Effects of early light environment on the photic response of the circadian system

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Abstract

Early light environment has been shown to alter locomotor activity behaviour in adult rats and mice when exposed to constant light (LL), constant darkness (DD) or 12:12 hour light-dark cycles (LD). In particular, exposure to LL during lactation results in an increased ability to cope with exposure to LL as adults, implying that the ability to interpret light information is altered depending on early light environment. Therefore, the aim of this project was to explore how early light environment affects the photic response of the circadian system.
The retina forms the first component of the photic response of the circadian system. We wanted to know whether being raised in DD, LD or LL would alter retinal function or structure in adult CD1 (albino) and C57BL/6J (pigmented) mice. We found that in CD1 mice, being raised in LL caused significant retinal damage and a significant reduction in retinal function. In C57BL/6J mice, we saw no such changes, implying that any changes that we see in behaviour would be due to alterations further downstream, such as the suprachiasmatic nucleus (SCN), site of the master circadian clock in the mammalian brain. We next exposed C57BL/6J mice to LL and found that mice raised in DD had significantly longer taus than mice raised in LL, implying that mice raised in DD are more sensitive to light than mice raised in LL.
Previous work has shown that early light environment alters neuropeptide and astrocyte expression in the SCN of C57BL/6J mice. Furthermore, early light environment produces opposite behavioural responses in CD1 and C57BL/6J mice when exposed to LD as adults. We therefore examined how neuropeptide and astrocyte expression would be affected by early light environment in CD1 mice and whether this would reflect the differential behavioural response. We found that neuropeptide and astrocyte expression in the SCN seemed to be affected by the level of retinal damage and/or the type and intensity of the light source used. This sensitivity to lighting environment makes CD1 mice unsuitable for further studies on the photic response of the circadian system. Pigmented mice were used for the remainder of this project.
The photic response of the circadian system can be quantified using a phase response curve (PRC) which measures behavioural responses to light pulses administered at different times of day. We measured the effect of a light pulse on the delay and advance portion of the PRC and found no differences due to early light environment, implying that the phasic effect of light is not altered by early light environment. Light pulses administered during the subjective night result in the upregulation of SCN intracellular photic signalling pathways. After a light pulse given during the early subjective night, we found no differences in the upregulation of different components of the photic signalling pathway due to early light environment indicating early light environment does not seem to affect the initial photic signalling pathway in the SCN.
Finally, recent advances in molecular biology allow for real-time monitoring of clock gene expression in vitro. Using mPer2::luc mice, we monitored in vitro PER2::LUC expression to determine the effects of early light environment on clock gene expression. In the SCN, we found that the amplitude of PER2::LUC expression was significantly reduced in mice raised in DD compared to mice raised in LD and LL. These results suggest that early light environment affects the coupling strength between SCN neurons and this may be the mechanism mediating the changes in behaviour we have measured. In peripheral tissue, we found altered PER2::LUC expression due to early light environment in the heart, lung and spleen, implying that early light environment not only alters behaviour but may also affect heart and lung function and the immune system.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree of this or any other university or other institute of learning.

Copyright Statement

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Thank you!

Abbreviations

ADP	Anterodorsal preoptic nucleus
AVP	Arginine vasopressin
cAMP	Cyclic adenosine monophosphate
CCG	Clock controlled gene
CCK	Cardiotrophin-like cytokine
CHX	Cycloheximide
CK1ε	Casein kinase 1ε
CRE	cAMP response element
CREB	cAMP response element-binding
CT	Circadian time
DD	Constant darkness
dLGN	Dorsal lateral geniculate nucleus
DMH	Dorsomedial hypothalamus
E	Embryonic day
ERG	Electroretinography
GABA	γ-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GRP	Gastrin releasing peptide
HRP	Horseradish peroxidase
IGL	Intergeniculate leaflet
i.p.	Intraperitoneal
IPL	Inner plexiform layer
ipRGC (or pRGC)	Intrinsically photosensitive retinal ganglion cell
JNK	c-Jun N-terminal kinase
LD	12:12 hour light-dark cycle
LED	Light-emitting diode
LL	Constant light
M1	Type 1 melanopsin-expressing retinal ganglion cell
M2	Type 2 melanopsin-expressing retinal ganglion cell
M3	Type 3 melanopsin-expressing retinal ganglion cell
M4	Type 4 melanopsin-expressing retinal ganglion cell
M5	Type 5 melanopsin-expressing retinal ganglion cell
MAPK	Mitogen-activated protein kinase (MAPK)
MDL	N-(Cis-2-phenyl-cyclopentyl) azacyclotridecan-2-imine-hydrochloride
MSG	Monosodium glutamate
NMDA	N-methyl-D-aspartate
ONL	Outer nuclear layer
OPN	Olivary pretectal nucleus
P	Postnatal day
PB	Phosphate buffer

PBX	Phosphate buffer containing 0.03% Triton X-100
PCH1	Power content of the first harmonic
PLR	Pupil light reflex
PRC	Phase response curve
PV	Percentage of variance
PVN	Paraventricular nucleus
qPCR	Quantitative polymerase chain reaction
RGC	Retinal ganglion cell
RHT	Retinohypothalamic tract
RPE	Retinal pigment epithelium
s.c.	Subcutaneous
SON	Supraoptic nucleus
SPZ	Subparaventricular zone
TBS	Tris-buffered saline
TGF-α	Transforming growth factor-α
TH	Tyrosine hydroxylase
TTX	Tetrotodoxin
SCN	Suprachiasmatic nucleus
VIP	Vasoactive intestinal polypeptide
VMH	Ventromedial hypothalamus
ZT	*Zeitgeber* time

Preface

The author has a BSc (Hons) degree from the University of Manchester in Neuroscience. She is first author on the following journal article which can also be found in Appendix 2:
"Differential hypothalamic tyrosine hydroxylase distribution and activation by light in adult mice under different light conditions during the suckling period" Brooks, E; Waters, E; Farrington, L; Canal, MM. Brain Structure and Function 2011, Nov; 216(4):357-370.

She has presented posters at the Gordon Conference of Chronobiology in June 2011 and at the UK Clock Club in April 2012. She has also presented a 10-minute talk at the UK Clock Club in April 2011.

1. Introduction

1.1 Biological rhythms

1.1.1 Circadian rhythms

Every 24 hours, our planet spins once on its axis, resulting in a daily alternation between darkness and light. These two dramatically different environments, day and night, provide challenging surroundings to which almost all species on the planet have adapted to ensure maximal survival. To do this, they have developed biological rhythms that match the rhythms of the external environment. The most common are circadian rhythms (from the Latin *circa dies* meaning "about a day") which are defined as rhythms which occur once every 24 hours approximately and persist even in the absence of external cues. Thanks to circadian rhythms, animals have developed the ability to be active at certain times of day - times when their predators may not be active. For example, some species will only come out at night (nocturnal) while their predators may only be active during the day (diurnal). Other species only come out at dawn or dusk (crepuscular). The importance of circadian rhythms for survival in the wild has been demonstrated by DeCoursey and colleagues (DeCoursey and Krulas, 1998; DeCoursey et al., 2000). Wild chipmunks, a diurnal species, were given a lesion of the suprachiasmatic nucleus (SCN), the master clock in the mammalian brain, or were sham-operated before being released back into the wild. The team found that chipmunks that had lesions to the SCN and thus lacked circadian rhythms were not able to survive in the wild for as long as chipmunks that had received sham surgery because the chipmunks which had no SCN were not able to restrict their time outside the burrow to daytime only and thus put themselves at risk of predation by nocturnal animals. Indeed, the team established that many of the SCN-lesioned chipmunks had been killed by weasels, which are largely nocturnal. These studies demonstrate the importance of circadian rhythms for survival in the wild (DeCoursey and Krulas, 1998; DeCoursey et al., 2000).

As mentioned above, circadian rhythms are rhythms which have a period close to 24 hours and persist even in the absence of external cues. Internal circadian rhythms are never exactly 24 hours so they must be reset everyday to the external environment. External cues which reset the circadian clock are termed *Zeitgebers* (time-givers in German) of which the most important is the 24-hour light-dark cycle. When the circadian clock is reset to the external time, it is said to be entrained to the external environment.

In the absence of external cues, the circadian clock is said to be free-running and has a period which is close to but not exactly 24 hours. Therefore, the measurement of time depends on whether an animal is entrained or free-running. If an animal is entrained, the measurement of time used is *Zeitgeber* Time (ZT) and corresponds to the time of the external clock. In a standard 12:12 hour light-dark cycle (LD), ZT0 corresponds to the time that the lights are turned on and ZT12 corresponds to the time when the lights are turned off. In the absence of external cues the measurement of time is defined as Circadian Time (CT) and corresponds to the free-running period (tau or τ) of the animal. A circadian day is divided into 24 equal parts (circadian

hours) which can be shorter or longer than the hours on a clock. CT0 corresponds to the beginning of the active phase in diurnal animals while CT12 corresponds to the beginning of the active phase in nocturnal animals (Jud et al., 2005).
Here I will introduce circadian and other biological rhythms, the circadian system, how it is influenced by light, how it develops and the effect of light on its development.

1.1.2 History of circadian rhythms

In the 18th century, Jean Jacques Ortous de Mairan, a French astronomer, observed that mimosa leaves droop at night and are rigid during the day. To test whether this was a direct result of external cues such as light, he placed the mimosa plant in a dark room and checked on it regularly. He noted that even in the absence of light the mimosa plant's leaves still followed a rhythm of being rigid followed by drooping, and this occurred at the same time in all the leaves (de Mairan, 1729). His study was the first to show that plants have rhythms which are not dependent on external cues but may be mediated by an internal clock.
It was not until the second half of the 20th century that interest in circadian rhythms really took off and the field of chronobiology came into existence thanks to work by Jürgen Aschoff, Colin Pittendrigh and Serge Daan. Using many different animal models and humans (in Aschoff's famous bunker experiments), some of the fundamental rules of chronobiology were set out. These include setting out the rules that define a circadian rhythm: 1) the rhythms must occur once a day; 2) the rhythms must be endogenous (they persist in the absence of external cues); 3) the rhythms must be able to entrain to external cues; 4) the rhythms must be temperature compensated (Pittendrigh, 1954; Pittendrigh et al., 1959). Other rules which were set out include Aschoff's rule which states that in nocturnal animals, in constant light conditions, the higher the light intensity, the longer the tau. Conversely, in diurnal animals, tau shortens as light intensity increases (Aschoff, 1960). Further, Pittendrigh and Daan showed that in nocturnal animals placed in constant darkness (DD), a light pulse given during the early subjective night (when they are active) resulted in a phase delay while a light pulse given during the late subjective night resulted in a phase advance (Daan and Pittendrigh, 1976a). In the same series of papers, these authors formulated many other rules on circadian rhythms which will not be mentioned here in detail (Daan and Pittendrigh, 1976a, 1976b; Pittendrigh and Daan, 1976a, 1976b, 1976c).
As the field of chronobiology expanded, it became clear that an important leap forward in the field would be to locate the master clock in the brain. As it had been established that the circadian system must be able to perceive light in order to entrain to the external environment, the primary accessory visual pathways were destroyed, but this did not result in a loss of the ability to entrain to LD (Stephan and Zucker, 1972a). Recent evidence about a new pathway from the retina to the hypothalamus (the retinohypothalamic tract - RHT) (Moore and Lenn, 1972), and more particularly to the SCN led to a series of studies in which the SCN was lesioned. SCN lesions resulted in a loss of rhythmic drinking behaviour (Stephan and Zucker, 1972b) and rhythms in adrenal corticosterone release (Moore and Eichler, 1972). These studies indicated that the SCN played a central role in the circadian system although it had not yet been confirmed whether it was the central pacemaker or just a relay station.

Using radiolabelled 2-deoxyglucose to study metabolism in the brain it was shown that in rats the SCN had high levels of metabolism during the day and low levels at night which were increased after acute light exposure. Furthermore, these oscillations persisted in the absence of light signals, demonstrating that SCN oscillations were endogenous (Schwartz and Gainer, 1977). Using multiunit electrical recordings in rats, and later in hamsters, it was shown that electrical activity in the SCN was higher during the day than during the night and that the SCN was still able to oscillate in vivo even when it was isolated from the rest of the brain (in "hypothalamic islands") (Inouye and Kawamura, 1979; Yamazaki et al., 1998). It was also shown that other areas of the brain could also oscillate, but in antiphase to the SCN, with highest activity during the night. Furthermore, when the SCN was isolated, other areas of the brain were not able to maintain their circadian rhythms, implying a role for the SCN in driving rhythms in the rest of the brain (Inouye and Kawamura, 1979; Yamazaki et al., 1998). It was also shown that the SCN could oscillate in vitro demonstrating that the SCN contained an autonomous clock (Green and Gillette, 1982; Shibata et al., 1982).

Confirmation that the SCN was indeed the central oscillator of the mammalian circadian system came thanks to work using the golden hamster. Golden hamsters have a tau which is very close to 24 hours. In a routine delivery of hamsters, one hamster was found to have a tau of 22 hours, instead of 24 hours. This hamster was named the *tau* mutant hamster. It was bred and the inheritance of the mutation was found to occur in a Mendelian fashion, with half the offspring exhibiting a 22-hour free-running rhythm (heterozygous) and the other half exhibiting a 24-hour free-running rhythm (wild-type). When two heterozygous hamsters were mated they obtained heterozygous hamsters and homozygous hamsters with a 20-hour free-running rhythm (Ralph and Menaker, 1988). When these hamsters were given SCN lesions they became arrhythmic. Interestingly, implanting foetal SCN from wild-type hamsters into mutant hamsters that had received an SCN lesion restored 24-hour free-running rhythms to the mutant hamsters. Conversely implanting SCN from heterozygous or homozygous mutant hamsters into wild-type hamsters that had received an SCN lesion resulted in the wild-type hamsters regaining a rhythm, but the tau was that of the donor hamster (22-hour or 20-hour respectively) (Ralph et al., 1990). These studies demonstrated that the SCN is the central mammalian pacemaker and that it dictates the period of the clock. The *tau* mutation is the result of a point mutation in the *casein kinase 1 epsilon (CK1ε)* gene, which was identified by mapping the mutation in the hamster then locating conserved loci between the hamster, mouse and humans to reveal a candidate locus (a method the authors termed positional syntenic cloning) (Lowrey, 2000; Lowrey and Takahashi, 2000).

A key step forward in chronobiology was the identification of clock genes, which led to the discovery of autoregulatory transcriptional/translational feedback loops which mediate 24-hour oscillations of cells in the SCN (for review see Hastings et al., 2008). The identification of key core clock genes such as *Per1* and *Per2* and recent advances in molecular biology have allowed for the development of rat and mouse strains with reporter genes fused to clock genes of interest (such as the *Per1-luc* rat (Yamazaki, 2000) and the *mPer2::luc* mouse (Yoo et al., 2004)) to allow for the imaging of gene expression in real time instead of sampling at distinct time points only. Advances such as these have led to the discovery that circadian oscillations of gene expression occur not only in the SCN but in other brain areas (Abe et al., 2002) and also

in other tissues throughout the body (Yamazaki, 2000; Yoo et al., 2004), which reveals the importance of circadian rhythms in metabolism, the immune response and heart and lung function. Much like a conductor and his orchestra, the SCN sends out signals to the peripheral clocks about time of day in order to synchronise them to the external environment (Antle and Silver, 2005). Although the exact nature of these signals has yet to be firmly established, it is believed that food (Damiola et al., 2000) and glucocorticoids (Balsalobre et al., 2000) may play an important role in synchronising peripheral clocks. The circadian rhythms in body temperature may also play an important role in synchronising peripheral clocks as it has been shown that cultured peripheral tissue can be entrained to temperature cycles within physiological temperature ranges while the SCN cannot (Buhr et al., 2010). Further work is needed to determine how different signals contribute to synchronising peripheral clocks.
Chronobiology is still a rapidly growing and dynamic field and recent advances in molecular biology promise more exciting discoveries in the years to come.

1.1.3 Other biological rhythms

Although circadian rhythms form the main focus of this report, it is important to mention that other rhythms exist in nature. These are termed ultradian rhythms when the tau is shorter than 20 hours and infradian rhythms when the tau is longer than 28 hours. Examples of ultradian rhythms can be found in sleep, where a sleep cycle lasts between 90 and 110 minutes (Voss, 2004) and in cortisol release, where the circadian rhythm of cortisosterone release is overlaid by an hourly pulse of cortisosterone release (Windle et al., 1998). Intertidal rhythms are also classed as ultradian as they occur once every 12.4 hours. Some species of plants and crab, such as the fiddler crab (*Uca pugnax*) show intertidal rhythms where they dig burrows and feed at low tide and hide in their burrows at high tide, behaviours which persist in constant conditions (Palmer, 2000).
Examples of infradian rhythms include the menstrual cycle which can also be classed as a circalunar rhythm as it occurs approximately every 29.5 days (Baker and Driver, 2007). Seasonal rhythms are also infradian rhythms and allow animals to breed so that their offspring are born at times when they are most likely to survive. Interestingly, it has been shown that arctic reindeer have very weak circadian rhythms, but strong seasonal rhythms driven directly by photic information on day length in the summer and absence of light in the winter (Lu et al., 2010).
These examples of other types of rhythms demonstrate how species are able to adapt to the environment in which they live in order to ensure maximum survival and efficiency in metabolic activities.

1.2 The circadian system in mammals

The circadian system can be very simply described as a central clock which receives inputs from the environment and sends output signals to the rest of the body (Figure 1.1).

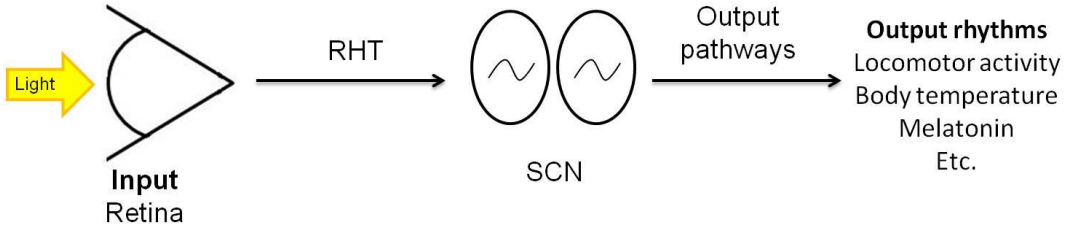

Figure 1.1: Simple schematic diagram of the circadian system. The circadian system can be described as a simple input-output system. Here, light information is received by the retina, which transmits this photic information to the suprachiasmatic nucleus (SCN) via the retinohypothalamic tract (RHT). The SCN processes this information and sends information about time of day to other areas of the brain and body via output pathways, thereby driving circadian rhythms such as locomotor activity behaviour, body temperature rhythms and melatonin rhythms.

1.2.1 Inputs

1.2.1.1 Retinal organisation

In mammals, it has been shown that photic entrainment is mediated solely through the eyes. This was done by enucleating both diurnal and nocturnal rodents then exposing them to LD cycles provided by direct bright sunlight (approximately 55,000 lux). Sighted animals were able to entrain to these light conditions. However, animals that had been enucleated were unable to entrain to these bright light conditions and instead free-ran with their endogenous periods (Nelson and Zucker, 1981). The retina, located in the eye, is the sole photoreceptive area in mammals. It contains all three types of photoreceptors: rods, cones and the recently discovered intrinsically photosensitive retinal ganglion cells (ipRGCs). Figure 1.2 shows the structure of the retina. The rod and cone photoreceptors are located at the back of the eye, adjacent to the retinal pigment epithelium (RPE) which, in pigmented mice, absorbs any light not captured by the retina in order to prevent light-induced retinal damage (Tessier-Lavigne, 2000). Light enters the eye and travels through the different layers of the retina before being detected by the outer segments of the rods and cones. These then send light information to the retinal ganglion cells (RGCs) via the interneurons located within the inner nuclear layer (bipolar cells, horizontal cells and amacrine cells). There are two types of bipolar cell: ON-bipolar cells (which are activated by increments of light) and OFF-bipolar cells (which are activated by decrements of light), which synapse with RGCs in different sublayers of the inner plexiform layer (IPL). Cone ON-bipolar cells synapse with ON-ganglion cells in the ON-sublayer of the IPL while cone OFF-bipolar cells synapse with OFF-ganglion cells in the OFF-sublayer of the IPL. Rod bipolar cells are all ON-cells which synapse with amacrine cells before information is sent to the RGCs. RGCs send photic information to the brain via their axons which form the optic nerve (Tessier-Lavigne, 2000).

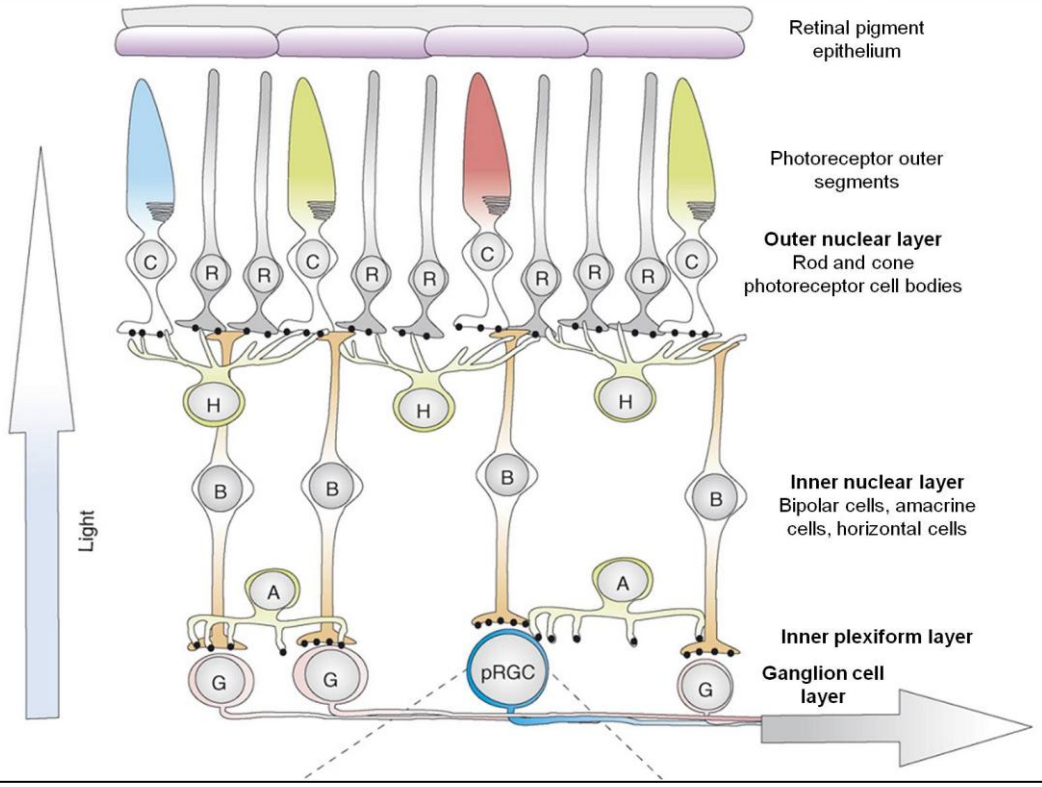

Figure 1.2: Schematic diagram of the retina. Light enters the eye and travels through the cell layers of the retina before being detected by the outer segments of the rod and cone photoreceptors. Light information is then transmitted back through the retina to the ganglion cell layer via the interneurons which compose the inner nuclear layer. The axons of the retinal ganglion cells form the optic nerve. C - cones (colours represent the wavelengths which each type of cone detects); R - rods; H - horizontal cells; B - bipolar cells; A - amacrine cells; G - retinal ganglion cells; pRGC - photosensitive retinal ganglion cells. Figure adapted from Hankins et al (2008).

1.2.1.2 The role of the retina in photic entrainment

In the search to determine which photoreceptors mediate photic entrainment, mutant mice lacking one or more photoreceptor were used. It was found that mice lacking rods and most cones (*rd/rd* mutant mice) were still able to entrain to LD and shift after a light pulse (Foster et al., 1991). As these mice still had some functional cones it was deemed possible that circadian photic entrainment may be mediated by cones. A mouse model that lacked all rods and cones was then developed (*rd/rd cl* mutant mouse). However, despite the complete loss of the outer layers of the retina, these mice were also shown to be able to entrain to LD and showed unattenuated phase shifts in response to a 15-minute monochromatic light pulse (Freedman et al., 1999; Lucas et al., 1999). It was therefore hypothesised that there may be a third type of photoreceptor in the retina. Previous work had shown that the RHT provides a direct pathway from the retina to the SCN (Moore and Lenn, 1972). Further work showed that the RHT actually originated from a distinct subset of RGCs (Moore et al., 1995). These RGCs were shown to be intrinsically photosensitive, while adjacent RGCs which did not project to the SCN were not photosensitive (Berson et al., 2002; Sekaran et al., 2003). The photopigment responsible for the

intrinsic photosensitivity of these RGCs was identified as a recently discovered opsin: melanopsin (Lucas et al., 2001). Melanopsin was found to be localised in all the RGCs that project to the SCN, and knocking out melanopsin resulted in a loss of photosensitivity in these RGCs (Lucas et al., 2003). Furthermore it was shown that expressing melanopsin in non-photosensitive RGCs resulted in these cells becoming photosensitive (Melyan et al., 2005). Interestingly, a mutant mouse strain lacking only melanopsin was still able to entrain to LD, indicating that melanopsin is not the sole mediator of photic entrainment (Panda et al., 2002). Indeed, the ability to entrain to LD was only lost once a mutant mouse was produced that lacked rods, cones and melanopsin (Hattar et al., 2003). ipRGCs are not only photoreceptors themselves, but also receive synaptic input from the rods and cones (Perez-Leon and Warren, 2006; Wong et al., 2007), leading to the ability to entrain to LD if one or two photoreceptors are missing, but not all three. It has also been shown that knocking out ipRGCs resulted in mice that were unable to entrain to LD, as these form the RHT and thus the direct link from the retina to the SCN (Güler et al., 2008).

1.2.1.3 Intrinsically photosensitive retinal ganglion cells

Rods and cones are crucial for image-forming vision and as discussed above are also important for non-image forming vision such as the pupil light reflex and circadian photoentrainment, while ipRGCs are mainly involved only in non-image forming vision (Berson et al., 2002; Hattar et al., 2002; Provencio et al., 2002; Rollag et al., 2003). Recent studies have shown that there are different types of melanopsin-expressing RGCs. Type 1 cells (M1) have dendrites that stratify exclusively in the OFF-sublayer of the IPL. Type 2 cells (M2) have dendrites that stratify exclusively in the ON-sublayer of the IPL. Type 3 cells (M3), which are less widely expressed, have dendrites that stratify in both sublayers of the IPL (Hattar et al., 2006; Viney et al., 2007). Two further melanopsin cell subtypes have recently been identified which express melanopsin too weakly to be identified using the classic antibodies. These are the M4 and M5 melanopsin cells, with dendrites stratifying in the ON-sublayer of the IPL. Both types show weak intrinsic light responses but M4 cells can be distinguished by their large radiated dendritic arbour while M5 cells can be distinguished by their compact, highly branched dendritic arbour (Ecker et al., 2010). M1 cells are the melanopsin-expressing cells which are most responsive to light, while M2 cells seem to be more responsive to signals coming from rods and cones (Ecker et al., 2010; Hughes et al., 2012). Furthermore, two different melanopsin isoforms, resulting from alternative splicing of the mouse *Opn4* gene (Pires et al., 2009) have been discovered which are differentially expressed in M1 and M2 cells. M1 cells express both the long and short isoforms, Opn4L and Opn4S, while M2 cells only express Opn4L (Pires et al., 2009; Hughes et al., 2012). The majority of ipRGCs projecting to the SCN are M1 cells (Baver et al., 2008), but they also project to other areas of the brain such as the shell of the olivary pretectal nucleus (OPN), which mediates the pupil light reflex (Trejo and Cicerone, 1984), the intergeniculate leaflet (IGL) and the habenular (Baver et al., 2008; Ecker et al., 2010). M2 cells have also been shown to project to the SCN and the core of the OPN (Baver et al., 2008; Ecker et al., 2010) and non-M1 melanopsin-expressing cells also project to the IGL, dorsal lateral geniculate nucleus (dLGN), superior colliculus and posterior pretectal nucleus (Hattar et al., 2006; Ecker et

al., 2010). M1 and M2 cells seem to play an important role in circadian entrainment and the pupil light reflex (Baver et al., 2008) but there is still much to learn on how the different subtypes of melanopsin cells contribute to non-image forming vision.

