was sufficient to result in arrhythmic behaviour, albeit with transient rhythmicity observed immediately after release into DD from LD (Lee et al., 2015). My own work has demonstrated that deletion of Bmal1 from 45% of VIP cells, resulting in an overall reduction of Bmal1 in the SCN of only ~10%, is sufficient to cause arrhythmicity in some mice, while in VpacCre-Bmal1, Bmal1 was reduced in the SCN by 15-20% which led to split behaviour or arrhythmicity in most mice. Remarkably, when Bmal1 is removed from DarCre cells which contain most of the VIP cells as well as AVP cells (and therefore some VPAC2 cells), resulting in only 31% of the SCN remaining positive for Bmal1, no phenotype is seen, raising the interesting possibility of compensatory mechanisms when the entire microcircuit is compromised.

The transient or permanent rhythmicity seen in DD in many of the VipCre- and VpacCre-Bmal1 mice, as well as NMS-Bmal1 mice (Lee et al., 2015), is intriguing, not least because phenotypes emerge after different lengths of time in DD both between mice as well as within mice (1<sup>st</sup> DD vs. 2<sup>nd</sup> DD). Presumably this rhythmicity emerges from non-VIPergic cells compensating for loss of cell-autonomous oscillators in the VIPergic microcircuit by intercellular coupling. Obvious candidate neuropeptides for this are AVP and GRP, both of which have been shown to be capable of inducing rhythmicity in VPAC2-null slices (Maywood et al., 2011). Given that many AVP cells also express VPAC2, this may remove AVP as an effective coupling factor in
VpacCre-Bmal1 mice, leaving only GRP, which has been shown to be able to sustain rhythmicity in VPAC2-null slices transiently before (Brown et al., 2005; Maywood et al., 2006). This could account for the more severe and consistent phenotypes often observed in VpacCre-Bmal1 mice, although arrhythmic AVP cells alone are not sufficient to induce whole organism arrhythmicity (Mieda et al., 2015). It is interesting to note that VIP signalling has previously been shown to be able to compensate for TTFL deficiencies (Maywood et al., 2011) and that intercellular coupling generally can result in erratic rhythms in Bmal1-null slices (Ko et al., 2010), thus this may be the cause of the partial rhythmicity and split behaviour seen in many VpacCre-Bmal1 mice.

As well as a lack of synchronisation factors, the phenotypes observed in both VipCre- and VpacCre-Bmal1 mice may arise from the role of GABA in desynchronisation across the SCN circuit. GABA\textsubscript{A} signalling appears to act counter to VIP signalling: while VIP is a potent synchronising signal, GABA actively causes desynchronisation (Evans et al., 2013). It is possible that VIP is unable to act as a synchronising signal in VipCre- and VpacCre-Bmal1 SCN due to arrhythmic VIP signalling (sending or receiving), yet GABA can still desynchronise cells because GABA expression is ubiquitous across the SCN (Abrahamson and Moore, 2001), although GABA expression has not yet been explicitly investigated in these genotypes.

To date, the VipCre line is the only line which has been demonstrated as being important to rhythmicity, but not to periodicity. This is in contrast to both AVP-Cre and DarCre lines, which can both affect period (Mieda et al., 2016; Smyllie et al., 2016) but not rhythmicity (Mieda et al., 2015). This is presumably due to the synchronising role of VIP: if signalling is disrupted, then so too is circuit synchrony, whereas due to being readily reset by light (Nagano et al., 2003), it is less predictable and therefore less robust as a
determiner of period. Rhythmicity seen in DarCre-Bmal1 mice may be the result of the remaining VIP and AVP cells which DarCre does not cover, and this may account for the differences seen with the arrhythmic behaviour of NMS-Bmal1 mice, which contains almost 100% of VIP and AVP cells.

4.4.3 Effects of light on chimeric circuits

Despite the often severe phenotypes observed in DD in the experiments presented in this chapter, no differences were observed in LD cycles (12:12 or 10:10) between experimental groups and controls in any study.

VipCre-Ck1ε\textsuperscript{Tau/Tau} mice were unable to entrain to 12:12 LD cycles, despite these retinorecipient cells being 24 h and thus resonant with the lighting schedule, as well as with the retina itself, which is known to express VIP in some cell types (Akrouh and Kerschensteiner, 2015; Cellerino et al., 2003) and thus presumably also contained 24 h cells. This is in clear contrast to DarCre-Tau mice, which exhibited plasticity in being able to entrain to both 12:12 and 10:10 LD cycles, with entrainment to 10:10 hypothesised as being a result of 20 h non-Cre GRP (or other retinorecipient) cells dominating when the 24 h VIP cells represented by DarCre lost coherence in the non-resonant lighting schedule (Smyllie et al., 2016). This provides evidence that resonant lighting schedules are not sufficient to allow the VIP cells to dominate period, suggesting entrainment to 12:12 LD in DarCre-Tau mice was not merely the act of 24 h VIP cells.

All VipCre- and VpacCre-Bmal1 mice showed WT-like rhythmicity in 12:12 LD, suggesting that while light is unable to drive mice at a period outside of their range of entrainment, it can induce rhythmicity in otherwise arrhythmic mice. Furthermore, phenotypes did not immediately emerge in DD for most mice, with many demonstrating transient rhythmicity as previously
discussed. This suggests that the effects of light in preserving network synchrony persists for at least some time once in DD. GRP-mediated intercellular coupling is a good candidate for this, as they are light-responsive (Dardente et al., 2002, 2004) and do not appear to express VIP or VPAC2, and GRP signalling can sustain VPAC2-null rhythms, although only transiently (Maywood et al., 2006).

Reverting to an LD cycle after several weeks in DD immediately stopped any disrupted behaviour that had been observed, reinforcing the idea that entraining cues can overcome arrhythmicity. However, releasing the mice back into DD (2nd DD) did not necessarily result in a return of the phenotype. Both VpacCre-Bmal1 mice investigated in this paradigm that had shown a phenotype in 1st DD showed one in 2nd DD, although the exact presentation did differ, while none of the VipCre-Bmal1 mice demonstrated disrupted rhythmicity in 2nd DD, regardless of their 1st DD phenotype. Thus it appears that the effects of light on the chimeric circuit have a stochastic element to them, revolving around a tipping point that may or may not be reached once in DD. Once the circuit is disrupted, however, it does not revert back to coherent rhythmicity without the influence of an entraining signal such as light.

4.4.4 Variability between and within mice

The exact cause of the large amount of variability between mice in Bmal1 experiments presented here is not clear, however the differences observed in the presentation and timing of the emergence of a phenotype in VpacCre-Bmal1 mice in 2nd DD, or the absence of it in VipCre-Bmal1 mice, may be able to provide clues.
First, intra-mouse variability, in particular in VipCre-Bmal1 mice, cannot be explained by penetrance of the Cre (inefficient removal of Bmal1), because Bmal1 cannot be “undeleted” once removed by Cre recombinase. However, this may provide an explanation for variability between mice. Given that VIP cells have previously been sub-categorised based on expression of other proteins, rhythmicity of VIP mRNA expression, GRP colocalisation and retinal innervation (Geoghegan and Carter, 2008; Kawamoto et al., 2003), it is conceivable that the deletion of Bmal1 predominantly in one subpopulation may have more pronounced effects than in another.

Another factor relating to the emergence (or lack thereof) of a phenotype could simply be time, whereby rhythmicity in the circuits of some mice was preserved long enough following the transition from LD to DD that the experiment had finished before rhythmicity was lost. This does not of course explain why some circuits manage to remain intact for so much longer than those of other mice. Indeed, the only apparent explanation for this observation, and for the variability within mice, is that some level of stochasticity is involved, similar to the emergence of rhythms in some, but not all, Cry1\textsuperscript{-/-}Cry2\textsuperscript{-/-} SCN slices (Maywood et al., 2011). Stochastic rhythms in Bmal1\textsuperscript{-/-} SCN slices have been suggested to be the result of intercellular coupling and noise at the molecular level (Ko et al., 2010).

Another example of variability was variability between ages, where SCN slices taken from pups showed a different phenotype to those taken from adults in all genotypes where this comparison was made. VpacCre-Ck1ε\textsuperscript{Tau/-} and VpacCre-Bmal1 pup slices were no different to their respective controls in terms of period, amplitude or RAE, whereas both showed strong phenotypes as adult slices. In contrast, both VipCre- and DarCre-Bmal1 pup slices showed a short period in at least some slices (coincident with a low amplitude in VipCre-Bmal1), whereas VipCre-Bmal1 adult slices showed a slightly worse
RAE and DarCre-Bmal1 adult slices showed no phenotype. Although the expression of VIP and VPAC2 do not appear to qualitatively change over time, an explanation for these results could be that the relative dominance of cell types within the circuit is altered over time. Given the role of VIP cells in conveying retinal and light information, it is conceivable that VIP cells are proportionately more important during postnatal development when the eyes are opening, for example ensuring that mice are quickly entrained to lighting conditions rather than to maternal rhythms as in neonatal stages (Viswanathan, 1999). The nature of intercellular coupling is also known to change throughout development, for example TTX appears to have a more potent effect on pup compared to adult slices (Baba et al., 2008), thus the nature of VIP signalling could change throughout development. Alternatively, the role of Bmal1 itself may change with development. For example, it is known that profiles of *Per1, Per2, Cry1, Bmal1*, and Clock mRNA change with development (Sládek et al., 2004), although given their highly developed rhythmic profile by P10 (the age at which pup slices were taken in these experiments), this is less likely to explain my findings.

**4.4.5 SCN slices do not always reflect behaviour**

Despite the SCN being the master mammalian pacemaker, slice rhythmicity as reported by bioluminescent constructs does not always directly reflect behavioural circadian periodicity or rhythmicity. With respect to TTFL deficiencies, *Cry1<sup>−/−</sup>Cry2<sup>−/−</sup>* slices have been shown to demonstrate stochastic rhythmicity despite whole animal behaviour being arrhythmic (van der Horst et al., 1999; Maywood et al., 2011; Ono et al., 2013), as have Bmal1<sup>−/−</sup> slices (Bunger et al., 2000; Ko et al., 2010). With regards to intercellular communication, VPAC2-null slices demonstrate rhythmicity for at least several days, whereas mouse behaviour is often arrhythmic (Maywood et al.,
Genetic manipulation of VIP and VPAC2 cell populations

2006; Sheward et al., 2007). Furthermore, recent intersectional approaches have also found that slices do not necessarily reflect behaviour: the arrhythmic behaviour of both NMS-Per2 and NMS-Bmal1 mice is not reflected at the slice level, with ensemble rhythms appearing intact (Lee et al., 2015), while the lengthening of period seen in AVP-CK1δ and AVP-Bmal1 mice is also not observed in slices (Mieda et al., 2015, 2016). In my own work, VpacCre-Ck1ε\textsuperscript{+/-} slices do not show the period lengthening seen in behavioural recordings, and VipCre-Bmal1 slices show robust rhythms even from arrhythmic mice. It is possible that the intact structure of the SCN is disrupted during the dissection process. Inevitably, connections between neurons will be severed when slices are made because a 300 µm slice only represents a portion of the whole SCN. With the manipulation of certain subpopulations within the SCN, if, for example, the effects of arrhythmic VIP cells are primarily mediated through cells at a different point along the rostro-caudal axis, this may not be evident in the slice.

An alternative explanation is that extra-SCN clock cells have a reinforcing effect \textit{in vivo} on the SCN and its outputs, which is subsequently lost \textit{ex vivo}. One instance of extra-SCN inputs determining \textit{in vivo} rhythmicity can be seen in arrhythmic VPAC2-null mice, which display 24 h rhythms when entrained to a scheduled voluntary exercise paradigm, an effect that persists on release into DD (Power et al., 2010). In the current study, retinal inputs may be relevant: the retina has been known to influence some aspects of SCN rhythmicity (Lee et al., 2003; Shibata et al., 1984), and retinal cells are known to express VPAC2 receptors (D’Agata and Cavallaro, 1998), thus the combination of 24 h retinal input and 24 h SCN VPAC2 cells may be sufficient to dictate wheel running period. This retinal input would then be lost on slice preparation. However, the wide variability seen in VpacCre-Ck1ε\textsuperscript{+/-} slice periods more strongly supports disruption of the circuit, and therefore
rearrangement of cellular dominance hierarchies. Indeed, given the chimeric nature of the circuit, the act of slicing may sever synaptic connections, resulting in paracrine intercellular signalling bearing more weight, allowing 22 h VIP cells to exert a stronger influence over the period of the slice as shown previously (Maywood et al., 2011). Subsequently, new synaptic connections are formed within the adult VpacCre-Ck1ε<sup>Tau/</sup> slice, which show a lengthening of period over time of almost 30 minutes per day. This may allow the long-period pacemaking VPAC2 cells to once again exert influence over the period of the whole slice. A key prediction of this hypothesis that future work could test would be that if adult VpacCre-Ck1ε<sup>Tau/</sup> slices were treated with TTX to inhibit synaptic communication, this period lengthening would not be seen.

VipCre-Bmal1 slices also do not correspond to the disrupted phenotype observed in wheel running activity, with robust rhythmicity seen in most slices, although a slightly worse RAE was also evident compared to controls. Once again, the retina expresses VIP (Akrouh and Kerschensteiner, 2015; Cellerino et al., 2003), therefore arrhythmic cues originating from the retina may reinforce arrhythmic retinorecipient VIP cells within the SCN. This effect was not observed in VipCre-Ck1ε<sup>Tau/Tau</sup> mice, but given the apparent differences between dictating rhythmicity and periodicity, this cannot be excluded. It may not be necessary, however, to invoke reinforcing inputs: Ko et al. (2010) demonstrated that even global Bmal1 knock-out slices, stochastic quasi-circadian rhythmicity can be seen, despite Bmal1 knock-out mice being arrhythmic (Bunger et al., 2000). Therefore it is perhaps unsurprising that in a circuit in which only 10% of the cells are genetically arrhythmic, strong rhythms are seen, with only a small effect on RAE. Indeed, what is therefore more remarkable is that VpacCre-Bmal1 adult slices do show highly disrupted rhythms, despite only 20% of cells in the slice lacking Bmal1,
providing strong evidence that VPAC2 cells in the SCN are vital to rhythmicity.

An alternative explanation to the lack of a strong effect in both VpacCre-Ck1ε\textsuperscript{Tau\textsuperscript{-}} and VipCre-Bmal1 slices is that direct outputs from segregated regions in the SCN are as important as whole SCN coherence for circadian behaviour. For example, the dorsomedial region of the SCN is known to be an important output region, primarily via AVP and PK2 (Kalsbeek et al., 2010; Zhou and Cheng, 2005), with most peripheral targets of the SCN being in phase with the shell rather than the core (Evans et al., 2015), potentially explaining the effect of VpacCre-Ck1ε\textsuperscript{Tau\textsuperscript{-}} cells on behavioural period. While the shell is known to be important for outputs, VIP cells in the ventrolateral region do also directly output to certain areas of the brain, including regions containing dorsal efferents, albeit different specific cell types (Abrahamson and Moore, 2001; Kalsbeek and Buijs, 2002; Watts and Swanson, 1987; Watts et al., 1987). Some areas of the brain have been shown to receive direct inputs from SCN VIP cells, such as gonadotropin releasing hormone (GnRH) cells (van der Beek et al., 1993), the subparaventricular zone, lateral septum anteroventral periventricular nucleus, preoptic nucleus and medial preoptic nucleus (Kalló et al., 2004a), with the paraventricular nucleus (PVN) in hamsters primarily entrained to the core (Yan et al., 2005). Many of these areas can in turn project throughout the central nervous system. Thus arrhythmic VIP cells have a large number of potential output regions to influence directly, without needing to dictate the rhythmicity of the entire SCN. Alternatively, VIP and VPAC2 cells are found throughout the brain, which potentially will be equally as affected by intersectional genetics as the SCN. The way that recipient regions respond to SCN-derived cues could depend on their own phase, period or rhythmicity. For example, a forebrain-specific Bmal1 deletion resulted in memory defects, suggesting that
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regardless of SCN coherence, recipient cell rhythmicity is also important for clock-dependent functions (Snider et al., 2016).

4.4.6 Technical difficulties

The primary technical difficulties encountered during the course of the experiments presented here constellated around the use of Cre recombinase: either insufficient or too much Cre-mediated recombination.

Bmal1 appears to be unusually difficult to delete by Cre-mediated recombination. In my own studies, fewer than 50% of Cre-expressing VIP cells, and approximately 65% of VPAC2 cells, managed to effectively remove Bmal1. DarCre was the most successful Cre in removing Bmal1, with only 17% of DarCre neurons remaining Bmal1-positive. Previous studies have also struggled to completely ablate Bmal1 expression, with heterozygous null approaches being used (Husse et al., 2011) to improve Cre efficiency. NMS-Cre and AVP-Cre also showed incomplete removal, with approximately 20% of NMS-positive and 23% of AVP-positive cells still expressing Bmal1 (Lee et al., 2015; Mieda et al., 2015) as detected by immunostaining. The exact causes of this difficulty and of variability in different Cre driver efficiencies are unknown, although chromosomal location of loxP sites, the age at which Cre is expressed and the epigenetic context within different cell types have been shown to play a role in recombination efficiency in other studies (Hameyer et al., 2007; Liu et al., 2013; Long and Rossi, 2009; Vooijs et al., 2001). Chromosomal location of loxP sites may explain general difficulties of removing Bmal1, while age effects and epigenetic context in different cell types could explain variability. Future experiments could investigate the methylation status of the Bmal1 locus, and explore alternative approaches to
reducing Bmal1 expression such as shRNA or CRISPR knock-downs (Tso et al., 2017).

Problems were also experienced with the expression of Cre in VpacCre lines, whereby mice unexpectedly contained a null allele of either Ck1ε or Bmal1 depending on the line, as well as affecting the use of fluorescent reporters (data not shown). This is now thought to be due to Cre expression in developing spermatocytes in the testes (Krempels et al., 1995; Usdin et al., 1994), thus leading to deletion of one allele prior to fertilisation (Schmidt-Supprian and Rajewsky, 2007).

A final difficulty emerged in assessing the efficacy of Ck1ε\textsuperscript{Tau/Tau} deletion. Unlike Bmal1, no reliable antibody specific for Ck1ε exists, and, despite much time and effort, no specific signal could be generated for Ck1ε using fluorescent in situ hybridisation (FISH; data not shown). Thus it cannot currently be ruled out that the absence of a phenotype in VipCre-Ck1ε\textsuperscript{Tau/Tau} mice, nor that an incomplete reversion to a 24 h period in VpacCre-Ck1ε\textsuperscript{Tau/Tau} mice were not just due to a lack of recombination. For VipCre-Ck1ε\textsuperscript{Tau/Tau} mice at least this would seem unlikely, as VIP cells were also unable to affect period in VIP-Clock\textsuperscript{Δ19} mice (Lee et al., 2015; also see Chapter 5.3.6 for a VipCre-Ck1ε\textsuperscript{Tau/Tau} phenotype).

**4.4.7 Conclusions**

To conclude, I have demonstrated that VPAC2 cells, not VIP cells, are capable of exerting a strong influence on behavioural free-running period, while both cell populations are able to manipulate the stability of circadian rhythmicity. Interestingly, DarCre cells, previously shown to be period setters in the SCN, have almost no effect on rhythmicity, potentially suggesting different cellular subpopulations within the SCN contribute to different circadian parameters.
The exact nature of the period-setting neuropeptide remains to be determined; although AVP may appear to be a strong candidate for this, the differences between DarCre-Ck1εTau/Tau and VpacCre-Ck1εTau/- with regards to period determination, along with their respective coverage of the AVP cell population in the SCN, suggests that this is not the case. Known issues of working with Cre recombinase systems are likely to explain some of the variability seen between mice, but intra-mouse variation argues that the SCN circuit is highly plastic, albeit in a somewhat stochastic manner. Furthermore, interesting differences seen between pup and adult SCN slice phenotypes are not likely to be due to Cre as both VipCre and VpacCre are effectively expressed in pup slices (see Chapter 3), providing evidence that circuit-level communication in the SCN can change throughout development. Finally, the surprising finding that SCN slices do not always correspond to the behavioural phenotype observed in wheel-running behaviour may suggest that different regions of the SCN output directly to behavioural control centres in the brain, thus circadian behaviour represents a composite of SCN output signals, as opposed to a single coherent output. Further studies should therefore investigate the output regions of VIP and VPAC2 cell populations using recent neural tracing techniques (Callaway and Luo, 2015) and their relative contributions to circadian behaviour.
5.1 Introduction

Despite the ubiquity of cellular oscillators throughout mammals, photosensitivity is restricted to the cells of the retina (Bedont and Blackshaw, 2015), hence in order to synchronise the majority of cells to photoperiod (and thereby to each other), a central mediator is required. The SCN is uniquely suited to this task: not only is it an exceptionally robust oscillator due to strong interneuronal coupling (reviewed in Welsh et al., 2010) with extensive synaptic and neuroendocrine outputs (Kalsbeek et al., 2006), it also receives input from the retina via the retinohypothalamic tract (RHT; Morin and Allen, 2006), which it then integrates with its own intrinsic oscillation to arrive at a suitable phase.

Photic input, such as from a light pulse during the night, is capable of leading to behavioural and physiological shifts through the induction of core clock proteins PER1 and PER2, as well as the immediate early gene c-Fos (Albrecht et al., 2001; Kuhlman et al., 2003; Rusak et al., 1990; Shigeyoshi et al., 1997), particularly in the retinorecipient ventrolateral region of the SCN (Yan and Okamura, 2002). The ventrolateral SCN is characterised by expression of a number of neuropeptides, including VIP, GRP and calbindin (Abrahamson and Moore, 2001), of which VIP is the best characterised with regards to SCN function and light responses (Vosko et al., 2007). VIP signals are received by cells expressing the VPAC2 receptor, which reside predominantly in the dorsomedial SCN (Kallo et al., 2004; and see Chapter 3). VIP and VPAC2 are important for SCN synchronisation, with \( Vip^{−/−} \) and \( Vipr2^{−/−} \) mice and slices
displaying weakened rhythmicity, fewer rhythmic neurons, damped cellular oscillations and greater phase dispersal (Brown et al., 2007; Ciarleglio et al., 2009; Colwell et al., 2003; Harmar et al., 2002; Maywood et al., 2006). VIP application to SCN slices or injection in vivo is capable of shifting rhythms in SCN physiology and mammalian behaviour (An et al., 2011; Piggins et al., 1995; Reed et al., 2001; Watanabe et al., 2000), as well as inducing PER1 and PER2 expression in the SCN (Nielsen et al., 2002), while daily application of VIP or a VPAC2 agonist can entrain SCN rhythms (An et al., 2011; Aton et al., 2005). Furthermore, mice deficient in either VIP or VPAC2 show aberrant light-responsiveness at the behavioural and molecular levels, with VIP expression being required for appropriate temporal gating of light responses (Colwell et al., 2003; Dragich et al., 2010; Sheward et al., 2007). Thus, VIP is likely to be responsible for phase shifting and SCN entrainment to light, although as described previously, GRP is also likely to play a role.

The exact mechanism for how VIP entrains the rest of the SCN has not been fully elucidated. VIP is known to induce PER gene expression (Nielsen et al., 2002), and loss of VIP significantly reduces the level of PER1 increases in the shell of the SCN in response to light, as well as long term induction in the core, although initial induction in the core is unaffected (Vosko et al., 2015). With regards to cellular transduction cascades, VIP has been shown to act through cAMP/PKA and phospholipase C (PLC) second messenger pathways, which subsequently phase shift rhythmicity and affect electrical firing rhythms (An et al., 2011; Kudo et al., 2013; Nielsen et al., 2002). This may lead to alterations in shell neuropeptide expression (Watanabe et al., 2000), which could then contribute to feedback from the shell to the core, ultimately arriving at a stable, coherent phase, as has been suggested by jet-lag paradigms in mice lacking AVP receptors (Yamaguchi et al., 2013). Surprisingly, given its role in synchronisation of neurons, bolus delivery of
VIP also appears to be able to reduce oscillator amplitude (An et al., 2011, 2013). Reduced amplitude of rhythmicity has previously been shown to allow greater phase resetting and entrainment to Zeitgebers (Abraham et al., 2010; Buhr et al., 2010; Vitaterna et al., 2006). Thus a reduction in either the amplitude of cellular oscillation or in phase synchrony amongst oscillators offers an attractive mechanism by which VIP could mediate photic input to enhance entrainment. However, given that only a subset of neurons within the SCN express the VPAC2 receptor (Kalamatianos et al., 2004; Kallo et al., 2004; and see Chapter 3), it is not clear how rhythmicity across the whole circuit is damped. For example, how is information transmitted between VPAC2 and non-VPAC2 cells? Furthermore, the observation that activation of Gq-dependent pathways in VIP cells can cause changes to the spatiotemporal wave of circadian clock gene expression in SCN slices (Brancaccio et al., 2013) might suggest that VIP is capable of altering intercellular communication and cellular phase relationships in addition to cell-autonomous oscillations.

The experiments presented in this chapter explore the means by which VIP mediates SCN responses to light input. By applying VIP to SCN slices in combination with pharmacological and genetic manipulations, the effects of VIP on cell-autonomous oscillation compared to circuit-level rhythmicity and the mechanisms through which these effects are achieved are investigated. I demonstrate that VIP works through multiple pathways in the SCN at both the cell- and the circuit-level, and that the effects of VIP application are highly persistent. I also show that VIP has the capacity to act not only through VPAC2, but also through the non-canonical PAC1 receptor in SCN slices.
5.2 Methods

5.2.1 Treatment of SCN slices

All pharmacological agents used in this chapter can be found in Table 5.1. Drugs were dissolved in their designated solvent, aliquotted and stored at \(-20^°C\) until use, at which point they were bath-applied to SCN slice culture medium. Slices were treated with VIP and Bay 55-9837 at CT10 (defining the peak of PER2::LUC as CT12), and with zebularine, and PACAP6-38 (P6-38) 6 hours and 30 minutes before VIP treatment respectively. Tetrodotoxin (TTX) was given either 24 h before VIP or several days after (independent of phase), depending on the experiment. Cycloheximide (CHX) treatment was performed irrespective of phase. Due to their arrhythmic nature, \(\text{Cry}^{1/-}\text{Cry}^{2/-}\) slices were treated based on \(\text{Cry}^{1/-}\) littermate phases.

<table>
<thead>
<tr>
<th>Pharmacological agent</th>
<th>Supplier, catalogue number</th>
<th>Concentration used</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIP</td>
<td>Tocris, 1911</td>
<td>10-10000 nM</td>
<td>Air medium (AM)</td>
</tr>
<tr>
<td>Bay 55-9837</td>
<td>Tocris, 2711</td>
<td>50 nM or 5 µM</td>
<td>AM</td>
</tr>
<tr>
<td>Zebularine</td>
<td>Tocris, 2293</td>
<td>50 µM</td>
<td>AM</td>
</tr>
<tr>
<td>CHX</td>
<td>Sigma, C7698</td>
<td>10 µg/ml</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>TTX</td>
<td>Sigma, T8024</td>
<td>1 µM</td>
<td>Water</td>
</tr>
<tr>
<td>P6-38</td>
<td>Tocris, 3236</td>
<td>1 µM</td>
<td>AM</td>
</tr>
</tbody>
</table>

Table 5.1 Pharmacological agents used on SCN slices to investigate VIP-related mechanisms of action

5.2.2 Analysis of bioluminescent recordings

*RMS change and \(\Delta\)PER2 for \(\text{Cry}^{1/-}\text{Cry}^{2/-}\) slices*

Due to the arrhythmic nature of \(\text{Cry}^{1/-}\text{Cry}^{2/-}\) slices, relative amplitude and fold induction could not be calculated. As a replacement for the amplitude of PER2::LUC signal, root mean square (RMS) was calculated instead. Raw \(\text{Cry}^{1/-}\text{Cry}^{2/-}\) bioluminescence traces were first normalised to the highest value within each dataset (to account for absolute gain differences between PMTs),
before performing a 24 h rolling baseline subtraction so that the signal oscillated around zero. Values 48 h before treatment and 48 h after treatment (allowing a 24 h interval which was not included immediately post-treatment) were squared, and the mean for each interval was calculated. The square root of these means was taken and a percentage change between the two intervals was calculated for each slice.

PER2::LUC fold induction could not be calculated because there were no peaks to extrapolate from. Thus ΔPER2 was calculated instead by subtracting the normalised bioluminescence value immediately before treatment from the highest value within the 6 h recording window after treatment for each slice.

**Slope change**

The gradient of PER2::LUC bioluminescence before or after pharmacological treatment was calculated by dividing the change in amplitude in the two hours before or after treatment by time (i.e. two hours). The post-treatment gradient was then divided by the pre-treatment gradient to calculate slope change. Thus a value of 1 represents an unchanged slope.
5.3 Results

5.3.1 VIP is capable of attenuating all circadian parameters, which cannot be fully washed off

The effects of pharmacological addition of different concentrations of VIP neuropeptide to SCN slices *in vitro* were reproduced based on previous literature, utilising CT10 (two hours before the peak of PER2::LUC, defined as CT12) as the phase of addition due to it eliciting the strongest response (An et al., 2011). A clear PER2::LUC induction, phase shift and amplitude change were all readily visible from the bioluminescence recordings (Figure 5.1A), and, interestingly, this ‘VIP-induced state’ could not be washed off even with multiple full medium changes over several weeks (Figure 5.1B). Upon quantification, period increase, amplitude change, PER2::LUC fold induction and phase shift all altered in a dose-dependent manner, and phase shifting was highly correlated with PER2 induction (Figure 5.1C-G). Washing incompletely restored SCN oscillation to a pre-VIP state, particularly with regards to amplitude (Figure 5.1B-D).

As expected, these responses appear to be mediated through VPAC2, the canonical SCN VIP receptor. Application of specific VPAC2 agonist Bay 55-9837 (Tsutsumi et al., 2002) at 50 nM or 5 µM concentrations resulted in a copy of the VIP-induced state described in Figure 5.1 (Figure 5.2A), with responses increasing in a dose-dependent manner (Figure 5.2B-E). Furthermore, for all circadian parameters examined, there were no significant differences between the effects of Bay 55-9837 and comparable VIP concentrations (Figure 5.2B-E), suggesting that VIP is acting via the VPAC2 receptor. Similarly to VIP, a strong correlation was observed between the phase shift and PER2::LUC fold induction following Bay 55-9837 application (Figure 5.2F). Based on the results presented in Figures 5.1 and 5.2 and on previous literature (An et al., 2011, 2013), VIP concentrations of 1 or 10 µM
Figure 5.1 VIP induces considerable changes to rhythmicity in SCN slices

(A and B) Representative PER2::LUC bioluminescence rhythms of vehicle-, 100 nM VIP- or 10 µM VIP-treated slices with media change (A) and three separate 10 µM VIP-treated slices followed by multiple media changes (B). VIP was bath applied at CT10 of the 5th cycle (marked by +) and washed off after 10 days (marked by §). Bioluminescence has been normalised to the first peak. (C-E) Dose-dependent responses in period change (C), relative amplitude (D) and PER2::LUC fold induction (E) (mean ± SEM) induced by VIP applied at CT10. Values are calculated based on data 4 days after VIP application and 4 days after wash-off. Data were fitted with a nonlinear regression. (F) Dose-dependent phase shifts induced by VIP applied at CT10, measured at the first (immediate) or third (delayed) peak after VIP application. Data were fitted with a nonlinear regression. (G) Scatterplot of phase shifts vs. PER2::LUC fold induction following VIP treatment, demonstrating a significant correlation (****P < 0.0001, Pearson’s correlation). Furthermore, a linear regression was computed: \( r^2 = 0.72, Y = -8.811X + 8.737 \). n = 4-6 slices per concentration of VIP.
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Figure 5.2 VPAC2 agonist Bay 55-9837 induces similar responses to VIP

(A) Representative PER2::LUC bioluminescence rhythms of 50 nM Bay 55-9837- or 5 µM Bay 55-9837-treated slices. Bay 55-9837 was bath applied at CT10 after 4 cycles (marked by +). Bioluminescence has been normalised to the first peak. (B-E) Period change (B), relative amplitude (C), phase shift (D) and PER2::LUC fold induction (E) responses (mean ± SEM) to Bay 55-9837 (50 nM or 5 µM) or VIP (100 nM or 10 µM) applied at CT10. Slices treated with 5 µM Bay 55-9837 (n = 5) showed significantly stronger responses in all parameters to those treated with 50 nM Bay 55-9837 (n = 4). No significant differences were seen between 5 µM Bay 55-9837 and 10 µM VIP (n = 5) or between 50 nM Bay 55-9837 and 100 nM VIP (n = 5) for any parameter. (F) Scatterplot of immediate phase shifts vs. PER2::LUC fold induction following Bay 55-9837 treatment, demonstrating a significant correlation (Pearson’s correlation). Furthermore, a linear regression was computed: $r^2 = 0.81$, $Y = -6.581X + 6.081$. All tests one-way ANOVA with Tukey’s multiple comparisons test unless otherwise specified, ns = not significant, *$P < 0.05$, ****$P < 0.0001$. 

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were used for all subsequent experiments to investigate the mechanisms by which VIP affects SCN rhythmicity.

5.3.2 SCN slice responses to VIP depend on duration and phase of treatment, and age of slice

VIP is known to elicit different phase shifts in SCN slices depending on the phase at which it is applied, partially reflecting the phase response curve to light of mice (An et al., 2011). However, the phase-dependence of other characteristics of the VIP-induced state has not been investigated. Thus VIP was applied to SCN slices at CT22, 12 h away from the previously used CT10 (Figure 5.3). As expected, phase shifts were largely absent in slices treated at CT22 (Figure 5.3A-C), however period and amplitude changes both persisted (Figure 5.3D and E), demonstrating that two key characteristics of the VIP-induced state are not phase-dependent. PER2 expression was significantly induced, albeit at a lower level to that of CT10 slices (Figure 5.3F), suggesting that PER2 induction alone is not sufficient to cause phase shifts in slices.

Duration of VIP exposure was investigated next, to determine if VIP being present in the culture media for a reduced amount of time would constrain the subsequent VIP-induced state (defined as a phase delay, period lengthening, amplitude reduction and baseline increase). Given that the half-life of VIP in culture medium (as opposed to in vivo) is approximately 2 hours (An et al., 2011), full medium changes were given at either 2 h or 6 h after VIP treatment at CT10 (Figure 5.4A and B), at which points the concentration of VIP would be approximately 50% or 12.5% of the starting concentration. In the 2 h wash condition, no significant phase shift or period change was observed, however there was a significant amplitude reduction compared to vehicle (Figure 5.4C-E). In contrast, if VIP was washed off after 6 h, a small (~2
h) phase delay was observed and amplitude was significantly reduced, although there was still no period increase compared to vehicle-treated slices. Thus it appears that the duration of VIP treatment has a significant effect on the emergence of the VIP-induced state, with different parameters (e.g. phase
Figure 5.4 Duration of VIP exposure affects the VIP response

(A and B) Representative PER2::LUC bioluminescence rhythms of SCN slices treated with 1 µM VIP or vehicle at CT10 and washed off after 2 h (A) or after 6 h (B). Treatments were bath applied after 4 cycles (marked by +) and washed by 4 full media changes (marked by §). Bioluminescence has been normalised to the first peak. (C-E) Delayed phase shift (C), period change (D) and relative amplitude (E) responses (mean ± SEM) to VIP followed by wash-off after 2 h (n = 3), 6 h (n = 3) or with no wash (n = 3) with vehicle controls (2 h: n = 3; 6 h: n = 3, no wash: n = 6). No significant phase shift was observed following VIP treatment and wash-off after 2 h compared to vehicle control. A significant shift was observed following VIP treatment and wash-off after 6 h, although this was significantly less than slices that were not washed. Slices washed after 2 h or 6 h showed no significant period changes compared to vehicle controls, but they did show significant amplitude reductions, albeit significantly less than VIP-treated slices that were not washed. All tests two-way ANOVA with Tukey’s multiple comparisons test, ns = not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
shift vs. period change) appearing to require different incubation times to achieve their maximal effect.

The age of the mouse from which an SCN slice was derived also appeared to alter the extent of some aspects of the VIP response, with slices taken from adult mice (3-6 months) displaying reduced responses (Figure 5.5A and B). Although phase shift and PER2 induction appeared to be unaffected by age, both amplitude and period changes were reduced in adult slices, although they were still significant compared to vehicle-treated adult slices (Figure 5.5C-F).

5.3.3 VIP affects SCN rhythmicity at both the cell and the circuit level

Time-lapse imaging was used to visualise the effects of VIP on SCN slices. Regions of interest (ROIs) were identified using Semi-Automated Routines for Functional Image Analysis (SARFIA; Dorostkar et al., 2010) in Igor Pro (Figure 5.6A), and subsequent analysis revealed that application of VIP had dramatic effects on the rhythmicity and amplitude of all oscillators (Figure 5.6B-E). ROIs showed very little phase coherence (Figure 5.6C and D), and the majority of ROIs displayed an increased period, although period dispersal was also much greater (Figure 5.6F-H). Together these results suggest exogenous application of VIP affects all cells in the SCN, and that the reduction in amplitude observed at the ensemble level is not merely the result of phase dispersal, although this does contribute considerably.

Given the role of VIP in intercellular communication within the SCN, network dynamics of PER2::LUC bioluminescence were next analysed using centre of mass (CoM) analysis as described in Chapters 2 and 3 and in Brancaccio et al. (2013). A clear disruption of the spatiotemporal wave was observed immediately after VIP application across all slices analysed (Figure 5.7A-C).
Not only was the overall displacement of the CoM reduced, as shown by CoM perimeter measurements (Figure 5.7D), but the directionality of the CoM was consistently altered: all four slices observed with time-lapse imaging displayed more of a dorsolateral to ventromedial, rather than the stereotypical dorsomedial to ventrolateral, displacement after VIP (Figure 5.7B). This may be in part due to the very dorsal tip of the slice displaying a...
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high baseline of bioluminescence but very little oscillation (Figure 5.7A). These results suggest that VIP affects the phase relationships between oscillators of the SCN in a predictable, non-random manner. The predictability of the CoM after VIP application, and the higher baseline of PER2::LUC further suggests that the loss in amplitude is not due to cell death in the slice (such as due to excitotoxicity), which would be expected to have

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|**Figure 5.6 VIP application affects all oscillators in the SCN**
(A) Regions of interest (ROI) determined by SARFIA mapped onto SCN phase image. (B and C) Representative raster plot (B) and associated Rayleigh plot (C) of PER2::LUC bioluminescence rhythms of one of 4 slices treated with 10 µM VIP imaged on CCD camera (same slice as in (A)) VIP treatment marked by *. (D) Length of Rayleigh plot vector (mean ± SEM, example in (A-C)) before and after VIP treatment to demonstrate significant loss of phase coherence. (E and F) Amplitude (E) and periods (F) for all oscillators across the 4 slices (mean ± SEM). (G) Standard deviation of periods (mean ± SEM) to demonstrate period dispersal amongst oscillators following VIP treatment. (H) Relative cumulative frequency (mean ± SEM, %) of ROIs with a given period before and after VIP treatment. All tests paired t-test between pre- and post-treatment data, **P < 0.01, ****P < 0.0001.
random effects on the spatiotemporal wave (unless certain cells were more susceptible than others to a putative ‘VIP-induced cell death’) and to reduce the baseline.
5.3.4 VIP differentially affects core clock genes, although Cry is not necessary for PER2 induction

The effects of VIP specifically at the cell-autonomous oscillator level were next investigated in a series of experiments to determine differential effects of VIP on core clock genes, the necessity for functional cell-autonomous rhythmicity for the responses, and to determine if the VIP-induced state of the network could be reverted back to a ‘pre-VIP state’ through pharmacological manipulation.

Two TTFL gene reporter lines (Per1-Luc and Cry1-Luc) were used in addition to PER2::LUC to establish if all core clock genes responded identically to a VIP pulse, if VIP selectively altered only some components of the TTFL, or if TTFL genes were affected at different rates. Reporters were treated at the same time in their oscillation (that is, at a time equivalent to CT10 for PER2::LUC based on phase mapping in Brancaccio et al., (2013)), equating to 0.2 h after the Per1Luc peak and 2.7 h before the Cry1Luc peak. None of the reporters showed any response to vehicle treatment (Figure 5.8A), however on VIP treatment there were obvious differences in the responses of the slices (Figure 5.8B). PER2 and Cry1 phase shifted a similar amount (4-5 h), while Per1 was delayed significantly less (although it was still delayed by 2-3 h) (Figure 5.8C). There was also a difference in reporter induction: PER2 showed a dramatic increase in the peak following VIP application, while Per1 and Cry1 did not, although Cry1 did show a broadened peak which may represent a delayed induction (Figure 5.8B and D). Per1-Luc did, however, show an increased baseline following VIP application (Figure 5.8B), thus it may be that the “induction” of Per1 simply shows different dynamics to that of PER2. No significant differences were observed for period change, while there was a significant difference between Per1Luc and Cry1Luc for relative amplitude, but this may simply reflect different dynamics of the three
reporters as significant differences were also seen in vehicle-treated controls (Figure 5.8E and F). Moreover, the exact waveform of the VIP-induced state in the three reporters differed: as stated, Per1-luc showed an increased baseline,
however it did not show a reduced peak progression, while Cry1-Luc showed reduced peaks after VIP but no increased baseline (Figure 5.8B). PER2 displayed both as previously shown. These results demonstrate that VIP application to SCN slices does not affect all TTFL genes in the same way, and that it is capable of disrupting the established phase relationship of these genes as evidenced by their different steady-state phases after VIP compared to before.

The ability of VIP to affect TTFL oscillations does not necessarily mean that a rhythmic cellular oscillator is required for VIP to induce gene changes. To test this, Cry1−/−Cry2−/− (Cry double knock out or CryDKO) slices were treated with VIP. Given the arrhythmic nature of most CryDKO slices (Figure 5.9A), the

**Figure 5.9 A cell-autonomous oscillator is not necessary for the VIP response**

(A) Representative PER2::LUC bioluminescence rhythms of Cry1−/−Cry2−/− (CryDKO) SCN slices treated with vehicle or 1 µM VIP. Treatments were bath applied after 5 cycles at CT10 determined by Cry1−/− littermate slices (marked by +; Cry1−/− slice data not shown). Bioluminescence has been normalised to the highest value in the recording. (B) Detrended (24 h baseline subtraction) PER2::LUC bioluminescence rhythms of slices in (A). The VIP-treated slice has been offset on the y-axis to aid visualisation. (C) Change in levels of PER2::LUC bioluminescence following treatment (mean ± SEM), calculated by subtracting the pre-VIP value from the highest value within the subsequent 6 h recording window. (D) Relative RMS change (mean ± SEM) following treatment. RMS was calculated based on data 48 h before and after treatment, excepting a 24 h window immediately after treatment, by taking the square root of averaged normalised, detrended and squared data. All tests unpaired t-tests, **P < 0.01.
phase of application was matched to CT10 of rhythmic Cry1⁻/⁻ littermate SCN oscillations (data not shown). A clear immediate induction of PER2::LUC can be seen following addition of VIP compared to vehicle (Figure 5.9A-C), suggesting that the signalling transduction pathway from VPAC2 is not clock-dependent. Furthermore, analogous to an amplitude reduction in WT slices following VIP, the root mean square (RMS) of PER2, acting as a measure of noise of the “oscillating” PER2::LUC signal in CryDKO slices, significantly decreased compared to vehicle (Figure 5.9D), as can be visualised by the relative smoothness of the signal after VIP compared to before (Figure 5.9A and B).

5.3.5 VIP-induced state is highly resistant to epigenetic and translational blockade

The VIP-induced state is long lasting and is not reversed by wash-off. In light of these observations and recent literature suggesting that lighting schedule-induced changes to mouse periodicity are the result of stable DNA methylation (Azzi et al., 2014, 2017), it was possible that VIP, a mediator of photic input, was also causing long-term DNA methylation changes, and thus a block of methylation during VIP addition would alter the emergence of the VIP-induced state. Zebularine is a nucleoside analog of cytidine and binds covalently to the active site of DNA methyltransferases, disrupting their activity (Azzi et al., 2014; Champion et al., 2010). Zebularine, which is relatively stable at 37°C (Champion et al., 2010), was added to culture medium 6 h before VIP or vehicle application. By itself, zebularine had a minimal effect on PER2::LUC bioluminescence (Figure 5.10A), and it did not clearly affect the VIP-induced state (Figure 5.10B and C). On quantification, however, although phase-shifting, PER2 induction and amplitude change were all similar to slices treated solely with VIP, SCN that had been treated
with VIP in the presence of zebularine showed a greater period change (Figure 5.10 D-G). Interestingly, this is actually an opposite effect to what has been observed previously where infusion of zebularine reduced period changes to different lighting schedules (Azzi et al., 2014), although considerable other differences exist between the experimental designs of the studies as well. Nevertheless, it appears that the VIP-induced state is not
caused by aberrant DNA methylation, and indeed that methylation may play a role in restricting the effect of VIP on SCN period.