1.2.1.4 Other inputs

The RHT is the principle pathway which conveys photic information to the SCN. However, there are also indirect pathways which convey light information such as the IGL which itself receives inputs from RGCs (Pickard, 1985) and utilises γ-aminobutyric acid (GABA) and neuropeptide Y as its main neurotransmitters (Moore and Speh, 1993; Moore and Card, 1994). The IGL may play a role in responses to light pulses and behaviour when placed in constant light (LL), as well as playing a role in non-photic phase shifts mediated by arousal or locomotor activity (Pickard et al., 1987; Biello et al., 1994; Janik and Mrosovsky, 1994; Biello, 1995; Maywood et al., 1997). The SCN also receives serotonergic input from the median raphe which appears to play a role in the magnitude of light-induced phase shifts (Bosler and Beaudet, 1985; François-Bellan and Bosler, 1992; Glass et al., 1995; Pickard et al., 1996; Pickard and Rea, 1997; Weber et al., 1998; van Esseveldt et al., 2000).

1.2.2 The pacemaker

1.2.2.1 Organisation

The SCN is composed of a pair of nuclei consisting of about 10,000 densely packed neurons at the base of the hypothalamus, above the optic chiasm. In the mouse, each nucleus can be broadly subdivided into the ventrolateral "core" SCN and the dorsomedial "shell" SCN (Abrahamson and Moore, 2001) (Figure 1.3). The terms "core" and "shell" very loosely describe the subdivisions within the SCN, which vary between species and are not clearly defined in mice (Morin and Allen, 2006). For the purpose of this report, the terms core and shell are suitable and broadly separate the ventral and dorsal SCN. The core sits adjacent to the optic chiasm and receives direct innervation from the RHT. It is characterised by the presence of the neuropeptide vasoactive intestinal polypeptide (VIP). Other neuropeptides are also present in the core SCN, although in smaller amounts, such as gastrin releasing peptide (GRP), calretinin and neurotensin (Abrahamson and Moore, 2001). The core SCN has dense projections to the shell SCN, while the shell only has sparse projections to the core (Leak et al., 1999). The shell is characterised by the presence of arginine vasopressin (AVP). Other neuropeptides present in the shell include met-enkephalin and angiotensin II (Abrahamson and Moore, 2001). The principle neurotransmitter in the SCN is GABA (Moore and Speh, 1993) and most synapses are GABA-ergic (Strecker et al., 1997) (Figure 1.3).
Individual SCN cells are able to oscillate autonomously when placed in culture (Welsh et al., 1995) and have periods ranging from 20 to 28 hours (Honma et al., 1998). Furthermore, isolated SCN neurons are able to spontaneously switch between being rhythmic or arrhythmic (Webb et al., 2009).

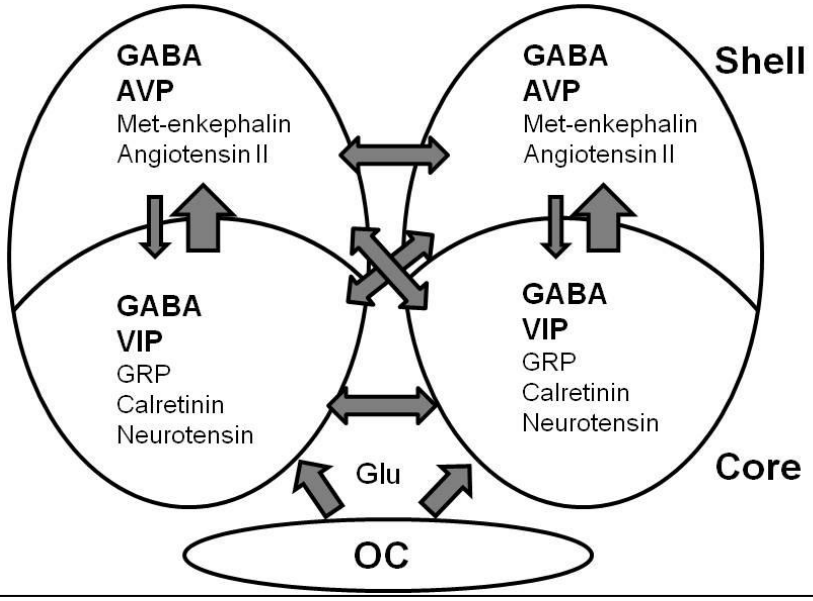

Figure 1.3: Basic organisation of the SCN. The SCN can be broadly subdivided into the core and shell. Retinal innervations reach the SCN via the RHT which signals to the core SCN using glutamate. The core SCN is mainly characterised by the presence of cells expressing the neuropeptide VIP. GRP, calretinin and neurotensin can also be found in smaller amounts. The shell SCN is characterised by the presence of cells expressing AVP. Met-enkephalin and angiotensin II can also be found in smaller amounts. The main neurotransmitter in the SCN is GABA. Projections are shown using arrows. There are dense projections from the core SCN to the shell SCN, but weak projections from the shell to the core. There are also projections between the adjacent SCN. OC - optic chiasm; Glu - glutamate; GABA - γ-aminobutyric acid; VIP - vasoactive intestinal polypeptide; GRP - gastrin releasing peptide; AVP - arginine vasopressin; SCN - suprachiasmatic nucleus.

Cells in both the core and shell SCN have been shown to oscillate but it seems that the oscillations in the core SCN, where photic information is received, are weaker than in the shell SCN. It has been hypothesised that this makes it easier for VIP-expressing cells to entrain to photic signals (Nakamura et al., 2001; Yan and Okamura, 2002; King et al., 2003). Rhythms in VIP and AVP expression have been shown to be synchronised in culture indicating that the oscillators in the core and shell are coupled (Shinohara et al., 1994). Applying antimitotic drugs results in the core and shell uncoupling from each other, indicating a separate function for these two areas of the SCN (Shinohara et al., 1995).

In DD, light exposure at any circadian time can alter the firing rate of circadian neurons (Meijer et al., 1992; Inouye and Shibata, 1994). However clock gene expression in the core SCN can only be increased after light pulses given during the subjective night (Albrecht et al., 1997; Shearman et al., 1997; Zylka et al., 1998). Meanwhile, in the shell SCN, neurons display circadian rhythmicity of *Per1*, *Per2* and c*Fos* gene expression but are not directly responsive to light pulses (Sumova et al., 1998; Hamada et al., 2001; Yan and Okamura, 2002). Thus the two broad compartments of the SCN seem to be separated according to their role. In the core SCN, photically induced gene expression is gated so as to happen only during the subjective night. Light information is then passed on to the shell SCN where oscillating neurons are reset to the external environment. The shell SCN can then send output signals to the rest of the brain and

peripheral tissues about time of day. It has also been shown that when the SCN is split into compartments, the shell SCN cannot sustain its circadian rhythmicity, indicating a crucial role for the core SCN in driving rhythms in the shell SCN (Yamaguchi et al., 2003).
It has been shown that circadian rhythms of locomotor activity can be maintained even if the SCN is in a capsule preventing neuronal connections from forming, implying a role for diffusible signals in signalling time of day to the rest of the brain (Silver et al., 1996). AVP is a key SCN output, sending projections to many areas of the brain such as the basal forebrain, thalamus and other areas of the hypothalamus such as the paraventricular nucleus (PVN), the dorsomedial hypothalamus (DMH) and the ventromedial hypothalamus (VMH) (Abrahamson and Moore, 2001). Other SCN outputs include prokineticin 2 (Cheng et al., 2002), transforming growth factor-α (TGF-α) (Kramer et al., 2001) and cardiotrophin-like cytokine (CCK) (Kraves and Weitz, 2006) which may all play a role in regulating locomotor activity behaviour. However, it seems that diffusible signals are not sufficient for maintaining circadian rhythms as abolition of efferent neuronal connections using tetrotodoxin (TTX) or knife cuts generally results in a loss of circadian rhythms (Inouye and Kawamura, 1979; Schwartz et al., 1987).

1.2.2.2 Synchronisation within the SCN

In dispersed SCN neuronal cultures, SCN cells oscillate with a wide variation of circadian periods (Welsh et al., 1995). However, in dense SCN neuronal cultures, SCN cells synchronise their periods to one another (Nakamura et al., 2001; Aton et al., 2005). Synchronisation between SCN neurons, also known as coupling, is essential to allow the SCN to entrain to the external light environment and send output signals about time of day to the rest of the brain and body (Abraham et al., 2010). It has been shown that synaptic transmission is important for SCN neuronal synchronisation as application of TTX, which disrupts Na^+-dependent synaptic transmission results in a desynchronisation of SCN neurons (Yamaguchi et al., 2003). In addition it has been shown that GABA plays an important role in synaptic transmission-mediated synchronisation of SCN neurons (Liu and Reppert, 2000). However, synaptic transmission may not be the only synchronising agent in the SCN. It has been shown that some level of neuronal synchronisation can occur even after application of TTX and this may be mediated by gap junctions (Bouskila, 1993; Jiang et al., 1997). Furthermore, VIP has also been shown to be important in maintaining synchrony among SCN neurons as knocking out *Vip* or its receptor *Vpac2* results in a loss of synchrony between neurons (Aton et al., 2005; Maywood et al., 2006b; Brown et al., 2007; Hughes et al., 2008).
Astrocytes may also contribute to the overall function of the SCN as it has been shown that neuronal somata tend to be separated by glial cells which completely enclose synaptic junctions within the ventrolateral SCN (Güldner and Wolff, 1996). Astrocytes have been shown to exhibit circadian rhythms of clock gene expression (Prolo et al., 2005) and mice lacking glial fibrillary acidic protein (GFAP - a marker for astrocytes) show altered locomotor activity behaviour when placed in LL (Moriya et al., 2000) implying that astrocytes may play an important role in the maintenance of circadian rhythms in the SCN. It has also been suggested that astrocytes may play a role in SCN clock function and in coupling oscillators in the SCN (Prosser et al., 1994; Welsh and Reppert, 1996), and application of antimitotics, which are sometimes used to reduce

the number of glial cells, results in desynchronisation between the core and shell SCN (Shinohara et al., 1995). Finally, it has been proposed the astrocytes may play a role in stabilising coupling within the SCN (Diez-Noguera, 1994).

1.2.2.3 Clock genes

SCN neuronal rhythmicity is mediated by the expression of core clock genes such as *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Clock*, *Bmal1*, *Casein kinase 1ε* (*CK1ε*) and *Rev-erbα*. Together, these genes oscillate with a 24-hour rhythm and determine the rhythmic properties of SCN neurons. To do this they form autoregulatory transcriptional/translational feedback loops, at the centre of which a CLOCK-BMAL1 protein complex drives the expression of the *Per* and *Cry* genes via E-box DNA regulatory sequences. This leads to the accumulation of PER and CRY protein complexes in the nucleus of SCN neurons which inhibit the expression of *Per* and *Cry*. PER and CRY are eventually degraded, thus disinhibiting the expression of *Per* and *Cry* and allowing the cycle to start again. This loop is stabilised by auxiliary loops involving REV-ERBα, and RORA which are also regulated by the CLOCK-BMAL1 protein complex (Maywood et al., 2006a). Figure 1.4 is a schematic diagram representing the basic feedback loops involved in the molecular circadian clock. The primary role of core clock genes is to regulate the rhythmic expression of clock controlled genes (CCGs). It has been shown that 5-10% of the SCN transcriptome is regulated by core clock genes (Ueda et al., 2002; Hastings et al., 2008), indicating an important role for the SCN in circadian regulation of gene expression.
The autoregulatory transcriptional/translational feedback loops set the period of the clock, making it crucial for all aspects of circadian rhythms. Indeed, application of the protein synthesis inhibitor cycloheximide (CHX) results in a slowing of the clock (Feldman, 1967). Furthermore, the *tau* mutation mentioned previously is a result of a point mutation in CK1ε, which plays a role

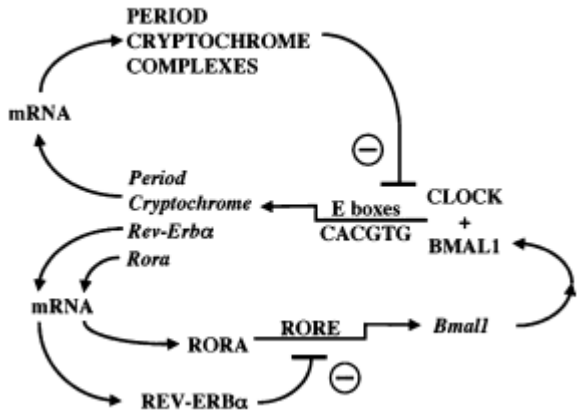

Figure 1.4: Schematic diagram of the basic feedback loops involved in the molecular circadian clock. The CLOCK-BMAL1 protein complex drives the expression of *Per* and *Cry* genes which leads to the accumulation of PER and CRY in the nucleus. These inhibit *Per* and *Cry* until the proteins are degraded and a new cycle can start. CLOCK-BMAL1 also regulates gene products involved in stabilising the core loop including *Rev-Erbα* and *Rora*. Figure taken from Maywood *et al.* (2006)

in delaying the negative feedback signal within the autoregulatory transcriptional/translational feedback loop. Thus the *tau* mutation leads to a speeding up of the clock (Lowrey, 2000). Mutations in other clock genes also result in altered periods. In the case of *Bmal1*, mutations in this gene result in an immediate and complete loss of circadian rhythms (Bunger et al., 2000). Mutations in *Per2* and *Clock* lead to a speeding up or slowing of the clock respectively, before an eventual loss in circadian rhythms (Vitaterna et al., 1994; Zheng et al., 1999). Mutations in *Cry1*, *Cry2*, *Per1* and *Per3* also result in alterations in period (Vitaterna et al., 1999; van der Horst et al., 1999; Shearman et al., 2000; Bae et al., 2001; Cermakian et al., 2001; Zheng et al., 2001). Therefore each component of the autoregulatory transcriptional/translational feedback loops is important for maintaining stable circadian rhythms.

1.2.3 Outputs

The SCN projects to many areas of the brain, including the basal forebrain, thalamus and other areas of the hypothalamus (Abrahamson and Moore, 2001) which have also been shown to oscillate (Abe et al., 2002; Guilding et al., 2009, 2010). AVP-positive fibres have been shown to project to hypothalamic areas such as the subparaventricular zone (SPZ), the PVN and the DMH, amongst other areas (Abrahamson and Moore, 2001). These areas have been shown to be involved in the integration of circadian signals coming from the SCN (Lu et al., 2001; Saper et al., 2005), the stress response (Cullinan et al., 1995; Kiyohara et al., 1995; Baffi and Palkovits, 2000), feeding behaviour (Chou et al., 2003), melatonin secretion (Kalsbeek et al., 2000) and regulation of the sleep-wake cycle (Aston-Jones et al., 2001; Chou et al., 2003). Thus, the SCN projects to areas involved in many aspects of physiological function, indicating a wide influence of the SCN on the body.

Indeed, many physiological functions have been shown to have circadian rhythms. These include locomotor activity behaviour, body temperature, heart rate, blood pressure, melatonin secretion, digestion etc. (for review see Richards and Gumz, 2012).

As mentioned earlier, output rhythms such as body temperature rhythms may function to synchronise clocks in peripheral tissue (Buhr et al., 2010). Furthermore, although the SCN is temperature compensated and thus not influenced by changes in body temperature (Buhr et al., 2010), other circadian rhythms such as wheel-running and melatonin release do feed back on the clock and affect SCN function (Yamada et al., 1990; Edgar et al., 1991; Liu et al., 1997), allowing the SCN to dynamically respond to signals from the rest of the body.

1.3 Light

1.3.1 Types of light

For the purpose of circadian experiments, animals are housed in artificial light conditions which are sometimes far removed from natural daylight. By measuring the spectral irradiance of different light sources it is possible to determine the type of light which is most similar to daylight and also how different photoreceptors are affected by different light sources (Enezi et al., 2011). The xenon arc lamp is a light source which has been shown to have a similar spectral irradiance

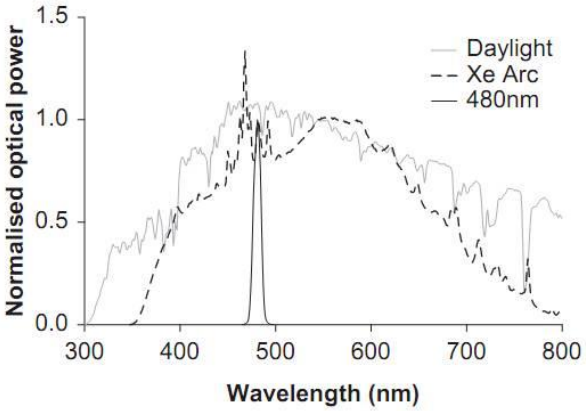

Figure 1.5: Normalised spectral irradiance profiles of daylight, an unfiltered xenon arc lamp and a 480-nm monochromatic light stimulus. The spectral irradiance of daylight was taken in Manchester, UK (53° 21' N 2° 16' W, elevation 78m) at midday under thick, low clouds, 3 weeks after the autumnal equinox. Figure taken from Enezi et al. (2011).

to daylight in Manchester (53° 21' N 2° 16' W, elevation 78m) under thick, low clouds (Figure 1.5) but for practical reasons cannot be used as a light source for long-term circadian experiments. Indeed, they produce a lot of heat and are not effective at emitting diffused light so many would be needed in an animal housing environment.

For most circadian experiments, a white fluorescent light source is used, which has a spectral irradiance profile which is vastly different from that of daylight (see Figure 1.6). For my project, the most commonly used type of light was white LED light (LED - light-emitting diode), which again has a different spectral irradiance profile (see Figure 1.6). The consequence of using these different light sources is that, as shown on the various spectral irradiance profiles, the irradiance at different wavelengths can be very similar, or very different. Different photoreceptors can therefore be affected more or less depending on what type of light is used. This is an important factor to take into consideration when comparing data from different papers.

There are also different methods to measure light intensity. The most commonly used unit is the "lux", which in most papers refers to photopic lux, or the measure of how much light the cones are receiving. This measurement is most relevant to humans, who have excellent cone vision,

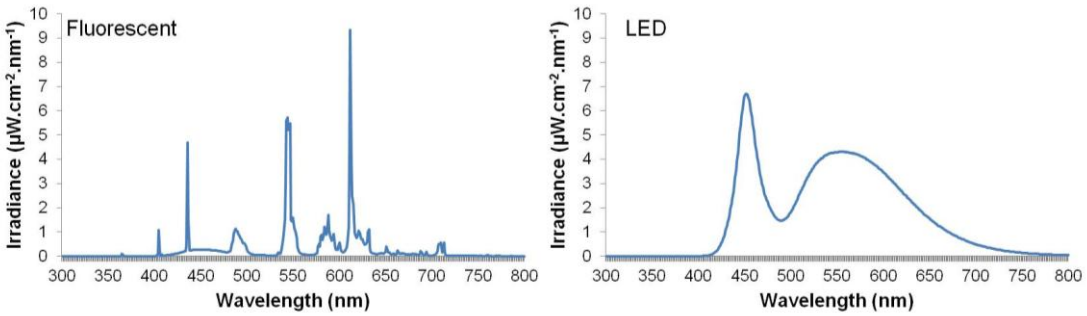

Figure 1.6: Example traces of the spectral irradiance of fluorescent light and LED light.

but is commonly used in rodent experiments, even though they have poor cone vision. A more relevant lux measurement for rodents would be the "scotopic lux" which measures illuminance levels for rods. Recently, a new lux measurement has been developed: the "melanopic lux" which measures the illuminance levels for ipRGCs (Enezi et al., 2011). These different lux measurements can be obtained by measuring the spectral irradiance of the light source and applying formulae to calculate the corrected sensitivity for different photoreceptors (for more information, visit http://lucasgroup.lab.ls.manchester.ac.uk/research/measuringmelanopicilluminance/) (Enezi et al., 2011). Light intensity can also be measured, not by measuring how it affects the subject, but by measuring the power of the light source itself (in $\mu W/cm^2$) which does not take into account the effect of different types of light on different photoreceptors. These different methods of measuring light intensity should be taken into account when comparing studies and designing new experiments.

Finally, most experiments use lighting protocols where the lights come on and are turned off suddenly, which is different to what happens in the wild. However, it has been shown that entrainment is altered when animals are subjected to dawn and dusk (Boulos et al., 2002; Comas and Hut, 2009). Therefore, care and consideration must be taken in designing experiments to take into account the possible effects of sudden or gradual changes in lighting conditions.

1.3.2 Exploring the effects of light on behaviour

Light is the most powerful *Zeitgeber* and has direct effects on locomotor activity behaviour such as causing a decrease in behaviour when lights are switched on (this behaviour is known as "masking", see below), shifting locomotor activity behaviour when light pulses are administered during the subjective night and lengthening tau in constant light (Aschoff, 1960; Daan and Pittendrigh, 1976a). Here I will introduce how locomotor activity behaviour can be used as a tool to measure the effects of light on behaviour.

As previously mentioned, there are many different output rhythms, such as melatonin secretion, body temperature rhythms and locomotor activity rhythms. The most commonly used measure of output rhythms is locomotor activity behaviour. In rodents, there are two main ways of monitoring locomotor activity behaviour. Many laboratories use wheel-running behaviour, where a wheel is placed in the cage and individually housed nocturnal rodents run in this wheel mainly during the dark period, when they are active. Wheel-running only records voluntary behaviours so behaviour such as grooming, feeding and drinking are excluded from this data, making the final data cleaner (Jud et al., 2005). However, wheel-running behaviour feeds back on the clock, which may result in effects on behaviour which are not directly due to the variable studied but instead due to wheel-running itself (Yamada et al., 1988, 1990; Edgar et al., 1991; Marchant and Mistlberger, 1996; Yamazaki et al., 1998). Another method to study locomotor activity behaviour is to measure all movement using an infra-red detection system. In this setup, animals are housed individually in cages which are crossed by infra-red beams. When an animal breaks the beam, it is recorded as movement. This method records all movement made by an animal, including grooming, feeding and drinking behaviour, resulting in more noisy data.

However, the advantage of this data is that there is no extra feedback of behaviour on the clock. Both of these methods use animals which are housed individually. It has been suggested that behaviour can be affected by whether animals have been group housed or housed individually. However, studies exploring the effects of social cues on behaviour have shown that although social effects are visible, the effects of light are much more powerful and override social cues (Davis et al., 1987; Cambras et al., 2011).

For the purpose of this study, we used locomotor activity behaviour monitored with infra-red. Therefore when describing methods of analysing this data, we will mainly be referring to this method of collecting data.

Locomotor activity behaviour is commonly presented in double-plotted actograms which allow us to visualise the behaviour of individual animals throughout the experiment (see Figure 1.7 for example).

This data can then be analysed to obtain information on the function, strength and stability of the clock. One of the first variables to be measured is the **period**. In 24-hour LD, wild-type animals should have a period of 24 hours, as they are entrained to the external light environment. Thus, if the period under LD is not 24 hours, it can be interpreted as an inability of the animal to entrain. An example of this can be seen with the tau mutant hamster which could

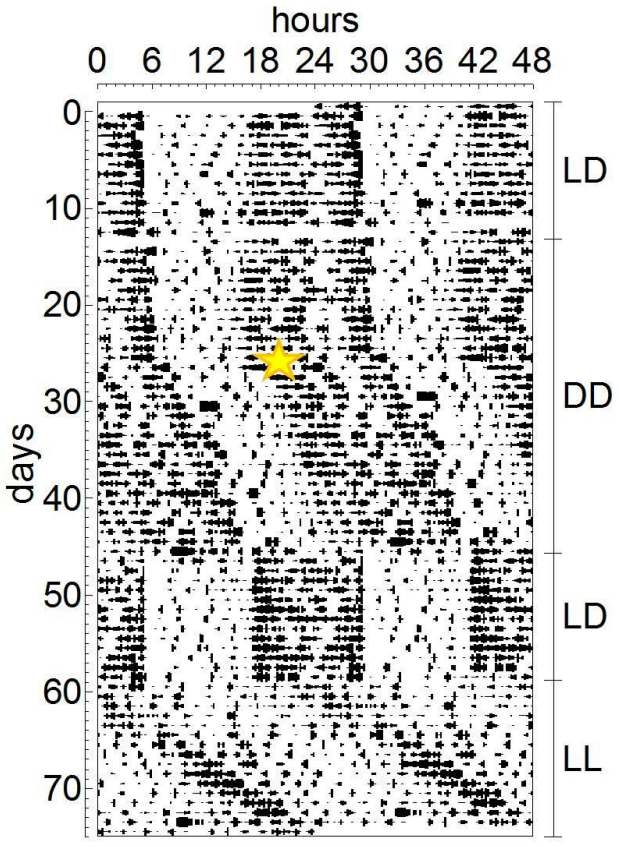

Figure 1.7: Example actogram showing locomotor activity behaviour in LD (lights on at 06:00h, lights of at 18:00h), DD and LL. When this mouse was in DD, a light pulse was administered at CT16 (shown by the yellow star) resulting in a phase delay. When placed in LL, the mouse lengthened its tau, consistent to what has been described by Aschoff's rule. DD - constant darkness; LD - 12:12 hour light-dark cycles; LL - constant light; CT - circadian time.

not entrain to LD (Ralph and Menaker, 1988). Another way of verifying that an animal is entrained is to measure the **phase angle difference (ψ)** which measures the difference between the onset of activity and the onset of the rhythm of the external environment. Entrainment is described as a stable phase angle difference between the internal and external rhythms (Jud et al., 2005). The phase angle difference can also be used when studying light cycles that are not 24 hours. These are known as T-cycles and the phase angle difference is used to measure whether an animal can entrain to various T-cycles. Finally, the phase angle is characteristic of the species and the *Zeitgeber*, so alterations in the phase angle would indicate changes in clock function (Pittendrigh and Daan, 1976b).

In constant darkness (DD) the free-running period (or **tau**) is genetically determined by the clock genes which form the autoregulatory transcriptional/translational feedback loops but can have minor variations due to previous light experience, age and environmental conditions (Pittendrigh and Daan, 1976a). In constant light (LL), tau has been shown to lengthen as light intensity increases, a phenomenon known as Aschoff's rule (Aschoff, 1960). At high intensities of LL, many animals are unable to maintain circadian rhythms of locomotor activity behaviour and become arrhythmic.