Although DNA methylation does not appear to play a major role in the VIP response, the differential effects of VIP on the genes of the TTFL and resulting phase mismatch are evidently persistent – this misalignment of normal core clock gene rhythmicity may therefore be the cause of disrupted SCN rhythmicity. Thus it may be expected that ‘restarting’ the TTFL by translational inhibition could realign the genes to their usual phases and revert the slice back to a pre-VIP-like oscillation. Cycloheximide (CHX), a protein synthesis inhibitor, was applied for 48 h almost two weeks after VIP application (Figure 5.11A and B). CHX application did not reduce the period

Figure 5.11 VIP-induced amplitude reduction can be restored by translational inhibition (A and B) Representative PER2::LUC bioluminescence rhythms of SCN slices treated with 10 µg/ml cycloheximide (CHX) after vehicle or 10 µM VIP (A) or VIP followed by CHX or vehicle (B). VIP was bath applied after 4 cycles (marked by +). CHX was bath applied for 48 h two weeks after the initial treatment. Bioluminescence has been normalised to the first peak. (C and D) Period (C) and normalised amplitude (D) over time following treatments (mean ± SEM). Veh/veh (n = 5), Veh/CHX (n = 6), VIP/veh (n = 7), VIP/CHX (n = 8). CHX following VIP partially restores rhythmicity to a level not significantly different from CHX treatment alone. Alone, CHX has a period lengthening effect. All tests two-way ANOVA with Tukey’s multiple comparisons test, significance values indicate differences from Veh/Veh slices. ns = not significant, *P < 0.05, ***P < 0.001, ****P < 0.0001.
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of VIP-treated slices, and indeed had a period-lengthening effect itself (Figure 5.11C). It did, however, restore amplitude to a level that was not significantly different from vehicle/CHX-treated slices, albeit not to a vehicle/vehicle level (Figure 5.11D). This amplitude restoration could be the result of restoration at the cellular level, or of resetting the phase-relationship between regions of the SCN and thus reducing phase dispersal. To address this, VIP- and CHX-treated slices were imaged on CCD camera (Figures 5.12 and 5.13). ROI analysis (each ROI = 5x5 pixels) revealed that, following CHX, individual cellular amplitude was indeed restored, and cells were initially reset to a similar phase (Figure 5.12A). However, several days after CHX wash-off, aberrant phase relationships returned, showing no improvement in phase vector length over the three slices visualised (Figure 5.12B and C). Periods were significantly increased after CHX as expected, however interestingly the period dispersal of SCN oscillators was reduced (Figure 5.12D-F).

Network dynamics were next investigated using CoM analysis. The CoM of VIP- and CHX-treated slices was highly reminiscent of VIP-treated slices, where the dorsal tip of the slice dominated and the CoM moved in a ventromedial direction rather than ventrolateral (Figure 5.7 and 5.13A and B). Furthermore, while the displacement of the CoM was considerable immediately after CHX wash-off, this reduced in size several days later (Figure 5.13C) and was not significantly different from post-VIP CoM perimeters (Figure 5.13D). Taken together, these results indicate that protein synthesis inhibition did restore amplitude to cellular oscillators at least initially, but was unable to restore the spatiotemporal wave. Treating WT slices with CHX has previously revealed that the spatial programme of gene expression observed in SCN slices is intrinsic to the network, rather than being stochastic or externally regulated (Yamaguchi et al., 2003), thus given that the network of VIP-treated slices similarly rearranged itself after CHX,
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albeit in a disrupted way, this suggests that VIP has stably altered intercellular communication in the SCN circuit.
5.3.6 Disrupted cellular communication alters the VIP response

Tetrodotoxin (TTX) is a highly potent voltage-gated sodium channel blocking agent, preventing sodium ion movement into the neuron and hence inhibiting...
action potentials. TTX has previously been applied to SCN slices to remove synaptically mediated intercellular communication, which can allow cell-intrinsic periods to be displayed (Patton et al., 2016; Schwartz et al., 1987; Smyllie et al., 2016; Yamaguchi et al., 2003). Here it was hypothesised that the way in which VIP stably alters intercellular communication may be mediated electrically, and thus blocking this form of communication may reduce the appearance of the VIP-induced state. TTX was applied to slices 24 h before VIP application, however slices treated with both drugs still elicited a strong response to VIP that could not visibly be washed off (Figure 5.14A and B). Pre-treatment with TTX actually resulted in a significantly greater immediate phase shift, although there was no difference in delayed phase shift with the VIP-only treatment, which appears to be due to the second peak after VIP appearing significantly earlier (Figure 5.14C and D). Period change was significantly reduced in TTX/VIP-treated slices compared to slices treated with VIP alone, although a significant effect was still observed compared to TTX/veh-treated slices (Figure 5.14E). No significant differences were observed for PER2::LUC induction or amplitude change, and quantification of amplitude after wash-off confirmed that this could not be restored (Figure 5.14F-H).

If TTX was added after VIP instead of before it, there was no effect on any aspect of the VIP-induced state (Figure 5.15A-D), including period (Figure 5.15C) and amplitude (Figure 5.15D). The lack of an effect on amplitude is particularly surprising given the strong reduction in amplitude seen when TTX is applied to untreated slices, and may suggest that intercellular communication within a VIP-treated slice is already completely disrupted.

Slices in which intercellular communication was compromised genetically, rather than pharmacologically, were also treated with VIP. VipCre-CkIε^{Tau/Tau} chimeric slices, described in detail in Chapter 4, contain 24 h VIP cells while
Figure 5.14 Disrupted synaptic communication potentiates the VIP-induced phase shift

(A and B) Representative PER2::LUC bioluminescence rhythms of SCN slices treated with 10 µM VIP after vehicle or 1 µM tetrodotoxin (TTX) (A) or TTX followed by VIP or vehicle, plus subsequent wash (marked by §; B). TTX was bath applied after 4 cycles (marked by *), and VIP was bath applied 24 h later (marked by +). Bioluminescence has been normalised to the first peak. (C and D) Phase shifts (C) and peak-to-peak period (D) following treatments (mean ± SEM). TTX/VIP-treated slices (n = 7) immediately shifted significantly more than those treated with VIP alone (n = 5), however this difference was abolished later on by a significantly shorter inter-peak interval between the first two peaks after VIP. Vehicle controls showed no phase shifts (veh: n = 6; TTX/veh: n = 5). (E-G) Period change (E), PER2::LUC fold induction (F) and relative amplitude (G) responses after treatments (mean ± SEM). Period change was significantly attenuated in the presence of TTX. (H) Relative amplitude after treatments and wash-off show that the amplitude effects of TTX alone, but not TTX and VIP combined, can be washed off. All tests two-way ANOVA with Tukey’s (C, E-G) or Sidak’s (D and H) multiple comparisons test, ns = not significant, *P < 0.05, **P < 0.01, ****P < 0.0001.
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the rest of the SCN (and indeed mouse) has a 20 h cell-intrinsic period. This is due to the Tau mutation of Ck1ε, which results in a faster degradation of PER1 and PER2. Cells expressing Cre recombinase remove a floxed exon within Ck1ε, resulting in a phenocopy of the Ck1ε<sup>−/−</sup> genotype, which has a 24 h period (Meng et al., 2008). Both in vivo and ex vivo, reverting the VIP cells to 24 h appeared to have no effect on periodicity, suggesting that the VIP cells are not pacemaker cells in the SCN (see Chapter 4). However, it was hypothesised that individual oscillators within these SCN slices may be less tightly coupled due to a disrupted VIPergic circuit. Application of VIP to

Figure 5.15 Disruption of synaptic signalling does not affect an existing VIP-induced state

(A and B) Representative PER2::LUC bioluminescence rhythms of SCN slices treated with 1 µM TTX after vehicle or 10 µM VIP (A) or VIP followed by TTX or vehicle (B), with subsequent wash-off (marked by §). VIP was bath applied after 5 cycles (marked by +). TTX was bath applied 6 days after the initial treatment (marked by *). Bioluminescence has been normalised to the first peak. (C and D) Period (C) and normalised amplitude (D) over time following treatments (mean ± SEM). VIP/TTX-treated slices (n = 6) show no significant differences from VIP/veh-treated slices (n = 8) in period or amplitude during treatment or after wash-off. Veh/veh (n = 10), Veh/TTX (n = 4). All tests two-way ANOVA with Dunnett's multiple comparisons test, significance values indicate differences from Veh/Veh slices. **P < 0.01, ***P < 0.001, ****P < 0.0001.
Figure 5.16: VIPergic chimerism prevents VIP-induced phase delay

(A) Representative PER2::LUC bioluminescence rhythms of VipCre-Ck1ε<sup>WT/WT</sup> and VipCre-Ck1ε<sup>Tau/Tau</sup> treated with 1 µM VIP. VIP was bath applied at CT10 after 5 cycles (marked by +). Bioluminescence has been normalised to the first peak. (B) Immediate and delayed phase shifts (mean ± SEM) in VipCre-Ck1ε<sup>WT/WT</sup>, VipCre-Ck1ε<sup>WT/Tau</sup> and VipCre-Ck1ε<sup>Tau/Tau</sup> slices following vehicle or VIP treatment. Significant differences between immediate and delayed shifts were observed for VipCre-Ck1ε<sup>WT/Tau</sup> (n = 7) and VipCre-Ck1ε<sup>Tau/Tau</sup> slices (n = 5) treated with VIP, but none was seen for VipCre-Ck1ε<sup>WT/WT</sup> (n = 3) nor vehicle controls (VipCre-Ck1ε<sup>WT/WT</sup>: n = 2; VipCre-Ck1ε<sup>WT/Tau</sup>: n = 7; VipCre-Ck1ε<sup>Tau/Tau</sup>: n = 3). (C) Peak-to-peak periods in VipCre-Ck1ε<sup>WT/WT</sup> and VipCre-Ck1ε<sup>Tau/Tau</sup> slices treated with VIP. Peak immediately following VIP treatment is shown in red. (D and E) Period change (D) and PER2::LUC fold induction (E) responses (mean ± SEM) following VIP treatment to chimeric slices. Chimerism significantly reduced the period change following VIP. All tests two-way ANOVA with Sidak’s (B) or Tukey’s (D and E) multiple comparisons test. (F) Bioluminescence signal slope change (mean ± SEM) following VIP treatment to chimeric slices. One-way ANOVA with Tukey’s multiple comparisons test. ns = not significant, **P < 0.01, ****P < 0.0001.
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VipCre-Ck1ε<sup>Tau/Tau</sup> SCN slices resulted in an immediate PER2 induction and acute phase-shift, but the rhythm was not phase-shifted when examined several days after treatment (Figure 5.16A and B). This arose because of a shorter inter-peak duration between the first and second peaks after VIP than in wild-types, and indeed it was shorter than the expected cell-intrinsic period of any cell within the VipCre-Ck1ε<sup>Tau/Tau</sup> slice (Figure 5.16C). The period did increase in VipCre-Ck1ε<sup>Tau/Tau</sup> slices, but it was nevertheless a significantly smaller increase compared to VipCre-Ck1ε<sup>WT/WT</sup> slices (Figure 5.16D): even when normalised to circadian hours, the period change in controls was 1.7 circadian hours but 1.1 circadian hours in VipCre-Ck1ε<sup>Tau/Tau</sup> slices. There was no significant differences in the magnitude of PER2::LUC induction (Figure 5.16E), although the speed at which PER2 was induced immediately following VIP application, as measured by the slope change in bioluminescence signal, was significantly greater in VipCre-Ck1ε<sup>Tau/Tau</sup> slices (Figure 5.16F). These results from slices with aberrant (VipCre-Ck1ε<sup>Tau/Tau</sup>) or disrupted (TTX-treated) intercellular communication suggest that the response to VIP, in particular the phase shift, period change and PER2 induction, are partly mediated at the circuit level in SCN slices.

5.3.7 VIP can act through VPAC2-independent pathways

Although several circadian parameters of the VIP response appear to be mediated at the circuit level, amplitude in particular is unaffected by disrupted circuit-level communication both before and after VIP application, and all oscillators in the SCN appear to be affected by VIP application even in the absence of synaptic signalling. This suggested that either recipient VPAC2 cells are communicating with non-VPAC2 cells non-synaptically, or that VIP was acting through receptors other than VPAC2 that are present throughout the SCN. To address one of these possibilities, Vipr<sup>2−/−</sup> slices were treated with
VIP. *Vipr2*−/− slices demonstrated weak rhythms pre-VIP, as seen previously, due to a lack of VIPergic signalling and thus weakened intercellular communication (Maywood et al., 2006). Interestingly, application of VIP caused a strong response in *Vipr2*−/− slices (Figure 5.17A), including a

Figure 5.17 *Vipr2*−/− slices respond to VIP
(A) Representative PER2::LUC bioluminescence rhythms of *Vipr2*+/+ and *Vipr2*−/− slices treated with 1 µM VIP. VIP was bath applied at CT10 after 4 cycles (marked by +). Bioluminescence has been normalised to the first peak. (B) Phase shifts following treatments (mean ± SEM). *Vipr2*−/− slices (n = 4) immediately shifted significantly more than *Vipr2*+/+ slices (n = 5) after VIP, however this difference was abolished later on. Vehicle-treated *Vipr2*−/− slices (n = 4) showed a slight delayed shift compared to *Vipr2*+/+ (n = 8). (C) Peak-to-peak period following VIP (mean ± SEM). (D-F) Relative amplitude (D), period change (E) and PER2::LUC fold induction (F) responses (mean ± SEM) in *Vipr2*+/+ and *Vipr2*−/− slices following treatments. Relative amplitude was increased, rather than decreased, in *Vipr2*−/− slices following VIP. All tests two-way ANOVA with Tukey’s multiple comparisons test, ns = not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
significantly longer immediate phase shift than in wild-type slices, although the phase delay was the same as in controls after three days (Figure 5.17B), which again was due to a short inter-peak duration between the first two peaks after VIP treatment (Figure 5.17C). The most dramatic effect of the Vipr2⁻/⁻ phenotype after VIP application was that VIP actually increased, rather than decreased, the amplitude of PER2::LUC oscillation while the baseline remained unchanged (Figure 5.17A and D) in contrast to what has been observed in all other experiments presented in this chapter. There was no effect of VIP on the period of Vipr2⁻/⁻ slices, although due to difficulty in establishing a stable Vipr2⁻/⁻ slice period, this was highly variable (Figure 5.17E). Finally, the fold change of PER2::LUC induction was significantly greater in Vipr2⁻/⁻ slices than WT controls (Figure 5.17F), potentially reflecting the weakened coupling within the circuit as in TTX pre-treated and VipCre-Ck1ε Tau/Tau slices.

A VIP response, albeit noticeably different to the WT response, in Vipr2⁻/⁻ slices prompted the obvious question of non-canonical VIP receptor identity. The PAC1 receptor is a close relative of the VPAC2 receptor and is expressed widely throughout the brain, although it is generally thought to be specific to pituitary adenylate cyclase-activating polypeptide (PACAP) signalling, having previously demonstrated a significantly greater affinity for PACAP over VIP of approximately 100-fold (reviewed in Vaudry et al., 2000). However, affinity for and activation of PAC1 by VIP varies widely depending on model system used, with EC₅₀ values for PAC1 activation by VIP determined in cAMP assays being as low as 40 nM (Buscail et al., 1990; Cauvin et al., 1990; Dickson and Finlayson, 2009; Dickson et al., 2006). Furthermore, PAC1 is known to be expressed in the SCN, colocalising with both VIP and AVP neurons (Cagampang et al., 1998b; Kalamatianos et al., 2004), unlike the third receptor of this family, VPAC1, which is not found in
the SCN (Usdin et al., 1994). The established role of PAC1 in the SCN is to act as a co-transmitter in transmitting information from the retina to the SCN alongside glutamate (Hannibal, 2002; Hannibal et al., 2001; Michel et al., 2006). Thus PAC1 appeared to be a likely candidate for non-canonical VIP responses in Vipr2−/− slices. To investigate this, both WT and Vipr2−/− slices were treated with the PAC1 antagonist PACAP 6-38 (P6-38; Chen et al., 1999; Hannibal et al., 1997; Robberecht et al., 1992) 30 minutes before VIP treatment (Figure 5.18A and B). P6-38 appeared to have no effect in isolation, nor did it attenuate the VIP response in WT slices for any parameter measured (Figure 5.18C-G), although there was a trend to reduce the delayed phase shift in WT slices (P = 0.07). In contrast, P6-38 significantly reduced the phase shift and amplitude change in Vipr2−/− slices to the extent that they were no longer significantly different to control Vipr2−/− slices (Figure 5.18C-E). Period change and PER2::LUC fold induction were unaffected by P6-38 (Figure 5.18F and G; although period change was again variable). Thus PAC1 appears to be capable of mediating VIPergic signalling in Vipr2−/− slices, although given the lack of effect of P6-38 in WT slices, it may not make a large contribution to the typical VIP-induced state.
Figure 5.18 Antagonism of the PAC1 receptor blocks the effects of VIP in Vipr2−/− slices
(A and B) Representative PER2::LUC bioluminescence rhythms of Vipr2+/+ (A) and Vipr2−/− (B) slices treated with 1 µM VIP in the presence or absence of 1 µM PACAP6-38 (P6-38). P6-38 was bath applied 30 minutes before VIP, which was applied at CT10 after 2-3 cycles (marked by +). Bioluminescence has been normalised to the first peak. (C and D) Immediate (C) and delayed phase shifts (D) following treatments (mean ± SEM). P6-38 treatment significantly reduced phased shifts in Vipr2−/− slices following VIP (n = 3) compared to those treated with VIP alone (n = 4), but not Vipr2+/+ slices (VIP/veh: n = 6; VIP/P6-38: n = 3). P6-38 had no effect alone in either genotype (Vipr2+/+ veh/veh: n = 8; Vipr2+/+ P6-38/veh: n = 3; Vipr2−/− veh/veh: n = 4; Vipr2−/− P6-38/veh: n = 3). (E-G) Relative amplitude (E), period change (F) and PER2::LUC fold induction (G) following treatments (mean ± SEM). The amplitude-increasing effect of VIP on Vipr2−/− slices was significantly reduced in the presence of P6-38, however it had no effect on period change or PER2::LUC fold induction. All tests two-way ANOVA with Tukey’s multiple comparisons test, ns = not significant, **P < 0.01, ***P < 0.001, ****P < 0.0001.
5.4 Discussion

The SCN has two primary functions: ensuring the synchrony of dispersed cellular oscillators within the organism, and ensuring that those oscillators maintain an adaptive phase relationship with the external environment. For the latter, photic input from the retina and subsequent signalling from VIP cells in the ventrolateral SCN is critical (Vosko et al., 2007), however the mechanisms through which VIP mediates SCN responses to light input remain to be fully elucidated. Here, by combining application of VIP to SCN slices with pharmacological and genetic manipulations, I present an extensive characterisation of the effects of VIP on SCN slices. I demonstrate that the VIP-induced state, characterised by a phase shift, period lengthening, amplitude reduction and baseline increase, is highly persistent, being resistant to washing, disruption of electrical signalling, translational inhibition and epigenetic blockade. Moreover, exogenous VIP affects multiple circadian parameters in the SCN slice, mediated at both the cell- and the circuit-level, and the effects on different parameters are at least partly independent of each other, suggesting that VIP acts through numerous pathways. Finally, I demonstrate that VIP application to the SCN is capable of eliciting a response from Vipr2−/− slices, which it achieves by acting through the non-canonical PAC1 receptor.

5.4.1 Characterisation of the VIP response

Application of VIP to slices was previously shown to induce PER2::LUC and to have significant effects on phase and amplitude of circadian oscillation (An et al., 2011, 2013). In order to establish a reliable protocol to further investigate the mechanisms of VIP on the SCN, I first set out to replicate these results. CT10 was the phase chosen for almost all subsequent experiments due to it
appearing to be at the peak of the phase-delaying region of the VIP phase response curve (PRC; An et al., 2011), and a subsequent dose response curve (DRC) was carried out using this phase. The DRC broadly reflected the results found by An et al., but with a number of key differences. First, I observed a significant dose-dependent period change following VIP addition, which was not reported previously (An et al., 2011). Secondly, I found a significant amplitude reduction at all concentrations of VIP tested (down to 10 nM), whereas previously an amplitude reduction was only seen at concentrations above 100 nM (An et al., 2013). This may argue that, contrary to what was previously stated, VIP has a desynchronising influence at all concentrations, at least when applied at CT10.

High concentrations of VIP have been utilised previously to investigate VIP-related transduction cascades (An et al., 2011; Nielsen et al., 2002), although the exact physiological concentration of VIP in the SCN is not known and is more difficult to calculate than that of neurotransmitters due to the contribution of non-synaptic release (Castel et al., 1996; Maywood et al., 2011). However, given that mammals have been shown to be able to phase shift by 2-3 h from a single saturating light pulse (Nelson and Takahashi, 1991), it seems likely that the high concentrations of VIP used in these experiments can be reached in vivo in artificial paradigms, when accounting for the extra difficulty of phase shifting the SCN in vivo due to potential mediating influences of extra-SCN tissue.

The duration of VIP treatment clearly had an impact on the strength of the response, although there was some differentiation between the circadian parameters examined. Following just 2 h of treatment with VIP, only amplitude was significantly affected, while after 6 h of treatment, a larger amplitude reduction as well as a phase shift were observed. However, only when VIP was not washed off was a period change seen. Although the
stronger effects seen in the ‘no wash’ condition may be due to VIP present at different phases, this is unlikely. Given the half-life of VIP in culture is approximately 2 h (An et al., 2011), by 6 h there is approximately 10%, or 100 nM (from a starting concentration of 1 µM) VIP left. While this is not an insignificant amount, the VIP DRC demonstrates that 100 nM VIP treatment is not sufficient to elicit an amplitude reduction of the magnitude seen in the ‘no wash’ condition (at any time, given the phase-independence of amplitude and period change). Thus the duration of VIP exposure appears to be important, which is consistent with what has been shown in vivo in response to light (Morin and Studholme, 2014). Given that the VIP-induced phase-shift is at least partly mediated by Per gene induction, prolonged activation (i.e. in the ‘no wash’ condition) of CREs in the Per promoter will result in greater protection of CRY from degradation (Akashi et al., 2014), allowing subsequently longer inhibition of CLOCK:BMAL1 at E-boxes. An alternative explanation is that the downstream transduction cascades activated by VIP take time to achieve their full, long-lasting effects (even following complete VIP degradation), such as through immediate early genes followed by a transcriptional cascade, and that an early (2 or 6 h) wash-off disrupts this by providing an opposing cue.

In addition to characterising the response of SCN slices from neonatal pups to VIP, slices from adult mice were also investigated. The amplitude reduction and period change were significantly less in adult slices, while phase shifting and PER2::LUC induction showed no difference. The nature of intercellular communication is believed to change throughout development, for example the disruption of synaptic signalling with TTX has a stronger effect on pup slices than adult (Baba et al., 2008), thus it is conceivable that responses to neuropeptidergic communication also change. The pups from which my slices were derived were P8-P10 in age, during a critical period for the mouse
visual system (Hooks and Chen, 2007), thus it might be expected that light input has a greater effect on the circadian system at this time.

5.4.2 Persistence of the VIP-induced state

One surprising feature of the VIP-induced state was its persistence: the low amplitude and period increase were maintained despite repeated washes. This amplitude reduction and persistent state do not occur when GRP is added at a similar time (An et al., 2013), implying that this effect is specifically mediated by the VPAC2 receptor. Furthermore, the persistence of the state even in the absence of VIP in the medium following washing suggests that VIP induces long-term changes to SCN rhythmicity, which may require a new Zeitgeber or other factor to overcome. Although VIP is known to signal via cAMP second messenger pathways (An et al., 2011; Kudo et al., 2013), the persistent effects of VIP are unlikely to be mediated by this because although treatment of slices with forskolin, an activator of adenylate cyclase, and the cAMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), to increase cAMP levels causes a similar reduction in amplitude, this can be washed off (O’Neill et al., 2008). It remains to be seen whether activation of both cAMP and PLC pathways simultaneously recapitulates the long-lasting effects of VIP.

The long-lasting nature of the VIP-induced state may suggest that gene expression was stably altered, such as through epigenetic mechanisms. However, application of zebularine prior to VIP did not reduce the response, and in fact potentiated the period change, suggesting that this was not the cause. A future experiment should wash off the zebularine/VIP combination to determine if the lack of DNA methylation has any impact on the persistence of the VIP-induced state following wash off. Inhibitors of other
forms of epigenetic modification, such as histone methylation or acetylation, could also be examined. Zebularine was previously shown to have an effect on periodicity following different lighting schedules, although this was actually to suppress period changes rather than exacerbate them (Azzi et al., 2014, 2017). Clearly numerous differences exist between the experimental designs of the studies (such as in vivo vs. ex vivo or rhythmic VIP vs. a single application) that might explain the differences seen. Nevertheless, the idea that DNA methylation induced by light or VIPergic input can manipulate period is consistent.

A potential mechanism that was not examined in this project relates to VPAC2 receptor desensitisation and internalisation. Agonist addition induces VPAC2 receptors to internalise following phosphorylation (McDonald et al., 1998), which is mediated through activation of PKA and GPCR kinase (GRK; McDonald et al., 1998; Murthy et al., 2008). After internalisation, VPAC2 receptors appear to be trafficked to a single accumulation site within the cell (McDonald et al., 1998), however they are then returned to the cell membrane within 2 hours (Langlet et al., 2004). This suggests that permanent internalisation of VPAC2 following VIP application in the current experiments, particularly after washing (and therefore in the absence of VIP), is an unlikely explanation for the persistence of the VIP-induced state.

In addition to resistance to reversal, the VIP-induced state did not appear to require synaptic signalling to be maintained. Remarkably, addition of TTX to slices following VIP had no visible impact on the rhythm at all. This suggests that VIP application has already completely disrupted intercellular communication within the SCN, and that the persistence of this state is due to effects at the cell-autonomous level. Visualising VIP-treated slices on camera supports the disruption of circuit-level communication, where the spatiotemporal wave was completely abolished, albeit in a predictable
manner. Given the lack of an effect of TTX on this state, this could suggest that the stable phase relationship seen after VIP occurs as a result of intrinsic period differences, although this would result in the cellular oscillators of a VIP-treated slice ultimately going completely out of phase, which has never been observed. Hence, although synaptic signalling is disrupted, a non-synaptic coupling factor of an undetermined nature may be maintaining cellular phase relationships following VIP.

The idea that some level of intercellular communication remains after VIP is supported by CHX experiments. Yamaguchi et al. (2003) argue that, in previously untreated slices, the re-emergence of a stereotypical spatiotemporal wave of clock gene expression following CHX wash-off suggests that this wave is an intrinsic property of the network, rather than due to stochastic events. In VIP-treated slices, a similar phenomenon is observed, whereby the spatiotemporal wave seen before CHX re-emerges after CHX. Therefore translation does not appear to be required for the maintenance of the VIP-induced state at the circuit level, while at the cellular level the amplitude of oscillation also remains low. Despite VIP resulting in out-of-phase core clock genes, resetting them to the same phase with CHX is not sufficient to restore oscillation amplitude, suggesting that disruption of phase relationships between Per and Cry genes is not responsible for the VIP-induced state.

If removal of VIP from the medium, epigenetic blockade, synaptic inhibition and translational inhibition are not involved, what is required to restore VIP-treated slices to a robust oscillation? One possibility yet to be investigated relates to long-lasting phosphorylation of core clock genes CLOCK and BMAL1. CLOCK/BMAL1 have relatively long half-lives (compared to PER) and hence are still present even after 36 h of translational inhibition (Yamaguchi et al., 2003). Furthermore, phosphorylation of these two proteins
by a number of different kinases has been shown to have significant effects on the cellular oscillation, as well as activity, stability and nuclear localisation of these proteins (Kondratov et al., 2003; Lipton et al., 2015; Matsumura et al., 2014; Sahar et al., 2010; Sanada et al., 2002; Tamaru et al., 2009, 2015; Yoshitane et al., 2009). VIP application can increase the rate of entrainment both in vivo and in vitro, with no obvious reduction in rhythm amplitude (An et al., 2013), implying that Zeitgebers are sufficient to restore oscillations to baseline levels following VIP or light input (Azzi et al., 2014). However, the cellular transduction mechanisms through which entrainment occurs are poorly understood (Tataroglu et al., 2015). Thus it is possible that a single bolus of VIP leads to hyperphosphorylation of these clock proteins, while an opposing signal (initiated by a rhythmic Zeitgeber) is capable of counteracting this, utilising the disrupted core TTFL protein oscillations to establish a new phase. Future work could characterise CLOCK and BMAL1 phosphorylation states following VIP treatment or apply VIP in combination with specific kinase inhibitors to explore the potential role of CLOCK/BMAL1 post-translational modifications in entrainment. Clearly this approach would only address the effects of VIP at the cell-autonomous level; the nature of the mechanism(s) holding cells in the aberrant spatiotemporal configuration observed following VIP remains to be determined.

5.4.3 Roles of cells and circuits in the VIP response

As alluded to several times, VIP is capable of influencing SCN rhythmicity through cell-autonomous oscillators and through alteration of intercellular coupling, components which have been previously shown to have different mechanistic bases (Pauls et al., 2014). Clearly these influences are difficult to completely separate out, although the emergence of a predictable
Mechanisms of VIP in SCN entrainment

spatiotemporal wave after CHX provides strong evidence that VIP disrupts the SCN circuit.

To investigate the role that the SCN circuit plays in mediating the response to VIP, TTX is a useful tool to isolate effects independent of synaptic signalling. While synaptic signalling is not important for the maintenance of the VIP-induced state, the immediate phase shifting response to VIP is potentiated in the presence of TTX, although this shift is ‘reined in’ when examined two days later. A decoupled SCN might be expected to shift to a greater degree as coupling grants a great deal of protection against perturbation (Welsh et al., 2010), hence the receptive VPAC2 cells may respond more strongly if they are not subject to the mediating effects of non-receptive cells. This difference is short-lived, however, despite synaptic communication still being disrupted. This could mean that while electrical communication is important for acute responses, extra-synaptic signalling can also facilitate core-shell communication, but at a slower rate. This result is supported by what is seen when applying VIP to VipCre-Ck1εTau/Tau slices, in which intercellular coupling may be weakened due to different intrinsic periods. These slices are completely resistant to the longer-term phase shifting effects of VIP. The exact mechanism for this is not clear, but the 24 h VIP cells in the slice may exert a strong influence over the phase of the VPAC2 cells non-synaptically (i.e. more slowly), which is why an immediate shift to exogenous VIP occurs, but is not maintained.

At the level of the cell, VIP disrupts core clock genes in different ways. The most striking difference is that the previous phase relationship of the genes is permanently altered, with Per1-Luc phase shifting a significantly smaller amount than both PER2::LUC and Cry1-Luc. Differences between PER and CRY in shifting to light pulses has been observed before (Reddy et al., 2002), while separable effects of PER1 and PER2 are less common (Nielsen et al.,
2002). However, it has previously been seen that PER1 is important for phase advances, while PER2 is important for phase delays (Albrecht et al., 2001; Yan and Silver, 2002), thus this may explain the lack of Per1-Luc induction in the data presented here, given that a phase-delivering stimulus was applied. Nonetheless, this does not necessarily explain the consistent and aberrant phasing of Per1 and PER2 seen here, of which the significance is not clear.

The presence of an intracellular clock was not necessary for the VIP response. CRY1 and CRY2 are vital for rhythmic oscillation in the SCN and in vivo (van der Horst et al., 1999; Kume et al., 1999), however application of VIP to CryDKO slices still elicited a strong PER2::LUC induction. This is consistent with what has previously been seen when giving CryDKO mice a light pulse (Okamura et al., 1999). Although phase and periodicity clearly cannot be investigated in CryDKO slices, an analogous measure of amplitude, the root mean square (RMS) of the PER2::LUC signal, was reduced after VIP, which may imply that the suppressive effects of VIP act specifically on PER2 transcription and translation, rather than affecting rhythmicity per se.

5.4.4 VIP can act through the PAC1 receptor in the absence of VPAC2

As mentioned above, my own data do not support the idea that low concentrations of VIP are synchronising compared to higher concentrations, given the reduction in amplitude caused by all concentrations of VIP down to 10 nM. Nevertheless, a clear jump in the magnitude of response can be seen between 100 nM and 1 µM. The data presented here reveal a potential mechanistic basis for this separation, which is that VIP is capable of acting via the PAC1 receptor, which could conceivably be activated by VIP concentrations above 100 nM based on biochemical assays. Vipr2^-/- slices showed a strong response to VIP, resulting in a PER2 induction, large phase
delay and an increase in amplitude, with evidence that this was PAC1-mediated emerging from studies utilising the PAC1 antagonist P6-38.

VPAC2 has no strong preference when responding to VIP and PACAP, whereas PAC1 is canonically believed to respond primarily to PACAP over VIP, displaying a greater affinity for PACAP of approximately two orders of magnitude (Harmar et al., 2012; Vaudry D. et al., 2000). However, the EC\textsubscript{50} values for PAC1 by VIP in cAMP assays can vary widely, being as low as 40 nM (Buscail et al., 1990; Cauvin et al., 1990; Dickson and Finlayson, 2009; Dickson et al., 2006). Thus at phase shifting concentrations of VIP (>10 nM), activation of PAC1 could be possible \textit{in vivo}, although a full dose response curve will need to be performed on \textit{Vipr2}\textsuperscript{-/-} slices to determine likely physiological relevance. SCN slices offer a useful tool to investigate this particular interaction due to the lack of confounding PACAP/PAC1 activity that would be found \textit{in vivo} in communication between the RHT and SCN. Indeed, this interaction may never before have been seen precisely because of that confound.

Clearly the VPAC2-mediated response is stronger than that of PAC1, as no increase in SCN slice amplitude is seen in WT slices, and blocking PAC1 with P6-38 when treating with VIP in WTs did not significantly affect any parameter measured compared to sole VIP application. This is supported by previous observations that application of a PAC1-specific agonist, maxadilan, did not have the capacity to phase shift electrical firing rhythms in the SCN (Reed et al., 2001). The ability for VIP to act through PAC1 seemingly only in the absence of VPAC2 does not have a clear mechanism, but VPAC2/PAC1 heterodimers have previously been indicated, with the suggestion that the receptor that is more highly expressed can suppress the activity of the other (Langer, 2012). The existence of VPAC2/PAC1 heterodimers has not been
studied, but may provide a possible explanation if a similar interaction is occurring.

The relevance of this pathway with respect to the VIP-induced state requires further investigation. Given that PAC1 expression is found in both AVP- and VIP-expressing cells (Kalamatianos et al., 2004), it may offer a mechanism by which the effects of VIP are experienced by all SCN oscillators (if VIP can act through PAC1 in the presence of VPAC2). VIP acting through PAC1 also has the potential to explain several findings relating to VPAC2-null mice that either do not apply or have not yet been observed in VIP-null mice. Firstly is the observation that constant light can promote stronger rhythms in VPAC2-null mice (Hughes et al., 2015). Constant light may increase overall VIPeric tone, which then signals via PAC1 to increase clock amplitude as I have shown here. Another study found that VPAC2-null, but not VIP-null, could entrain to a scheduled voluntary exercise (SVE) paradigm (Power et al., 2010). While the input mechanism for SVE into the SCN is not known, given that the VIP-expressing region receives many photic and non-photic inputs, SVE could stimulate VIP release, which could then act through PAC1 receptors. This would of course not be possible in VIP-null mice.

5.4.5 Conclusions

To conclude, I have found that VIP application to SCN slices has profound and persistent effects on all aspects of circadian rhythmicity. Interestingly, each parameter (period, phase shift etc.) can be selectively manipulated with different forms of pharmacological or genetic manipulation, suggesting that the effects of VIP are multi-faceted and are transduced through multiple, distinct pathways, rather than merely relying on the induction of PER proteins. At least some of these distinct effects are mediated at the cellular
level, circuit level or both, consistent with the role of VIP as a strong coupling factor in the SCN, and others may be mediated by the non-canonical receptor PAC1, although the significance of this awaits further investigation. While I was unsuccessful in restoring a ‘pre-VIP-like’ oscillation after VIP application through either washing or pharmacology, the fact that the VIP-induced state renders slices and mice more amenable to Zeitgeber input suggests that this state may offer a useful model in which to investigate the mechanisms of cellular entrainment. Finally, the protocol established here for investigating the mechanisms through which VIP works has allowed me to undertake a detailed dissection of the effects of VIP on the SCN transcriptome by microarray to identify novel VIP-regulated transcripts, the results of which will be discussed in the following two Chapters.
6.1 Introduction

The mechanisms by which the SCN is entrained to the environmental photoperiod, or influenced by light at night, remain to be fully elucidated. Following release of glutamate and PACAP (Hannibal, 2002) from the retinohypothalamic tract (RHT), retinorecipient SCN neurons undergo an influx of Ca^{2+} (as well as Ca^{2+} release from internal stores), activating multiple kinases which ultimately converge on Ca^{2+}/cAMP response element (CRE) binding protein (CREB; Gau et al., 2002; Ginty et al., 1993). CREs within the promoters of genes are bound by phosphorylated CREB, inducing their transcription. These light-induced genes within the SCN core include numerous immediate early genes (IEGs) such as Fos, Egr3 and Nr4a1 (Morris et al., 1998; Porterfield et al., 2007), the MAP kinase phosphatase Dusp1 (Doi et al., 2007) as well as Per1 and Per2 (Travnickova-Bendova et al., 2002), which provide a link to the cellular oscillator.

While early work into the mechanism of light-induced phase shifts focused on Fos induction and subsequent attribution of this to glutamate release (Aronin et al., 1990; Ebling, 1996; Kornhauser et al., 1990; Meijer et al., 1988; Rea, 1989; Rusak et al., 1990, 1992), later studies have concentrated on the induction of the Per genes (Albrecht et al., 1997; Kuhlman et al., 2003; Shigeyoshi et al., 1997; Tischkau et al., 2003; Wakamatsu et al., 2001; Yan and Silver, 2002), presuming this to be a sufficient mechanism through which light can influence the circadian oscillator. This is, however, likely a gross simplification. Firstly, this viewpoint primarily focuses on the retinorecipient
cells of the SCN, despite a clear, albeit delayed, response of the dorsal SCN following light exposure (Dardente et al., 2002; Yan and Silver, 2004). It also provides no mechanism for integration of light signals with the existing circadian oscillation at the circuit level, whereby the core must influence the shell, a stronger oscillator, to arrive at a new phase that is adaptive to the external environment. Neuropeptidergic communication is known to be critical in this process (Sheward et al., 2007; Vosko et al., 2015). Furthermore, CREB is capable of acting at several thousand sites in vivo (Zhang et al., 2005), suggesting that, even if CREB were the only mediator of light responsiveness, a large array of transcripts are likely to be induced. This indicates that a far more complex, multigenic network beyond merely the Per genes is required (Zhu et al., 2012) in order to produce a coherent output to the periphery reflective of the light input.

To address this latter issue, techniques such as microarrays (Jagannath et al., 2013; Porterfield et al., 2007), RNA-seq (Bedont et al., 2017) and high-throughput qPCR (Zhu et al., 2012) have been performed in order to identify individual genes as well as transcriptional circuits that may play a role in the circadian light response and its integration with oscillatory components within the cell. These techniques have also been used to assess rhythmic transcripts within the SCN and beyond (Panda et al., 2002; Zhang et al., 2014). It is important to note that these two populations of genes (light-inducible and rhythmic) are not the same, although identification of transcripts that are both light-inducible and rhythmic may provide further mechanistic insight into the integration of these two signals (Araki et al., 2006), in addition to the actions of Per1 and Per2.

Transmission of light-related information from the retinorecipient cells to the dorsomedial region of the SCN is known to involve neuropeptidergic communication, particularly VIP. VIP is capable of inducing Per gene
expression (Nielsen et al., 2002) and VIPergic signalling is essential for phase shifting in response to presentation of light at night (Colwell et al., 2003). Transcriptional networks regulated by VIP have been investigated through the use of mice deficient for VIP, or deficient for the VIP regulator LHX1 specifically in the hypothalamus (Bedont et al., 2017). This study demonstrated that VIP-dependent transcripts were largely clock-controlled, including neuropeptides (such as AVP), mediators of transduction cascades, and core clock genes. This work provided valuable insight into the mechanisms of VIP in core-shell communication, however it is possible that, due to using genomic knockout mice, transcriptional circuits were dysregulated, or that genes typically induced by VPAC2 activation were missed due to compensatory mechanisms. Furthermore, the genes observed as being regulated by VIP in the Bedont study might not necessarily be the same as those induced by VIP in response to light at night that subsequently produce phase shifts in behaviour and SCN rhythmicity.

Utilising the reliable phase delaying protocol established in the previous chapter, the experiments presented here use microarray analysis to explore transcriptional networks regulated by VIP over time in the context of an acute pulse of VIP to organotypic SCN slices. I demonstrate that VIP largely up-regulates rather than down-regulates transcripts in the SCN and that there are many common transcripts between those induced by light (in the core) and those induced by VIP (in the shell). Functional annotation and clustering reveals that VIP has extensive effects on multiple pathways, including immediate early genes (e.g. Fos and Egr1) and MAP kinase regulators (e.g. Dusp1 and Dusp4), as well as chemokines and steroid synthesis transcripts. Furthermore, significant enrichment of CRE-regulated genes and rhythmic genes was found, and separate functional annotation of these groups reveals
that VIP regulates multiple transcriptional circuits through distinct cellular transduction cascades.
6.2 Methods

6.2.1 DNA Microarray

Organotypic SCN slices were treated as described in Chapter 5, and divided into four groups for subsequent RNA extraction and microarray analysis to determine the acute effects of VIP treatment. The CT10 group was untreated and would serve as a baseline comparison; CT12 Vehicle slices were treated with vehicle (air medium) at CT10 and harvested at CT12 (the peak of PER2::LUC bioluminescence); CT12 VIP slices were treated with 10 µM VIP at CT10 and harvested at CT12; CT16 VIP slices were treated with 10 µM VIP at CT10 and harvested at CT16 (the approximate new peak of PER2::LUC following VIP treatment based on previous experiments). The CT16 VIP group served as a phase control in case differences observed comparing CT12 Vehicle with CT12 VIP were just the result of a difference in phase (peak vs. rising phase respectively) rather than treatment. It would also provide some information on the longer-term effects of VIP treatment.

Following total RNA extraction (Section 2.3.10), samples were sent to Cambridge Genomic Services (Department of Pathology, University of Cambridge) for further processing and microarray analysis. Here the total RNA was checked on an Agilent Bioanalyzer 2100 for integrity, with all samples demonstrating an RNA Integrity Number (RIN) of >9 apart from two (RIN 7.1 and 8). Total RNA was amplified using the Ovation Pico WTA v2 kit (NuGEN Technologies), and the resulting cDNA was fragmented and biotinylated using the BiotinIL kit (NuGEN Technologies). Concentration, purity and integrity of this labelled cDNA were determined using the Nanodrop ND-1000 (Thermo Scientific) and by Bioanalyzer before the cDNA was hybridised to a MouseWG-6 v2 BeadChip overnight. After washing, hybridisation was visualised by staining with streptavidin-Cy3 and scanned using a Bead Array Reader (Illumina).
A number of technical controls were included during hybridisation to determine the consistency and quality of the microarray. Low-, medium- and high-level hybridisation controls were spiked in, which should return low, medium and high intensity signals respectively. ‘Perfect match’ and ‘mismatch’ probes were included to ensure stringency, whereby the ‘perfect match’ probes should have a higher signal than ‘mismatch’ although the ratio of the two should remain fairly constant across samples. Finally, negative (no biotinylation) and positive samples were included to show staining specificity.

6.2.2 Microarray Analysis

Initial Processing

Raw data were loaded into R using the Bioconductor lumi package (Du et al., 2008) and segregated into pairs (such as CT12 Vehicle vs. CT10) for later comparison. These subgroups were then filtered using the detection p-value from Illumina to exclude any non-expressed probes, defined as not being significantly different (p > 0.01) from negative controls. Data were then transformed using the Variance Stabilisation Transformation (VS.T; Lin et al., 2008) and then normalized using quantile normalisation to remove technical variation between arrays. A global normalisation to allow visualisation of all groups was also performed. The limma package (Smyth, 2004) was used for comparisons between sample groups, and results were corrected for multiple testing using False Discovery Rate (FDR) correction. Correlations and clustering of the datasets from each sample were then performed to identify potential outliers and assess the quality of the data. Pairwise comparisons CT12 VIP vs. CT12 Veh and, to a lesser extent, CT16 VIP vs. CT12 VIP were chosen for the vast majority of analysis presented in this chapter.
Volcano plot and heat map generation

Volcano plots were created using Prism (Graphpad) by plotting the log fold change of a given transcript against its corresponding –log adjusted p value. Heat maps were generated using Morpheus software (Broad Institute, software.broadinstitute.org/morpheus/), and clustering was carried out using the in-built Hierarchical Clustering function in Morpheus.

Functional Annotation Analysis

Transcripts found to be significantly regulated by VIP were examined using the DAVID ontology software (Huang et al., 2009a, 2009b) to identify significantly regulated pathways (using gene ontology (GO) terms) through the functional annotation chart function. These significant GO terms were then clustered and visualised using the enrichment map plugin (Merico et al., 2010) for Cytoscape (v 3.5; Shannon et al., 2003). Only terms significant at $P < 0.01$ and $q < 0.05$ were visualised.

Transcript regulation analysis

Transcripts were assessed for circadian oscillation using the publicly available Circadian Expression Profiles Database (CircaDB, which can be found at http://circadb.hogeneschlab.org/, Pizarro et al., 2013). The embedded JTK algorithm estimates the probability of a given transcript from a dataset being rhythmic (JTK p-value) as well as a corrected probability accounting for false discovery rate (JTK q-value). Transcripts from the current investigation were interrogated against the Mouse 1.OST SCN 2014 dataset and considered circadian if they met a JTK-q-value cut-off of 0.05.
Transcripts were also examined for the presence of cAMP regulatory elements (CRE) in their promoters utilising the dataset from Zhang et al. (2005). Transcripts that were not found in that dataset were excluded from this analysis.