To determine the strength of the rhythm the **amplitude** of locomotor activity behaviour can be measured. This calculates the difference between the level of activity during the day (when mice are inactive) and the night (when mice are active). A high amplitude of locomotor activity behaviour means that mice restrict their movements mainly to the night, with very little movement during the day. Another method used to measure the strength of the circadian rhythm is the Fourier analysis. The Fourier analysis measures the contribution of individual simple waveforms to an overall complex waveform and how important the complex waveform is compared to the simple waveforms. In circadian rhythms, the complex waveform, which is known as the first harmonic, has the same period (T) as the overall rhythm, so typically 24 hours. The second harmonic has a period of T/2, the third harmonic has a period of T/3 etc. and these harmonics describe the ultradian content of the waveform. By measuring the **power content of the first harmonic**, the strength of the circadian rhythm and how important it is compared to the ultradian content is measured.

The stability of the circadian rhythm can be measured using the **percentage of variance**, which measures how variable the rhythm is from day to day.

Another important variable to measure when studying circadian rhythms is the **duration of the active phase** (known as α) and the duration of the inactive phase (known as ρ) and is particularly interesting to measure when the animals are free-running. Furthermore, measuring the **amount of activity during α** and comparing it to the total amount of activity can give a further indication of strength of the rhythm.

Light can affect the circadian pacemaker differently depending on time of day, and this is illustrated by the **phase response curve** (PRC) (Moore, 1997). In nocturnal animals, a light pulse given during the early subjective night results in a phase delay, as the animal interprets it as a delay in the end of the day. A light pulse given during the late subjective night results in a phase advance, as the animal interprets it as an advance in the beginning of the day. A light pulse given during the subjective day has no effect, as this is when the animal would normally be exposed to light (Daan and Pittendrigh, 1976a). There are two types of PRC: the Type 1

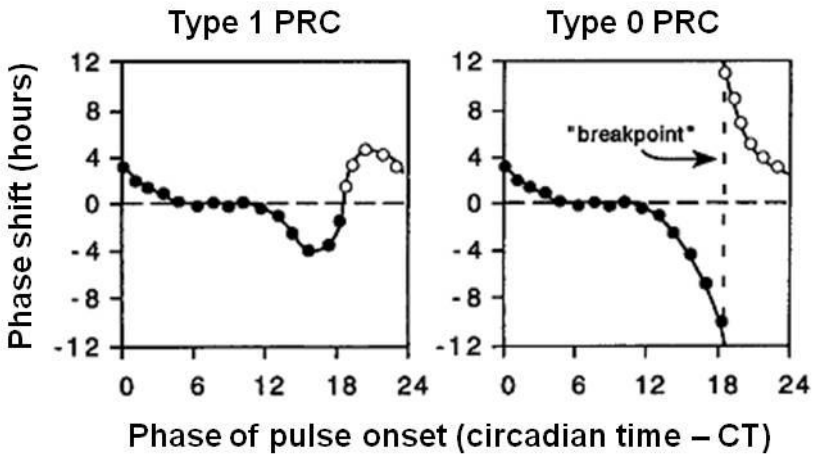

Figure 1.8: Schematic diagrams of the two types of PRC obtainable after light pulses administered at different circadian times. A light pulse given during the early subjective night (CT12 to CT18) results in a phase delay (closed circles) while a light pulse given during the late subjective night (CT18 to CT24) results in a phase advance (open circles). PRC - phase response curve; CT - circadian time. Figure modified from Johnson (1999).

PRC consists of relatively small phase shifts (less than 6 hours), while the Type 0 PRC consists of large phase shifts (Figure 1.8). The size of the phase shift can be influenced by the intensity and duration of the light stimulus (Johnson, 1999).

To create PRCs, light pulses must be administered and the subsequent response measured in DD. However, it is possible to administer light pulses to animals that have just come out of LD. This method is known as the Aschoff Type 2 protocol. The advantage of this method is that as animals have not had a chance to free-run, they are still all at the same CT and the light pulse can be administered to all animals at the same time (Jud et al., 2005). However, the disadvantage of this method is that the resulting phase shifts are usually quite small. Indeed, it has been shown that the longer an animal is kept in DD before a light pulse is administered, the longer the phase shift, as animals have had more time to dark adapt (Refinetti, 2001, 2003, 2007). In the Aschoff Type 2 protocol animals have not had a chance to dark adapt, hence the small response. In the Aschoff Type 1 protocol, on the other hand, animals are placed in DD for a few weeks to dark adapt and their responses to light pulses are therefore usually larger. As they are all free-running, the CT of each individual animal must be calculated and light pulses given to each animal at their individual CT. This generally means that the cage will have to be moved to a light area to administer the light pulse as other animals in the same room may not be at the same CT. Thus the effects of the mechanical stimulus must be taken into account, on top of the effect of the light pulse (Jud et al., 2005).

Light can have parametric and non-parametric effects. Parametric effects are generally defined as effects caused by long-term light signals such as LL (also known as tonic effects). Non-parametric effects are defined as effects caused by brief light signals such as light pulses (also known as phasic effects) (Pittendrigh and Daan, 1976a). For many years the main school of thought was that entrainment was mediated by non-parametric effects, but it is now believed that both parametric and non-parametric effects mediate circadian entrainment (Daan, 2000).

Finally, it is important to mention the phenomenon of masking, which is defined as an exogenous influence on the measured rhythm (i.e. not mediated by the SCN) (Mrosovsky, 1999). Light can have an important masking effect on the measured rhythm. In nocturnal animals, negative masking is described as a decrease in the amount of activity as a result of light exposure and positive masking is described as an increase in the amount of activity in the absence of light exposure. Masking can occur in the absence of a genetically functional clock (van der Horst et al., 1999) or an intact SCN (Redlin and Mrosovsky, 1999) and it is argued that masking may contribute to entrainment (Mrosovsky, 1999). It is therefore important to be aware of it so as to analyse locomotor activity data as accurately as possible (some evidence of masking can be seen in Figure 1.7).

1.3.3 The effects of light on the SCN.

As previously discussed, when animals are placed in DD, light pulses given at different times affect the SCN in different ways. Here I will mainly discuss the effects of a light pulse given during the subjective night on the pacemaker.

Photic signals from the retina are transmitted via the RHT to the SCN. The main neurotransmitter involved in signalling between the RHT and the SCN is believed to be glutamate (Ding et al., 1994; Shirakawa and Moore, 1994) acting via N-methyl-D-aspartate (NMDA) and non-NMDA receptors (Abe et al., 1992; Rea et al., 1993). NMDA has been shown

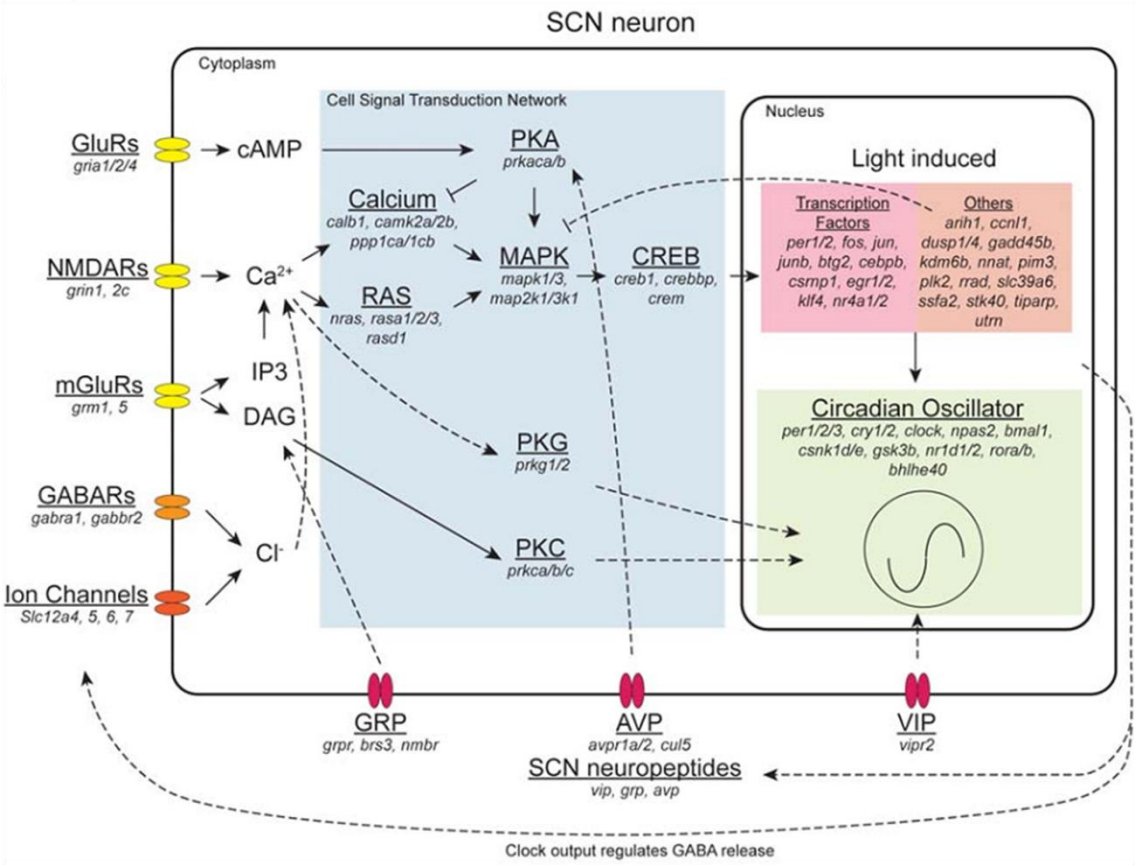

Figure 1.9: Schematic diagram of the light signalling pathway within an SCN neuron. Definitive gene functions are shown in solid arrows while implied gene functions are shown in dashed arrows. Figure taken from Zhu et al. (2012).

to mimic the phase shifting effects of light (Mintz and Albers, 1997). The activation of NMDA receptors in the SCN leads to intracellular calcium release (Colwell, 2001). Intracellular calcium release after a light pulse leads to the phosphorylation of the mitogen-activated protein kinase (MAPK) signalling pathway (Obrietan et al., 1998). This in turn leads to the phosphorylation of the transcription factor cyclic adenosine monophosphate (cAMP) response element-binding (CREB) (Obrietan et al., 1998) which binds to the cAMP response element (CRE) in the promoter region of photically inducible clock genes such as *Per1* and *Per2* as well as immediate early genes such as *cFos* (Ginty et al., 1993; Obrietan et al., 1999; Gau et al., 2002). This is one of the main pathways activated after a light pulse. Figure 1.9 illustrates this pathway and shows others also involved in the response after a light stimulus.

The ability of the SCN to respond to light differently depending on time of day is a result of its ability to gate its responses to light. One of the first factors to be affected is the effectiveness of glutamate on NMDA receptors, with increased NMDA receptor activity during the subjective night (Colwell, 2001; Pennartz et al., 2001). Within the photic signalling pathway, some elements are important in both the response during the early night and late night. Thus, inhibiting the MAPK signalling pathway results in an inability to shift after light pulses both in the early and late night (Butcher et al., 2002; Hainich et al., 2006). Furthermore, CREB targets are only inducible during the subjective night (Obrietan et al., 1999). Light pulses given at any time of the subjective night result in an increase in cFOS expression but this does not occur if light pulses are administered during the subjective day (Colwell and Foster, 1992). The core clock gene *Per1* is upregulated after both early and late night light pulses (Albrecht et al., 1997) while *Per2* is only upregulated after a light pulse given during the early night (Albrecht et al., 1997; Zylka et al., 1998).

After a light pulse, neurons in the core SCN are activated in response to glutamate release by the RHT. These then send signals to oscillating neurons within the shell SCN which can adjust their oscillations to the external environment.

In normal situations, cells within the SCN are tightly synchronised (or coupled). However, exposure to LL can result in a desynchronisation between SCN neurons (Ohta et al., 2005) which may reflect the effects of LL on behaviour, as it has been shown that LL often leads to "splitting" of rhythms or arrhythmicity.

Here I have shown that the SCN is well prepared to respond to light stimuli in order to allow for proper entrainment. However, light can also have a detrimental effect if presented constantly, particularly on the retina.

1.3.4 The effects of light on the retina

In the retina, environmental light can sometimes cause damage. This is especially the case in albino animals where the lack of pigment in the eye results in a loss of protection for the retina. Indeed, pigment absorbs any light which has not been picked up by the retina, and prevents light bouncing back through the retina. Furthermore, a lack of pigment means that light can enter the eye not only through the pupil (which in pigmented mice can constrict to up to 0.1mm) but also through other parts of the eye, resulting in more light available to damage the eye (Rapp and Williams, 1980). Retinal damage occurs in stages, starting in the rods, followed by

the cones. This includes changes in rod structure, reduced rhodopsin levels and a loss of rhythmic outer disc shedding. After damage to the outer segments of the retina has occurred, damage to the inner segments of the retina begins (Lanum, 1978). As damage occurs first in the outer layers, measuring the outer nuclear layer of the retina is a good measure of how much damage there is in the retina.

Albino rats are extremely susceptible to light damage. Indeed, four strains of albino rat (CDC, CDF, Sprague-Dawley and Wistar) kept under fluorescent light between 1200-2500 lux covered in green plastic for only 24 hours were found to have significant and persistent damage to their retinas. However, Long-Evans rats (a pigmented strain) kept in the same conditions did not suffer damage, even when their pupils were dilated by atropine (Noell et al., 1966). Indeed, Long-Evans rats kept in LL for up to 16 days did not suffer retinal damage unless their pupils were dilated, while Sprague-Dawley rats suffered light-induced retinal damage within one or two days (Rapp and Williams, 1980). Similarly, pigmented mice have been shown to be highly resistant to retinal damage. Indeed, to induce retinal damage, the pupils of C57BL/6J mice had to be dilated and the mice subjected to light intensities of 15000 lux (10 times brighter than the light intensities used for my studies) (Grimm et al., 2000). Strain also seems to play an important role in the level of light-induced retinal damage. Indeed, three weeks in fluorescent light at 1500 lux caused different levels of damage depending on the strain of albino mouse. In particular, the C57BL/6J-c^{2J} strain which is genetically identical to the C57BL/6J mouse except for being homozygous in the mutant albino gene was shown to be highly resistant to light-induced retinal damage (LaVail et al., 1987a, 1987b).

This data indicates that albino animals are highly susceptible to retinal damage and this must be taken into account when choosing strains for experiments involving light conditions which may be damaging. In albino animals, this seems to be quite low light intensities as damage has been shown to be induced in LD of only 50 lux (Terman et al., 1991).

The retina has been shown to have its own circadian clock which can oscillate independently of the SCN (Tosini and Menaker, 1996; Tosini et al., 2008). The retinal clock may contribute to circadian rhythmicity as the behaviour of hamsters raised in DD was found to be different to that of hamsters that were enucleated one day after birth, with a wider range of taus in the enucleated hamsters (Yamazaki et al., 2002). Indeed, there is evidence that the eye contributes to some aspects of the circadian phenotype (Lupi et al., 1999; Lee et al., 2003).

1.4 The development of the circadian system

A summary of the development of the circadian system can be found in Figure 1.10.

1.4.1 The retina and retinohypothalamic tract

In the retina, development occurs at different rates for different photoreceptors and multiple components are needed before photoreceptors are fully functional. Thus, rod cells differentiate at embryonic day 12 (E12) (Young, 1985) but the rod photopgiment, rhodopsin, is not expressed until postnatal day 5 (P5) (Bibb et al., 2001). Similarly, cone cells differentiate at E10

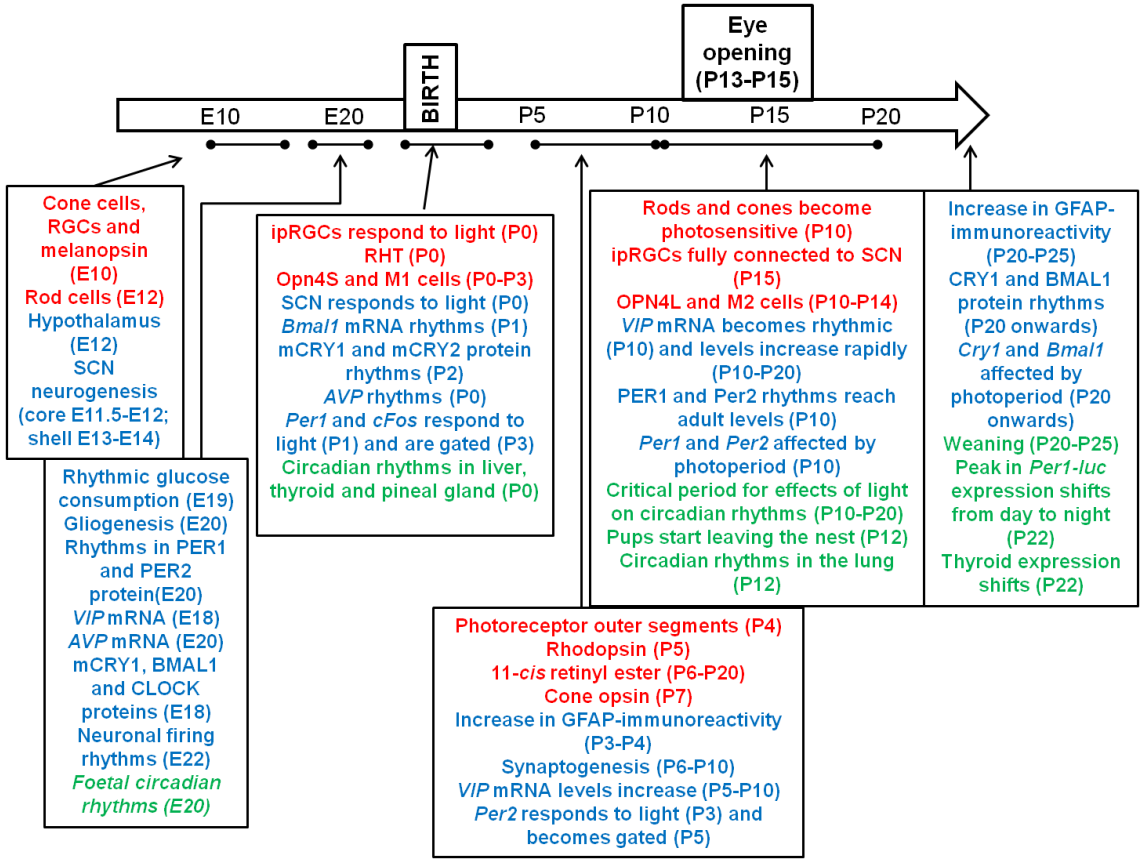

Figure 1.10: Development of the circadian system. Summary of the different stages of development in the retina (red), SCN (blue) and circadian rhythms (green). E - embryonic day; P - postnatal day; RGC - retinal ganglion cell; SCN - suprachiasmatic nucleus; VIP - vasoactive intestinal polypeptide; AVP - arginine vasopressin; RHT - retinohypothalamic tract; GFAP - glial fibrillary acidic protein; Opn4S and Opn4L - melanopsin isoforms; M1 and M2 - melanopsin cell subtypes.

(Young, 1985) but the cone photopigment, cone opsin, is not expressed until P7 (Fei, 2003). Photoreceptor outer segments, which detect light begin to develop from P4 but are not fully developed until P20 (Olney, 1968; Fei, 2003). Furthermore, these photopigments require 11-*cis* retinaldehyde to function. Its precursor, 11-*cis* retinyl ester, only begins to accumulate in the retina from P6 up until P20 (Carter-Dawson et al., 1986). From P10, enough of each element has developed to allow rods and cones to begin to be photosensitive (Tian and Copenhagen, 2003) but full photosensitivity does not occur until P20, with the eye opening at around P13-15 (Harada et al., 1998).

Retinal ganglion cells differentiate at E10 (Drager, 1985) and, unlike rhodopsin and cone opsin, melanopsin, the photopigment expressed in ipRGCs, begins to be expressed from E10.5, coinciding with the appearance of the first RGCs (Tarttelin et al., 2003; Sekaran et al., 2005). By P0, 13% of ipRGCs are already responsive to light and the RHT, which links the retina to the SCN, is already present (Speh and Moore, 1993; Hannibal and Fahrenkrug, 2004; Lupi et al., 2006; McNeill et al., 2011) although the level of connectivity will not reach adult levels until P15 (Speh and Moore, 1993; Seron-Ferre et al., 2001). It has further been demonstrated that the SCN is able to receive and respond to light information from the retina from P0 (Sekaran et al., 2005; Lupi et al., 2006). Furthermore it has been shown that different melanopsin isoforms

develop at different times (Hughes et al., 2012). Opn4S is present throughout postnatal development, with an increase in protein expression between P0 and P3, reaching adult levels at P10, while Opn4L shows an increase in expression at P14 (Hughes et al., 2012). Thus, M1-type melanopsin cells expressing both Opn4S and Opn4L are present from birth and are the dominant ipRGC subtype while M2-type melanopsin cells expressing only Opn4L mature between P10 and P14 (Hughes et al., 2012). Interestingly, in melanopsin knock-out mice, functional innervations of the SCN do not occur until P14, when rods and cones become photosensitive, further indicating that rods and cones also play a role in innervating the SCN (Sekaran et al., 2005; Lupi et al., 2006).

1.4.2 The SCN

In mice, the hypothalamus begins to form at around E12. SCN neurogenesis occurs between E12 and E14.5, with the core forming first from E11.5 to E12 followed by the shell between E13 and E14. Cells in the posterior SCN develop at the same time as the shell while cells in the anterior SCN are produced last, with neurogenesis completed 5 days prior to birth (Kabrita and Davis, 2008). Synaptogenesis, on the other hand, occurs mainly postnatally, primarily from P6 to P10, reaching adult levels at about P10 (Moore and Bernstein, 1989; Moore, 1991).
Gliogenesis has been shown to begin from E20, when GFAP-immunoreactivity is first detected. There is a rapid increase in GFAP-immunoreactivity from P3 to P4, then again from P20 to P25 (Munekawa et al., 2000). Interestingly, it seems that the development of astrocytes may be mediated by the RHT as enucleation at birth results in a lack of increase in GFAP-immunoreactivity from P20 to P25 (Munekawa et al., 2000).
Oscillations in the SCN have been shown to occur before birth. Rhythmic glucose consumption has been found in the SCN before synaptogenesis occurs, from E19 onwards (Reppert and Schwartz, 1984) and the SCN shows rhythmic firing patterns before birth, at E22 (Shibata and Moore, 1987). In the mouse, circadian rhythms of PER1 and PER2 protein expression have been found from E18, with the amplitude of the rhythms reaching adult levels between P2 and P10 (Ansari et al., 2009). mCRY1 protein is found at E18, but only becomes rhythmic after birth, while mCRY2 protein is only detected from birth onwards, when they have also been found to be rhythmic (Ansari et al., 2009). BMAL1 and CLOCK protein have also been found from E18 onwards in the mouse SCN, but rhythmic expression was not seen until adulthood (Ansari et al., 2009). In rats, *Bmal1* mRNA rhythms have been found from P1 onwards (Kovacikova et al., 2006; Sumova et al., 2006b). Interestingly, a recent study has shown that there does not seem to be circadian oscillations of clock genes in peripheral tissues of the embryo, but that the mechanisms for circadian oscillations are in place as, if embryonic peripheral tissues are placed in culture, they show circadian rhythms of PER2::LUC expression (Dolatshad et al., 2010). Therefore it seems that during embryonic development, clock gene development occurs but is not rhythmic within the whole tissue until some entraining signal occurs during postnatal development.
Neuropeptide expression also oscillates. *AVP* mRNA is detected from E20 and shows rhythmic expression from birth (Kovacikova et al., 2006; Ansari et al., 2009). *VIP* mRNA is detected from E18, but does not shown circadian rhythms until P10. The levels of *VIP* mRNA increase

between P5 and P10, with a sharp increase between P10 and P20, coinciding with the time when photic signals reaching the SCN increase (Ban et al., 1997).
Another key characteristic of some clock genes and immediate early genes is their ability to respond to light. It has been shown that *Per1* and *cFos* are responsive to light pulses from P1. Furthermore, the responses to light pulses become gated (they only respond to light pulses during the subjective night) from P3 (Matejů et al., 2009). Light responsiveness of *Per2* develop slightly later, become apparent from P3 and not fully gated until P5 (Matejů et al., 2009). It has also been shown that photoperiod (LD 16:8 or LD 8:16) affects *Per1* and *Per2* expression from P10 onwards, *Cry1* expression from P20 onwards and *Bmal1* expression after weaning, indicating that full entrainment to external light environment only occurs in adult animals (Kováciková et al., 2005).

1.4.3 Circadian rhythms

Circadian rhythms have been shown to already exist in the foetus. These rhythms are very rudimental and are not entrained by light directly but instead are entrained by the maternal circadian system (Reppert and Schwartz, 1983; Davis and Gorski, 1988).
After birth, circadian rhythms gradually get stronger and maternal entrainment still seems to play a role in synchronising circadian rhythms (Davis and Gorski, 1985, 1988; Bellavía et al., 2006; Sumova et al., 2006a), but light has been shown to play a more important role in the development of circadian rhythms (Cambras et al., 1997).
Circadian rhythms in peripheral tissues such as the liver, thyroid and pineal gland have been found to be rhythmic from birth (Yamazaki et al., 2009). Interestingly, the peak in *Per1-luc* expression in the liver shifted from day to night at around P22, coinciding with weaning. This data indicates that the liver is entrained to feeding time, as pups tend to nurse during postnatal development during the day when their mother is on the nest, then switch to eating solid food during the night after weaning (Yamazaki et al., 2009). A similar shift in peak *Per1-luc* expression in the thyroid was found, indicating a possible role of food in entraining the thyroid (Yamazaki et al., 2009). Rhythms were found in the adrenal gland throughout development, with an increase in amplitude from P25. Rhythms in the lung were not found until P12, coincident with the time that pups start leaving the nest (Yamazaki et al., 2009).
As mentioned above circadian rhythms are present from birth and are entrained both by maternal rhythms at the very start of life before being more influenced by light in later developed. Now the effects of light on the development of the circadian system will be discussed.

1.5 Effects of early light environment on the development of the circadian system

1.5.1 Locomotor activity behaviour

After a few days or weeks in LL, the circadian rhythms of body temperature, wakefulness, locomotor activity behaviour and plasma corticosterone in rats become arrhythmic (Honma and Hiroshige, 1978; Eastman and Rechtschaffen, 1983). However, when rats were placed in LL

from birth, they began to show circadian rhythms of locomotor activity about 10 days after weaning (P35) (Cambras and Diez-Noguera, 1991). In contrast rats raised in DD from birth until weaning (P21) then placed in LL as adults became behaviourally arrhythmic (Cambras et al., 1997, 1998) and this response was not influenced by maternal rhythms. Both rats raised in LL or DD were able to entrain to LD and showed free-running rhythms in DD, with τ shorter than what has been recorded in rhythmic rats in LL (24.4 hours in DD *vs.* 25.8 hours in LL). Furthermore, rats raised in LL were able to retain circadian rhythms when placed back in LL for up to a year, indicating that early light environment has long term effects on locomotor activity behaviour (Cambras et al., 1998).

Next, rats were exposed to LL for different durations during postnatal development. It was found that rats exposed to LL for less than 12 days during postnatal development became arrhythmic when placed in LL as adults, while being exposed to LL for more than 12 days during postnatal development resulted in rats that were able to maintain circadian rhythms when they were placed in LL as adults. This same study showed that rats raised in LL during postnatal development had longer phase shifts after a light pulse administered at CT15 than rats raised in DD. Furthermore it was shown that light exposure must occur between P8 and P20 to see the effects of LL during postnatal development on locomotor activity behaviour later in life (Canal-Corretger et al., 2000). A later study was able to narrow down the critical period to P10 to P20, demonstrating that rats exposed to LL during this time period were able to maintain circadian rhythms of locomotor activity when exposed to LL as adults (Canal-Corretger et al., 2001a). Interestingly, the critical period coincides with the time in postnatal development when the SCN receives increasing amounts of light information from the retina (see Figure 1.10), demonstrating the importance of light in this phenomenon.

Light during postnatal development has not only been shown to affect the ability to remain rhythmic in LL but also the percentage of variance in DD, as rats raised in DD had a significantly higher percentage of variance compared to rats raised in LL (Canal-Corretger et al., 2001a). Furthermore the power content of the first harmonic in LD was higher in rats raised in DD compared to rats raised in LL (Canal-Corretger et al., 2003a). Interestingly, in T-cycles of 25 hours or less (down to T22), rats raised in LL had a higher phase angle of entrainment than rats raised in DD, indicating that rats raised in LL were able to entrain to T-cycles easier than rats raised in DD (Canal-Corretger et al., 2003b). The ability to remain rhythmic in LL implies that rats raised in LL were able to cope better with this light environment. However, in LD the data seems to indicate that rats raised in DD had stronger rhythms of locomotor activity behaviour than rats raised in LL as they had a higher percentage of variance and a higher power content of the first harmonic. Furthermore, the ability to entrain to T-cycles may be associated with weaker coupling between SCN neurons (Abraham et al., 2010) so it is possible that rats raised in DD also had stronger coupling within the SCN compared to rats raised in LL.

Mice are less susceptible to becoming arrhythmic when placed in LL as adults. However, early light environment has also been shown to affect locomotor activity behaviour when mice were placed in LL as adults. Indeed, albino mice raised in DD had longer taus than mice raised in LL (Canal-Corretger et al., 2001b). Aschoff's rule states that in LL, the higher the light intensity the longer the tau (Aschoff, 1960). Here all the mice were housed in the same intensity of LL, yet the mice raised in DD displayed longer taus, implying that they were detecting a higher light

intensity than the mice raised in LL and may therefore have been more sensitive to light. These albino mice also had decreased amplitudes of locomotor activity if they were raised in LL compared to if they were raised in DD. As these mice were albino, and we have seen that light can cause damage to the retinas of albino animals, it is possible that mice raised in DD seemed more sensitive to light than mice raised in LL because mice raised in LL suffered light-induced retinal damage. Furthermore, a study in C57BL/6J mice (a pigmented strain) showed that the amplitude of locomotor activity was higher in mice raised in LL compared to mice raised in DD, an opposite response to what was shown in the albino mice (Smith and Canal, 2009). As pigmented mice are much less susceptible to retinal damage than albino mice, it is possible that the difference between the two experiments is due to different levels of retinal damage as a result of light during postnatal development.

An earlier study on the contribution of the retina to the effects of early light environment on the SCN found that enucleating adult mice raised in DD, LD or LL still resulted in mice raised in DD having seemingly stronger rhythms than mice raised in LL, indicating that even if retinal damage had occurred due to light during postnatal development, early light environment still seemed to affect circadian rhythms in adult rats (Canal-Corretger et al., 2003b).

1.5.2 The hypothalamus

Early light environment has been shown not only to affect locomotor activity behaviour but also hypothalamic physiology. Indeed, expression of AVP and VIP was shown to be lower in C57BL/6J mice raised in LL compared to mice raised in DD, indicating that the processing of photic information in the SCN may have been altered by early light environment (Smith and Canal, 2009). The same study also found that mice raised in DD had higher levels of AVP in the PVN and the supraoptic nucleus (SON) compared to mice raised in LL, indicating that the effects of early light environment may be more far-reaching than initially thought. This was confirmed by a further study which showed that tyrosine hydroxylase (TH) and cFOS expression were affected by early light environment in other areas of the hypothalamus such as the PVN, anterodorsal preoptic nucleus (ADP), SPZ and DMH and that dopamine may play a role in mediating these effects (see Appendix 2 - Brooks et al., 2011). In the SCN it has also been shown that astrocytes are affected by early light environment. Indeed, C57BL/6J mice raised in LL had reduced GFAP expression compared to mice raised in DD. However, mice raised in LL had higher numbers of GFAP-expressing cells compared to mice raised in DD, indicating that early light environment may play a role in structural remodelling of astrocytes (Canal et al., 2009). As VIP may play a role in coupling within the SCN, it is possible that early light environment may alter coupling strength in the SCN. Alternatively, it may alter how photic information is received, processed and communicated within the SCN. Altered AVP expression may result in altered output signals from the SCN which could be mediating the changes we see in locomotor activity behaviour, but also could affect signalling to the rest of the brain and body. Indeed, the alterations shown in TH and cFOS expression in the hypothalamus seem to indicate that early light environment affects other areas of the hypothalamus which may play a role in the stress response and feeding behaviour, and this may be mediated by changes in AVP signals to the rest of the brain. Finally, altered GFAP expression in the SCN may affect coupling strength

within the SCN, as astrocytes have been hypothesised to play a supporting role in coupling (Diez-Noguera, 1994).

1.5.3 The retina

As previously mentioned it is possible that light during postnatal development (such as exposure to LL) may cause retinal damage. Indeed, we have already seen that albino animals are highly susceptible to light-induced retinal damage, while pigmented animals are not. A recent study has shown that light during postnatal development can affect the amount of melanopsin-expressing cells in the retina of albino mice. Indeed, exposure to DD during postnatal development resulted in an increase in the number of melanopsin positive cells, with mice raised in LD also showing an increase, but reduced compared to mice raised in DD. In contrast, mice raised in LL showed no increase in the number of melanopsin-expressing cells in the retina. Further studies showed that it was in fact the M2 melanopsin cells which were most affected by light during postnatal development (González-Menéndez et al., 2010b). However, in C3H/He mice (a pigmented strain) the number of melanopsin-positive cells in the retina was not affected by early light environment (González-Menéndez et al., 2010a, 2010b). Furthermore, a recent study in our lab showed that early light environment did not affect cFOS expression in the SCN of C57BL/6J mice after a light pulse (see appendix 2 - Brooks et al., 2011), implying that the amount of light information reaching the SCN was the same in all three groups. These results highlight a differential response of the retina to early light environment depending on whether mice are albino or pigmented and show the importance of determining how early light environment affects the retina and how this may contribute to the effects of early light environment on locomotor activity behaviour and hypothalamic morphology.

1.6 Aims

The aim of this project was to determine how early light environment affects the development of the photic responses of the circadian system. The aims of this project thus followed the basic structure of the circadian system:

1. The first aim of this project was to look at how early light environment affects the development of the retina. There has been much speculation about how the retina is affected by early light environment, but no definitive study on the subject. Thus the aim was to determine how the functionality and morphology of the retina of both albino and pigmented mice was affected by early light environment.
2. Secondly, it has been shown that behaviour in LL is a key factor affected by early light environment, so the second aim of this project was to determine how early light environment affected the locomotor activity behaviour of C57BL/6J mice when placed in LL as adults.
3. Based on the differential responses of albino and pigmented mice to early light environment, the third aim of our project was to determine whether neuropeptide and GFAP expression in the SCN of CD1 mice was altered by early light environment and/or strain.

4. The photic response of the circadian system is characterised by the ability of the circadian system to respond to light pulses. Therefore the fourth aim of this project was to determine how early light environment affected the response of C57BL/6J mice to phase delaying and phase advancing light pulses. Light pulses also induce the photic signalling pathway in the SCN so the effects of early light environment on the activation of the photic signalling pathway in the SCN after a phase delaying light pulse were also studied.
5. Recent advances in molecular biology have allowed for real-time monitoring of clock gene expression, giving us a clearer picture of circadian oscillations of clock genes. Therefore the fifth aim of this project was to look at how early light environment affected PER2::LUC rhythms in the SCN in order to examine the function of the molecular clock. To explore whether being placed in LL as adults altered clock gene expression in the SCN, PER2::LUC rhythms were monitored in mice that had been in LL for a prolonged duration.
6. Finally, it is known that peripheral tissues oscillate and that early light environment affects AVP, TH and cFOS expression in other areas of the hypothalamus. The final aim of this project was therefore to study PER2::LUC oscillations in peripheral tissues to determine whether early light environment may have affected other aspects of physiology such as metabolism and stress.

2. Materials and methods

2.1 Animals

All experimental procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Throughout the experiment mice were kept under a controlled ambient temperature of 21°C (±2°C) with water and food (B&K Universal, Hull, UK) available ad libitum. Cages were changed every fortnight at random times during the day. Infrared goggles were used in the routine maintenance of the animals in the dark.
Unless otherwise stated cages were placed in Light Tight Cabinets specially modified to be used in circadian studies (Techniplast, London, UK). Mean light intensity at cage floor level was 330µW/cm^2 provided by white LED (LED - light-emitting diode) light (VLM, Relco Group, Italy). Each experiment began with the same basic design. Pregnant female mice were placed in constant darkness (DD), 12:12 hour light-dark cycles (LD) or constant light (LL) two or three days before they were due to give birth. Pups were raised in these light conditions from day of birth (postnatal day 0 – P0) till P21 and were weaned between P21 and P25. As adults all three groups followed the same experimental design in order to determine how early light environment affects various aspects of the circadian system.

2.2 Light measurements

Light measurements over the duration of the experiment were performed using three different instruments. In a first instance, irradiance was measured in µW/cm^2 using an optical power meter (Macam PM203, Macam Photometrics, Livingstone, Scotland). We also measured photopic lux using a light meter (DT-1300, CEM, Shenzhen, China). Finally, we measured the spectral irradiance profiles in all light environments used between 300 and 800nm in W/m^2/nm with a spectroradiometer fitted with a cosine diffuser (Bentham Instruments, Reading, UK). The spectral irradiance allowed us to calculate the melanopic illuminance using the function provided by the Lucas lab and available online at http://lucasgroup.lab.ls.manchester.ac.uk/research/measuringmelanopicilluminance/ (Enezi et al., 2011). Using similar functions kindly provided by Tim Brown, we were also able to extrapolate the scotopic and photopic illuminance from the spectral irradiance.

2.3 Electroretinography

The electroretinography (ERG) experiment was conducted as previously described (Cameron et al., 2008a; Cameron and Lucas, 2009). Mice were dark-adapted overnight before beginning the ERG experiment. All work during this experiment was performed under dim red light (<0.2µW/cm^2) between circadian time 2 (CT2) and CT10.
Mice were anaesthetised with an initial dose of 70mg/kg ketamine (Ketaset, Fort Dodge Animal Health Ltd., Southampton, UK) and 7mg/kg xylazine (Rompun, Bayer HealthCare, Newbury, UK) given intraperitoneally (i.p.) followed by a top-up of 72mg/kg ketamine and 5mg/kg xylazine

given subcutaneously (s.c.) to maintain a sufficient level of anaesthesia. Once anaesthetised body temperature was maintained by placing the mice in a custom made heat chamber connected to a constant temperature water source. Mydriatics, 1% tropicamide and 2.5% phenylephrine, and 0.3% hypromellose (FDC Ltd, Fareham, UK) were applied to the eye before placing a corneal electrode contact lens on the left eye. A silver wire bite bar was used to stabilise the head and act as ground. A reference needle electrode was placed in the right cheek, 5mm from the base of the contralateral eye, to prevent signal interference. The electrodes were connected via a signal conditioner (Model 1902 Mark III, CED, Cambridge, UK; signal differentially amplified, x3000, and band-pass filtered 0.5 to 200Hz), and digitised (model 1401, CED) to a Windows PC (sampling rate 2kHz) running the Signal 2.16 software (CED). Before recording began, stability of the electrodes was confirmed over a period of 10 minutes. Dark-adapted irradiance responses were elicited by white flash stimuli from a xenon arc source (Cairn Research Ltd, Faversham, UK) reflected in a custom-made Ganzfeld dome and attenuated with neutral density filters (Edmund Optics, York, UK) to obtain irradiances ranging from -4.9 to 3.1 $\log_{10}\mu W/cm^2$. An electrically controlled mechanical shutter (Cairn Research Ltd) was used to apply a series of single 15ms flashes, starting 200ms after sweep onset. Table 2.1 describes the sequence of stimuli applied to the mouse eye over the course of the dark-adapted ERG experiment.

Figure 2.1 shows an example waveform obtained during an ERG experiment. The a-wave (when present) was quantified from the trough to the baseline in the unfiltered traces. To measure the amplitude of the b-wave, the ERG wave was filtered by removing any signal below 5Hz, thereby removing the influence of oscillatory potentials. The b-wave amplitude was quantified by summing the absolute a-wave (unfiltered) amplitude and the b-wave amplitude when filtered. The implicit time for the a-wave was measured from the stimulus onset to the trough of the a-wave. The implicit time for the b-wave was measured from the stimulus onset to the peak of the b-wave.

	Light intensity ($\log_{10}\mu W/cm^2$)	**Number of flashes applied**	**Time between flashes (seconds)**
1	-4.9	30	1.5
2	-3.9	30	1.5
3	-2.9	15	3
4	-1.9	15	3
5	-0.9	15	6
6	0.1	8	15
7	1.1	6	25
8	2.1	6	35
9	3.1	6	35

Table 2.1: Sequence of stimuli applied to the eye of the mouse during the dark-adapted electroretinography experiment.

Cone-driven activity (photopic ERG) was isolated by measuring the responses to a series of bright white flashes (Grass Model PS33 Photic Stimulator, Astro-Med, Inc., West Warwick RI, fitted with a 400nm high pass filter, 10µs duration, peak corneal irradiance 1logµW/cm^2) applied at a frequency of 0.75Hz against a uniform white background light (metal halide source) of sufficient intensity (400µW/cm^2) to saturate rods but not cones. The background light was left on and the ERG was recorded continuously at 0.75Hz for 20 minutes. Average waveforms were obtained for every 25 frames and filtered (low pass 5Hz) to exclude oscillatory potentials. The

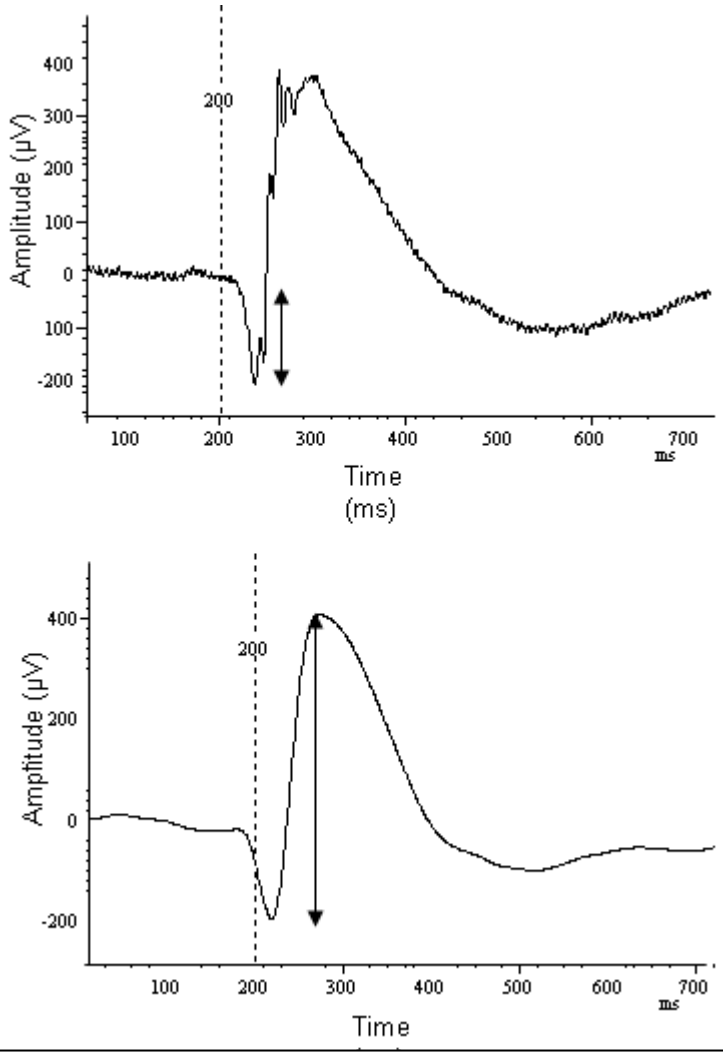

Figure 2.1: Examples of waveforms obtained during electroretinography (ERG) experiments. The arrows indicate the amplitude of the wave measured. The marker at 200ms indicates the moment the flash of light was administered to the mouse. **A:** The unfiltered waveform is used to calculate the amplitude and implicit time of the a-wave (shown by the arrow). The implicit time is measured from the 200ms point to the maximum of the wave. **B:** The filtered waveform is used to calculate the amplitude and implicit time of the b-wave (shown by the arrow) for both the dark-adapted ERG and the photopic ERG. In the dark-adapted ERG the value of the a-wave is deducted from the total b-wave amplitude. In the photopic ERG the full amplitude of the b-wave is used as the a-wave cannot be measured due to the artefact produced by the light stimulus. The implicit time of the b-wave is measured from the 200ms point to the maximum of the wave.

peak of the b-wave was identified from the averaged traces and its amplitude and implicit time (from the start of the flash) were calculated.

2.4 Pupil light reflex

Pupillometry was conducted as previously described (Lall et al., 2010; Enezi et al., 2011). Mice were entrained to a 12:12 hour light-dark cycle then dark-adapted for 1 to 1.5 hours prior to the beginning of the experiment. Measurements were taken between *Zeitgeber* time 5 (ZT5) and ZT8. Mice were gently restrained so that the right eye sat at the aperture of an integrating sphere capable of illuminating the whole eye. Pupillary responses were elicited by applying a light stimulus (200µW/cm^2) provided by Xenon arc lamp (Cairn Research Ltd.) filtered with a 480nm monochromatic interference filter (half bandwidth ≤10 nm) and transmitted to the integrating sphere using a quartz fibre optic. Consensual pupil constriction could then be monitored in the left eye by illuminating it with infrared (>900nm) and recording it with a CCD camera fitted with a 140mm lens in a parallel plane to the cornea. The eye was recorded for two seconds with no light stimulus, then for a further 58 seconds as the light stimulus was presented.
Pupil area was measured in images captured from the videotaped records using VirtualDub (web-based software package, VirtualDub.org) and Matlab R2008a (The Mathworks, Cambridge, UK). To correct for individual variation in dark-adapted pupil area data were normalised to pupil area immediately preceding light onset.

2.5 Retinal histology

When mice were culled, one eye from each mouse studied was removed and the cornea was pierced to allow full penetration of fixative before placing in 4% paraformaldehyde (Sigma-Aldrich, Dorset, UK) at 4°C for 2 days. The eyes were then placed in a 30% sucrose solution (sucrose in phosphate buffered saline solution, Sigma-Aldrich) for a further 2 days for cryoprotection. Lenses were removed from the eye cups and eyes were placed in tissue freezing medium (Jung tissue freezing medium, Leica Microsystems, Milton Keynes, UK) on dry ice to freeze. Eyes were stored at -20°C until processed.
Transverse sections (10µm) were sliced using a cryostat (Leica 3050S, Leica Microsystems) at -21°C and sections were thaw mounted onto gelatine coated slides (gelatine – Sigma-Aldrich) and stored at -20°C until needed.
To stain the retinal sections, slides were defrosted before being washed in 70% ethanol (Fisher Scientific, Loughborough, UK) for 1 minute. They were then rinsed in distilled water for 1 minute. Slides were then incubated in cresyl violet (Sigma-Aldrich) for 5 seconds before being rinsed in distilled water. Slides were then incubated for 1 minute in 70% ethanol followed by 1 minute in 95% ethanol. They were then dipped in 95% ethanol containing a few drops of acetic acid until differentiated. Slides were then dehydrated in increasing gradients of ethanol before washing in Histoclear (National Diagnostics, Fisher Scientific) and coverslipping using DPX mounting medium (Fisher Scientific).

Sections were observed under a microscope (Leica DM2000, Leica Microsystems) at x40. Pictures were taken of each section using a camera (Leica DFC295, Leica Microsystems) mounted onto the microscope and the Leica Application Suite software package (v3.2.0, Leica Microsystems). Photographs of at least 6 retinal sections per mouse were analysed using ImageJ (version 1.43, NIH, Bethesda, MD, USA) by measuring the width of the outer nuclear layer (ONL) at 6 different points of the retinal section and taking the average width per section. The average ONL width per mouse could then be calculated (using an average of at least 36 measurements) and analysed for statistical differences. To obtain a more detailed insight into the structural integrity of the ONL, the density of the cells could be assessed by isolating the ONL from the rest of the image. The photograph of the ONL could then be converted to an 8-bit greyscale image. To isolate the cells from the rest of the image, a threshold was set (172 on ImageJ) which converted the image to black and white, with anything which was darker than the threshold in black (the cells), and anything lighter in white. The density of the cells could then be assessed by measuring the area of the black particles, which we noted as the optical density of the ONL.

2.6 Immunohistochemistry

Immunohistochemistry was conducted as previously described (Smith and Canal, 2009; Brooks et al., 2011). Mice were culled and their brains were carefully removed and placed in fixative (4% paraformaldehyde; Sigma-Aldrich) for two days at 4°C. The brains were then transferred to 30% sucrose (Sigma-Aldrich) for cryoprotection. They were then rapidly frozen on dry ice and stored at -80°C until cut. Using a sledge microtome (Bright Instruments Ltd., Huntingdon, UK) coronal sections of 30μm were cut throughout the whole suprachiasmatic nucleus (SCN) and paraventricular nucleus (PVN) (0.22-1.06mm posterior to bregma) according to the mouse brain atlas (Paxinos and Franklin, 2004).

Antibody	Concentration	Source
Anti-cFOS made in rabbit (polyclonal)	1:10000	Santa Cruz Biotechnology Inc., CA, US
Anti-pERK made in rabbit (monoclonal)	1:5000	Cell Signaling Technology, Beverly, MA, US
Anti-mPER2 made in rabbit	1:5000	Kind gift from Hajime Tei
Anti-AVP made in rabbit (polyclonal)	1:10000	Fitzgerald Industries International, Inc., Concord, MA, US
Anti-VIP made in rabbit (polyclonal)	1:2000	Enzo Life Sciences, Exeter, UK
Anti-GFAP made in rabbit (polyclonal)	1:1000	Sigma-Aldrich

Table 2.2: List of antibodies used for immunohistochemistry and their concentrations.

Free-floating sections were washed in 0.1M phosphate buffer (PB; Sigma-Aldrich) followed by 0.1M PB containing 0.03% Triton X-100 (PBX; Sigma-Aldrich) to permeabilise the cells. Sections were then incubated in 1.5% hydrogen peroxide made in PBX. They were then washed again in PB followed by PBX before being incubated in a blocking solution of 5% normal goat serum (Sigma-Aldrich) made in PBX. Sections were then incubated in primary antibody for 2 nights at 4°C (see Table 2.2 for the antibodies used and their concentrations). Sections were then washed before being incubated in biotinylated IgG goat anti-rabbit (diluted 1:400 in PBX; Vector laboratories, Inc., Burlingame, CA, USA) for 90 minutes. Sections were washed and the reaction was amplified using the avidin-biotin complex solution (diluted 1:200 in PBX; Vector Laboratories) before being washed twice more. The staining was visualised with diaminobenzidine intensified with a nickel solution as a chromogen (DAB substrate kit; Vector laboratories) and mounted onto gelatine-coated slides (gelatine – Sigma-Aldrich). Control sections followed the same protocol as described above but with no primary antibody resulting in no staining.

Sections were observed under a microscope (Leica DM2000, Leica Microsystems) at x20. Pictures were taken of each section using a camera (Leica DFC295, Leica Microsystems) mounted onto the microscope and the Leica Application Suite software package (v3.2.0, Leica Microsystems). Two investigators naive to the experimental groups determined the optical density of the staining in the SCN by drawing a line around the boundary of the SCN using ImageJ. An area outside of the SCN was used to determine the background level of staining, which was subtracted from the value of the staining in the SCN to obtain a normalised optical density. The number of positively stained cells was also determined in the SCN. The average of the data collected by the two investigators was taken for subsequent calculations. If sections were stained for AVP immunoreactivity the density of staining and number of positively stained cells was also calculated in the PVN.

2.7 Behavioural analysis

Locomotor activity was analysed using the software package El Temps (A. Diez-Noguera, Universitat de Barcelona, Spain). For each light condition, the period of the rhythm was calculated using the χ^2-periodogram. The stability of the circadian rhythm of locomotor activity was measured using the percentage of variance explained by the highest peak of the periodogram (PV value). Fourier analysis was used to calculate the amplitude and power content of the first harmonic (PCH1) of the circadian rhythm. The mean waveform was produced by smoothing the waveform using a 12-hour moving average. The mean waveform of daily activity was used to study the characteristics of alpha (active phase). Alpha refers to the period of time during the daily cycle in which most of the activity levels are above the median. The duration (hours) and intensity (area under the curve) of activity during alpha were compared between groups. The percentage of activity occurring during alpha in relation to the total daily activity levels was examined as an indicator of the pattern of distribution of activity throughout the circadian cycle. In the LD stage the phase angle difference (φ) was analysed to study entrainment characteristics. This was done by calculating the difference in minutes between the onset of darkness and the onset of activity from the mean waveform. A negative

value indicates that mice began moving before the lights were turned off and were therefore entrained to the light environment. A positive value indicates that mice only started moving after the lights had been turned off and may therefore not have been entrained to the light environment but instead were reacting to the lights turning on.

2.8 Tissue culture

For baseline tissue culture experiments, mPer2::luc mice were raised in DD, LD or LL from day of birth (P0) till weaning and were then group housed and used from P50 through to six months of age. Mice were culled by cervical dislocation between ZT4 and ZT5 and tissues (brain, heart, lung, liver and spleen) were removed and placed in ice-cold Hank's Balanced salt solution supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 10mM HEPES and 352.5µg/ml sodium bicarbonate (Sigma Aldrich). The SCN and peripheral tissue were microdissected and cultured in a 35mm petri dish containing 1ml of culture medium containing DMEM supplemented with 50U/ml penicillin, 50µg/ml streptomycin, 10mM HEPES, 352.5µg/ml sodium bicarbonate, 3.5mg/ml D-glucose (Sigma), B27 (Invitrogen, Paisley, UK) and 0.1mM Beetle Luciferin (Promega, Madison, WI, US). Bioluminescence recordings were measured at 10 minute intervals using a Lumicycle (Actimetrics, Wilmette, IL. USA). The rhythms were analysed using a method based on previously published work (Abe et al., 2002). The data was detrended using a 24-hour running average and smoothed using a 3-hour running average of the detrended data. The 3-hour running average was used to calculate the phase, amplitude, period and damping rate of the rhythm. The phase was calculated as the first peak of the data and compared to ZT12. The amplitude of the rhythm was determined using the first two peaks and troughs of the data and calculated using the average of the amplitudes of the half cycles (peak to trough). The period was calculated by taking the average of the periods between the first three peaks and the first three troughs. The amplitude of each peak and trough was taken and plotted on a graph to calculate the damping rate. A linear trendline with its equation was fitted to the data and the point at which the trendline crossed the x-axis (y=0) was taken as the damping rate in days. We were also able to calculate the percentage of the initial amplitude to normalise the data if needed. The level of rhythmicity was determined by applying an autocorrelation analysis to the data using Matlab R2008a (Mathworks, Natlick, MA, US) which enabled us to measure how stable the rhythms were. For SCN tissue analysis, we were also able to calculate the goodness of fit of the waveform by applying a sinusoidal wave to the data which taking into account the damping of the data using Matlab and including any samples with a goodness of fit above 0.8.

2.9 Statistical analysis

Data in the text and in tables is presented as mean ± standard deviation. Data in figures is presented as mean ± standard error. Statistical analysis was carried using SYSTAT (Version 10, SPSS Inc., Chicago, IL, USA). More information is available in the results.