**Chi-squared analysis**

Once the circadian or CRE regulation of transcripts had been established, significant enrichment (e.g. for CRE regulation amongst VIP-up-regulated transcripts) was tested using the chi-squared test. The observed number of genes regulated in a given way was compared with expected values calculated based on the ratios seen when looking at all transcripts in the CT12 VIP vs. CT12 Vehicle dataset. Graphs were then plotted utilising the observed/expected frequency, so a value of 1 would mean that there was no difference between the observed and expected frequencies.
6.3 Results

6.3.1 Transcriptome regulation by VIP

To investigate the signalling pathways involved in the VIP response, the transcriptomes of WT slices treated with VIP were examined 2 or 6 h after treatment by microarray. 1165 significant, distinct transcripts were found across all comparisons, with CT12 VIP vs. CT12 Veh being the comparison with the greatest number of significant genes (Table 6.1). Only one gene was found to be significant in the CT12 Veh vs. CT10 comparison (Figure 6.1A), which was Rasl11b, a member of the small GTPase protein family. The majority (65% of 738 genes) of transcripts regulated by VIP after 2 h were up-regulated, and they also tended to show a greater fold change in abundance (Figure 6.1B).

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Number of detected probes</th>
<th>Significant results at $P &lt; 0.01$</th>
<th>Significant results at $P &lt; 0.05$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT12 Veh vs. CT10</td>
<td>16847</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>CT12 VIP vs. CT10</td>
<td>17658</td>
<td>318</td>
<td>497</td>
</tr>
<tr>
<td>CT12 VIP vs. CT12 Veh</td>
<td>17605</td>
<td>422</td>
<td>738</td>
</tr>
<tr>
<td>CT16 VIP vs. CT10</td>
<td>18763</td>
<td>269</td>
<td>569</td>
</tr>
<tr>
<td>CT16 VIP vs. CT12 Veh</td>
<td>18724</td>
<td>314</td>
<td>668</td>
</tr>
<tr>
<td>CT16 VIP vs. CT12 VIP</td>
<td>19110</td>
<td>148</td>
<td>342</td>
</tr>
</tbody>
</table>

*Table 6.1 Summary results for each pairwise comparison made in the microarray analysis*

Transcripts showing significant differences in the CT16 VIP vs. CT12 VIP comparison would comprise genes which continued to be up-regulated or down-regulated by VIP, those which had been attenuated after 2 h and were subsequently returning to baseline, and those which showed a delayed, possibly downstream, response to VIP. Genes which were unaffected by VIP
Figure 6.1 VIP-regulated transcription in the SCN

(A-C) Volcano plots displaying log fold change against –log adjusted p value for 3 comparisons: CT12 Veh vs. CT10 (A), CT12 VIP vs. CT12 Veh (B) and CT16 VIP vs. CT12 VIP (C). An adjusted p value cut off of $P < 0.05$ was used to determine significance. $n = 4$ slices per group. (D) Globally normalised probe expression levels across time and treatment (mean ± SEM) of genes with known involvement in the circadian TTFL. Significantly altered transcripts in CT12 VIP vs. CT12 veh comparison (Rora, Dec1, Dbp, Per2 and Per1) have coloured lines, the others black. (E) Hierarchically
but which oscillated over time could also have been found. Thus considerable manual interrogation of the dataset was necessary throughout all subsequent analysis. Overall, however, the proportion of up-regulated compared to down-regulated genes was fairly equal (46% and 54% respectively; Figure 6.1C), although many of those down-regulated genes did indeed incorporate immediate early genes that had been up-regulated at 2 h, such as Fosl2 and Egr1.

Up-regulated genes of potential interest across time points included Dusp1 and Dusp4, two members of the dual specificity phosphatase family previously shown to be induced by light (Doi et al., 2007; Jagannath et al., 2013), clock genes (such as Per1, Per2, Rora and Dec1; Figure 6.1D) and proteins involved in intercellular communication such as cocaine- and amphetamine-regulated transcript prepropeptide (Cartpt) and gap junction protein beta 2 (Gjb2, also known as connexin 26). Genes that were down-regulated by VIP included D site albumin promoter binding protein (Dbp), a protein known to be regulated by core TTFL genes (Yamaguchi et al., 2000; Figure 6.1D).

In order to view how transcript levels varied with time and treatment as well as to cluster genes that responded with similar dynamics, a heatmap of the 300 genes with the most significant P values (at the CT12 VIP vs. CT12 Veh comparison) was generated (Figure 6.1E) using Morpheus software (Broad

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**Figure 6.1 continued**

clustered heatmap of top 300 most significantly altered genes in CT12 VIP vs. CT12 veh comparison across time and treatment using globally normalised probe expression levels. Two regions are enlarged: the top displays genes acutely upregulated 2 h after VIP treatment before returning to baseline levels, the bottom shows genes which continue to be upregulated after 6 h. Within each gene row, transcript levels have been normalised to the minimum (green) and maximum (red) values for that given gene.
Institute) based on globally normalised values. In a reflection of the volcano plot (Figure 6.1A), very little difference could be seen between the CT10 and CT12 Veh groups, while samples within the CT12 VIP and CT16 VIP groups showed clear segregation. The majority of selected genes were up-regulated 2 h after VIP treatment, with clusters then forming based on either their decreased levels (including Per1 and Dusp4 which clustered closely), or their consistent or increased levels (including Cartpt) at CT16.

To validate the results of the microarray, 11 genes based on a number of criteria which will be further explored throughout this chapter (plus RNS18 as a control; Figure 6.2A) were chosen for qPCR analysis, using SCN samples independent of those used for the microarray. The same groups were utilised along with the inclusion of a CT16 Vehicle group to aid interpretation. Determination of strong or specific expression in the SCN was based on in situ hybridisation images from the Allen Mouse Brain Atlas (Lein et al., 2007). qPCR data reflected what was observed in the microarrays, and could again be separated into clusters based on kinetics of response: Dbp showed down-regulation in response to VIP, Per2, Cartpt and Vgf continued to increase at CT16, and the remainder increased acutely before subsequently declining, with some returning to baseline levels (Figure 6.2B). Based on this analysis, the microarray data were considered to be reliable and valid.

6.3.2 VIP regulates diverse cellular pathways

In order to gain an understanding of broad cellular processes that VIP was regulating in the SCN, gene ontology (GO) term analysis was performed with subsequent clustering for visualisation (Figure 6.3). The largest group of transcripts regulated after 2 h contained transcriptional regulators, including transcription factors and nuclear receptors (Figure 6.3A). This group was
Figure 6.2 qPCR validation of VIP microarray
(A) Flow chart of steps taken to arrive at genes of potential interest for qPCR validation and further testing (see Chapter 7). (B) Relative mRNA expression levels (mean ± SEM) over time and VIP treatment assessed by qPCR. All transcript levels except RNS18 are normalised to RNS18 quantity to control for total RNA variation. Two way ANOVA with Tukey’s multiple comparisons test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ ****$P < 0.0001$. 

The VIP-regulated transcriptome
closely connected to proteins involved in cellular transduction cascades relating to kinases, in particular MAPK pathways. The relevance of some unexpected clusters, including steroid synthesis, heparin binding and cytokine signalling remains to be clarified. The most significant clusters after 6 h included the down-regulation of previously up-regulated transcription factors and MAPK-related transcripts (Figure 6.3B).

Figure 6.3 Cluster pathway analysis of VIP-regulated genes
(A and B) Functional annotation of genes significantly altered by VIP treatment after 2 h (A; 738 genes) and after 6 h (B; 342 genes) using DAVID functional annotation and visualised using the enrichment map plugin for Cytoscape.
CREB binds to CREs in gene promoters in order to initiate transcription, and has a known role in light responses and phase shifting (Gau et al., 2002; Ginty et al., 1993; Lee et al., 2010a). Furthermore, the induction of cAMP- and PKA-dependent pathways as a result of VPAC2 activation (An et al., 2011; Kudo et al., 2013; Nielsen et al., 2002), which could subsequently result in CREB phosphorylation, suggested that VIP is also likely to work via CREB-dependent pathways. The transcripts in the current dataset were therefore compared to the dataset of Zhang et al. (2005) to identify the extent of CREB-mediated transcription of VIP-regulated genes (Figure 6.4). Chi-squared analysis revealed that there was a significant enrichment for genes up-regulated after 2 h and down-regulated after 6 h to have a CRE within 300 bp of the TATA box (CRE-TATA; Figure 6.4A). Subsequent cluster analysis showed that these genes were predominantly from the large group of transcriptional activators and MAPK-related transcripts (Figure 6.4B and C). VIP did not solely act through CRE regulation, however, with down-regulated genes at 2 h and those up-regulated at 6 h showing no enrichment for CREs (Figure 6.1A). Furthermore, genes that were regulated by VIP but did not contain CREs separated out into distinct clusters, for example cytokine-related transcripts showed no CRE regulation (Figure 6.4B and C). Whether these transcripts are directly regulated by VIP or instead by VIP-induced genes remains to be determined.

The proportion of genes regulated by VIP which oscillate in a circadian manner was next examined by comparing genes to the Circadian Expression Profiles Database (CircaDB; Pizarro et al., 2013). Circadian genes were significantly over-represented in up-regulated at 2 h and down-regulated at 6 h groups, similar to CRE-TATA genes (Figure 6.5A). Nevertheless, this was a relatively small proportion of all VIP-regulated transcripts (Figure 6.5B).
Figure 6.4 CRE occurrence in promoters of VIP-regulated transcripts

(A) Enrichment of CREs in the promoters of significantly regulated genes, displayed as observed/expected frequency, with expected values based on the appearance of promoter elements across all detected genes at CT12 VIP vs. CT12 Veh. CRE-TATA: CRE within 300 bp of TATA box; CRE-NoTATA: CRE in promoter further upstream; Others: No CRE present in promoter (promoter defined as 3 kb upstream to 300 bp downstream of the transcription start site). Acutely upregulated genes are significantly enriched for CRE-TATA elements showing the importance of CREB-dependent transcription in the VIP response. Chi-squared test, ****P < 0.0001. (B and C) Functional annotation of genes significantly altered by VIP treatment after 2 h (B) and after 6 h (C) segregated based on promoter elements.
Figure 6.5 Oscillatory natures of VIP-regulated transcripts

(A) Enrichment of oscillatory transcripts among genes significantly regulated by VIP, displayed as observed/expected frequency, with expected values based on the proportion of circadian genes across all detected genes at CT12 VIP vs. CT12 Veh. Acutely upregulated genes are significantly enriched for circadian transcripts, as are downregulated genes at CT16. Chi-squared test, *P < 0.05. (B) Proportion of genes that demonstrate a circadian oscillation within the whole SCN transcriptome (all detected genes at CT12 VIP vs. CT12 Veh) and transcripts significantly regulated by VIP at different time points.
6.4 Discussion

6.4.1 VIP directly influences TTFL transcriptional regulation

The protocol used here to assess VIP regulation of the SCN transcriptome was based on experiments presented in the previous chapter utilising organotypic slices containing luciferase reporters of core clock genes. Thus, the observation that both Per1 and Per2 were significantly up-regulated by VIP after 2 h when assessed by microarray is not surprising, particularly in light of extensive research demonstrating the effects of light pulses or VIP regulation on Per gene expression (Bedont et al., 2017; Dardente et al., 2002; Yan and Silver, 2004). Despite this, the effect on their transcription was relatively small: the fold changes of Per1 and Per2 were 1.29 and 1.22 respectively (in comparison to the genes with the largest inductions: Fosl2, Adams1 and Dusp1, which had a >4 fold induction), although the low fold inductions seen in the microarray analysis may partly represent differences in dynamic range to, for example, qPCR (Allanach et al., 2008; Etienne et al., 2004). The canonical clock gene with the greatest up-regulation was Bhlhb2, also known as Dec1, which increased by a factor of 1.63 after 2 h. The only other clock gene detected as being significantly regulated by VIP in this study was Rora, which was induced at a similar level to the Per genes.

The fact that VIP regulates several auxiliary TTFL genes is important because it suggests that the effect of VIP, and thus potentially of light, on the cell-autonomous oscillator is not merely through direct regulation of the Per genes, but also indirectly. Rora is a positive regulator of Bmal1 expression (Sato et al., 2004b), which in turn will activate transcription of Per and Cry genes. In contrast, Dec1 is seen as a negative regulator of CLOCK/BMAL1-depedent transcription of Per and Cry (Honma et al., 2002; Sato et al., 2004a), thus VIP appears to induce a limiting mechanism on clock gene expression as well as the Per genes themselves. This theme of activation of a pathway
followed by the induction of a suppressive mechanism is common in cellular transduction cascades and was observed several times in the current dataset. Moreover, the induction of \textit{Dec1} may be sufficient to explain the differences observed between \textit{Per1} and \textit{Per2} responses to VIP. Differential responses were observed using luciferase reporters (Chapter 5) and by qPCR in this chapter, and \textit{Dec1} has previously been shown to control the phase of \textit{Per1} through interactions with E boxes, but not with \textit{Per2} through the E’ box in its promoter (Nakashima et al., 2008).

These core TTFL genes were not the only circadian genes to be affected, as VIP-regulated transcripts were significantly enriched for oscillatory genes. Some of these may provide key nodes of integration for oscillatory signals (from the TTFL) and VIP signals (from the core) and therefore provide another mechanism by which stimuli can feed into the cellular oscillator.

\textbf{6.4.2 Genes induced by light and VIP}

There were a striking number of similarities between the current dataset and microarray profiles obtained following light pulses \textit{in vivo} (Jagannath et al., 2013; Morris et al., 1998; Porterfield et al., 2007; Zhu et al., 2012), some of which may be the result of genes induced by VIP \textit{in vivo} particularly early in the aforementioned studies, while others will represent common pathways to stimulus inputs. The most obvious similarities were the immediate early genes, including \textit{Fos, Fosl2, Egr1, Egr3, Klf4} and \textit{Nr4a2}, all of which were rapidly up-regulated at 2 h but had returned to baseline by 6 h. Many of these also contain CREs in their promoters, representing another commonality between VIP- and light-induced genes: both primarily function through phosphorylated CREB. Light and VIP also both induce negative regulators of this CREB-dependent pathway, primarily in the form of dual specificity
phosphatases, which dephosphorylate kinases of the MAP kinase pathway leading to their inactivation (and thus inability to phosphorylate CREB; Patterson et al., 2009). *Dusp1, Dusp4, Dusp10* and *Dusp14* were all significantly up-regulated after 2 h in this dataset, with *Dusp4* in particular having been repeatedly observed as light-induced (Jagannath et al., 2013; Zhu et al., 2012) and demonstrating strong expression in the SCN according to the Allen Mouse Brain Atlas. *Dusp4* will be discussed more in Chapter 7.

Another class of phosphatases of interest that was up-regulated by VIP are some members of the protein phosphatase 1 (PP1) family. PP1 is one of the major phosphatases in the cell, affecting diverse cellular processes through numerous substrates, but achieving specificity through an array of regulatory subunits (reviewed in Ceulemans and Bollen, 2004). Two of these subunits, *Ppp1r3c* and *Ppp2r1b* were up-regulated 2 h after VIP addition. Furthermore, PP1 is known to work in concert with CK1δ/ε to determine PER protein stability (Gallego et al., 2006; Lee et al., 2011), and has been found to be a negative regulator of light-induced phase shifting (Schmutz et al., 2011). Thus VIP-induced up-regulation of specific PP1 subunits may play a role in limiting the phase shifting of the VIP-receptive cells of the SCN to nocturnal light through actions on PER protein levels.

There were also many differences between the VIP- and light-regulated transcriptomes. Overall, for example, light has been noted as down-regulating many genes (Jagannath et al., 2013), while VIP was predominantly up-regulatory. A number of key light-induced regulators were also not observed, such as *Sik1* (Jagannath et al., 2013). Other strongly up-regulated transcripts in this dataset, such as *Adams1* and *Mat2a*, have not been seen in light-induced gene sets. *Adams1*, part of the ‘a disintegrin and metalloproteinase with thrombospondin motifs’ family, cleaves components of the extracellular matrix (Lemarchant et al., 2013), while *Mat2a* (methionine
adenosyltransferase 2a) is essential for generating S-adenosylmethionine (SAM). SAM is the key methyl donor within the cell and has been previously shown to oscillate in a circadian manner with regards to the oscillation of pineal melatonin biosynthesis (Kim et al., 2005), however the relevance of Mat2a or Adams1 up-regulation by VIP in the SCN is unclear.

In contrast to previously observed regulation of AVP by VIP (Bedont et al., 2017; Watanabe et al., 2000), no canonical SCN neuropeptides (e.g. AVP, GRP, Prok2) were observed as being regulated by VIP. There was, however, a strong up-regulation of the neuropeptides Vgf (non-acronymic) and Cartpt after 2 h, the levels of which continued to rise at 6 h. Galanin (Gal), a neuropeptide more closely associated with the ventrolateral preoptic nucleus (VLPO) and its role in sleep-promotion (Saper et al., 2005; although it has been found in the SCN previously (Hatcher et al., 2008)), was up-regulated after 6 h only. The observation that Vgf was up-regulated by VIP is consistent with a previous observation that it is light-induced, but with delayed kinetics (Wisor and Takahashi, 1997). Cartpt, a neuropeptide with an established role in appetite control (Kristensen et al., 1998), has been found in peptidomic studies of the SCN (Lee et al., 2010b, 2013), yet there is no existing work on its function with regards to circadian time-keeping. No receptors for VGF-derived peptides nor CART have currently been identified, thus it is unknown whether their up-regulation by VIP is likely to represent an output pathway (to the brain and periphery) or a mechanism of intercellular feedback to the SCN core.

6.4.3 Limitations of DNA microarrays

Despite providing large amounts of valuable information, there are some clear limitations of DNA microarrays. First and foremost, changes in RNA
levels do not necessarily correlate to changes in protein levels, as has been noted before in SCN proteomics studies (Deery et al., 2009), thus up-regulated transcripts will not necessarily reflect up-regulated proteins. The benefit of assessing transcripts, however, is that they are often considerably more sensitive than proteomic analyses, which tend to find more highly expressed proteins. RNA-seq is more sensitive than microarrays, however for differential gene expression studies (rather than studies wishing to identify novel genes or alternative splice forms), analysing microarrays is computationally less intensive than RNA-seq analysis.

The use of microarrays in circadian experiments can be difficult as it requires controls for both ‘time since treatment’ and phase of oscillation. Based on phase delaying experiments in Chapter 5, slices were harvested 2 h after treatment to identify genes that were acutely up-regulated, while others were harvested after 6 h (the new peak of PER2::LUC) in order to control for the 4 h phase differences between slices. Based on the lack of differentially regulated transcripts between CT10 and CT12 Veh, it appears that ‘time since treatment’ likely has a far greater impact on differential expression than phase does, particularly over relatively short timescales, with the effect of a strong VIP stimulus outweighing normal circadian oscillation. Therefore, for qPCR validation, an additional group of CT16 Veh was included to aid interpretation of CT16 VIP group data. Nonetheless, the CT16 VIP microarray group provided highly valuable information, such as in identification of IEGs, and providing insight into potential output signals from the VIP-receptive shell region.

Although it is predominantly the VPAC2 cells that are initially activated by VIP (along with a negligible activation of PAC1, based on experiments in Chapter 5), the time resolution utilised here means that other cell types, including glia, could be contributing to the genes regulated by VIP following
downstream activation by VPAC2 cells. In particular, it is unknown if the chemokine-related gene set seen here are neuronal or astrocytic/microglial in origin. VIP has been shown to shift the rhythms of astrocytes previously (Marpegan et al., 2009), and glia are generally more associated with inflammation signals than neurons. Chemokines have been found as neural messengers previously though (de Haas et al., 2007), and evidence suggesting that they may be neuronally derived can be found in their kinetics, as glial-derived chemokines tend to peak on a timescale of days, compared to neuron-derived chemokines which are detectable after hours (Che et al., 2001). The latter case was found in the present study. Nonetheless, the significance of chemokine up-regulation in SCN slices after VIP is not clear, but may be indicative of excitotoxicity, although this would be surprising as VIP is generally regarded as neuroprotective against inflammation (Brenneman, 2007).

6.4.4 Conclusions

The work presented here identifies previously unknown VIP-regulated genes in the SCN in the context of an acute pulse of VIP, expanding our knowledge of transcriptional networks involved in phase shifting. Similarly to light-pulses, VIP works predominantly via CRE promoter elements and induces many of the genes that light does, including many negative feedback mechanisms, suggesting common pathways in response to a strong stimulus. Key differences exist, however, including the induction of neuropeptides that have been little studied in a circadian context, but which may represent output pathways from the SCN, or feedback pathways from the SCN shell to the core.
CHAPTER 7

DOWNSTREAM MEDIATORS OF VIP SIGNALLING

7.1 Introduction

As demonstrated in the previous two chapters, VIP affects SCN oscillations by influencing both the cell-autonomous oscillator and intercellular communication. At the level of the cell, a clear theme of pathway activation followed by negative regulation of that pathway can be observed. At the circuit-level, the VIP-recipient cells must transmit information to non-receptive cells both within the SCN and further afield. The microarray analysis undertaken in Chapter 6 identified several interesting and unexpected candidates for further study to investigate the mechanisms through which VIP exerts its effects. Specifically, proteins that regulate phase shifting of cells, the SCN and the whole organism in response to light, as well as previously unidentified proteins that are involved in the steady state oscillation, would be of great interest to further understanding of the mammalian circadian system. The experiments presented in this chapter explore the potential roles of two candidates chosen for further study: dual specificity phosphatase 4 (DUSP4) and cocaine and amphetamine regulated transcript (CART).

DUSP4

DUSP4, encoded by the Dusp4 gene and also known as mitogen-activated protein kinase (MAPK) phosphatase 2 (MKP2), is a negative regulator of the MAPK pathways. These pathways are a collection of intracellular signal
Downstream mediators of VIP signalling

Transduction cascades, transmitting information in response to a variety of external signals (growth factors, peptides, cytokines etc.), and mediating a diverse array of cellular responses, primarily through phosphorylation of transcription factors such as cAMP response element-binding protein (CREB; Goldsmith and Bell-Pedersen, 2013; Johnson and Lapadat, 2002; Owens and Keyse, 2007). MAPK cascades are typically three-tiered, with the MAP kinase itself representing the last stage of the pathway; extracellular signal–regulated kinases 1 and 2 (ERK1/2) are examples of MAPKs. Situated upstream of the MAPKs are the MAPK kinases (MAPKKs), such as MAPK/ERK Kinase 1 (MEK1), which phosphorylate MAPKs to lead to their activation. They in turn are phosphorylated and activated by MAPKK kinases (MAPKKKs), such as RAF-1, which often respond to receptor activation. Aside from the ERK1/2 pathway (of which MEK1 and RAF-1 are components), the other two canonical MAPK pathways are the c-Jun amino-terminal kinase (JNK) and the p38 cascades.