3. Effects of early light environment on retinal structure and function in pigmented and albino mice.

3.1 Introduction

Light plays an important role in synchronising the internal clock to the external environment (Moore, 1997). It is detected by rod and cone photoreceptors in the retina, which convey image-forming visual information (Hattar et al., 2002), along with the most recently discovered photoreceptors, the intrinsically photosensitive retinal ganglion cells (ipRGC) which express melanopsin and play an important role in circadian entrainment (Berson et al., 2002). Light information is transmitted to the suprachiasmatic nucleus (SCN) via the retinohypothalamic tract (RHT) (Moore and Lenn, 1972) and can then reset the SCN to the external environment, thus preventing a loss of synchrony between the internal clock and the outside world.

Light also plays an important role in the development of the circadian system. Adult rats placed in constant light (LL) are unable to sustain rhythmic locomotor activity behaviour. However, research has shown that rats that were raised in LL from birth until postnatal day 21 (P21), then placed back into LL later on in life retained their circadian rhythms of locomotor activity behaviour, while rats raised in normal 12:12 hour light-dark cycles (LD) or constant darkness (DD) did not. This was the first evidence that light environment during postnatal development could affect circadian rhythms later on in life (Cambras et al., 1997, 1998).

More recently, similar findings on the effects of light during postnatal development have been shown in mice. As it is rarer for mice to become arrhythmic in LL, the changes seen due to early light environment were more subtle. For example, when placed in LL as adults, CD1 mice (an albino strain) raised in DD had a significantly longer tau than mice raised in LL (Canal-Corretger et al., 2001b). In nocturnal animals placed in LL, Aschoff found that tau length increased with light intensity (Aschoff, 1960). The CD1 mice in the study mentioned here were all placed in the same intensity of LL, yet the mice raised in DD had a longer tau than the mice raised in LL, which could lead us to hypothesise that the mice raised in DD were more sensitive to light than the mice raised in LD and LL. Alterations in locomotor activity behaviour due to early light environment have also been shown in CD1 mice when they were in LD as adults. In LD, mice raised in DD had a higher amplitude of locomotor activity behaviour than mice raised in LD or LL (Canal-Corretger et al., 2001b). Interestingly, a later study in C57BL/6J mice (a pigmented strain) found an opposite response to what was found in the CD1 mice, with C57BL/6J mice raised in LL having the higher amplitude of locomotor activity behaviour compared to mice raised in DD (Smith and Canal, 2009). This result led us to wonder what could cause the difference in response between the two strains. One clear difference is that one strain is albino and one is pigmented. We hypothesised that being raised in LL may cause damage to the retinas of our albino mice but not our pigmented mice, and that this may contribute to the differences we see in locomotor activity behaviour.

Therefore, we set out to test how early light environment would affect retinal function and structure in C57BL/6J and CD1 mice. To assess retinal function, we used electroretinography (ERG) as it can be used as a sensitive assay of retinal damage (Cameron et al., 2008b). We

also measured the pupil light reflex (PLR) as it is a measure of how much the pupil constricts in response to light and can be used to determine the non-photic visual response (Lucas et al., 2001).

Changes in retinal structure were assessed histologically using cresyl violet staining. We quantified these changes by measuring the width of the outer nuclear layer (ONL), which contains the nuclei of the rods and cones, as this provided us with the clearest marker of retinal damage (Noell et al., 1966; LaVail et al., 1987b; Harada et al., 1996).

Next, we wanted to assess the effect of early light environment on locomotor activity behaviour of C57BL/6J mice in LL to see whether we obtained similar results to what has previously been published in CD1 mice (Canal-Corretger et al., 2001b). Therefore we placed the C57BL/6J mice in LL, decreasing the intensity of LL every few weeks, before placing them in DD, then back in LD. To explore the effect of long-term exposure to LL on the retina of C57BL/6J mice, we then assessed retinal function and structure again using the methods previously described.

3.2 Experimental design

Tables 3.1 and 3.2 show the experimental design for the pigmented (Table 3.1) and albino (Table 3.2) mice used to study whether early light environment affects retinal function and structure in adult mice.

	C57BL/6J group 1 (n=5 males in each light condition)	**C57BL/6J group 2 (DD – n=7 males; LD – n=5 males and 2 females; LL – n=7 males)**
P0	Lactation (DD, LD, LL)	Lactation (DD, LD, LL)
P21	All animals placed in LD Animals were weaned and individually housed between P21 and P25.	All animals placed in LD Animals were weaned and individually housed between P21 and P25.
P25	Locomotor activity in LD	Locomotor activity in LD
	PLR and ERG (ERG data not used due to complications with the anaesthetic) (P39-60)	ERG (P35-55)
P64	Locomotor activity in LL	P66: Mice culled at ZT14, brains and eyes collected
P98	Locomotor activity in LL (37.9µW/cm^2 provided by LED light)	
P115	Locomotor activity in LL (6.9µW/cm^2 provided by LED light)	
P130	Locomotor activity in DD	
P156	Locomotor activity in LD	
	ERG and PLR (P169-177)	
P184	Mice culled at ZT14, brains and eyes collected	

Table 3.1: Experimental design for the C57BL/6J mice used to determine the effects of early light environment on the retina and locomotor activity behaviour. Unless otherwise stated, mean light intensity was 330µW/cm^2 provided by LED light. DD – constant darkness; LD – 12:12 hour light-dark cycle; LL – constant light; PLR – pupil light reflex; ERG – electroretinography; ZT – Z*eitgeber* time; LED - light-emitting diode

	CD1 group 1 (n=5 males in each light condition)	CD1 group 2 (n=7 males in each light condition)
P0	Lactation (DD, LD, LL)	Lactation (DD, LD, LL)
P21	All animals placed in LD Animals were weaned and individually housed between P21 and P25.	All animals placed in LD Animals were weaned and individually housed between P21 and P25.
P25	Locomotor activity in LD	Locomotor activity in LD
	PLR and ERG (ERG data not used due to complications with the anaesthetic) (P39-60)	ERG (P35-55)
P64	Locomotor activity in LL – n numbers too low to be able to use data	P66: Mice culled at ZT14, brains and eyes collected
P98	Animals housed in LD	
P147	Mice culled at ZT16 – brains and eyes collected	

Table 3.2: Experimental design for the CD1 mice used to determine the effects of early light environment on the retina and locomotor activity behaviour. Mean light intensity was 330µW/cm^2 provided by LED light. DD – constant darkness; LD – 12:12 hour light-dark cycle; LL – constant light; PLR – pupil light reflex; ERG – electroretinography; ZT – *Zeitgeber* time; LED - light-emitting diode

At the end of the experiment mice were culled and their eyes were processed for histology using cresyl violet as described in the materials and methods (Chapter 2). The integrity of retinal structure was assessed by measuring the width of the outer nuclear layer (ONL).
Statistical analysis was carried out by means of several ANOVA of general linear models using SYSTAT software.
For all experiments the independent variable was lactation (DD, LD or LL). When analysing the dark-adapted ERG the dependent variables were a-wave amplitude, b-wave amplitude, a-wave implicit time and b-wave implicit time. When analysing the photopic ERG the dependent variables were b-wave amplitude and b-wave implicit time. When analysing the PLR the dependent variable was normalised pupil area. When analysing the histology the dependent variable was ONL width. When analysing the locomotor activity behaviour the dependent variables were period, percentage of variance, amplitude, power content of the first harmonic, duration of alpha, area under the curve, percentage of total area and phase of entrainment. We also compared the dark-adapted ERG, photopic ERG, PLR and histology in mice that had been in long-term LL compared to mice that had not and whether this was affected by early light environment in C57BL/6J mice.

3.3.1 Effects of early light environment on the retinal function of C57BL/6J mice

A total of 21 male and female C57BL/6J mice were born in DD, LD or LL and kept in these conditions until P21, when they were transferred to LD. They were weaned between P21 and P25, housed individually and their locomotor activity behaviour was monitored to ensure that they were entrained to LD. Their retinal function was assessed using ERG and PLR from P35 onwards.

We first measured the dark-adapted ERG which measures changes in field potential associated with extracellular currents in the retina in response to flashes of light of increasing intensity in mice that have been dark-adapted overnight (Cameron et al., 2008b). This allows us to assess the functionality of rods and cones (using the a-wave) and ON-bipolar cells (using the b-wave). We found no significant difference in a-wave amplitude (the response of the rods and cones) due to early light environment (Figure 3.1A). At the highest intensity light stimulus ($3.1\log_{10}\mu W/cm^2$), the mean a-wave amplitude was 381.79±154.84μV in mice raised in DD, 368.46±183.01μV in mice raised in LD and 298.55±59.92μV in mice raised in LL. We found no significant differences in the b-wave amplitude (the bipolar cell response) due to early light

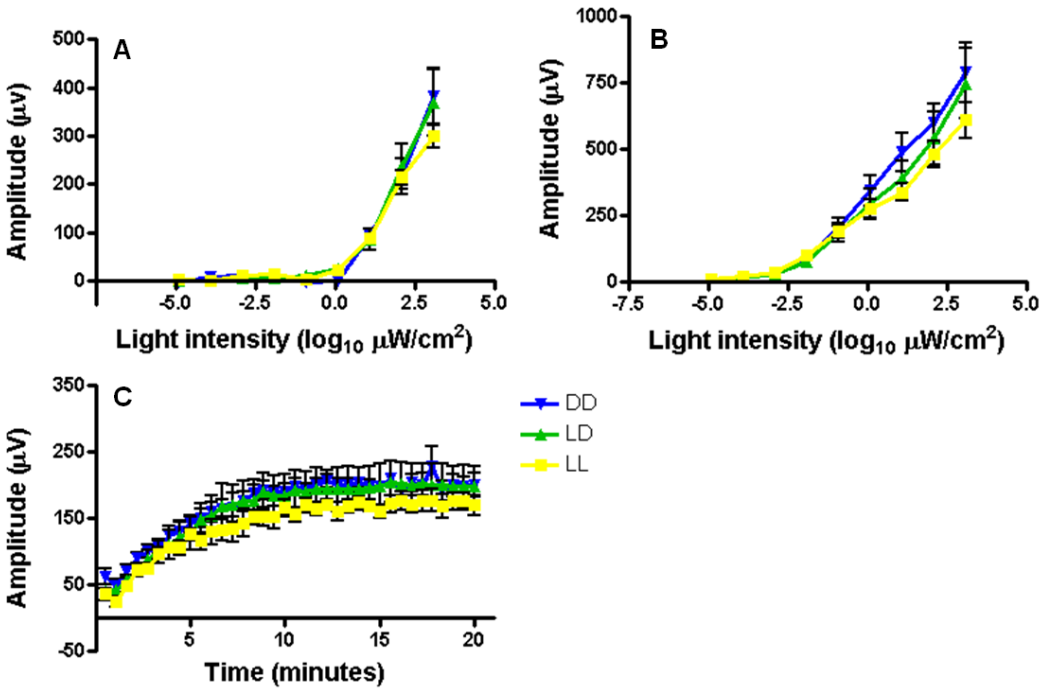

Figure 3.1: Effects of early light environment on the retinal function of C57BL/6J mice. Mice were raised in DD, LD or LL from birth until P21 then placed in LD. We found no difference in the amplitude of the a-wave (**A**) or b-wave (**B**) due to early light environment when using the dark-adapted ERG. Similarly, we found no differences in the adaptation of cones to light over 20 minutes using the photopic ERG (**C**). ERG – electroretinography; DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light.

environment (Figure 3.1B). At the highest intensity light stimulus (3.1$\log_{10}$µW/cm^2) the mean b-wave amplitude was 790.11±296.76µV in mice raised in DD, 747.60±353.03µV in mice raised in LD and 608.59±161.30µV in mice raised in LL. Finally we found no significant differences in a-wave implicit time or b-wave implicit time due to early light environment (data not shown).
We also isolated the cone response (the photopic ERG) by exposing the retina to a constant rod-saturating background light and overlaying it with a series of very bright flashes over 20 minutes and measured the adaptation of cones to this light stimulus. We found no significant difference in the b-wave amplitude due to early light environment (Figure 3.1C). After 20 minutes of light adaptation, the mean b-wave amplitude was 200.59±45.38µV in mice raised in DD, 198.00±76.54µV in mice raised in LD and 169.35±35.14µV in mice raised in LL. We found no significant differences in the b-wave implicit time due to early light environment (data not shown). We can therefore conclude that early light environment does not seem to affect rod, cone or bipolar cell function in C57BL/6J mice.

3.3.2 Effects of early light environment on the retinal function of CD1 mice

A total of 21 male CD1 mice were born in DD, LD or LL and raised in these conditions until P21,

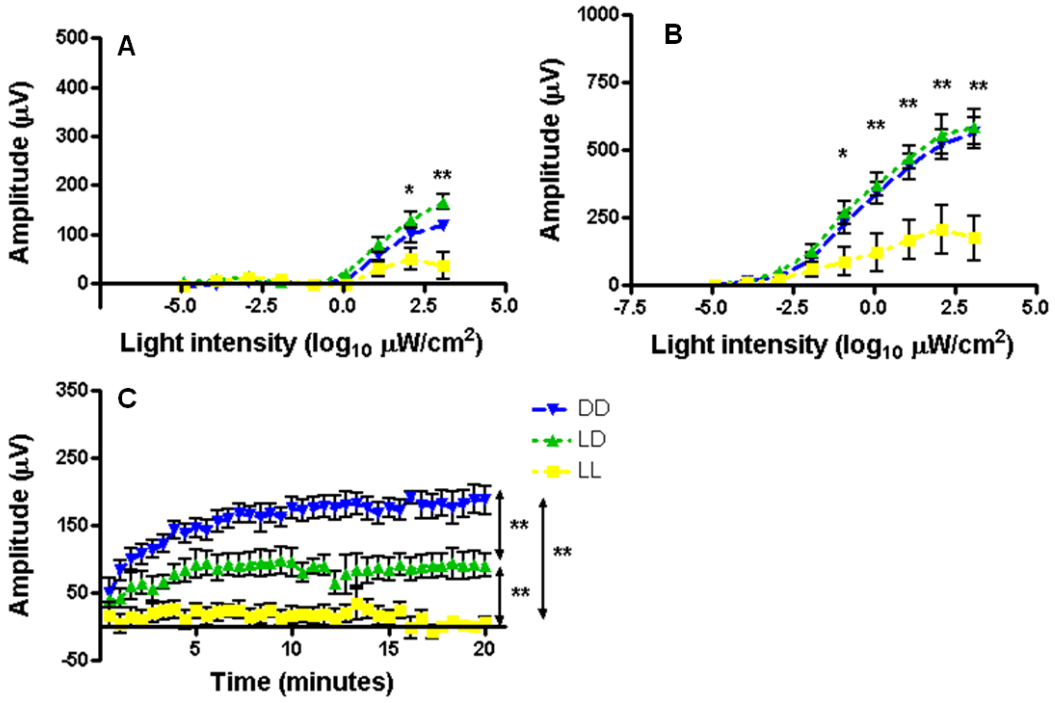

Figure 3.2: Effects of early light environment on the retinal function of CD1 mice. Mice were raised in DD, LD or LL from birth until P21 then placed in LD as adults. Mice raised in LL had significantly reduced a-wave (**A**) and b-wave (**B**) amplitudes compared to mice raised in LD and DD when measuring the dark-adapted ERG. Furthermore, when measuring the photopic ERG, we found that mice raised in LL and LD had significantly reduced b-wave amplitudes compared to mice raised in DD (**C**). ERG – electroretinography; DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light. * $p<0.05$ compared to DD and LD; ** $p<0.01$ compared to DD and LD

when they were all placed in LD. They were weaned between P21 and P25, housed individually and their locomotor activity was monitored to ensure that they were entrained to LD. Their retinal function was assessed from P35 onwards.

When measuring the dark-adapted ERG, we found that CD1 mice raised in LL had significantly reduced a-wave amplitudes (the rod and cone response) compared to mice raised in DD and LD ($p<0.05$; Figure 3.2A). At the highest intensity light stimulus used ($3.1 \log_{10} \mu W/cm^2$) the mean a-wave amplitude was 118.99±23.40μV in mice raised in DD, 165.54±38.36μV in mice raised in LD and 35.46±70.58μV in mice raised in LL. Furthermore mice raised in DD had significantly reduced b-wave amplitudes (the bipolar cell response) compared to mice raised in DD and LD ($p<0.01$; Figure 3.2B). At the highest intensity light stimulus used ($3.1 \log_{10} \mu W/cm^2$) the mean a-wave amplitude was 564.11±152.27μV in mice raised in DD, 583.89±158.65μV in mice raised in LD and 173.59±214.12μV in mice raised in LL. We found no differences in the a-wave or b-wave implicit time due to early light environment (data not shown). When we measured how cones adapt to light over 20 minutes using the photopic ERG, we found that mice raised in LL had a significantly reduced b-wave amplitude over 20 minutes compared to mice raised in LD and DD ($p<0.01$; Figure 3.2C) and that mice raised in LD had a significantly smaller b-wave amplitude than mice raised in DD ($p<0.01$; Figure3.2C). After 20 minutes of light adaptation, the mean b-wave amplitude was 187.92±52.08μV in mice raised in DD, 91.16±34.41μV in mice raised in LD and 6.07±19.12μV in mice raised in LL. We found no differences in the b-wave implicit time (data not shown).

From this data we can conclude that in CD1 mice, being raised in LL causes a large reduction in retinal function, as measured by the dark-adapted and photopic ERG.

3.3.3 Effects of early light environment on the pupil response of C57BL/6J mice

A total of 15 male C57BL/6J mice were born in DD, LD or LL and kept in these conditions until P21 when they were all placed in LD. At P35 their pupil response to a 480nm-wavelength light

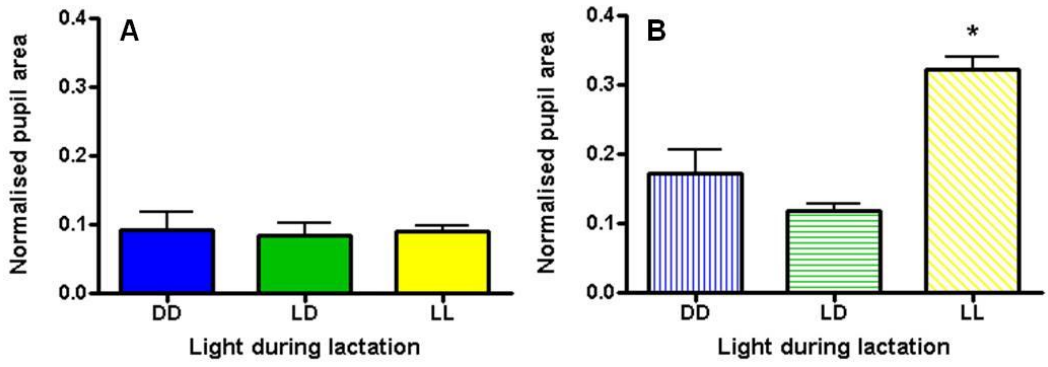

Figure 3.3 Effects of early light environment on the pupil response. C57BL/6J mice (**A**) and CD1 mice (**B**) were raised in DD, LD or LL from birth until P21 then all placed in LD. Their pupil light reflex was measured in response to a 480nm-wavelength light stimulus presented for 1 minute. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light. * $p<0.05$ compared to LD

stimulus, the optimal wavelength for melanopsin activation, was assessed. This allowed us to measure the effect of early light environment on the non-photic visual response.
We found no differences in the pupil response of C57BL/6J mice due to early light environment (Figure 3.3A). The normalised pupil area of mice raised in DD was 0.09± 0.06, 0.08±0.04 in mice raised in LD and 0.09±0.02 in mice raised in LL. This data indicates that early light environment does not seem to affect the non-photic visual response of C57BL/6J mice.

3.3.4 Effects of early light environment on the pupil response of CD1 mice

A total of 15 male CD1 mice were born in DD, LD or LL and kept in these conditions until P21 when they were all placed in LD. At P35 their pupil response to a 480nm-wavelength light stimulus was assessed.
We found that CD1 mice raised in LL had a significantly higher normalised pupil area in response to the light stimulus compared to CD1 mice raised in LD ($p<0.05$, Figure 3.3B). The normalised pupil area of mice raised in DD was 0.17±0.07, 0.12±0.02 in mice raised in LD and 0.32±0.04 in mice raised in LL. This data indicates that CD1 mice raised in LL were unable to constrict their pupils as much as the mice raised in LD in response to a 480nm-wavelength stimulus. We can therefore conclude that being raised in LL reduces the non-photic visual response of CD1 mice.

3.3.5 Effects of early light environment on the retinal structure of C57BL/6J mice

At total of 21 male and female C57BL/6J mice were born in DD, LD or LL and kept in these conditions until P21. They were then placed in LD, weaned, housed individually and their locomotor activity was monitored to ensure that they were entrained to LD. Their ERG and PLR was measured, then they were culled at P66 and their eyes removed and processed with cresyl violet to visualise the structure of the retina. We measured the width of the ONL, site of the nuclei of the rod and cone photoreceptors, and found that mice raised in DD had a mean ONL width of 60.55±6.14μm, mice raised in LD had a mean ONL width of 49.81±4.35μm and mice raised in LL had a mean ONL width of 62.71±3.88μm. There were no statistically significant differences in the width of the ONL due to early light environment in C57BL/6J mice (Figure 3.4A, C, E and G). We can therefore conclude that early light environment does not seem to affect the structural integrity of the retinas of C57BL/6J mice.

3.3.6 Effects of early light environment on the retinal structure of CD1 mice

A total of 21 male CD1 mice were born in DD, LD or LL and kept in these conditions until P21. They were then placed in LD, weaned, housed individually and their locomotor activity was monitored to ensure that they were entrained to LD. Their ERG and PLR was measured, then they were culled at P66 and their eyes removed and processed with cresyl violet to visualise the structure of the retina. We measured the width of the ONL, site of the nuclei of the rod and cone photoreceptors, and found that CD1 mice raised in LL had a mean ONL width of 9.15±10.95μm

which was significantly smaller than that of mice raised in LD and DD who had mean ONL widths of 41.92±9.21μm and 40.92±14.05μm respectively ($p<0.05$, Figure 3.4B, D, F and H). We can conclude from this data that in CD1 mice, being raised in LL from P0 to P21 causes significant damage to the rod and cone nuclear layer. When we combine this data with the ERG and PLR data we previously found in the CD1 mice, we can conclude that LL during postnatal development causes significant damage to the retina.

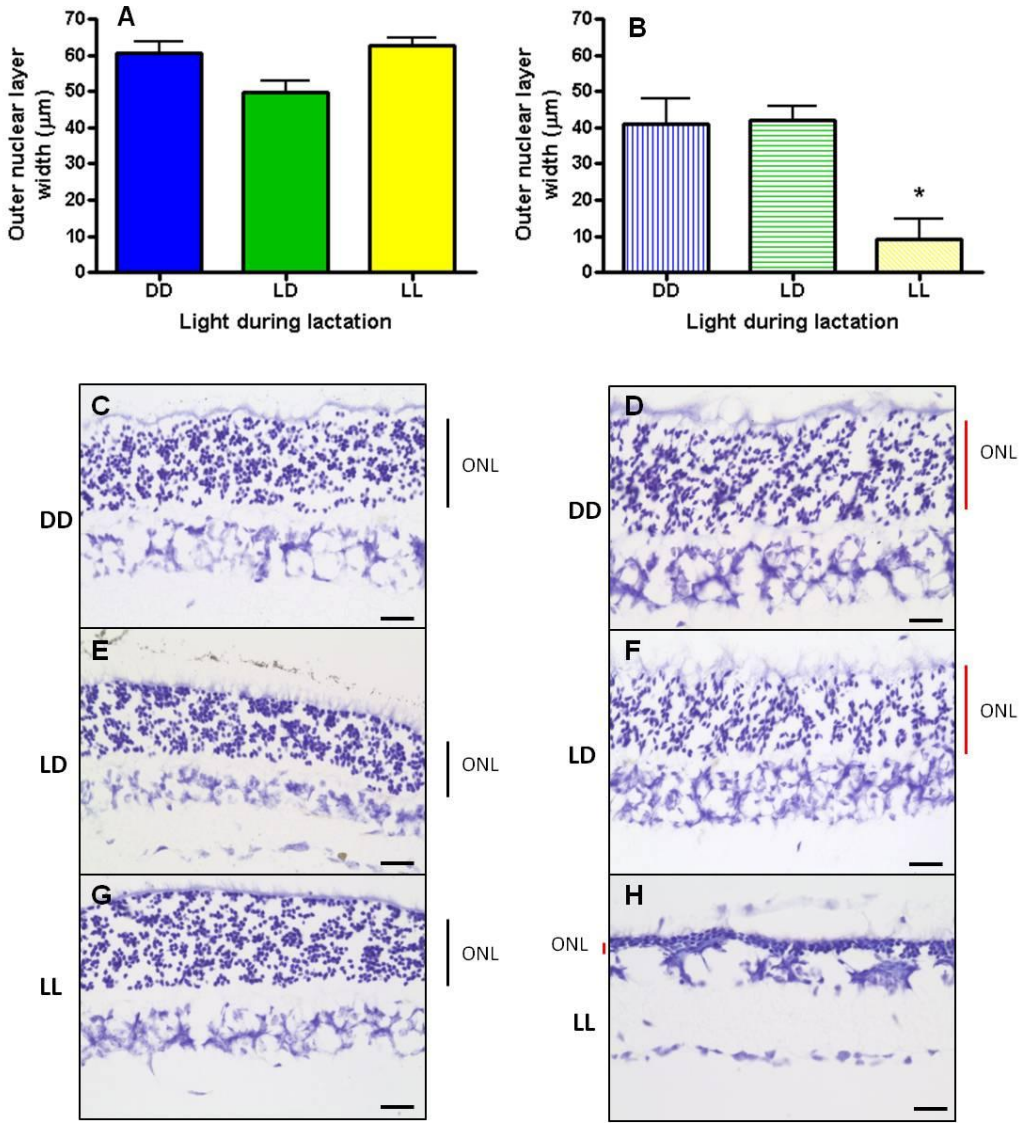

Figure 3.4 Effects of early light environment on retinal structure. C57BL/6J and CD1 mice were raised in DD, LD or LL from P0 to P21 then placed in LD from P21 onwards. Retinal structure was visualised with cresyl violet staining. We found no major differences in retinal structure due to early light environment in C57BL/6J mice (**A, C, E** and **G**). CD1 mice raised in LL had significantly reduced ONL widths compared to the mice raised in DD and LD (**B**) as seen by the difference in structure in the mice raised in LL (**H**) compared to the mice raised in DD (**D**) and LD (**F**). Data presented as mean ± SEM. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light; ONL – outer nuclear layer. * $p<0.05$ compared to DD and LD.

3.3.7 Effects of early light environment on the locomotor activity behaviour of C57BL/6J mice

As we found no differences in retinal function or structure in the C57BL/6J mice we focused on this strain for the remainder of this study. We wanted to know how the locomotor activity behaviour of C57BL/6J mice was affected by early light environment when they were placed in LD, and also when they were placed in constant light of decreasing intensities, followed by DD. A total of 15 male mice were born in DD, LD or LL and kept in these conditions until P21, when they were all placed in LD. They were then weaned, housed individually and their locomotor activity behaviour was monitored. We found no differences in the locomotor activity behaviour of C57BL/6J mice due to early light environment when they were housed in LD (data not shown). We then placed them in LL of decreasing intensities, starting at 330µW/cm^2 for 34 days, followed by 37.9µW/cm^2 for 17 days, followed by 6.9µW/cm^2 for 15 days. They were then placed in DD for 26 days. We found that at all intensities of LL, mice raised in DD had significantly longer taus than mice raised in LL ($p<0.05$; Figure 3.5). At the highest intensity of LL, mice

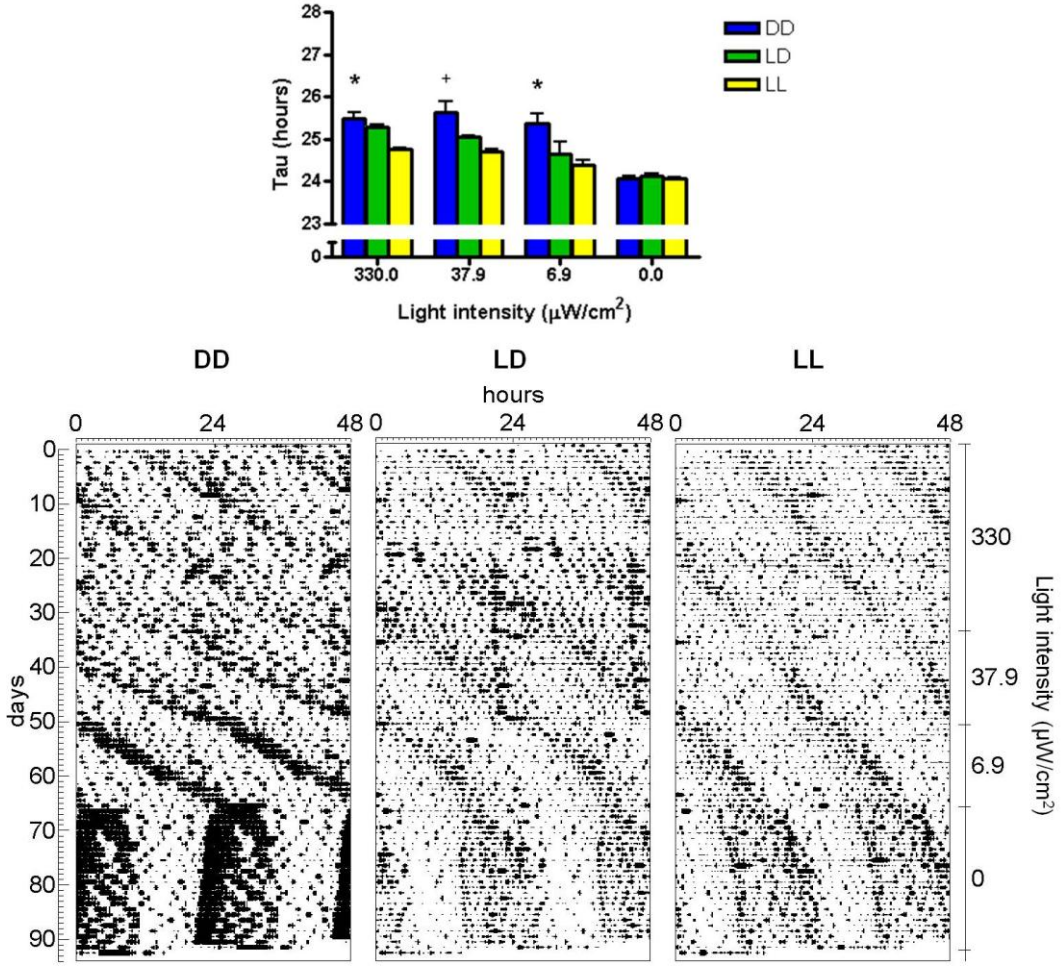

Figure 3.5: Effects of early light environment on locomotor activity behaviour in LL. Average tau length and representative actograms of C57BL/6J mice raised in DD, LD or LL from P0 to P21 then placed in LD before being placed in constant light conditions of decreasing intensities. Day 0 on the actogram represents the first day of locomotor activity behaviour monitoring after weaning. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light. * $p<0.05$ compared to DD; $^+$ $p=0.05$ compared to DD.

raised in DD had an average tau length of 25.48±0.33 hours, mice raised in LD had an average tau length of 25.29±0.06 hours and mice raised in LL had an average tau length of 24.75±0.10 hours. When we reduced the intensity of LL to 37.9μW/cm^2 mice raised in DD had an average tau length of 25.63±0.52 hours, mice raised in LD had an average tau length of 25.04±0.06 hours and mice raised in LL had an average tau length of 24.69±0.10 hours. When we reduced the light intensity further to 6.9μW/cm^2 mice raised in DD had an average tau length of 25.35±0.47 hours, mice raised in LD had an average tau length of 24.64±0.51 hours and mice raised in LL had an average tau length of 24.38±0.24 hours. At this intensity mice raised in LL also had significantly shorter periods than when they had been housed in the highest light intensity ($p<0.05$). Apart from tau length, we found no other differences in locomotor activity behaviour at any intensity of LL. When we placed the mice in DD, we found no differences in tau length or any of the other variables we measured due to early light environment. The average tau length in DD for mice raised in DD was 24.07±0.12 hours, 24.11±0.13 hours for mice raised in LD and 24.05±0.10 hours for mice raised in LL. The tau lengths for all mice in DD were significantly shorter than when the mice were in the highest intensity LL ($p<0.05$). Furthermore mice raised in DD and LL had significantly shorter periods in DD compared to all intensities of LL ($p<0.05$).

Our data has shown that at the intensities of LL we measured, mice raised in DD have longer periods longer than mice raised in LL. Aschoff's rule states that the higher the light intensity, the longer the tau. Here the mice are placed in the same intensity of LL, yet display different tau lengths depending on the light intensity in which they are raised. Indeed, it seems that mice raised in DD perceive a higher light intensity than mice raised in LL. We have previously shown that early light environment does not affect retinal function and structure in C57BL/6J mice so we can hypothesise that the changes in tau length may be due to how the SCN interprets the light information it receives.

3.3.8 Effects of early light environment on retinal function and structure of C57BL/6J mice after prolonged exposure to constant light

We wanted to see how the retinal function and structure of C57BL/6J mice was affected by being in LL for a prolonged period of time.

C57BL/6J mice that had been raised in DD, LD or LL from P0 to P21, then all placed in LD before being placed in LL of decreasing intensities followed by DD were placed back in LD. Two weeks after being placed in LD their retinal function was measured using ERG. When measuring the dark-adapted ERG, we found no differences in the a-wave amplitude due to early light environment (Figure 3.6A). At the highest intensity light stimulus measured ($3.1\log_{10}$μW/cm^2), mice raised in DD had a mean a-wave amplitude of 243.76±37.52μV, mice raised in LD had a mean a-wave amplitude of 230.62±28.87μV and mice raised in LL had a mean a-wave amplitude of 223.55±13.21μV. These values were significantly lower than the a-wave amplitude of C57BL/6J mice that had not been placed in long-term LL ($p<0.01$; see Figure 3.1A for comparison). When we measured the b-wave amplitude we found that being in LL for a prolonged duration caused the mice raised in LL to have a significantly lower b-wave amplitude

than mice raised in DD ($p<0.05$; Figure3.6B). Indeed, at the highest intensity light stimulus that we measured, mice raised in DD had a mean b-wave amplitude of 569.66±51.45μV, mice raised in LD had a mean b-wave amplitude of 535.60±40.34μV and mice raised in LL had a mean b-wave amplitude of 452.56±56.10μV. These values were significantly lower than the b-wave amplitude of C57BL/6J mice that had not been placed in long-term LL ($p<0.01$; see Figure 3.1B). We found no differences in the a-wave or b-wave implicit time due to early light

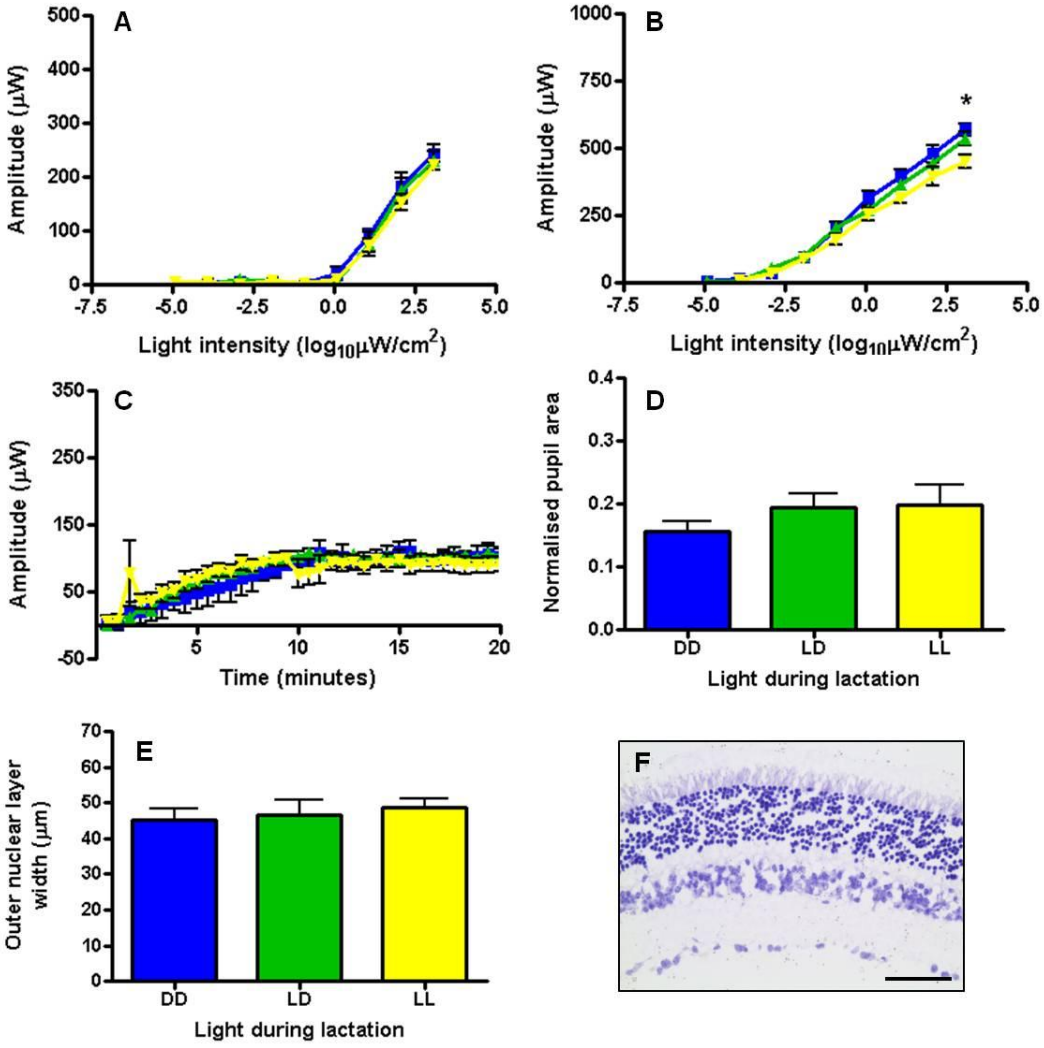

Figure 3.6: Effects of early light environment on retinal function and structure after prolonged exposure to LL. C57BL/6J mice were raised in DD, LD or LL from P0 to P21, then placed in LD at P21 before being placed in LL of decreasing intensities, followed by DD, then placed back in LD. When measuring the dark-adapted ERG we found no differences in the a-wave amplitude (**A**), but found that the b-wave amplitude of mice raised in LL was significantly lower than that of mice raised in DD (**B**). When measuring the photopic ERG we found no differences in the b-wave amplitude (**C**). We found no differences in the pupil response (**D**) or the ONL width (**E**). We found that the structural integrity of the retina remained intact despite being in LL for a prolonged duration (**F**).ERG – electroretinography; DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light; ONL – outer nuclear layer. * $p<0.05$ compared to DD. Scale bar - 50μm

environment or compared to mice that had not been in LL for a prolonged duration (data not shown).

We then measured the photopic ERG and found no differences in the b-wave amplitude due to early light environment (Figure 3.6C). After 20 minutes of light adaptation, mice raised in DD had a b-wave amplitude of 102.00±31.71μV, mice raised in LD had a b-wave amplitude of 102.99±21.21μV and mice raised in LL had a b-wave amplitude of 91.25±25.79μV. These values were significantly lower than the b-wave amplitude of C57BL/6J mice that had not been in LL for a prolonged period ($p<0.01$; see Figure 3.1C for comparison). After a prolonged duration in LL we found no differences in the b-wave implicit time due to early light environment or compared to mice that had not been in LL for a prolonged duration (data not shown).

Next we measured the pupil response and found no differences in the normalised pupil area due to early light environment (Figure 3.6D). Mice raised in DD had a normalised pupil area of 0.16±0.04, mice raised in LD had a normalised pupil area of 0.19±0.04 and mice raised in LL had a normalised pupil area of 0.20±0.07. These values were significantly higher compared to mice that had not been placed in LL for a prolonged duration ($p<0.01$; See Figure 3.3A for comparison).

Next we culled the mice, collected their eyes and processed them with cresyl violet to look at the structure of the retina. We found that the structural integrity of the retinas of C57BL/6J mice remained intact despite being placed in LL for a prolonged duration (Figure 3.6F). We measured the width of the ONL and found no differences in the ONL width due to early light environment (Figure 3.6E). Mice raised in DD had a mean ONL width of 45.23±7.21μm, mice raised in LD had a mean ONL width of 46.52±7.66μm and mice raised in LL had a mean ONL width of 48.55±5.97μm. These values were significantly lower than in mice that had not been placed in LL for a prolonged duration ($p<0.01$; see Figure 3.4A for comparison).

From this data we can conclude that being placed in LL for a prolonged duration does reduce retinal function and structure, but does not cause visible damage to the retina. Furthermore, in most cases all mice were affected in the same way, regardless of the light environment in which they were raised. The only exception was the b-wave amplitude in the dark-adapted ERG. This will be discussed further below.

3.4 Discussion

The primary purpose of this study was to determine how light during postnatal development affects retinal function and structure in adult C57BL/6J mice and CD1 mice. We also wanted to assess the locomotor activity behaviour of C57BL/6J mice in LL and whether this is affected by early light environment. Finally, we wanted to determine the effect of prolonged exposure to LL during adulthood on the retinal function and structure of C57BL/6J mice raised in different light environments.

3.4.1 CD1 mouse retinas are damaged by light during postnatal development

Our assessment of the retinal function of CD1 mice allows us to draw some conclusions about the effects of LL on the retina of albino mice during postnatal development. First of all we found that when measuring the dark-adapted ERG there was a significant reduction in the amplitude of the a-wave in mice raised in LL compared to mice raised in DD and LD. The a-wave represents the hyperpolarisation of the rod and cone photoreceptors in response to a light stimulus (Brown, 1968). Here, the reduction in the a-wave amplitude in mice raised in LL leads us to believe that the rods and cones may have been damaged by prolonged exposure to LL. We also found a reduction in the amplitude of the b-wave in the dark-adapted ERG in mice raised in LL compared to mice raised in LD and DD. The b-wave represents the depolarisation of the ON-bipolar cells in response to activation by the rods and cones (Stockton and Slaughter, 1989). A reduction in the ON-bipolar cell response may either be due to damage to the bipolar cells themselves, or may be due to a reduced signal from the rods and cones. As we have seen that the rod and cone response is reduced in mice raised in LL, it is likely that this has contributed to the reduced bipolar cell response, although we cannot rule out the bipolar cells also being damaged by exposure to LL during postnatal development.
Using the photopic ERG, we can isolate the response of cones to light to see how cones are affected by early light environment. As with the dark-adapted ERG, we found that mice raised in LL had a significantly reduced photopic response compared to mice raised in DD and LD. Furthermore, the response of mice raised in LD was significantly smaller than that of the mice raised in DD. From these results we can deduce that LL during postnatal development causes a loss of the ability of cones to adapt to light. Furthermore, mice raised in LD also seem to be affected, with a measurable, but smaller response than mice raised in DD. Previous work has shown that rods can influence the response of cones to light. Indeed mice lacking the α-subunit of rod transducin (a crucial component of the rod phototransduction cascade) have increased b-wave amplitudes during the cone light adaptation ERG than mice that have rods, as rods have a suppressive effect on the cone ERG (Cameron and Lucas, 2009). We could hypothesise here that mice raised in DD have less rod suppression of the cone ERG than mice raised in LD, perhaps because they have less rods than mice raised in LD. This seems surprising as we would expect mice raised in DD to have more rods to try to compensate for the lack of light during development. Our results may also be due to damage in cones of mice raised in LD.
Using the pupil light reflex we were able to measure the non-photic visual response which is mediated by ipRGCs but also receives some contribution from rods and cones (Lucas et al.,

2003). We found that mice raised in LL did not constrict their pupils as much as mice raised in LD in response to a one minute 480nm-wavelength stimulus. As we have ascertained that mice raised in LL had reduced photoreceptor function it is possible that this was contributing to the reduced pupil response we observed. It is also possible that ipRGC function had been directly affected by LL during postnatal development. A recent study has shown that early light environment can affect the number of M2 melanopsin cells in the retinas of CD1 mice (González-Menéndez et al., 2010b). Mice raised in DD or LD from P0 to P21 showed an increase in the number of M2 cells over this time period, while mice raised in LL did not show this increase. The number of M2 cells in the retinas of mice in LL could recover and increase if they were placed back in LD by P11 but it is not clear whether this recovery would occur if the mice were placed back in LD after P21, as in our experimental design. M2 cells have been shown to be less intrinsically photosensitive than M1 cells, and receive more innervations from rods and cones (Ecker et al., 2010; Hughes et al., 2012). It is possible that in LL, there is no increase in the number of M2 cells because of an alteration in the number of innervations from rods and cones. Furthermore, rods and cones mature from P10 onwards and, as the increase in the number of M2 cells can be rescued by placing mice back into LD at P11, this could indicate a role for rods and cones in the proliferation of M2 cells. The physiological role of M2 cells is unclear but it has been shown that M2 cells project to the core OPN (Baver et al., 2008; Güler et al., 2008; Ecker et al., 2010) and therefore may be involved in the pupillary light reflex (Trejo and Cicerone, 1984).

To complete our assessment of how light during postnatal development affects the retina we looked at retinal structure using cresyl violet staining. We measured the width of the ONL, which contains the nuclei of the rods and cones, and found that mice raised in LL had a significantly thinner ONL than mice raised in DD and LD. We can conclude from this result that mice raised in LL have significant visible damage to their retinas, while the mice in LD and DD do not.

When we take all our data together we can conclude that in CD1 mice, being raised in LL causes a significant reduction in retinal function and significant damage to the structure of the retina. The damaging effects of LL in adult albino animals have been well documented, with exposure for as little as 48 hours causing significant damage (Noell et al., 1966; Rapp and Williams, 1979, 1980; LaVail et al., 1987a, 1987b). However, a study looking at the effects of LL between P2 and P21 in albino rats found no damage in their retinas, but found damage if they were in LL from P2 to P35 (Harada et al., 1998). They suggest that retinal damage due to LL only occurs after P21 as the eyes open from P15 and there is not enough time for retinal damage to occur. In our experiment we placed mice in LL from P0 to P21 then placed them back in LD and found significant damage in the retinas of mice that were in LL up until P21. The differences between these two studies may be due to the type and intensity of light used. Harada et al. (1998) used 1000-1200lux light provided by tungsten lamps. We used 2000lux light provided by LEDs. As our light intensity is so much higher it is likely that this has resulted in retinal damage occurring more rapidly in our mice.

In this project we are interested in how early light environment affects the photic response of the circadian system. As the CD1 mice raised in LL have retinal damage, while the mice raised in DD and LD do not, we would be unable to distinguish effects due to retinal damage from effects

due to changes in other parts of the circadian system such as the SCN. Therefore CD1 mice do not seem to be an appropriate model for this project.

3.4.2 C57BL/6J mouse retinas do not seem to be affected by light during postnatal development

We assessed the effect of light during postnatal development on the retinal function and structure of C57BL/6J mice. We found no differences in the retinal function of C57BL/6J mice due to early light environment using the dark-adapted ERG, the photopic ERG or the pupil light reflex. We can therefore conclude that the function of the rods, cones, bipolar cells and ipRGCs in C57BL/6J mice is largely unaffected by early light environment.

When we looked at the structure of the retina using cresyl violet staining, we found no differences in the width of the ONL providing further evidence that early light environment does not greatly affect the retinas of C57BL/6J mice.

Light-induced retinal damage in pigmented animals most often occurs if their pupils are chemically dilated (Rapp and Williams, 1980; Grimm et al., 2000). In normal circumstances, the retinas of pigmented animals are protected by the pigment itself which has the ability to absorb light and the fact that the pupil can dilate to a width of 0.1mm, limiting the amount of light that can reach the retina (Rapp and Williams, 1980).Therefore these studies suggest that at least in adult C57BL/6J mice the retina is protected from the damaging effects of LL.

During development, C3H mice in LL from P0 to P21 have the same number of M2 melanopsin cells as mice in LD from P0 to P21 (González-Menéndez et al., 2010a, 2010b) indicating that melanopsin-expressing retinal ganglion cells do not seem to be affected by early light environment.

Taken together our data shows that early light environment does not seem to affect retinal function or structure. C57BL/6J mice therefore seem to be a good model for further studies on the effects of early light environment on the photic response of the circadian system. As there are no major changes in the retina due to early light environment, any changes we see in the photic response may be due to changes further downstream, for example in the SCN. Therefore for the remainder of the project I will use pigmented mice such as C57BL/6J mice and mPer2::luc mice raised on a C57BL/6J background.

3.4.3 The locomotor activity behaviour of C57BL/6J mice in LL is affected by early light environment

We wanted to assess the effects of early light environment on the locomotor activity behaviour of adult C57BL/6J mice placed in LL. We first monitored their locomotor activity in LD from P25 and found no differences in locomotor activity behaviour due to early light environment.

Previous studies have shown that in LD, C57BL/6J mice raised in LL had significantly higher amplitudes of locomotor activity than mice raised in DD and LD (Smith and Canal, 2009). It is important to note that there is a difference in the type of light used between the two studies. Smith and Canal (2009) used fluorescent light with a mean intensity of 57μW/cm^2 while our current study used LED light with a mean intensity of 330μW/cm^2. It is possible that the differences in the type and intensity of light used have led to the differences in locomotor activity

behaviour seen in these two studies. Further work would be needed to demonstrate whether the intensity and type of light used during postnatal development could alter locomotor activity behaviour later on in life.

We then placed the mice in LL (mean intensity 330μW/cm^2) from P64 to P98, then reduced the light intensity to 37.9μW/cm^2 and kept them in this condition from P98 to P115. We then reduced the light intensity once more to 6.9μW/cm^2 and monitored them from P115 to P130. Similarly to what has previously been found in CD1 mice (Canal-Corretger et al., 2001b), we found that at all intensities of LL mice raised in DD had a significantly longer tau than mice raised in LD or LL. The finding that mice raised in DD have a longer tau than mice raised in LD or LL when they are placed in LL as adults implies that mice raised in DD were more sensitive to light than mice raised in LD or LL. As we have found that in C57BL/6J mice early light environment does not affect the retina we can hypothesise that the differences in tau length are due to changes in how light information is processed by the SCN. Further work is needed to determine where these changes occur.

Aschoff's rule states that the higher the light intensity the longer the tau (Aschoff, 1960). Here we reduced the light intensity by one log unit at a time and did not find significant shortenings of tau as we reduced the light intensity. This may be due to the fact that we started our measurements at the highest intensity and worked our way down. Indeed, previous work in Wistar rats has shown that if animals are first placed in DD, then in low intensity LL and gradually increase the intensity, the tau length also increased, following Aschoff's rule (Canal-Corretger et al., 2003a). It is possible that in this study the amount of light caused a saturated response which was not able to immediately recover when the light intensity was reduced. The one significant shortening of tau that we found was between the mice raised in LL at 330μW/cm^2 and at 6.9μW/cm^2, a 2 log unit decrease in light intensity. Our data implies that mice raised in DD are more sensitive to light. Here the mice raised in LL, which seem to be less sensitive to light, seem to be able to respond to reductions in light intensity in a way which is closer to that predicted by Aschoff's rule. It is possible that the mice raised in LL have more stable rhythms when placed in LL as adults and can therefore cope better with changes in light intensity.

We then placed the mice in DD from P130 to P156 and found that all mice had significantly shorter taus than when they had been in LL but no differences due to early light environment, indicating that there were no differences in the core functioning of the circadian clock. We found no other differences in locomotor activity behaviour due to early light environment.

3.4.4 C57BL/6J retinal function and structure is reduced by exposure to LL for a prolonged duration

As the damaging effects of exposure to LL for a prolonged duration have been well documented in albino animals (Noell et al., 1966; LaVail et al., 1987a), we wanted to see how prolonged exposure to LL during adulthood would affect the retinas of C57BL/6J mice using dark-adapted ERG, photopic ERG, the pupil response and looking at retinal histology and whether early light environment would cause differential responses to prolonged exposure to LL.

We found a general reduction in the function of the retina and the width of the ONL of the retina due to long-term exposure to LL, indicating that the retinas of C57BL/6J mice can be affected by long-term LL. Previous work has shown that in 129sv/C57BL/6J mice (pigmented strain of mixed background) the ONL width is not affected by a year in LD or 3 weeks in LL (Chen et al., 1999). Here we see a reduction in the width of the ONL which may reflect a reduction in the amount of rows of cells in this layer. Our mice were in LL for 9 weeks, so it may be that more than 3 weeks is needed to begin to elicit retinal damage in pigmented mice. Of further note is the difference in the type of light used between our study and that performed by Chen and colleagues (1999). The light intensity in their study was between 1200 and 1500 lux provided by fluorescent light, while in our study the light intensity was approximately 2000 lux provided by LED light. It is possible that the different types of light may induce retinal damage at different rates. Finally it is worth noting the difference in strains between the two studies. Chen et al. (2009) used 129sv/C57BL/6J while we used C57BL/6J mice. Previous studies have shown that the genetic background of a mouse can affect the speed at which retinal damage occurs (LaVail et al., 1987a, 1987b).

Using dark-adapted and photopic ERG we found no differences in the rod and cone response or the adaptation of cones to light due to early light environment, indicating that the reduction in rod and cone function found was not dependent on the light environment in which the mice were raised. However we did find that mice raised in LL had a significantly reduced ON-bipolar cell response compared to mice raised in DD. The amplitude of the ON-bipolar cell response of mice raised in LL after a light stimulus of $3.1\log_{10}\mu W/cm^2$ was equivalent to the response of mice raised in DD after a light stimulus of $2.\ 1\log_{10}\mu W/cm^2$ indicating that the bipolar cell response of mice raised in LL is less sensitive to light by one log unit compared to mice raised in DD. We previously found that it takes a two log unit reduction in light intensity to see a significant shortening of tau in LL so it is unlikely that the reduction in bipolar cell response is mediating the changes we saw in tau length in LL.

We found no differences in the non-photic visual response and no differences in the structure of the retina due to early light environment, further confirming that although long-term exposure to LL causes an overall reduction in retinal function, the reduction is not facilitated by being raised in different environments during postnatal development.

3.4.5 Conclusions

This study has shown that CD1 mice raised in LL suffer a severe loss of retinal function and severe retinal damage compared to mice raised in LD and DD, making them unsuitable for further experiments exploring the role of early light environment on the photic response of the circadian system.