MAPKs have previously been shown to play a role in the circadian system, specifically in response to light. ERK pathway inhibitors attenuate phosphorylation of ERK, resulting in a reduced phase shift in wheel running following a light pulse (Coogan and Piggins, 2003). Relay of light information is also mediated by MAPK signalling, with injection of GRP (mimicking GRP release in response to a light pulse) into the 3rd ventricle increasing phosphorylated ERK (p-ERK) levels (Antle et al., 2005). This appears to be required for behavioural shifts normally observed in response to GRP (Antle et al., 2005). Involvement of MAPKs in rhythmicity is less clear: ERK phosphorylation state oscillates throughout the day (Obrietan et al., 1998), and BMAL1 and CRY1/2 have been identified as targets of p-ERK-mediated phosphorylation (Sanada et al., 2002, 2004), however deficits in ERK
signalling tend not to affect free-running behaviour (Antoun et al., 2012; Butcher et al., 2002).

The regulated dephosphorylation of critical threonine and tyrosine residues in MAPKs by a family of proteins known as dual specificity phosphatases (DUSPs) is key in determining the magnitude and duration of the MAPK response (Owens and Keyse, 2007). DUSP4, a nuclear-localised and inducible DUSP, is capable of dephosphorylating all three major MAPKs (ERK1/2, JNK and p38; Owens and Keyse, 2007), and has been found to be involved in a diverse number of cellular processes and pathologies, including neuronal differentiation (Kim et al., 2015), development (Niwa et al., 2007), gluconeogenesis (Berasi et al., 2006), cancer (Chitale et al., 2009), oxidative stress (Teng et al., 2007) and infection (Al-Mutairi et al., 2010).

With regards to cellular timekeeping, microarray and RNA-seq studies have found DUSP4 to be significantly rhythmic in the SCN, with its peak in the middle of the circadian day (CircaDB: Pizarro et al., 2013; Zhu et al., 2012), and several studies have identified it as light-induced (Jagannath et al., 2013; Zhu et al., 2012), along with the related DUSP1 (Doi et al., 2007; Porterfield et al., 2007). Furthermore, it appears to be VIP-regulated based on RNA-seq analysis of VIP-null SCN (Bedont et al., 2017), and one study found it to be upregulated in response to a light pulse in both the core and shell of the SCN (Zhu et al., 2012), suggesting that DUSP4 may have a regulatory role in both the immediate response to light as well as transmission of light information from retinorecipient neurons to the dorsomedial SCN. The data presented in Chapter 6 support this, with DUSP4 showing an acute up-regulation in response to VIP. Despite these observations, no study has specifically investigated the role of DUSP4 in circadian timekeeping. Indeed, the only study of negative regulation of MAPK pathways in relation to the circadian system to date investigated the role of RAF kinase inhibitor protein (RKIP;
Antoun et al., 2012). RKIP inhibits RAF1 activation of MEK1, and thus ultimately prevents ERK1/2 phosphorylation. RKIP knockout mice display greater phase shifts to light pulses and faster entrainment to an advanced lighting schedule, while at the molecular level greater phosphorylation of ERK1/2 and an increased induction of PER1 are seen (Antoun et al., 2012).

Given the above observations, DUSP4 was chosen as a protein of interest for further investigation. Its known role in regulating MAPK pathways, rhythmic oscillation and response to light in both the core and shell highlighted it as a likely regulator of MAPK with regards to photic input and downstream signalling, while its rhythmic nature suggested it may be capable of serving as a link between light and the core cellular oscillator. The hypothesis is that DUSP4 acts to limit the role of MAPK in phase shifting, providing a level of resistance to perturbation for the SCN as has been suggested previously for other negative regulators (Jagannath et al., 2013). Therefore, increasing or decreasing levels of DUSP4 might be expected to reduce or potentiate phase shifts, respectively.

CART

While DUSP4 was chosen as a target for its clear potential to influence the cell-autonomous oscillator, VIP was also shown to affect intercellular communication. Furthermore, any alteration to SCN rhythmicity induced by light would need to be conveyed to other tissues. Thus VIP-regulated genes were interrogated for signalling molecules, from which the neuropeptide CART was identified. CART, encoded by the Cartpt gene, is translated as propeptides before being cleaved into the active CART 55-102 and CART 62-102 forms in mice (Thim et al., 1999). Humans and other species express a shorter variant of the transcript and thus produce CART 42-89 and CART 49-
Downstream mediators of VIP signalling

89 instead, containing identical amino acids to the murine versions (Rogge et al., 2008). CART peptides are classically associated with feeding and energy regulation, acting as a satiety factor in close association with two other well-known neurohormones, leptin and neuropeptide Y (Kristensen et al., 1998), as well as in reward circuits such as in response to psychostimulants, as the CART name implies (Kuhar et al., 2002). However, anatomical studies have identified CART peptides in widespread (albeit discrete) regions across the brain and periphery (Couceyro et al., 1997; Koylu et al., 1998), suggesting functions beyond just feeding and reward, and indeed CART has been implicated in an array of processes including stress, pain and neural development (Rogge et al., 2008).

To date, a role for CART has not been identified in the circadian system, nor has it been identified by immunohistochemistry or in situ hybridisation as present in the SCN (Couceyro et al., 1997; Koylu et al., 1998). It has, however, been identified as an SCN neuropeptide by more recent neuropeptidomics studies (Lee et al., 2010b, 2013), suggesting either that the SCN was overlooked in previous analyses, or that it is present in very low levels only detectable by more recent techniques. These peptidomics studies have not revealed a function for CART, however the observations presented in Chapter 6 that Cartpt is upregulated in response to VIP at both 2 h and 6 h time points suggest that it could play a role in intercellular communication within the SCN, or that it functions as an output signal from the SCN. Cartpt contains CRE elements with its promoter (Dominguez et al., 2002) and is activated by dephosphorylated CREB-regulated transcription coactivator 1 (CRTC1; Altarejos et al., 2008), a mediator of light input to the SCN (Jagannath et al., 2013). The additional observation that E-box elements are also present in the Cartpt promoter (Dominguez et al., 2002) means that transcriptional activation
by both phosphorylated CREB and the circadian TTFL are possible, resulting in CART being ideally situated to serve a role in circadian timekeeping.

The experiments presented here thus represent preliminary work on the roles of DUSP4 and CART peptides in circadian rhythmicity and phase shifting responses. CART is investigated by knocking down its expression through the use of short hairpin RNA (shRNA) in SCN slices, while DUSP4 is examined more extensively through the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 gene-editing system and constitutive expression ex vivo, and genomic knockout in vivo. These results suggest that CART does not have a role in intra-SCN communication, however DUSP4 is capable of affecting steady-state period as well as phase shifting dynamics.
7.2 Methods

7.2.1 Commercial viruses and plasmids

A number of viruses and plasmids were used in this chapter that were commercially available. These can be found in Table 7.1.

<table>
<thead>
<tr>
<th>Vector name</th>
<th>Identifier</th>
<th>Use</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV.Dusp4-myc</td>
<td>MR222119</td>
<td>Overexpression of DUSP4; cloning</td>
<td>Origene</td>
</tr>
<tr>
<td>CMV.Cartpt-myc</td>
<td>MR222081</td>
<td>Overexpression of CART</td>
<td>Origene</td>
</tr>
<tr>
<td>hSyn.GFP::Cre</td>
<td>N/A</td>
<td>Neuronal Cre expression, AAV</td>
<td>UNC Vector Core</td>
</tr>
<tr>
<td>Mecp2.SpCas9</td>
<td>#60957</td>
<td>Neuronal Cas9 expression, AAV</td>
<td>Addgene</td>
</tr>
</tbody>
</table>

Table 7.1 shRNA 22-mer sequences tested for efficacy of knock down against Cartpt

7.2.2 Cartpt shRNA cloning

Cloning into vectors for cell validation

To establish a protocol whereby genes of interest (in this case Cartpt) could be knocked down using shRNA (Paddison and Hannon, 2002; Paddison et al., 2002), a protocol based on the Hannon Laboratory protocol was utilised (found at http://hannonlab.cshl.edu/GH_protocols.html), which designs shRNA constructs based on the human miR30 microRNA (miR; Premsrirut et al., 2011). The final construct MSCV.P2Gm.shRNA contains the following components: a murine stem cell virus (MSCV) backbone, two long terminal repeats (LTR), with the 5’ LTR acting as a promoter, a puromycin resistance cassette, a GFP marker, and the miR30 context region for cloning into.

22 nucleotide stretches of DNA (22-mers) were designed using the Hannon lab shRNA design tool (http://hannonlab.cshl.edu/GH_research.html) to find 22-mers complementary to Cartpt mRNA. The resulting 22-mers were input
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into NCBI’s Basic Local Alignment Search Tool (BLAST) to identify any off-target matches; any which had more than 15/22 matching bases to a transcript other than \textit{Cartpt} was not utilised. The 4 chosen 22-mers can be found in Table 7.2.

<table>
<thead>
<tr>
<th>shRNA Construct</th>
<th>22-mer antisense sequence</th>
<th>Location in \textit{Cartpt} mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>sh1</td>
<td>TCCTCTTGAAGTGCTTGTGAAG</td>
<td>420-441</td>
</tr>
<tr>
<td>sh2</td>
<td>CCTGAAGAAGCTCAAGAGTAA</td>
<td>268-289</td>
</tr>
<tr>
<td>sh3</td>
<td>CTGCAATTCTTTCTCTTGAG</td>
<td>409-430</td>
</tr>
<tr>
<td>sh4</td>
<td>GCCTGGCTTTAGCAACATAA</td>
<td>512-533</td>
</tr>
</tbody>
</table>

\textbf{Table 7.2} shRNA 22-mer sequences tested for efficacy of knock down against \textit{Cartpt}

Next, 97-mer oligonucleotides were synthesised (by Sigma) which contained the anti-sense and sense 22-mers for a given shRNA. These were linked by a 19-mer loop, alongside common 5’ and 3’ ends to serve as a PCR template, allowing amplification of any shRNA design using common primers: 5’-cagaaggtgagaaggtatat\textbf{TGCTGTTGACAGTGAGCG}-3’ and 5’-\textbf{tgaattCCGAGGCAGTAGGCA}-3’, where the red, uppercase bases represent complementary regions to the 97-mer, while the black, lowercase bases contain XhoI and EcoRi restriction sites respectively. 97-mers were PCR-amplified with these primers to produce 138-mers, which were subsequently digested with XhoI and EcoRI. This was ligated into the \textit{MSCV P2Gm +SwaI} vector (Addgene #22699), which had been similarly digested, to produce e.g. \textit{MSCV.P2Gm.Cartptsh2}. Successful ligations were confirmed by sequencing. sh2 was validated as successfully knocking down Cartpt expression, thus a scramble control plasmid was made using the 22-mer 5’-\textbf{GCCTGGCTTTAGCAACATAA}-3’.
Cloning into AAV vectors for SCN slice experiments

Following the validation of the sh2 construct, it was then cloned into an AAV backbone and, following re-validation, packaged into AAV particles for use in slices in a Cre-dependent manner. It was therefore cloned into \textit{pAAV-EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA} (Addgene #20297), details of which can be found in section 3.2.2 of Chapter 3. Restriction cloning was used to insert a TdTomato reporter into this plasmid, and subsequently place the miR30 context containing the sh2 sequence downstream of the TdTomato gene.

hChR2-mCherry was excised from \textit{pAAV-EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA} using Ascl and NheI. TdTomato was amplified by PCR from \textit{pAAV.CAG.hChR2-tdTomato}, kindly donated by Ernesto Ciabatti, using the forward primer 5\'-TAAGCAGCTAGCatggtgagcaagggcgaggag-3\' and the reverse primer 5\'-TTACGCGGCGCGCCgaattcgaatcgactagtcgc-3\', which contain restriction sites for NheI and Ascl respectively. After restriction digest with the appropriate enzymes, TdTomato was ligated into the digested vector to produce \textit{Ef1a.DiO.TdTom}. Next, the shRNA sequence within the miR30 context from the MSCV vector was amplified using the forward primer 5\'-TAAGCAACTAGTGCATTTAAATGGCCGCAAGCCT-3\' and the reverse primer 5\'-TTACGCGGCGCGCCgaattcgaatcgactagtcgc-3\', which incorporated restriction sites for SpeI and Ascl respectively. Digestion of the PCR product and of \textit{Ef1a.DiO.TdTom} with SpeI and Ascl allowed their ligation, producing \textit{Ef1a.DiO.TdTom-sh2} (or \textit{Ef1a.DiO.TdTom-Scram}).

7.2.3 \textit{Dusp4} CRISPR cloning

The CRISPR-Cas9 system (Hsu et al., 2014) was used to knock out \textit{Dusp4} in SCN slices. Guide RNA (gRNA) sequences were cloned into \textit{pAAV-
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U6sgRNA(SapI)_hSyn-GFP-KASH-bGH (Addgene #60958; Swiech et al., 2014), which contains a gRNA and gRNA scaffold sequence under the control of the human U6 promoter (an RNA polymerase III promoter), as well as a GFP reporter localised to the cytoplasm by a Klarsicht, ANC-1, Syne Homology (KASH) domain under the human synapsin promoter. gRNA sequences were designed using either the gRNA Design Tool by Atum (https://www.atum.bio/products/crispr#4) or the MIT CRISPR design tool (http://crispr.mit.edu/), the results of which can be seen in Table 7.3.

<table>
<thead>
<tr>
<th>CRISPR Construct</th>
<th>20 nt Target</th>
<th>Associated PAM</th>
<th>Location in Dusp4 gene</th>
<th>Design tool</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dusp4 g1</td>
<td>GCTGCAATACCATCGTGCGG</td>
<td>CGG</td>
<td>334-353</td>
<td>Atum</td>
</tr>
<tr>
<td>Dusp4 g2</td>
<td>GCGCCCTCTTAGCCCTCGC</td>
<td>TGG</td>
<td>15-34</td>
<td>MIT</td>
</tr>
<tr>
<td>Dusp4 g3</td>
<td>ACTGCGTTTGGCCGCACAA</td>
<td>TGG</td>
<td>101-120</td>
<td>MIT</td>
</tr>
<tr>
<td>Dusp4 g4</td>
<td>CTCCATCGTCAACCATGTCG</td>
<td>CGG</td>
<td>115-134</td>
<td>MIT</td>
</tr>
<tr>
<td>Dusp4 g5</td>
<td>GCGGCGGCGGCAGCGCGGG</td>
<td>CGG</td>
<td>192-211</td>
<td>MIT</td>
</tr>
<tr>
<td>Dusp4 g6</td>
<td>AACAAGAAGGAACCAGGC</td>
<td>GGG</td>
<td>30-49</td>
<td>MIT</td>
</tr>
</tbody>
</table>

Table 7.3 CRISPR gRNA sequences tested for efficacy against Dusp4

The sequences in Table 7.3, along with complementary 20 nt oligomers, were synthesised (by Sigma) and annealed together. Annealing was carried out by mixing 2 µg of each oligomer (dissolved in water at 100 µM) together with its counterpart, then making the final solution up to 50 µl with annealing buffer (10 mM EDTA, 10 mM Tris (pH 8) and 50 mM NaCl). This was heated at 95°C for 5 minutes and then allowed to cool to room temperature. The oligomers were designed such that there would be a 3 nt overhang at each end (ACC and AAC at the 5’ and 3’ ends respectively) so that, following annealing, the
double stranded product could be ligated into \textit{pAAV-U6sgRNA(SapI)_hSyn-GFP-KASH-bGH} digested with SapI to generate final constructs such as \textit{U6.Dusp4g1.hSyn.GFP-KASH} for validation in cells and subsequent packaging into AAVs. Slices were co-transduced with \textit{Mecp2.SpCas9}. This two-plasmid system was used due to packaging size constraints in AAVs.

\subsection*{7.2.3 DUSP4 overexpression cloning}

Conditional DUSP4 overexpression was achieved by cloning \textit{Dusp4} cDNA, alongside an mCherry tag, into \textit{pAAV-EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA} (Addgene #20297), details of which can be found in section 3.2.2 of Chapter 3. mCherry-P2A was amplified from \textit{Ef1a.DiO.mCherry-P2A-DtR} (see Chapter 3) using the forward primer 5’-TATGGTACCTTCATAGGGCCGGGATTCTCC-3’ and the reverse primer 5’-TATGTCGACCATGGTGAGCAAGGGCGAG-3’, containing KpnI and SalI restriction sites respectively. The product was digested with KpnI and SalI and ligated into a similarly digested \textit{CMV.Dusp4-myc} vector (Origene MR222119). mCherry-P2A-Dusp4 was amplified from the resulting vector using the forward primer 5’-TATGCTAGCCGTCGACCATGGTGAGCAA-3’ and reverse primer 5’-TATGGCGCGCCTACAGCTGGGGGAGGTGG-3’, which incorporated NheI and Ascl restriction sites respectively. After digestion with NheI and Ascl, the product was ligated into \textit{EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA}, which had been similarly digested, to produce \textit{Ef1a.DiO.mCherry-P2A-Dusp4}. This was validated in cells and subsequently packaged into AAV particles.
7.2.4 Mouse behaviour analysis

Onsets and offsets of behaviour were determined using the in-built onsets and offsets functions in Clocklab (ActiMetrix Inc.), with manual adjustment if necessary. Phase angles of entrainment were calculated based on these onsets in Microsoft Excel. 50% phase shift values (PS50s) were calculated by fitting a sigmoidal curve to onset (phase advance) or offset (phase delay) values using the interpolate function in Prism.
7.3 Results

7.3.1 Knockdown of *Cartpt* has no effect on SCN slice rhythmicity or VIP response

To investigate the role of *Cartpt* in SCN rhythmicity, knockdown constructs using shRNA were produced and first validated in cell culture to determine knockdown efficacy. *MSCV.P2Gm.Cartptsh1-4* plasmids (Figure 7.1A) were used for expressing shRNA constructs within a miR30 context. PurR, puromycin resistance; LTR, long terminal repeat. **(B)** Western blot using an anti-myc antibody for myc-tagged CART following transfection. HEK293 cells were co-transfected with CMV.*Cartpt-myc* and an shRNA construct designed against *Cartpt* (or GFP as a negative control; shGFP), treated with puromycin after 24 h, and harvested after 72 h. Actin serves as a loading control. No CART bands can be seen when transfected with sh2. n = 3 wells per condition. **(C)** Western blot using an anti-myc antibody for myc-tagged CART following transfection to compare sh2 with a scramble construct; same protocol as **(B)**. **(D)** General plasmid map of *Ef1a.DiO.TdTom-shRNA*; shRNA and miR30 context from **(A)** were transferred to a flexed AAV backbone with a TdTomato reporter. Triangles represent loxP sites. ITR, inverted terminal repeat. **(E)** Western blot using an anti-myc antibody for myc-tagged CART following transfection. HEK293 cells were co-transfected with a combination of CMV.*Cartpt-myc*, Cre::GFP, and an shRNA construct. sh2 successfully knocked down CART expression only in the presence of Cre recombinase. The nature of the smaller bands seen is not clear, but seems to be a result of the Cre::GFP plasmid or AAV backbone. n = 2 wells per condition and this result was duplicated in a separate experiment.

![Diagram of downstream mediators of VIP signalling](image)
transfected into HEK293 cells alongside CMV.Cartpt-myc, treated with puromycin 24 h later (to kill any cells not expressing an shRNA plasmid) and harvested 48 h after puromycin treatment to be assessed by western blot. The negative control plasmid, MSCV.P2Gm.shGFP, contained an shRNA sequence against GFP, and was kindly donated by Li Jin. No bands were visible using MSCV.P2Gm.Cartptsh2, and MSCV.P2Gm.Cartptsh3 also showed considerable knockdown (Figure 7.1B; MSCV.P2Gm.Cartptsh1 not shown). Thus, MSCV.P2Gm.Cartptsh2 was chosen for further experimentation, and was compared to a scrambled version (MSCV.P2Gm.Cartptsh2scram), which showed no knockdown (Figure 7.1C). Following transfer of the shRNA and miR30 context into an AAV backbone (Figure 7.1D), the constructs were re-verified to establish that backbone context was not important for knockdown efficacy, as well as to ensure Cre-dependence by co-transfection with a nuclear-localised Cre::GFP plasmid (kind gift from Ernesto Ciabatti). Ef1a.DiO.TdTom-sh2 showed a 100% knockdown of Cartpt in the presence of Cre as assessed by western blot, while the scramble had no visible effect (Figure 7.1E). Thus, Ef1a.DiO.TdTom-sh2 and the scramble counterpart were packaged into AAV particles for SCN slice transduction.

SCN slices were successfully transduced with Ef1a.DiO.TdTom-sh2 and Ef1a.DiO.TdTom-Scram, showing strong TdTomato expression in the presence of Cre (Figure 7.2A), however there was no visible effect on slice rhythmicity (Figure 7.2B). Steady state period was not significantly different (Figure 7.2C), and period change, phase shift, PER2::LUC induction and amplitude change following VIP application were similarly unaffected by Cartpt knockdown (Figure 7.2D-G). Therefore, despite being induced by VIP, Cartpt does not appear to play a critical role in SCN rhythmicity nor the response to VIP as measured here.
Figure 7.2 Knockdown of Cartpt has no effect in SCN slices

(A) Representative images of SCN slices transduced with hSyn.GFP::Cre followed one week later by either Effa.Dio.TdTom-sh2 or Effa.Dio.TdTom-scram, showing nuclear-localised GFP and flexed TdTomato expression. (B) Representative PER2::LUC bioluminescence rhythms of slices transduced as in (A) two weeks prior to the start of recording, and treated with 1 µM VIP (marked by +). VIP was bath applied at CT10 of the 6th cycle. Bioluminescence has been normalised to the first peak. (C) Steady state period of slices transduced as in (A). No significant differences were observed; n = 4 (scramble) or 5 (sh2). (D-G) Post-VIP period change (D), phase shift (E), PER2::LUC fold induction (F) and relative amplitude (G) responses (mean ± SEM) of slices transduced as in (A) and treated with VIP at CT10. No significant differences were observed for any parameter; n = 3 per group. All tests unpaired t-tests apart from phase shift, which was two-way ANOVA with Sidak’s multiple comparisons test.
7.3.2 CRISPR-mediated knockout of DUSP4 shortens SCN slice period and potentiates phase shifting

To knock out Dusp4 in SCN slices, 6 gRNA sequences were designed to target the Dusp4 gene and were separately cloned into an AAV vector backbone under the control of the U6 promoter (Figure 7.3A). They were then evaluated in N2A cells for their effectiveness at reducing DUSP4 expression when co-transfected with a Cas9 plasmid (Figure 7.3A). gRNA g1 showed a complete knockout against DUSP4 when assessed by western blot, g5 showed a partial knockout, and the remaining 4 gRNA sequences showed no visible effects (Figure 7.3B). Thus U6.Dusp4g1.hSyn.GFP-KASH was packaged into AAV particles for transduction of SCN slices.