On the other hand C57BL/6J mice do not experience major changes in retinal function or structure due to early light environment, making them a good model for exploring how early light environment affects the circadian photic response in adult mice. Furthermore we have shown that when placed in LL as adults, mice raised in DD have longer taus than mice raised in LD or LL, implying that mice raised in DD are more sensitive to light. This exposure of mice to LL during adulthood lead to an overall decrease in retinal function but this decrease was not

dependent on postnatal light environment. These results suggest that postnatal light environment affects the future sensitivity of the circadian system to light. The origin of this effect may not be at the retinal level, but may be at the clock level, due to changes in how the SCN processes light information. Further work will explore this in more detail.

4. Effects of early light environment on neuropeptide expression in the hypothalamus of albino mice

4.1 Introduction

Light plays a critical role in entraining the circadian clock to the external environment. Light information is transmitted from the retina to the suprachiasmatic nucleus (SCN) via the retinohypothalamic tract (RHT) (Moore and Lenn, 1972). The SCN is subdivided into two main regions: the core (ventromedial) and shell (dorsolateral). The RHT synapses directly onto the core SCN where neurons expressing vasoactive intestinal polypeptide (VIP) are located. These VIP-expressing neurons have fibres that project to the shell SCN, where neurons expressing arginine vasopressin (AVP) are located. AVP-expressing neurons send outputs to many other parts of the hypothalamus. Thus, it seems that VIP plays a role in transmitting external light information from the light-receiving part of the SCN to the rest of the SCN in order to reset the clock to the external light environment, while AVP plays a role in relaying information about the SCN to other parts of the brain (Abrahamson and Moore, 2001).

It has previously been shown that C57BL/6J mice raised in constant light (LL) from day of birth (P0) to P21 then kept in normal 12:12 hour light-dark conditions (LD) until P50 had significantly lower VIP and AVP expression in the SCN and paraventricular nucleus (PVN) compared to mice raised in LD or constant darkness (DD) from P0 to P21 then kept in LD until P50 (Smith and Canal, 2009). It was also shown using staining for glial fibrillary acidic protein (GFAP) that the astrocytic population in the SCN was affected by the light environment in which mice were raised. Indeed, mice raised in DD were found to have significantly higher density of GFAP staining, but a lower number of GFAP-expressing cells compared to mice raised in LD and LL (Canal et al., 2009). These data indicate that early light environment affects both neuropeptide expression and astrocyte structure in the hypothalamus of C57BL/6J mice.

Studies in locomotor activity behaviour have shown that C57BL/6J mice raised in LL had a higher amplitude of locomotor activity behaviour compared to mice raised in DD and LD (Smith and Canal, 2009). However in a study using CD1 mice, an albino strain, it was found that mice raised in LL had a significantly lower amplitude of locomotor activity behaviour compared to mice raised in DD and LD (Canal-Corretger et al., 2001b). It has been hypothesised that the altered response between the two strains of mice may be because one strain is pigmented (the C57BL/6J mice) while the other is albino.

In a previous study, I have shown that early light environment did not affect retinal function or structure in C57BL/6J mice but that CD1 mice raised in LL had significantly reduced retinal function and severely damaged retinal structure (see Chapter 3). This data indicates that in C57BL/6J mice, the changes seen in VIP, AVP and GFAP expression and in locomotor activity behaviour were not due to changes in the retina but may be a result of changes in the SCN itself. In view of the fact that CD1 mice raised in LL had significant damage to their retinas due to early light environment compared to mice raised in DD and LD and had different locomotor activity behaviour to C57BL/6J mice, we wanted to see how VIP, AVP and GFAP expression

was affected by early light environment in CD1 mice and whether the changes could explain the differences seen in locomotor activity behaviour in LD.

4.2 Experimental design

Table 4.1 describes the experimental design for the CD1 mice used during this experiment. After the brains were removed, they were processed for immunohistochemistry as described in the Materials and Methods (Chapter 2). Brains from CD1 group 1 mice were stained using anti-VIP (1:2000), anti-AVP (1:10000) and anti-GFAP (1:1000). Brains from CD1 group 2 mice were stained using anti-VIP (1:2000) and anti-AVP (1:10000). Staining in the SCN and PVN was quantified by measuring the density of the staining (using ImageJ) and counting the number of positively-stained cells in these two areas.

For animals in group 2, retinas were processed for histology using cresyl violet staining. The integrity of retinal structure was assessed by measuring the width of the outer nuclear layer (ONL) and calculating the density of the cells in the ONL.

Statistical analysis was carried out by means of an ANOVA of general linear models using SYSTAT software. For all statistical analysis the independent variable was light during lactation (DD, LD or LL). For immunohistochemistry, the dependent variables were density of AVP, VIP or GFAP staining and number of AVP- or GFAP-positive cells per mm^2. For the retinal histology, the dependent variables were ONL width and ONL optical density.

	CD1 group 1 (DD – n=7 males; LD – n=7 males; LL – n=7 males)	**CD1 group 2 (n=5 females in each light condition)**
P0	Lactation (DD, LD, LL) – LED light ($330\mu W/cm^2$)	Lactation (DD, LD, LL) – LED light ($330\mu W/cm^2$)
P21	All animals placed in LD – LED light ($330\mu W/cm^2$) Animals were weaned and individually housed between P21 and P25	All animals placed in LD – fluorescent light ($800\mu W/cm^2$) Animals were weaned and individually housed between P21 and P25
P25	Locomotor activity in LD	
P50	ERG (P35-55; see Chapter 3 for results)	Animals culled at ZT16 in complete darkness and brains and eyes collected
P66	Animals culled at ZT14 in complete darkness and brains and eyes collected.	

Table 4.1: Experimental design for the CD1 mice used to determine the effects of early light environment on neuropeptide expression in the suprachiasmatic nucleus (SCN) of CD1 mice. Up until P21, all mice were kept in Light Tight Cabinets (Techniplast), mean light intensity $330\mu W/cm^2$ provided by LED light. CD1 mice in group 1 remained in this type of light for the duration of the experiment. CD1 mice in group 2 were transferred to light-tight wooden boxes with fluorescent light, mean light intensity $800\mu W/cm^2$. DD – constant darkness; LD – 12:12 hour light-dark cycle; LL – constant light; ERG – electroretinography; LED - light-emitting diode.

4.3 Results

4.3.1 Light measurements

In this experiment we used two different types of light. We therefore measured the spectral irradiance of the light sources and calculated how these lights would affect the different photoreceptors (Enezi et al., 2011). Table 4.2 presents an overview of the results. Of particular interest was that despite the fact that the fluorescent light source emitted a higher intensity light, the melanopic lux (m-lux) value was considerably higher under LED light than under fluorescent light.

Type of light	Radiometric measure ($\mu W/cm^2$)	Scotopic lux	Photopic lux	Melanopic lux
LED	350	4000	2000	10000
Fluorescent	800	1800	1500	3900

Table 4.2: Light intensity measures and their relevance to the different photoreceptors.

4.3.2 Effects of early light environment on neuropeptide expression in the hypothalamus of mice housed under LED light throughout life (group 1)

Mice in this experiment were born in DD, LD or LL and kept in these conditions until P21 when they were all housed in LD until the end of the experiment. Mean light intensity throughout the experiment was 330$\mu W/cm^2$ provided by LED light.

VIP staining was mainly found in the ventrolateral SCN, above the optic chiasm. We found that staining levels were significantly higher in mice raised in LL compared to mice raised in LD and DD ($p<0.01$ vs. DD and $p<0.05$ vs. LD; Figure 4.1). The mean density of staining was 22.62±1.47 in mice raised in DD, 24.53±2.87 in mice raised in LD and 29.59±2.99 in mice raised in LL.

AVP-positive cells and fibres were mainly found in the dorsolateral SCN and throughout the PVN. In the SCN, we found no difference in the AVP staining levels due to early light environment although it seems that mice raised in DD tended to have lower staining levels than mice raised in LD and LL (Figure 4.2A). The level of staining in mice raised in DD was 25.80±6.66, 32.26±5.68 in mice raised in LD and 36.39±8.71 in mice raised in LL. When we counted the number of AVP-positive cells in the SCN we found that mice raised in LL had significantly more AVP-positive cells than mice raised in LD ($p<0.05$; Figure 4.2B). Mice raised in DD had 245.76±81.88 AVP-positive cells/mm^2, mice raised in LD had 199.24±37.16 AVP-positive cells/mm^2 and mice raised in LL had 396.24±162.51 AVP-positive cells/mm^2.

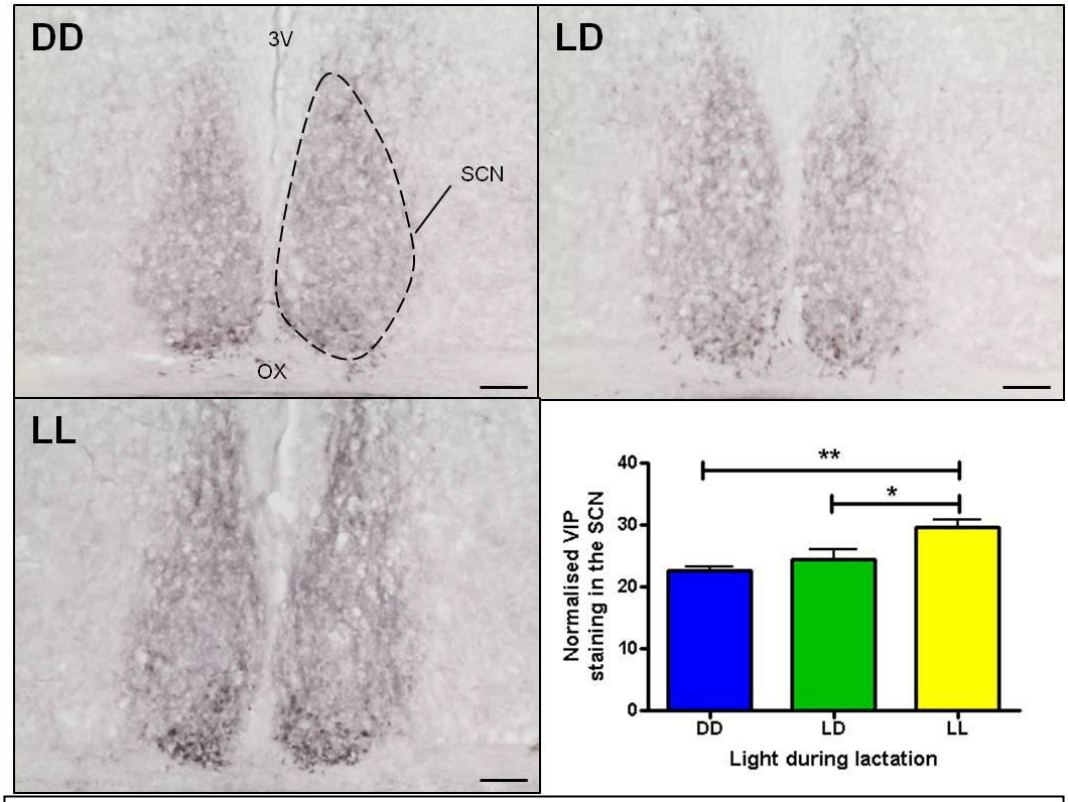

Figure 4.1: Effects of early light environment on VIP expression in the SCN - group 1. Mice were raised in DD, LD or LL from birth until P21, then placed in LD until P66. Mice were then culled at ZT14. Throughout the experiment mean light intensity was 330μW/cm^2 provided by LED light. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light; VIP – vasoactive intestinal polypeptide; SCN – suprachiasmatic nucleus; 3V - third ventricle; OX - optic chiasm; ZT - *Zeitgeber* time; LED - light-emitting diode. *p<0.05 compared to LD; ** p<0.01 compared to DD. Scale bar: 100μm.

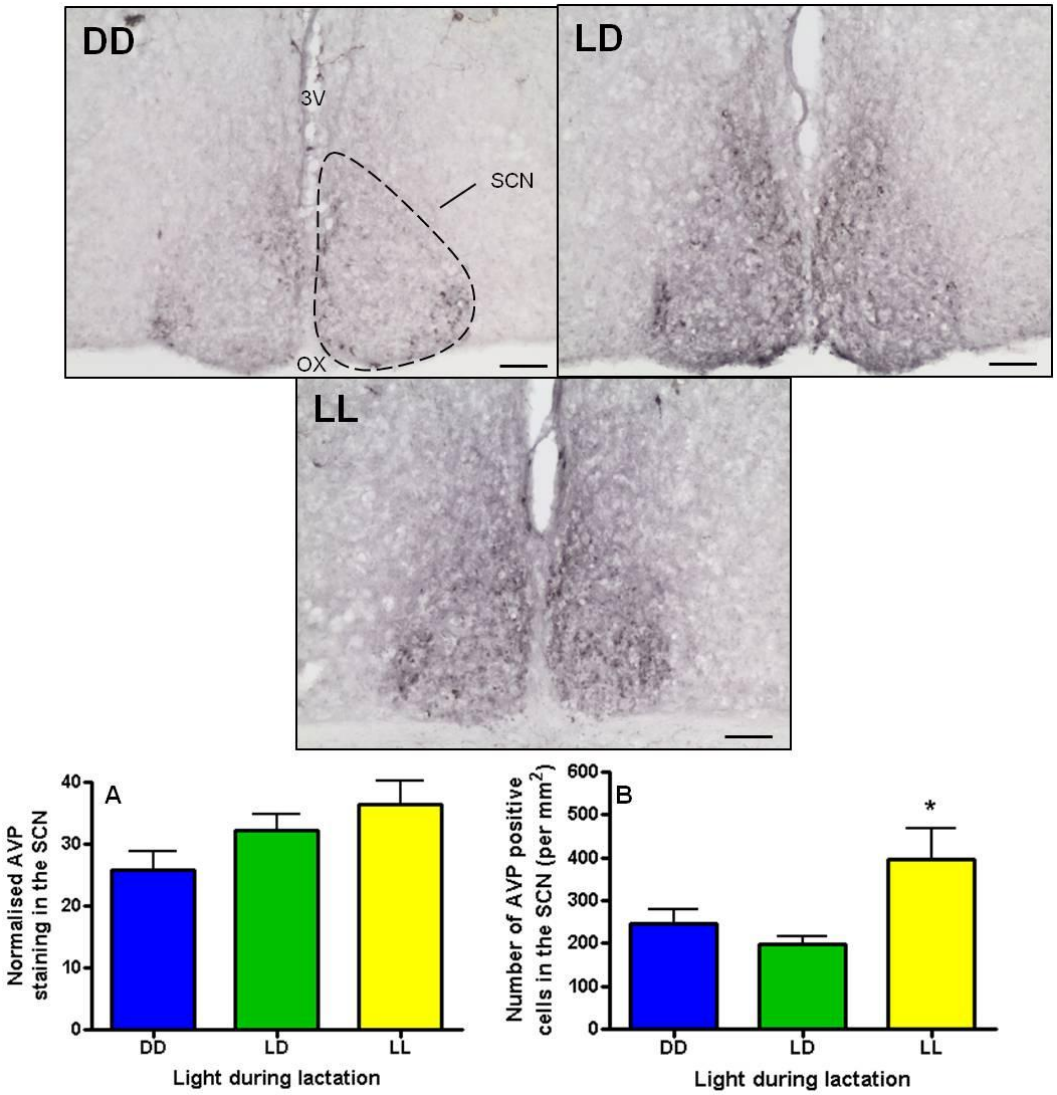

Figure 4.2: Effects of early light environment on AVP expression in the SCN - group1. Mice were raised in DD, LD or LL from birth until P21. They were then placed in LD and kept in these conditions until P66 when they were culled at ZT14. The staining levels (**A**) and number of AVP-positive cells (**B**) were quantified in the SCN. Throughout the experiment mean light intensity was 330μW/cm^2 provided by LED light. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light; AVP – arginine vasopressin; SCN – suprachiasmatic nucleus; 3V - third ventricle; OX - optic chiasm; ZT - *Zeitgeber* time; LED - light-emitting diode. *p<0.05 compared to LD. Scale bar: 100μm.

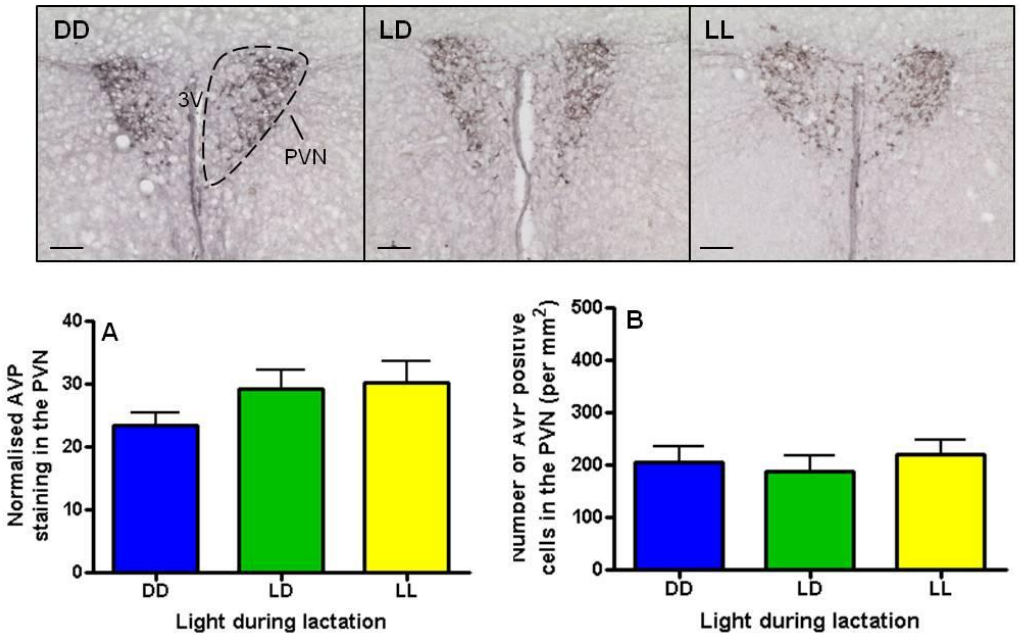

Figure 4.3: Effects of early light environment on AVP expression in the PVN - group 1. Mice were raised in DD, LD or LL from birth to P21. They were then placed in LD and kept in these conditions until P66 when they were culled at ZT14. The staining levels (**A**) and number of AVP-positive cells (**B**) were quantified in the PVN. Throughout the experiment mean light intensity was 330μW/cm^2 provided by LED light. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light; AVP – arginine vasopressin; PVN – paraventricular nucleus; 3V - third ventricle; ZT - *Zeitgeber* time; LED - light-emitting diode. Scale bar: 100μm.

In the PVN we found no difference in the level of AVP staining due to early light environment, although mice raised in DD seemed to have lower levels than mice raised in LD and LL (Figure 4.3A). Indeed, the level of staining in mice raised in DD was 23.50±4.96, 29.30±6.43 in mice raised in LD and 30.33±7.40 in mice raised in LL. We found no significant difference in the number of AVP-positive cells/mm^2 in the PVN due to early light environment (Figure 4.3B). Mice raised in DD had 204.79±75.21 AVP-positive cells/mm^2, mice raised in LD had 186.90±68.51 AVP-positive cells/mm^2 and mice raised in LL had 219.47±63.04 AVP-positive cells/mm^2.

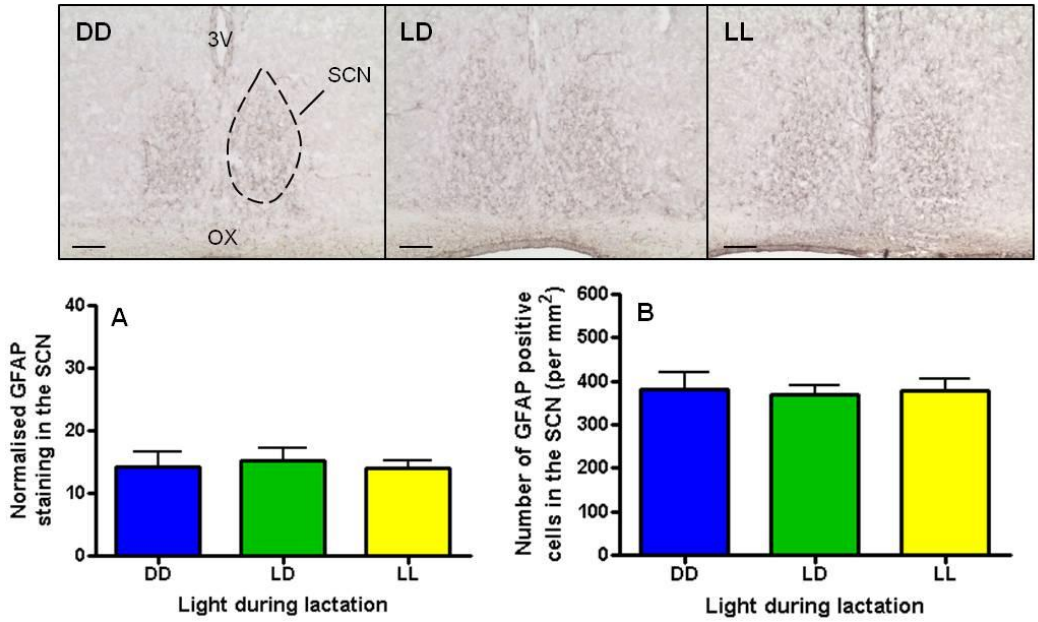

Figure 4.4: Effects of early light environment on GFAP expression in the SCN - group 1. Mice were raised in DD, LD or LL from birth until P21. They were then placed in LD and kept in these conditions until P66 when they were culled at ZT14. The staining levels (**A**) and number of GFAP-positive cells (**B**) were quantified in the SCN. Throughout the experiment mean light intensity was 330μW/cm^2 provided by LED light. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light; GFAP – glial fibrillary acidic protein; SCN – suprachiasmatic nucleus; 3V - third ventricle; OX - optic chiasm; ZT - *Zeitgeber* time; LED - light-emitting diode. Scale bar: 100μm.

GFAP staining was observed throughout the SCN, with the majority of GFAP-positive cells showing a typical stellate shape and multiple processes characteristic of astrocytes. We found no significant difference in the level of GFAP staining or the number of GFAP-positive cells in the SCN due to early light environment (Figure 4.4). The level of GFAP staining in mice raised in DD was 14.20±5.52, 15.29±4.48 in mice raised in LD and 14.03±2.74 in mice raised in LL. In the SCN, mice raised in DD had 380.95±90.99 GFAP-positive cells/mm^2, mice raised in LD had 369.49±48.46 GFAP-positive cells/mm^2 and mice raised in LL had 379.64±59.78 GFAP-positive cells/mm^2.

4.3.3 Effects of early light environment on neuropeptide expression in the hypothalamus of mice raised under LED light but transferred to fluorescent light as adults (group 2)

Mice in this experiment were born in DD, LD or LL provided by LED light (mean light intensity 330μW/cm^2) and kept in these conditions until P21. They were then all transferred to LD provided by fluorescent light (mean light intensity 800μW/cm^2) and kept in these conditions until the end of the experiment.

VIP staining was mainly found in the ventrolateral SCN, above the optic chiasm. We found no difference in VIP staining levels in the SCN due to early light environment (Figure 4.5). The level of VIP staining in the SCN was 26.93±9.59 in mice raised in DD, 22.49±4.27 in mice raised in LD and 28.20±5.57 in mice raised in LL.

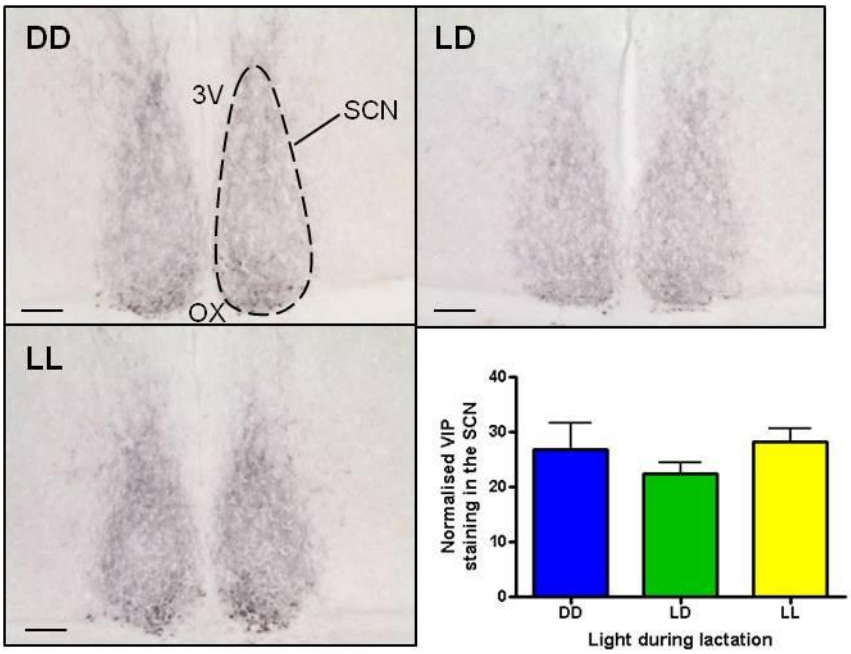

Figure 4.5: Effects of early light environment on VIP expression in the SCN - group 2. Mice were raised in DD, LD or LL from birth until P21, mean light intensity 330μW/cm^2 provided by LED light. At P21, mice were placed in LD, mean light intensity 800μW/cm^2 provided by fluorescent light. At P50, mice were culled at ZT16. DD - constant darkness; LD - 12:12 hour light-dark cycles; LL - constant light; VIP - vasoactive intestinal polypeptide; SCN - suprachiasmatic nucleus; 3V - third ventricle; OX - optic chiasm; ZT - *Zeitgeber* time; LED - light-emitting diode. Scale bar: 100μm.

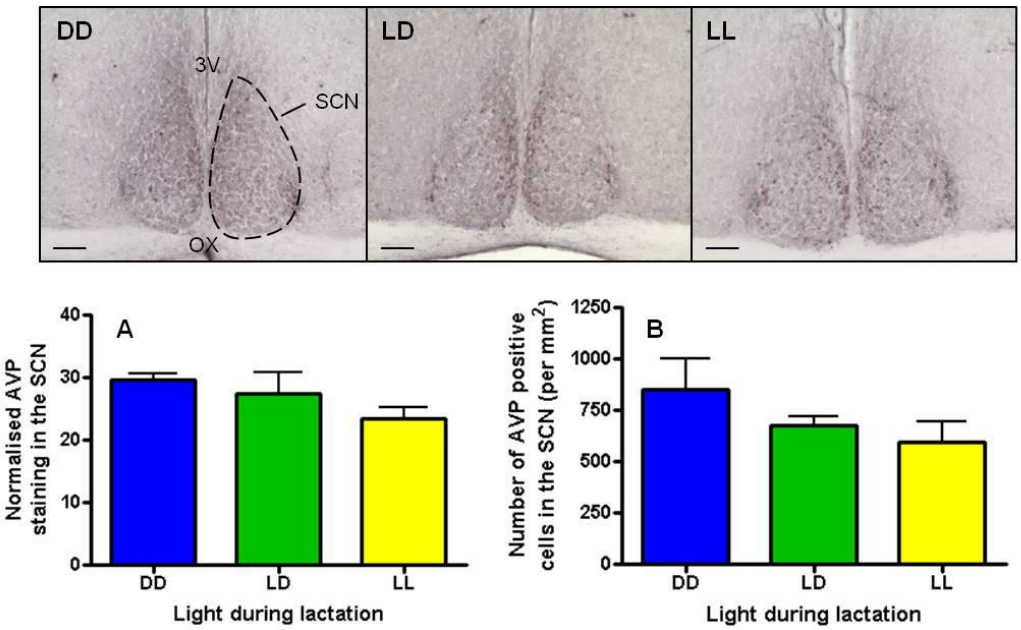

Figure 4.6: Effects of early light environment on AVP expression in the SCN - group 2. Mice were raised in DD, LD or LL from birth until P21, mean light intensity 330µW/cm^2 provided by LED light. At P21, mice were placed in LD, mean light intensity 800µW/cm^2 provided by fluorescent light. The staining levels (**A**) and number of AVP-positive cells (**B**) were quantified in the SCN. At P50, mice were culled at ZT16. DD - constant darkness; LD - 12:12 hour light-dark cycles; LL - constant light; AVP - arginine vasopressin; SCN - suprachiasmatic nucleus; 3V - third ventricle; OX - optic chiasm; ZT - *Zeitgeber* time; LED - light-emitting diode. Scale bar: 100µm.