Figure 7.3 Validation of Dusp4 CRISPR constructs
(A) General plasmid maps of U6.Dusp4gRNA.hSyn.GFP-KASH, e.g. U6.Dusp4g1.hSyn.GFP-KASH, and Mecp2.SpCas9. The gRNA is expressed along with a gRNA scaffold to direct Cas9-mediated cleavage. ITR, inverted terminal repeat; hSyn, human synapsin promoter; KASH, Klarsicht, Anc-1, Syne Homology domain; hGHpA, human growth hormone polyadenylation signal; Mecp2, methyl CpG binding protein 2 promoter; HA, hemagglutinin tag; pA, polyadenylation signal. (B) Western blot using an anti-myc antibody for myc-tagged DUSP4 following transfection. N2A cells were transfected with CMV.Dusp4-myc, Mecp2.SpCas9 and a gRNA construct designed against Dusp4 (or the original gRNA ‘acceptor’ plasmid template, Acc), and harvested 72 h later. Actin serves as a loading control. No DUSP4 bands can be seen when cells are transfected by g1. n = 2 wells per condition.
Mecp2.SpCas9, which should express Cas9 only in neurons through the methyl CpG binding protein 2 (MECP2) promoter, was transduced into SCN slices either in isolation (Cas9 slices) or followed one week later by U6.Dusp4g1.hSyn.GFP-KASH transduction (Dusp4CRISPR slices) to determine the effects of Dusp4 knockout on SCN slice rhythmicity and VIP responsiveness (Figure 7.4A and B). Dusp4 mRNA expression was almost completely abolished in Dusp4CRISPR slices when assessed by qPCR, but no significant difference was observed in total RNA expression based on RNS18 (Figure 7.4C and D). Unexpectedly, Dusp4CRISPR slices had a significantly shorter steady-state period (24.36 ± 0.11 h) compared to Cas9 slices (24.79 ± 0.06 h). No significant differences were observed for post-VIP period, period change, PER2 induction or amplitude, but Dusp4CRISPR slices were phase delayed by approximately 30 minutes more in response to VIP (Figure 7.4F-J).

7.3.3 Constitutive expression of DUSP4 shortens period and affects the VIP response

The role of Dusp4 was next investigated by Cre-dependent constitutive expression of DUSP4. Efla.DiO.mCherry-P2A-Dusp4 (Figure 7.5A) was validated in N2A cells to determine Cre-dependence and presence of DUSP4. No DUSP4 expression was seen in the absence of Cre assessed by western blot and immunocytochemistry, and fluorescence microscopy revealed predominantly nuclear localisation as expected (Figure 7.5B and C). Thus Efla.DiO.mCherry-P2A-Dusp4 was packaged into AAVs.

SCN slices were transduced with hSyn.GFP::Cre either in isolation (hSyn-Cre slices) or followed one week later by Efla.DiO.mCherry-P2A-Dusp4 (DUSP4Con slices; Figure 7.6A and B). Like the Dusp4CRISPR slices, DUSP4Con slices also had a shortened steady-state period (24.45 ± 0.04 h) compared to hSyn-Cre slices.
Figure 7.4 *Dusp4* knockout in SCN slices shortens period and potentiates phase shifts

(A) Representative images of SCN slices transduced with *Mecp2.SpCas9* followed one week later by *U6.Dusp4g1.hSyn.GFP-KASH*. Cas9 is shown using an anti-HA antibody, while GFP-KASH shows the expected cytoplasmic localisation. (B) Representative PER2::LUC bioluminescence rhythms of slices transduced with *Mecp2.SpCas9* in isolation or followed one week later by *U6.Dusp4g1.hSyn.GFP-KASH*, two weeks prior to the start of recording, and treated with 1 µM VIP (marked by +). VIP was bath applied at CT10 of the 8th cycle. Bioluminescence has been normalised to the first peak. (C) Relative RNS18 rRNA expression (mean ± SEM) assessed by qPCR, based on a standard curve (highest standard = 100). (D) Relative *Dusp4* mRNA expression (mean ± SEM) in SCN slices following transduction assessed by qPCR, normalised to RNS18 levels to account for total RNA differences. §: When transduced with both Cas9 and g1, no *Dusp4* expression could be detected in 2/3 samples, while the 3rd had very low levels. (legend continued on next page)
(25.04 ± 0.05 h; Figure 7.6C). Following VIP treatment, the post-VIP period was also shorter, which was due both to a shorter starting period as well as a reduced period change (Figure 7.6D and E). The amplitude decreased significantly less in response to VIP in \textit{Dusp4}^{+/+} slices than in \textit{hSyn-Cre} slices, and the oscillation was also more robust, as demonstrated by the post-VIP RAE (Figure 7.6F-H). There was no significant difference in phase shifting or \textit{PER2::LUC} fold change (Figure 7.6I and J).

\subsection*{7.3.4 Genomic DUSP4 knockout results in altered phase shifting dynamics \textit{in vivo}}

The role of \textit{Dusp4} was also investigated \textit{in vivo}, using a DUSP4\textsuperscript{−/−} mouse, which contains a targeted insertion of a lacZ reporter into the \textit{Dusp4} gene, creating a functional knockout (see Chapter 2.2.2). This mouse was first used to characterise where DUSP4 expression was found in the mouse brain using the lacZ reporter and an X-gal staining protocol (Figure 7.7). Positive staining could be seen in the SCN (Figure 7.7A and B), while strong staining was also observed in the hippocampus (particularly in the dentate gyrus) (Figure 7.7C). Finally, some positive cells were also found in the cerebral cortex (Figure 7.7D) as well as the piriform cortex (Figure 7.7E).

\textbf{Figure 7.4 continued}

expression: 0.1). Slices were harvested at CT6 (peak of \textit{Dusp4} mRNA expression). (E) Steady state (pre-treatment) period (mean ± SEM) of slices transduced as in (B). Cas9 + 1 slices had a significantly shorter period than slices transduced with Cas9 alone. n = 9 per group. (F-J) Post-treatment period (F), period change (G), immediate phase shift (H), \textit{PER2::LUC} fold induction (I) and relative amplitude (J) responses (mean ± SEM) of slices transduced as in (B) and treated with vehicle or VIP at CT10. Within treatment, Cas9 + 1 slices phase shifted significantly more than Cas9 slices. No significant differences were observed within treatment for other parameters. n = 4 for all vehicle-treated groups, n = 5 for all VIP-treated groups. Unpaired t-tests were used for (C-E), two-way ANOVAs with Sidak’s multiple comparisons were used for (F-J). *P < 0.05, **P < 0.01.
The wheel running behaviour of DUSP4+/+, DUSP4-/-, and DUSP4-/- mice was next examined. All three genotypes appeared to have normal, robust wheel running rhythms in both LD and DD (Figure 7.8A). No significant differences in period were observed (Figure 7.8B) but DUSP4-/- mice did have a significantly shorter phase angle of entrainment of approximately 18 minutes (Figure 7.8C), while alpha (length of active phase) was unaffected (Figure 7.8D).
Figure 7.6 Constitutive expression of DUSP4 in SCN slices shortens period and influences the VIP response

(A) Representative images of SCN slices transduced with hSyn.GFP::Cre followed one week later by Eif1a.Dio.mCherry-P2A-Dusp4. (B) Representative PER2::LUC bioluminescence rhythms of slices transduced with hSyn.GFP::Cre in isolation or followed one week later by Eif1a.Dio.mCherry-P2A-Dusp4, two weeks prior to the start of recording, and treated with 1 µM VIP (marked by +). VIP was bath applied at CT10 of the 7th cycle. Bioluminescence has been normalised to the first peak. (C) Steady state (pre-treatment) period (mean ± SEM) of slices transduced as in (B). Syn-Cre + DUSP4 slices had a significantly shorter period than slices transduced with Syn-Cre alone. Unpaired t-test, n = 5 per group. (D-J) Post-treatment period (D), period change (E), relative amplitude (F),
Following assessment of free-running behaviour (Figure 7.8), mice were re-entrained to a 12:12 LD cycle before subjecting them to 8 h phase advances and delays (Figure 7.9A) to determine the phase shifting dynamics of DUSP4+/− mice. Interestingly, differential responses were observed depending on the nature of the phase shift (Figure 7.9B-E). DUSP4+/− mice shifted significantly faster in response to a phase advance, with significant differences in onset time in the first two days following the advance (Figure 7.9B), although this was not significant when assessed using a 50% phase shift value (PS50; Figure 7.9D). In contrast, DUSP4+/− mice shifted significantly more slowly following a phase delay (Figure 7.9C and E), as evidenced by the time to execute 50% of the final shift.

**Figure 7.7 DUSP4 lacZ reporter expression across the brain**
(A-E) Representative images of X-gal stained sections from the DUSP4+/− mouse, containing a LacZ reporter gene inserted into the Dusp4 locus, in the SCN (A, close up in (B)), hippocampus (C), cerebral cortex (D) and piriform cortex (E) using a 10x (A), 20x (B) or 4x (C-E) objective. Dotted lines in (A) delineate the SCN and 3rd ventricle. Scale bars represent 100 µm (A and B) or 250 µm (C-E).
Figure 7.8 Global DUSP4 knockout has no effect on free-running wheel running behaviour

(A) Representative double-plotted actograms of wheel running behaviour of DUSP4+/+, DUSP4+/- and DUSP4-/- mice exposed to a 12:12 light:dark (LD) cycle followed by continuous dim red light (DD) conditions. Grey shading represents lights off. (B-D) Period in LD and DD (B), phase angle of entrainment (C) and free-running alpha (D) of DUSP4+/+ (n = 6), DUSP4+/- (n = 6) and DUSP4-/- (n = 5) mice (mean ± SEM). Phase angle was calculated as the difference between lights off during LD and the onset of activity (negative values mean activity began after lights off). DUSP4-/- mice had a significantly shorter phase angle than WT mice. Alpha (active phase duration) was calculated as the length of time between activity onset and offset in the first 5 days of DD. (B) used two-way ANOVA with Tukey’s multiple comparisons test, (C and D) used one-way ANOVA with Dunnett’s multiple comparisons test. *P < 0.05.
Figure 7.9 Global DUSP4 knockout modifies behavioural phase shifts
(A) Representative double-plotted actograms of wheel running behaviour of DUSP4\(^{+/+}\), DUSP4\(^{+/−}\) and DUSP4\(^{−/−}\) mice exposed to an 8 h phase advance and an 8 h phase delay in light:dark (LD) cycle. Grey shading represents lights off. (B and C) Activity onsets during the phase advance (B) and activity offsets during the phase delay (C) of DUSP4\(^{+/+}\) (n = 6), DUSP4\(^{+/−}\) (n = 6) and DUSP4\(^{−/−}\) (n = 5) mice (mean ± SEM). DUSP4\(^{−/−}\) mice shifted significantly faster in the phase advance but significantly slower in the phase delay, two-way ANOVA with Dunnett’s multiple comparisons test. (D and E) 50% phase shift values (PS50; mean ± SEM) of phase advances (D) and phase delays (E), one-way ANOVA with Dunnett’s multiple comparisons test. **P < 0.01, ***P < 0.001, ****P < 0.0001.
7.4 Discussion

7.4.1 CART does not have a role in SCN rhythmicity

Knockdown of Cartpt mRNA through virally transduced shRNA did not appear to have any effect on SCN rhythmicity nor on phase delaying in response to VIP when compared to a scramble knockdown. Clearly this is very preliminary work: vehicle controls were not presented due to currently insufficient n numbers of 1 and 2 slices for scramble and sh2 knockdowns respectively, and verification of Cartpt knockdown in SCN slices has not yet been performed due to time constraints. Nonetheless, the work presented here does not suggest an important role for CART-mediated signalling in intra-SCN communication.

In contrast, a role for CART signalling as an SCN output has not been ruled out, as this cannot be investigated using SCN slices. Indeed, many similarities between CART peptides and the SCN output signal prokineticin 2 (Prok2) exist: both are rhythmically expressed (Cheng et al., 2002; Vicentic et al., 2005), both respond to phase shifting paradigms (Cheng et al., 2002; own observations), both contain CRE and E-box elements within their promoters (Cheng et al., 2002; Dominguez et al., 2002), and yet both appear to be dispensable for SCN rhythmicity (Prosser et al., 2007; own observations). In mice lacking Prok2 or the Prok2 receptor (Prokr2), however, co-ordination of physiology and behaviour by the SCN are disrupted (Li et al., 2006; Prosser et al., 2007). Thus a role for CART in SCN communication with the periphery remains a possibility.

7.4.2 Future directions for the role of CART in the circadian system

As well as the aforementioned controls and verification needed to confirm the validity of current experimental results, further characterisation of CART
with respect to the circadian system is required. Fluorescent in situ hybridisation (FISH) for Cartpt following a light pulse would confirm physiological relevance as well as identify localisation of the neurons that express it. Given that Cartpt has not been noted in any transcriptome profiling studies following light pulses, it is likely that downstream neurons, such as those receptive to VIP or GRP, are the primary sites of Cartpt expression. Sufficiently sensitive FISH could also characterise a rhythm of Cartpt in the SCN, as only the hypothalamus as a whole (among several other brain regions) has been characterised thus far, in diurnal conditions, and with minimal temporal resolution (Vicentic et al., 2005).

Confirmation of the inability of CART to influence SCN rhythmicity would also be useful. Although shRNA experiments seem to suggest that it is not necessary, simple bath application of CART peptides to SCN slices may reveal that it is sufficient to induce changes. Unfortunately, a CART receptor has not yet been identified (Zhang et al., 2012), making it difficult to discern CART-receptive neurons both in the SCN and beyond. Ultimately, to test the hypothesis that CART serves as an SCN output signal, an in vivo investigation would be required, such as through stereotaxic injection of shRNA-expressing AAVs into the SCN.

7.4.3 Mechanism of DUSP4 influencing circadian rhythmicity

The role of the MAPK pathway in VIP-induced phase shifting is not necessarily an obvious one. VPAC2 is a GPCR traditionally coupled to the GαS subunit, which is linked to adenylate cyclase (AC) to stimulate cAMP production. However, it has also been shown to be able to work through Gαi and Gαq subunits, stimulating the activity of phospholipase C (PLC; MacKenzie et al., 2001) and increasing Ca²⁺ levels (Dickson et al., 2006).
Protein kinase C (PKC) is classically activated by this pathway (via increased Ca$^{2+}$ and diacylglycerol (DAG) as a result of PLC activation), and can subsequently phosphorylate RAF-1 in the MAPK pathway to initiate the cascade. CREB sits downstream of ERK1/2, thus this would appear to be the most direct pathway for MAPK to influence phase shifting, although clearly cAMP and PLC signalling both influence a large array of signalling cascades in the cell. Regardless of mechanism, MAPK activation is presumably occurring in order to require DUSP4-mediated negative regulation. Indeed, both DUSP4 overexpression and CRISPR-mediated knockout in SCN slices had significant effects on the VIP response: overexpression attenuated period, amplitude and RAE, while knockout potentiated phase shifting. The seemingly weaker phenotype of $Dusp^\text{CRISPR}$ slices compared to $Dusp^\text{Con}$ may be explained by some level of redundancy between DUSP4 and the related DUSP1, which is also involved in light-responsiveness (Doi et al., 2007; Porterfield et al., 2007).

Unexpectedly, both $Dusp^\text{CRISPR}$ and $Dusp^\text{Con}$ slices demonstrated a shorter period of approximately 30 minutes. This was not anticipated for two reasons: one is that ERK1/2 signalling has previously been shown not to have a large impact on steady-state rhythmicity (Antoun et al., 2012; Butcher et al., 2002), and the second is that manipulating DUSP4 levels in opposite directions had the same effect. With regards to the first point, it is possible that the period effect of DUSP4 was not through regulation of ERK1/2, but of other MAPK cascades such as JNK. JNK cascades have been shown to influence the stability of core clock proteins PER2 and BMAL1 (Uchida et al., 2012; Yoshitane et al., 2012), providing a potential link for DUSP4 to influence the circadian TTFL. In both of those studies referenced, however, JNK inhibition (as might be the expected result of DUSP4 overexpression) and deficiency resulted in a longer, rather than shorter, period. Thus the exact aetiology of...
the observed shortened period remains unclear, although the common feature of the two manipulations is that both should result in constant, rather than rhythmic, levels of MAPK activation. It is possible therefore that it is the rhythmicity, rather than the absolute levels of phosphorylated MAPK protein per se, that is affecting the cell-autonomous oscillator.

In vivo, the role for DUSP4 is complicated further. In contrast to the CRISPR-mediated Dusp4 knockout, DUSP4−/− mice did not display a shorter period. Furthermore, while they did show faster re-entrainment to a phase advanced lighting schedule, they were slower to entrain to an 8 h delay. The lack of a short period may be explained either by a compensatory mechanism of DUSP1, given that the genomic manipulation was present through development, or by greater variability between mouse periodicities than slices, resulting in a masking of the relatively minor period change. The differential response observed between phase advances and phase delays in the jetlag paradigm was also unexpected. It has been proposed that PER1 and PER2 have different roles in phase advances and delays (Albrecht et al., 2001; Yan and Silver, 2002), and therefore it is feasible that DUSP4 feeding into the TTFL, either via ERK1/2 or JNK, could also have differential responses. Nevertheless, the mechanism for, in particular, the slower phase delaying is not clear insofar as absence of DUSP4 would be expected to allow enhanced activation of the MAPK pathway.

7.4.4 Future directions for the role of DUSP4 in the circadian system

Molecular characterisation of the effects of manipulating DUSP4 is paramount to understanding the mechanism and contribution of MAPK pathways to phase shifting. This includes western blots for phosphorylated ERK and JNK following overexpression or knockout to help identify the most relevant
pathway, and to determine if, for example, a rhythm in ERK1/2 phosphorylation (Obrietan et al., 1998) still persists. Downstream effectors should also be investigated, such as the transcription factor Elk-1, which has been shown to be phosphorylated by ERK1/2 and may play a role in phase shifting (Coogan and Piggins, 2003). In one study, greater activation of ERK through DUPS4 knockdown resulted in reduced expression of voltage-gated calcium channel Cav1.2, subsequently reducing intracellular Ca\(^{2+}\) and downstream Ca\(^{2+}\)/calmodulin-dependent kinase (CaMK) activity (Kim et al., 2015). Given the role of cytosolic Ca\(^{2+}\) in the cell autonomous oscillator (Brancaccio et al., 2013), this is a feasible mechanism through which DUSP4 manipulation could affect the TTFL.

The constitutive overexpression of DUSP4 significantly reduced the severity of the VIP-induced state, where notably the amplitude of the post-VIP SCN oscillation was reduced less in the presence of more DUSP4. This provides evidence that the long-term effects of VIP may be related to the phosphorylation states of key proteins, although the identity of these proteins has not yet been discerned. Thus assessment of the phosphorylation state of core clock genes, in particular CLOCK and BMAL1 (bearing in mind their long half-lives and the persistent effects of VIP even after cycloheximide presented in Chapter 5), could be undertaken, and may consequently provide a mechanism through which VIP effects its long-term changes to SCN rhythmicity.

Finally, all of the experiments presented in this chapter have investigated the role of DUSP4 across the whole SCN. However, DUSP4 is upregulated in both the core and the shell in response to light (Zhu et al., 2012), presumably in response to glutamate/PACAP in the core, and later on VIP/GRP in the shell. To therefore dissociate any differential roles of DUSP4 in the immediate and delayed response to light, manipulation of DUSP4 should be carried out in
specific neuronal subpopulations utilising the VipCre and VpacCre lines. This will be facilitated by the “conditional-ready” nature of the DUSP4 mouse currently under development (see Chapter 2.2.2), while the viral constructs could be altered to be Cre-dependent (such as through a flexed Cas9), if they are not already.

7.4.5 Conclusions

To conclude, this chapter represents preliminary investigation into genes identified by microarray analysis that are promising candidates for TTFL and phase shifting regulators or mediators. Analysis of the role Cartpt may play in phase shifting is in its very early stages, however it does not appear to have a role in SCN rhythmicity, yet similarities between CART peptides and Prok2 hint at the tantalising prospect of a novel SCN output signal. In contrast, DUSP4 seems to regulate the TTFL as well as both light- and VIP-responsiveness, the exact mechanisms of which remain to be elucidated.
CHAPTER 8

GENERAL DISCUSSION

The suprachiasmatic nucleus is well established as the mammalian circadian pacemaker, acting to ensure internal phase alignment and synchronising endogenous rhythmicity with the external environment. Yet while inputs into the SCN are well understood, particularly with respect to light, the mechanisms of downstream integration of these inputs with its own robust oscillation, and subsequent output to control peripheral oscillators, have yet to be fully elucidated. Nevertheless, VIP has emerged as critical to this process, conveying retinal information to the rest of the SCN and playing a vital role in SCN synchrony. Therefore the primary aim of this thesis was to further understand the function of VIP in the mammalian circadian system. This was approached in two ways: first, through the use of intersectional genetics to establish differential functions of the VIP and VPAC2 cells, and second, to utilise pharmacological application of VIP to elucidate transduction mechanisms of VIP signalling, and how this may lead to changes in intercellular communication. In addition, this latter method was used to identify novel genes that may be involved in the response to VIP and/or in circadian rhythmicity. Individual results have been extensively considered within their respective chapters, therefore the purpose of this Discussion is to place the acquired results across experimental chapters in to a shared context, and to highlight common themes of VIP functionality.
8.1 Technical Considerations

Throughout this thesis, I have used a diverse array of modern and classic experimental techniques to approach the question of VIP in the SCN. Foremost among these was my use of both organotypic slices and in vivo behavioural recording. Organotypic slices offer many advantages of a reductionist system (such as ease of manipulation) while still maintaining the complexity of the intact SCN circuit. However, it is important to note that there is an inherent variability between slices because the technique does not allow for precise dissection of certain regions of the SCN, thus some slices will be more rostral or caudal than others, and the angle of cut may also vary. Measures were taken in all experiments in this thesis to match slices across treatment groups (to ensure results were not merely the consequence of differential responses across the rostro-caudal axis), however some variability would still inevitably remain. Another disadvantage of slices is that they do not allow investigation into SCN outputs, a drawback that was particularly highlighted in Chapter 4 when recording from VpacCre-Ck1εTau- and VipCre-Bmal1 slices. These mice demonstrated clear phenotypes in vivo, which were then predominantly lost ex vivo, suggesting a larger role for output tissue responsiveness than previously anticipated, particularly regarding period determination.

Cre recombinase has also been used extensively throughout this project, allowing the dissection of SCN subpopulation function. This was used either genomically to remove floxed alleles from certain populations, or in combination with AAVs to result in expression of flexed constructs. When employed genomically, off-target effects can be avoided (e.g. VpacCre or VipCre removal of Bmal1 compared to universal Bmal1 KO, which is typically in poor health), although developmental effects are still present unless further combined with an inducible system, such as tetracycline-controlled
transcriptional activation (Tet-On/Off). A further disadvantage is that of incomplete penetrance, such as in VipCre-Bmal1 mice where Bmal1 was not removed from all cells expressing Cre. Complete Bmal1 removal has been difficult to achieve in other studies (Barca-Mayo et al., 2017; Husse et al., 2011; Lee et al., 2015; Mieda et al., 2015), although the cause is unknown. The chromatin context of the Bmal1 locus is likely to be important, while variability between Cre lines may be due to age of Cre expression and epigenetic context at that time (Hameyer et al., 2007; Liu et al., 2013; Long and Rossi, 2009; Vooijs et al., 2001).

AAV transduction avoids confounds related to developmental effects, and expression of flexed constructs is typically shown in 100% of Cre-expressing cells due to the high copy number per cell. Furthermore, AAVs can allow even greater regional specificity. For example, in the planned use of my diphtheria receptor constructs in vivo, the flexed DtR AAV will be stereotactically injected into the SCN, resulting in only VIP or VPAC2 cells within the SCN expressing DtR, dependent on the Cre line used. Thus, when diphtheria toxin is injected, only VIP or VPAC2 cells within the SCN will be ablated, preserving non-SCN localised VIP or VPAC2 cells.

AAVs were used in the final experimental chapter to manipulate levels of protein expression through constitutive expression, CRISPR-mediated knockout or shRNA-mediated knockdown. The use of CRISPR here demonstrates the primary drawback of AAVs, which is their limited packaging capacity, preventing packaging of both the Cas9 protein and the gRNA (and a fluorescent reporter) into a single construct, thus requiring two separate AAVs. Recent use of a Cas9 protein from Staphylococcus aureus (SaCas9), which is ~1 kb smaller than the classically used Streptococcus pyogenes Cas9 (SpCas9), demonstrated that SaCas9 and the gRNA could be successfully transduced in a single construct (Ran et al., 2015). However,
SaCas9 has a larger protospacer adjacent motif (PAM) site (6 bases compared to 3 for SpCas9), greatly restricting the number of targets within a given gene. Nevertheless, the use of CRISPR to knockout *Dusp4* in slices was successful, and to the best of my knowledge represents the first use of CRISPR in SCN slice culture.

Recording organotypic slice bioluminescence and *in vivo* mouse behaviour in combination with Cre recombinase, AAV transduction and pharmacological manipulation has therefore allowed me to explore the functions of VIP at the cellular, circuit and whole organism levels, the results of which will be considered in the following sections.

8.2 Separable roles for VIP and VPAC2 neurons

Alteration of period or rhythmicity, or complete ablation of cell types, has revealed fundamentally different functions for the VIP and VPAC2 neurons within the circadian system. VPAC2 neurons are important for both periodicity and rhythmicity at the behavioural and the SCN level, while VIP cells are important for rhythmicity at the behavioural level and synchrony, but have no role to play in period determination (Figure 8.1A). Further, the observation that VipCre-Ck1ε^Tau/Tau^ slices do not phase shift in response to VIP strongly suggests that VIP cells can regulate the phase of SCN oscillation, and more broadly that ensemble phase is determined at the circuit level, not simply at the level of individual (VPAC2 cell) oscillators.

On one level, differential functionality is not surprising; VIP neurons have long been known to be involved in photic (and non-photic) input, while neurons in the dorsomedial area are generally regarded as robustly rhythmic and important for SCN outputs (Antle and Silver, 2005; Hamada et al., 2001; Nakamura et al., 2001). However, the roles of the cell types themselves, rather
Figure 8.1 Model of SCN subpopulation functions in constant conditions and after a light pulse

(A) Regions of the SCN regulate different circadian parameters in constant conditions. The robustly rhythmic dorsomedial cells (I) determine the period and rhythmicity (II) of the core region, resulting in rhythmic VIP release (III). VIP cells in turn enhance synchrony across the SCN and determine phase of oscillation, as well as having a smaller input into rhythmicity (IV). Both regions output to the brain. (B) Series of events leading to SCN and behavioural phase shifts. Glutamate and PACAP are released from the retinohypothalamic tract (I) to activate retinorecipient cells in the core (II), which are easily shifted. VIP release (III) impairs synchrony across the shell and reduces the amplitude of single cell oscillation (IV), making the shell easier to phase shift (although cell-cell coupling resists complete shifting). The shift is then passed on to the brain (V), possibly by specific light-induced output molecules. AVP, arginine vasopressin; GRP, gastrin-releasing peptide; Prok2, prokineticin 2; VIP, vasoactive intestinal peptide.
than the VIP neuropeptide or the VPAC2 receptor, has rarely been examined, with only two studies directly investigating VIP neuron function (Brancaccio et al., 2013; Lee et al., 2015). This is an important distinction, because the neuronal identity comprises not only the protein of interest (e.g. VPAC2 in VPAC2 neurons), but also the multitude of other cell-type specific protein expression (e.g. AVP in some VPAC2 neurons). This is highlighted most clearly in the difference between ablating VIP or VPAC2 neurons with Dtx compared with VIP- or VPAC2-null slices, where a genomic deletion of VPAC2 will predominantly only affect VIP communication, while an ablation of VPAC2 cells will affect VIP, AVP and Prok2 signalling amongst many other potential pathways. For this reason, it was surprising that ablating VIP cells had a stronger effect on SCN slices than did ablating VPAC2 cells, given VPAC2 cells are important for VIP signalling as well as other forms of communication, in addition to their greater cell number in the SCN. The observation that compensatory VIP signalling through PAC1 receptors increases synchrony and/or rhythm amplitude in VPAC2-null slices may provide an answer to this. Ablation of VIP cells completely removes VIP signalling, while killing VPAC2 cells leaves open the option of PAC1-mediated VIP signalling as PAC1 is found throughout the SCN (Kalamatianos et al., 2004), particularly in the retinorecipient core. Addition of a PACAP antagonist to VPAC2-ablated SCN slices could therefore be expected to significantly reduce synchrony.

Comparisons between SCN slices containing arrhythmic (BMAL1-deleted) VIP cells or no VIP cells (after Dtx) suggests that cell-autonomous rhythmicity, at least for some cell types, is not necessarily important for SCN oscillations. Arrhythmic VIP cells can likely be driven by the strongly oscillating shell, still resulting in rhythmic VIP release, while this is obviously not possible if the VIP cells have been ablated. This shell-mediated drive of
oscillation is supported by experiments with the Tau mutation, in which the VPAC2 cells determine the period of the core. How can this be reconciled with the period-determining VIP cells seen in grafting studies (Maywood et al., 2011)? One explanation could be as follows: in a single intact SCN slice, the period of VIP cells, and therefore of VIP release, is determined by the shell (Figure 8.1A). Rhythmic VIP release then feeds back to the oscillators of the shell, subsequently reinforcing the periods of the TTFLs present in VPAC2 cells. By presenting a VIP stimulus that is outside of this feedback loop, such as in co-culture experiments, the dorsomedial SCN of host slices has no means by which to determine the period of VIP release of graft slices, thus the TTFL in VPAC2 cells aligns to the period of the graft.

The flexed DtR can also be used in concert with Bmal1 or Tau manipulations. For example, one hypothesis for near-revertant periodicity in VpacCre-Ck1εTau−/− mice but not in slices is that a reinforcing stimulus, such as from 24 h retinal VPAC2 cells, may be helping to determine period but is then lost upon dissection. A direct test of this could be achieved through specific Dtx-mediated ablation of retinal VPAC2 cells. This approach could also be used to determine if retinal input is responsible for the residual 24 h rhythmicity seen in DD in both VpacCre- and VipCre-Bmal1 mice, despite later arrhythmicity or split periods.

One striking feature of the Bmal1 and Tau experiments was that slices often did not recapitulate what was observed in vivo, such as VpacCre-Ck1εTau−/− slices showing highly variable periods, and VipCre-Bmal1 slices showing no disruption of rhythmicity despite being derived from behaviourally arrhythmic animals. For VipCre-Bmal1 mice in particular, I have suggested that the rhythmicity of VIP cell outputs, or of recipient regions across the brain, may therefore be important in ultimately determining behavioural rhythmicity. The SCN shell is generally regarded as being the output region
of the SCN, although the core does have some influence (Abrahamson and Moore, 2001; van der Beek et al., 1993; Yan et al., 2005), however the relevance of different output pathways is not entirely clear. Given the fast-shifting nature of the core, is it beneficial for some tissue types to be in phase with it rather than the shell, and thus the core is the primary output to these regions? Different neuropeptidergic or synaptic inputs convey different signals and invoke different responses in the SCN (e.g. responding to glutamate/PACAP compared to neuropeptide Y, which convey different inputs (Maywood et al., 1999, 2002)), so it seems feasible that different outputs from the SCN are intended to convey different information. This may explain the notable absence of traditional SCN outputs (e.g. AVP and Prok2) as being significantly regulated by VIP in my microarray analysis, while some other putative output molecules, such as Vgf, Cartpt and Galanin were all upregulated, and CART at least does not seem to mediate shell to core feedback following VIP application. This, combined with the observation that Prokr2 is necessary for rhythmicity but not for photic responses (Prosser et al., 2007), may suggest that specific output peptides serve different functional roles within the circadian system, however a considerable amount of research is still required to test this hypothesis.

8.3 VIP mechanisms of action

Activation of VIP cells in response to light, as measured by c-FOS, p-ERK or Per expression (Hughes et al., 2004; Kuhlman et al., 2003; Vosko et al., 2015), is well established, however the downstream consequences of this, and subsequent integration of this light information with the endogenous SCN oscillation, are not well understood. Using pharmacological application of VIP, I have investigated its mechanisms of action in the SCN. The application
of VIP to slices had significant and persistent effects on all circadian parameters of bioluminescent rhythms, including period, phase and amplitude, as well as inducing PER2::LUC expression. Furthermore, microarray functional annotation analysis suggested that VIP works through a wide variety of mechanisms. VIP is primarily up-regulatory, inducing a number of immediate early genes via CREB (Figure 8.2). These genes include negative regulators of the cascades through which its signal was likely transduced, such as the MAPK pathway, however a large array of unexpected clusters were also significantly altered, including sterol synthesis and cytokine signalling.

The effects of VIP being mediated by a variety of mechanisms, rather than just a single one (such as Per induction) is supported by the observation that all parameters affected by VIP application were separable. For example, while phase shifts are generally regarded as a consequence of Per induction (and indeed I observed a strong correlation also), application of TTX prior to VIP resulted in a larger immediate phase shift, yet there was no difference in the induction of PER2, showing that at least some part of the phase shift is mediated at the circuit level (and not via neuronal firing). Similarly, treatment of adult slices with VIP showed the same magnitude of phase shift and PER2 induction, yet significantly weaker effects on period and amplitude compared to pup slices. Moreover, period and amplitude were differentially affected by the addition of DNA methyltransferase inhibitor zebularine, which enhanced period change following VIP, but not amplitude.

This extensive characterisation of the VIP response was intended to complement and add to what has already been reported (An et al., 2011, 2013), as well as to aid interpretation of how VIP cells may communicate with VPAC2 cells in intersectional genetics experiments. One interesting observation was the difference between pup slice responses to VIP and those
Glutamate and PACAP stimulate CREB-mediated transcription of Per genes and Dusp4 by increasing cAMP and Ca\textsuperscript{2+} levels in retinorecipient cells. VIP release from these cells to VPAC2 cells causes a similar transduction cascade, whereby VPAC2 signalling through Gas and Ga\textsubscript{q/i} increases cAMP and Ca\textsuperscript{2+} levels to activate kinases PKA, PKC and ERK1/2 amongst others. These pathways converge on CREB, which stimulates transcription of genes containing CREs in their promoters, including TTFL genes Per1, Per2, Rora and Dec1. RORA stimulates Bmal1 transcription, while DEC1 can inhibit CLOCK:BMAL1 at E-boxes but not E’-boxes (as in the Per2 promoter). CREB-mediated transcription of Dusp4 feeds back to inhibit the MAPK pathway, while output genes may play a role in signaling downstream tissues. VIP also stimulates other pathways, possibly through kinases such as PKA and MAPK, although the details of these cascades are not known. Additionally, non-CRE-mediated transcriptional activation occurs, such as with some members of the PP1 family, which can then feed into the TTFL by regulating PER protein stability. B, BMAL1; C, CLOCK; cAMP, cyclic adenosine monophosphate; CRE, cAMP response element; CREB (Cr), CRE binding protein; Dusp4, dual specificity phosphatase 4; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PACAP, pituitary adenylate cyclase-activating polypeptide; Per, Period; PKA, protein kinase A; PKC, protein kinase C; PP1, protein phosphatase 1; Rora, retinoic acid receptor- (RAR-) related orphan receptor; RRE, ROR response elements; TTFL, transcriptional-translational feedback loop; VIP, vasoactive intestinal peptide.
of adult slices. Numerous differences existed between pup and adult VipCre and VpacCre slices in both Bmal1 and Tau experiments, such as VpacCre-Bmal1 pup slices showing normal rhythmicity, whereas adult slices are highly disrupted. The observation that adult slices show a less severe response to VIP may imply that VIP cells hold less influence in the adult mouse than in pups, thus rhythmic VIP cells can compensate for arrhythmic VPAC2 cells at a young age but not in older mice. Clearly this is only one possible explanation out of several for how and why adults and pups may differ, as both intracellular rhythmicity and intercellular communication, albeit particularly in relation to VIP, have been shown to alter throughout life (Azzi et al., 2014; Banks et al., 2016; Chang and Guarente, 2013; Duncan et al., 2001; Kalló et al., 2004b; Kawakami et al., 1997; Li and Satinoff, 1998; Ono et al., 2013, 2016). Nevertheless, an age-related change in connection with the VIP-VPAC2 signalling axis appears to be a common theme.

A second aim of this work was to expand our current mechanistic insight into how light information (in this case light-mediated activation of VIP cells) is integrated with the endogenous circadian system. This was achieved by investigating not only the cellular transduction mechanisms of VIP, but also the effects of VIP at the circuit level. Cell-autonomous rhythmicity and circuit-generated rhythmicity are clearly difficult to dissociate, and even when only one is manipulated (such as in a CryDKO or VPAC2 KO genotype), this often influences the other (Maywood et al., 2006; Ono et al., 2016). Nevertheless, a slice regarded as oscillating using only cell-autonomous mechanisms could be a TTX-treated slice, while rhythms generated solely through coupling can be seen in BMAL1 KO and CryDKO slices (Ko et al., 2010; Maywood et al., 2011). An intact slice contains both of these mechanisms, and indeed requires both to produce consistent oscillations: TTX causes SCN cells to gradually drift out of phase and lose amplitude, while the rhythms of core clock gene knockout
slices are unpredictable. My work demonstrates that the effects of VIP at both the cellular and circuit level are likely to be important for phase shifting and the subsequent VIP-induced state. For example, the reduction in amplitude induced by VIP was not merely the result of phase desynchrony, but also a reduction in amplitude of the cellular oscillator, in contrast to what has been suggested previously (An et al., 2013). Further, the effects on the phase relationships between different regions of the SCN were consistent, not random.

The effects of VIP on individual cells were shown most obviously at the level of gene expression, where core clock genes reported by Per1-Luc, Cry1-Luc and PER2::LUC showed differential responses (Figure 8.2). Per1 demonstrated less of a phase delay than both PER2 and Cry1, implying that the phase relationship of core TTFL components has been altered (an observation that was supported by qPCR data in Chapter 6). Strong TTFL regulation was seen in the microarray analysis as well, where Per1, Per2, Rora and Dec1 were all significantly up-regulated. The ability of DEC1 to control Per1 phase through E-box elements, but not through the E’-box of Per2 (Nakashima et al., 2008), may be sufficient to explain the phase separation. Additionally, VIP down-regulated Dbp expression. DBP is an important output of the TTFL, regulating the transcription of many genes, including those involved with cellular physiology as well as components of the TTFL (Schrem et al., 2004; Takahashi, 2017). Thus VIP affects multiple interlocking loops of the cell-autonomous oscillator.

Transcriptome profiling also showed that VIP potentially affects TTFL protein stability and degradation through regulation of the expression of several phosphatases (Figure 8.2). These included Ppp1r3c and Ppp2r1b, two members of the protein phosphatase 1 (PP1) family, a family that has been implicated in PER protein stability (Gallego et al., 2006; Lee et al., 2011). Another family of
phosphatases, the DUSP family, also had several members up-regulated by VIP, and has been discussed at length in previous chapters. Future work is needed to determine whether these phosphatases are regulating the phosphorylation of TTFL proteins or of others, such as those involved in the VIP transduction cascade.

The exact mechanism by which VIP affected intercellular communication is not entirely clear, although some candidates do exist. First, due to its effects on the TTFL, any E-box regulated neuropeptides, including AVP, Prok2, CART and VGF (Cheng et al., 2002; Coulson et al., 1999; Dominguez et al., 2002; Di Rocco et al., 1997), may be affected. Both Cartpt and Vgf transcripts were up-regulated 2 and 6 h after VIP application. While shRNA-mediated knockdown of Cartpt suggested that this peptide was not involved in SCN rhythmicity, Vgf is yet to be investigated. Secondly, gap junction β-2 protein (Gjb2), also known as connexin 26, was up-regulated by VIP after 6 h. Gap junctions have been previously observed in the SCN, although connexins 32 and 36 are more typically implicated (Colwell, 2000b; Rash et al., 2007). Connexin 26 has been found to oscillate in a circadian manner in other tissues (Ihara et al., 2017; Temme et al., 2000), but it has not yet been studied in the SCN.

VIP simply having differential effects at the cellular level based on regional localisation, rather than affecting cell-cell communication per se, is of course a possibility, however the fact that VIP modulates the spatiotemporal wave of gene expression remains, regardless of mechanism. While the significance of this waveform is yet to be fully appreciated, it seems to be involved in encoding photoperiod, a function that VIP has been implicated in previously (Evans et al., 2013; Lucassen et al., 2012; Meijer et al., 2010; Vanderleest et al., 2007), and so may mediate the function of the SCN in seasonal responses.
A final aim of the VIP application experiments was to determine the nature of the pathway that was responsible for the highly persistent amplitude effects following VIP, which have previously been suggested to aid entrainment (An et al., 2013). This was approached by trying to prevent this state from occurring in the first place, or by trying to revert the VIP-treated slice back to a pre-VIP state. Experiments such as co-treatment with zebularine, an inhibitor of a known mediator of long-term change, were performed, as well as less directed approaches, such as subsequent treatment with CHX, which may have had resetting effects on the TTFL that would reverse the VIP-induced state. Unfortunately, a treatment that attenuated these persistent effects was not found, however DUSP4 experiments presented in Chapter 7 might provide a hint, as DUSP4 overexpression resulted in a much reduced amplitude effect of VIP. Overexpression of DUSP4 would reduce the activity of the MAPK pathway, a likely target of a VPAC2-mediated Ca²⁺ increase. Broadly speaking, this would suggest that phosphorylation is involved in the VIP-induced state, although overexpression of DUSP4 blocking the initial induction following VIP application, rather than the downstream effects, remains a possibility. The target of this phosphorylation is not known, although BMAL1 itself is known to be phosphorylated by ERK (Sanada et al., 2002). DUSP4 is involved in both the immediate light (glutamatergic/PACAP) response as well as the subsequent VIP response (Zhu et al., 2012), thus manipulation of DUSP4 levels within specific cell types is needed to elucidate the full involvement of this phosphatase.

8.4 Implications and future directions

The work presented in this thesis has solidified VIP signalling as being integral to SCN function. Manipulation of circadian properties at either end of
the VIP-VPAC2 axis, as well as application of VIP to SCN slices, had strong effects on behavioural and slice rhythmicity, periodicity and phase. This demonstrates both a sufficiency of VIP signalling to influence circadian timekeeping as well as a necessity, as ablation of either the VIP or VPAC2 cells results in attenuation of normal SCN oscillations. This work has also expanded on existing knowledge of mechanisms of VIP signalling in the SCN, and highlighted a number of possible pathways that will likely be important to the effects of VIP in entrainment to light. Finally, by separately manipulating periodicity and rhythmicity in these cell populations, and by systematically characterising the phase, period and amplitude effects of VIP in combination with other treatments, this work is well situated to show that different circadian parameters are not all determined by the same cell population in the SCN, nor by a single transduction mechanism. If correct, this renders the idea of “pacemaker cells” within the SCN overly simplistic and susceptible to the context, such as age, of the animal or slice. The highly heterogeneous nature of the SCN is therefore unlikely to be accidental: different cell populations serve different functions, such as sustaining rhythmicity and mediating inputs or outputs, all of which have a part to play in SCN function.

Specific experiments for prospective investigations have been suggested throughout this thesis, therefore the ideas posited here represent broad avenues for future research to pursue. Firstly, what is the nature of the period-determining signal emitted from VPAC2 cells, or indeed other pace-setting cells such as those expressing Drd1a or NMS? This is unlikely to be AVP (in VPAC2 and Drd1a cells) or NMS due to the absence of an effect of deleting these peptides on period. Generally speaking, multi-directional communication (i.e. rather than simply core to shell) has not been extensively researched, yet is clearly important in SCN coherence. Secondly is to
investigate outputs of the SCN, and the contribution of downstream SCN targets to the determination of period and rhythmicity. A greater understanding of where different regions of the SCN project, a goal that may be achieved through the use of novel Cre-dependent tracer viruses (Ciabatti et al., 2017), will assist this.

A greater emphasis could also be placed on transduction mechanisms of SCN neuropeptides. Large amounts of previous literature has focused on regions of the SCN and their respective neuropeptide outputs, however it is clear that the functions of these signalling molecules changes with time (on both circadian and developmental timescales) and context (such as lighting conditions). Only through an appreciation of the molecular mechanisms underlying intercellular communication will the full complexity of the SCN be understood, and provide greater clarity into how environmental inputs can be integrated with the endogenous circadian oscillator.

8.5 Concluding Remarks

The SCN offers a rare opportunity in neuroscience to study a circuit in which a full complement of events linking molecular mechanisms to overt behaviour can be illuminated. The interconnected SCN is robustly rhythmic, resistant to perturbation, yet resettable by light, and maintains the capability to influence a wide range of fundamental physiology such as sleep and feeding. The unique array of neuropeptides present in the SCN and the intercellular coupling that they engender are essential to its ability to generate coherent circadian timekeeping, and the role of the VIP signalling axis is central to this. VIP is active at all levels of the circadian hierarchy, from inputs to outputs, and the work in this thesis reinforces the importance of VIP to SCN pacemaking. By using a combination of intersectional genetics, virally
mediated circuit manipulation and pharmacological treatments, my work has shed light on SCN subpopulation functions and the integration of inputs with endogenous oscillations, and has therefore helped to elucidate organisational features of mammalian circadian timekeeping.
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