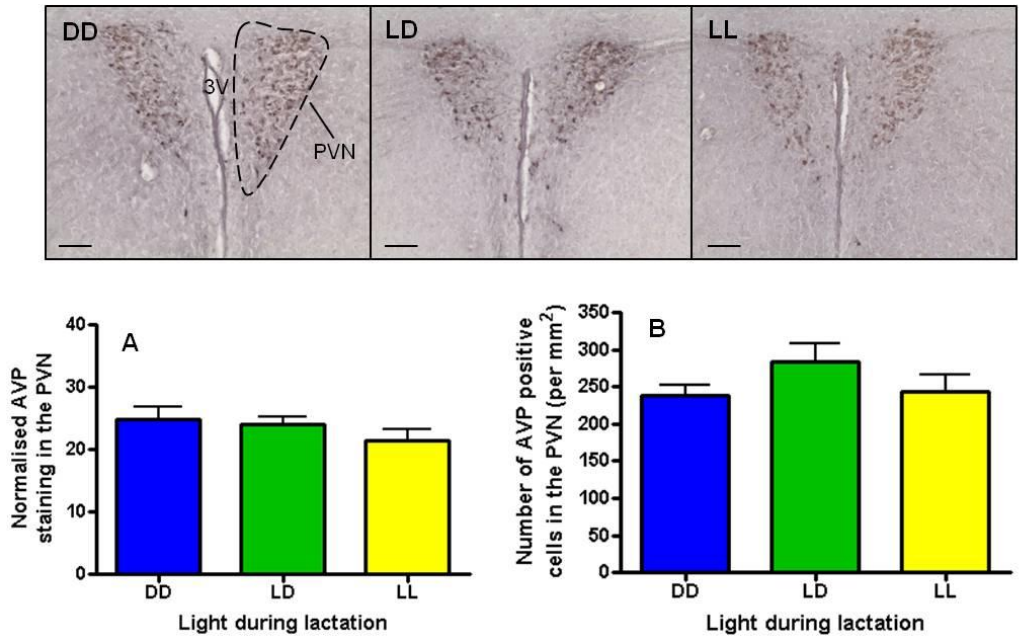

Figure 4.7: Effects of early light environment on AVP expression in the PVN - group 2. Mice were raised in DD, LD or LL from birth until P21, mean light intensity 330μW/cm^2 provided by LED light. At P21, mice were placed in LD, mean light intensity 800μW/cm^2 provided by fluorescent light. The staining levels (**A**) and number of AVP-positive cells (**B**) were quantified in the PVN. At P50, mice were culled at ZT16. DD - constant darkness; LD - 12:12 hour light-dark cycles; LL - constant light; AVP - arginine vasopressin; PVN - paraventricular nucleus; 3V - third ventricle; ZT - *Zeitgeber* time LED - light-emitting diode. Scale bar: 100μm.

AVP-positive cells and fibres were mainly found in the dorsolateral SCN and throughout the PVN. In the SCN, we found no difference in the level of AVP staining or the number of AVP-positive cells due to early light environment, although it seemed that mice raised in DD had higher staining levels and more positively stained cells than mice raised in LD and LL (Figure 4.6). The level of AVP staining in the SCN was 29.68±1.77 in mice raised in DD, 27.44±7.60 in mice raised in LD and 23.42±4.14 in mice raised in LL. In the SCN, mice raised in DD had 850.11±301.55 AVP-positive cells/mm^2, mice raised in LD had 674.50±101.48 AVP-positive cells/mm^2 and mice raised in LL had 594.32±218.93 AVP-positive cells/mm^2.

In the PVN, we found no difference in the level of AVP staining or the number of AVP-positive cells due to early light environment, although mice raised in DD seemed to have slightly higher staining levels than mice raised in LD and LL (Figure 4.7). Indeed, the level of AVP staining in the PVN of mice raised in DD was 24.82±3.98, 23.97±3.04 in mice raised in LD and 21.38±4.34 in mice raised in LL. In the PVN, mice raised in DD had 238.43±26.72 AVP-positive cells/mm^2, mice raised in LD had 283.32±55.45 AVP-positive cells/mm^2 and mice raised in LL had 243.18±51.28 AVP-positive cells/mm^2.

4.3.4 Effects of early light environment on the retinal histology of CD1 mice raised under LED light but transferred to fluorescent light as adults (group 2)

Previous work has shown that when CD1 mice were housed under LED light with a mean intensity of 330μW/cm^2 throughout life, mice raised in LL from P0 to P21 then placed in LD from P21 onwards suffered severe retinal damage compared to mice raised in DD and LD (see Chapter 3). We showed this using measures of retinal function such as electroretinography (ERG) and measuring the pupil light reflex (PLR) followed by looking at the retinal histology, which matched what we had seen in our retinal function experiments. Therefore, in this study, we looked at the retinas using only histology, to obtain a general overview of changes in the retina due to the light conditions in which these mice were housed. The mice studied were CD1 mice raised in DD, LD or LL provided by LED light (mean intensity 330μW/cm^2) then placed in LD provided by fluorescent light (mean intensity 800μW/cm^2) from P21 onwards.
We first measured the width of the ONL and found no difference in ONL width due to early light environment although it appeared that mice raised in LL had slightly smaller ONL widths than mice raised in LD and DD (Figure 4.6). Mice raised in DD had a mean ONL width of 40.17±5.24μm, mice raised in LD had a mean ONL width of 38.20±8.56μm and mice raised in LL had a mean ONL width of 30.86±7.29μm. During the analysis it was noted that although the width of the ONL did not seem to be affected by early light environment, there were some differences in the structure of the ONL. In particular it was noted that the ONL of mice raised in LL seemed to have more gaps (Figure 4.8). To quantify this we measured the density of the cresyl violet staining, thus taking a measure of the cell density in the ONL. We found that mice raised in LL had significantly reduced staining levels compared to mice raised in DD and LD ($p<0.05$). Indeed the staining level in the ONL of mice raised in DD was 8828.28±769.55, 8046.46±710.70 in mice raised in LD and 6481.67±992.29 in mice raised in LL. Thus, although these mice do not suffer retinal damage to the extent of the mice that had been housed under LED light throughout life (see Chapter 3) the structural integrity of the ONL was still affected by early light environment in CD1 mice.

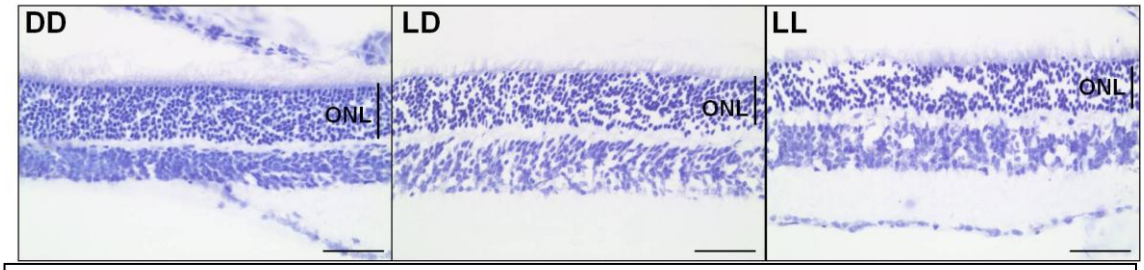

Figure 4.8: Effects of early light environment on retinal structure - group 2. Example retinal sections from mice raised in DD, LD or LL from P0 to P21 under LED light with a mean intensity of 330μW/cm^2. At P21 the mice were transferred to LD provided by fluorescent light with a mean intensity of 800μW/cm^2. The density of cells in the ONL was measured and found to be reduced in mice raised in LL, indicated by the larger gaps seen in the ONL pictured. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light; ONL – outer nuclear layer; LED - light-emitting diode. Scale bar: 50μm.

4.4.1 Summary of results

A summary of the results of this study is provided in Table 4.3, which also contains details of the results found in previous studies of changes in neuropeptide and GFAP expression in the hypothalamus due to early light environment (Canal et al., 2009; Smith and Canal, 2009). Previous work has shown that mice raised in LL had significantly lower AVP- and VIP- positive staining than mice raised in DD and LD (Smith and Canal, 2009). Furthermore it has been shown that the density of GFAP staining was significantly higher in mice raised in DD compared to mice raised in LD and LL, but the number of GFAP-positive cells was lower in mice raised in DD compared to mice raised in LD and LL (Canal et al., 2009). In this study we were unable to replicate the findings previously published. Furthermore our experiment consisted of two different experimental conditions. We were unable to obtain consistent results between our two experimental conditions. We must therefore consider the differences between these experiments to determine any reasons why we could not replicate the findings.

4.4.2 The contribution of strain to the different results

The first obvious difference between the current study and those published is that we used CD1 mice (an albino strain) while the published work used C57BL/6J mice (a pigmented strain). In our study using mice that were kept under LED light throughout life, we found that CD1 mice raised in LL until P21 then placed in LD from P21 onwards had significantly higher VIP staining levels and significantly more AVP-positive cells than mice raised in DD and LD. On the other hand, previous work has shown that C57BL/6J mice raised in DD had significantly higher VIP and AVP staining levels than mice raised in LD and LL. It seems therefore that strain differences have resulted in an opposite response in neuropeptide expression due to early light environment. Indeed, previous work on neuropeptide gene expression after food deprivation has found that the neuropeptide response to food deprivation can be affected by the strain of the mouse (Chua et al., 1991). For example Chua et al. (1991) found that in CD1 mice fasting caused an increase in enkephalin mRNA in the hypothalamus while in C57BL/6J mice, fasting caused a decrease in enkephalin mRNA. It is possible that alterations in AVP and VIP expression by early light environment are also strain dependent.

The current study, however, seems to indicate that strain may not be the only contributing factor to the different results we obtain. Although we saw significant differences in VIP and AVP expression due to early light environment in the mice kept under LED light throughout life, we found no significant differences in VIP or AVP expression in the CD1 mice raised under LED light then placed under fluorescent light from P21 onwards, but did find that all results looked similar to what had been published previously (but not statistically significant). Thus, within the same strain we were not able to obtain consistent results in the effect of early light environment on VIP and AVP expression.

		Results from this study		Results from Smith and Canal (2009) and Canal et al. (2009)
Strain		CD1	CD1	C57BL/6J
Sex		Male	Female	Male
Type and intensity of light		LED 330μW/cm^2 throughout life	LED 330μW/cm^2 from P0-P21 then fluorescent 800μW/cm^2 from P21 onwards	Fluorescent 57μW/cm^2 throughout life
Retina		Mice raised in LL have severe retinal damage (see Chapter 3)	Decrease in structural integrity of ONL in mice raised in LL compared to LD and DD	No data (we can hypothesise that there is no retinal damage as these mice are pigmented – see Chapter 3)
VIP in SCN	**Optical density**	**DD<LD<LL**	N.S	**DD,LD>LL**
AVP in SCN	**Optical density**	N.S. (visual observation shows slight DD<LD<LL)	N.S. (visual observation shows slight DD>LD>LL)	**DD>LD>LL**
	Number of cells	**LD<LL**	N.S. (visual observation shows slight DD>LD>LL)	No data
AVP in PVN	**Optical density**	N.S. (visual observation shows slight DD<LD<LL)	N.S. (visual observation shows slight DD>LD>LL)	**DD,LD>LL**
	Number of cells	N.S.	N.S.	N.S.
GFAP in SCN	**Optical density**	N.S.	No data	**DD>LD>LL**
	Number of cells	N.S.	No data	**DD<LD<LL**

Table 4.3: Summary of effects of early light environment on neuropeptide and GFAP expression in mice. Results obtained in this study are compared to previous published work. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light; N.S. – not significant; VIP – vasoactive intestinal polypeptide; AVP – arginine vasopressin; GFAP – glial fibrillary acidic protein; SCN – suprachiasmatic nucleus; PVN – paraventricular nucleus; LED - light-emitting diode.

4.4.3 The contribution of sex to the different results

In our study we housed CD1 mice in two different lighting conditions. Furthermore these mice were also separated according to sex. Thus the mice that were kept under LED light throughout life were male, while the mice that were kept under LED light until P21 then placed in fluorescent light were female. It is therefore possible that the sex of the mice contributed to the different results we found. Indeed previous work has shown that the circadian profile of VIP expression in rats was sexually dimorphic with the peak in VIP mRNA expression occurring during the subjective night in males and during the subjective day in females (Krajnak et al., 1998a, 1998b). To the extent of our knowledge there is no evidence of sexual dimorphism in AVP expression in rats or mice. In our study we saw significant effects of early light environment on VIP and AVP expression in male mice, but not female mice. However, previous work has shown opposite neuropeptide responses to early light environment, also in male mice. Thus it does not appear that sex dictates how neuropeptide expression is affected by early light environment.

4.4.4 The contribution of retinal damage to the different results

In our work we have taken a particular interest in how the retina is affected by early light environment. We have shown that in C57BL/6J mice early light environment did not appear to cause retinal damage, even at high intensities (see Chapter 3). However, when CD1 mice were housed in LED light throughout life, we saw significant retinal damage in mice raised in LL until P21 then placed in LD from P21 onwards compared to the mice raised in DD and LD. Here we were able to examine the retinas of CD1 mice raised under LED light until P21 then placed in fluorescent light. We found no severe damage due to early light environment, but the mice raised in LL had alterations in the structural organisation of the ONL, indicating some effects of LL on the ONL of CD1 mice, which may lead to altered processing of light information in the retina. What is interesting about these results is that these mice were in the same light conditions up until P21 as the CD1 mice that had sustained severe retinal damage due to being raised in LL. After P21 the mice that sustained severe damage remained under LED light while the mice that had only minor alterations in ONL structure were transferred to fluorescent light. These results imply that LL during postnatal development may not be as damaging as we had previously thought, but that it is the light conditions after P21 that mediate the damage. Previous studies have also made the observation that light during postnatal development may not cause retinal damage (Harada et al., 1998, 2000). Indeed one study has shown that in Wistar rats retinal degeneration due to LL only began after P21 (Harada et al., 1998). It therefore seems that retinal damage caused by being raised in LL provided by LED light can be rescued if the animals are placed in fluorescent light from P21 onwards.

Of interest to this current study is that we have mice that have different levels of retinal damage. The mice that were in LED light throughout life and were raised in LL had severe retinal damage while the mice that were in LED light up until P21 then transferred to fluorescent light had altered ONL structure but no apparent damage if they were raised in LL. We can hypothesise that the C57BL/6J mice in the published data did not have any retinal damage as they were

housed under fluorescent light throughout life and benefited from protection from the damaging effects of LL (see Chapter 3). When we put this together with the neuropeptide expression we find that the CD1 mice raised in LL that had severe retinal damage (housed in LED light throughout life) had increased VIP and AVP levels compared to the mice raised in DD and LD. Meanwhile the CD1 mice that had no apparent retinal damage but alterations in ONL structure due to early light environment had no significant alterations in neuropeptide expression due to early light environment but neuropeptide expression seemed to be going in the same direction as what had been published in C57BL/6J mice. Finally, the C57BL/6J mice that had no retinal damage had significantly reduced AVP and VIP expression when they were raised in LL compared to being raised in DD and LD (Smith and Canal, 2009). We can therefore hypothesise that in CD1 mice, when retinal damage occurs, there is an increase in AVP and VIP expression, perhaps to compensate for the reduced light signals reaching the SCN. When there is no retinal damage in the CD1 mice, we do not see any alterations in neuropeptide expression in the SCN due to early light environment. Changes in neuropeptide expression do seem to be in the direction of what has been published in C57BL/6J mice, although these were not significant. In the C57BL/6J mice, where there is no retinal damage, the lowest light signals the SCN are found in the mice raised in DD which leads to an increased neuropeptide expression in these mice to compensate for the reduced light information.
These data shows us that it is therefore possible that the amount of light reaching the SCN after weaning could dictate the neuropeptide response to early light environment.

4.4.5 The effect of the type of light on the melanopsin-expressing retinal ganglion cells

LED and fluorescent light have different spectral irradiance curves, which can result in differential activation of photoreceptors depending on the type of light used. We measured the spectral irradiance of the LED lights in our Light Tight Cabinets and the fluorescent lights in our wooden boxes and discovered that the LED lights had a much higher melanopic lux (m-lux) value than the fluorescent lights (10,000m-lux compared to 3,900m-lux) despite the fluorescent lights having a seemingly higher light intensity. Melanopic lux is a measure of the amount of light which activates the melanopsin-expressing retinal ganglion cells (RGCs). Melanopsin-expressing RGCs mediate the non-photic visual response which includes circadian entrainment as well as the pupil light reflex. We have previously measured the pupil light reflex to ascertain the non-photic visual response in C57BL/6J mice and CD1 mice housed in LED light throughout life (see Chapter 3). We found that early light environment did not affect the non-photic visual response in C57BL/6J mice, but that CD1 mice raised in LL had a reduced non-photic visual response, which may mean that there was also a reduction in the amount of melanopsin-expressing RGCs innervating the SCN. We have not measured the pupil light reflex of CD1 mice raised in LED light until P21 then transferred to fluorescent light, but based on the retinal histology, we can hypothesise that there would be very little reduction in the pupil response in these mice. We can therefore hypothesise that the m-lux levels of the light environment may affect neuropeptide expression in the SCN. In C57BL/6J mice that were housed in low-intensity fluorescent light throughout life, mice raised in DD received the lowest amount of m-lux, leading to an increase in neuropeptide expression in the SCN. In CD1 mice housed in high-intensity

LED light throughout life (receiving 10,000 m-lux throughout life), the mice raised in LL suffered retinal damage and therefore received the least m-lux leading to an increase in neuropeptide expression in the mice raised in LL. The mice raised in DD and LD still received high levels of m-lux during adulthood which could have resulted in a saturation of the light signals reaching the SCN. This may have lead to us not seeing any other differences in neuropeptide expression due to early light environment. Finally the CD1 mice raised in LED light then transferred to fluorescent light received an intermediate amount of m-lux (10,000 m-lux during lactation followed by 3,900 m-lux during adulthood) which may also have lead to a saturation of the m-lux signal to the SCN, which we saw as no difference in neuropeptide expression due to early light environment.
Because of the major difference in the amount of m-lux emitted by the two different light sources, it is important to take into consideration the effects this may have on the response of the SCN to light.

4.4.6 GFAP expression in the SCN is not affected by early light environment in CD1 mice

We found no differences in GFAP expression in the SCN due to early light environment in the CD1 mice regardless of the light conditions in which they were housed. Previous work has shown that GFAP expression was affected by early light environment in C57BL/6J mice housed in low intensity fluorescent light throughout life (Canal et al., 2009). The main difference between that study and ours was that we raised our mice in high intensity LED light. It is possible that the SCN had been saturated by the large amount of light signals, resulting in the mice raised in LD and LL having the same amount of GFAP expression as the mice raised in DD. It is also possible that, as mentioned above, strain contributed to how GFAP expression was affected by early light environment. Furthermore, it has been shown in hamsters and rats that enucleation at birth caused a decrease in GFAP expression in the SCN (Munekawa et al., 2000; Lavialle et al., 2001) compared to being raised in DD. Hamsters raised in DD and LD also showed rhythmic GFAP expression in the SCN while enucleated hamsters did not (Lavialle et al., 2001). It is therefore possible that in CD1 mice raised in LL, damage to the retinas has resulted in reduced GFAP expression, much like what occurs when hamsters were enucleated. This may result in effects of early light environment on GFAP expression being obscured by the effects of retinal damage.

4.4.7 Conclusion

Our data shows that the effects of early light environment on VIP, AVP and GFAP expression in the SCN are not robust and may be affected by factors such as retinal damage and type of light used. It is therefore important to consider the strain of mouse to be used in this type of experiment, as albino animals are particularly susceptible to light-induced retinal damage. As we have shown that C57BL/6J mice are resistant to light-induced retinal damage, they seem like an ideal model for exploring neuropeptide expression in the SCN as changes observed in the SCN would mainly reflect changes in SCN function and would therefore be better to use in our studies in which we try to find out what the long-term effects of early light environment are

on SCN function. Furthermore, these results also indicate that it is important to remain consistent when choosing the light conditions to use for experiments.

5. Effects of early light environment on locomotor activity behaviour and the photic signalling pathway in the SCN after light pulses

5.1 Introduction

In mice, early light environment has been shown to affect locomotor activity behaviour, along with neuropeptide and glial fibrillary acidic protein (GFAP) expression in the suprachiasmatic nucleus (SCN) (Canal-Corretger et al., 2001b; Canal et al., 2009; Smith and Canal, 2009). Of particular interest to this project is the finding that when adult mice are placed in constant light (LL), mice that were raised in constant darkness (DD) from birth (P0) to weaning (P21) have a significantly longer tau than mice raised in LL or 12:12 hour light-dark cycles (LD) (see Chapter 3 and (Canal-Corretger et al., 2001b)). Aschoff's rule states that in constant conditions increasing the light intensity also increases tau length (Aschoff, 1960). We have seen that at the same intensity of LL, mice raised in DD have a longer tau. We can therefore hypothesise that mice raised in DD are more sensitive to light than mice raised in LL.

My experiments have shown that early light environment does not seem to affect retinal function or structure in C57BL/6J mice (see Chapter 3) indicating that the changes we see in the

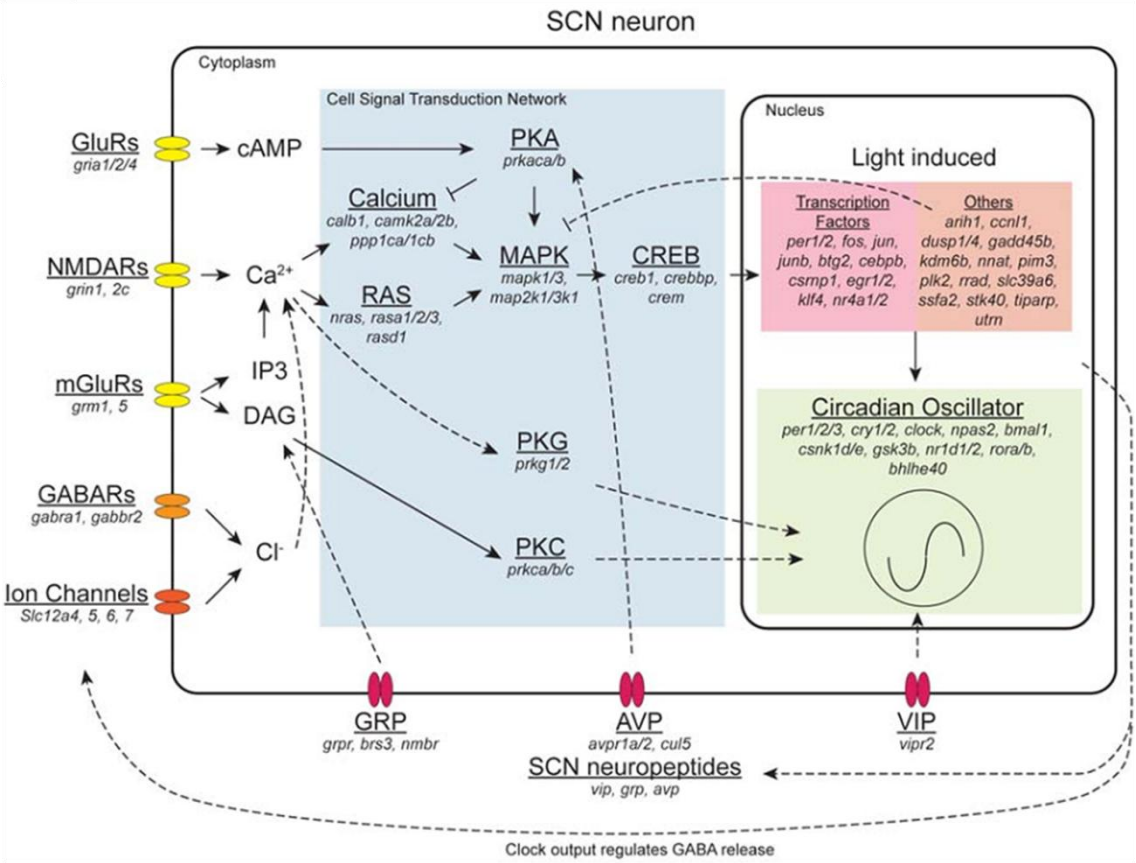

Figure 5.1: Light signalling pathway within an SCN neuron. Definitive gene functions are shown in solid arrows while implied gene functions are shown in dashed arrows. Figure taken from Zhu et al. (2012).

locomotor activity behaviour of adult mice placed in LL may be due to changes in how the SCN is interpreting light information.

A common method used to measure the circadian response to light is to place mice in DD and administer light pulses during their subjective night. A light pulse during the early subjective night will result in a phase delay and a light pulse in the late subjective night will result in a phase advance (Daan and Pittendrigh, 1976a). We hypothesised that if C57BL/6J mice raised in DD are more sensitive to light, they may shift their locomotor activity behaviour after a light pulse more than mice raised in LD or LL.

Within SCN neurons, light pulses result in changes in intracellular signalling pathways (Figure 5.1). Among other changes, activation of neurons by glutamate after a light pulse results in the phosphorylation of the mitogen-activated protein kinase (MAPK) signalling pathway (Obrietan et al., 1998). Furthermore, it has been shown that inhibition of the MAPK signalling pathway results in the loss of ability to shift after light pulses both in the early and late night (Butcher et al., 2002; Hainich et al., 2006) indicating a role for MAPK in phase shifting after light pulses. Activation of the MAPK signalling pathway leads to the phosphorylation of the transcription factor cyclic adenosine monophosphate (cAMP) response element-binding (CREB) (Obrietan et al., 1998) which binds to the cAMP response element (CRE) in the promoter region of photically inducible clock genes such as *Per1* and *Per2* as well as immediate early genes such as *cFos* (Ginty et al., 1993; Obrietan et al., 1999; Gau et al., 2002). *cFos* is upregulated in response to light pulses both in the early and late night (Colwell and Foster, 1992) and is commonly used as a marker of photic activation in the SCN. *Per1* and *Per2* are core clock genes which are also rapidly activated after a light pulse, with *Per1* being upregulated after both early and late night light pulses while *Per2* is upregulated only after light pulses in the early night (Albrecht et al., 1997; Zylka et al., 1998).

The aim of this experiment was to see whether early light environment would alter the size of phase shift after light pulses at CT16 (early night) or a light pulse at CT22 (late night). Furthermore we wanted to see whether upregulation of various components of the intracellular signalling pathway after a light pulse at CT16 would be affected by early light environment. We therefore monitored the locomotor activity behaviour of C57BL/6J mice in response to light pulses and stained the SCN for immunoreactivity against cFOS, PER2 and pERK (a member of the MAPK family) to look at various stages of the photic signalling pathway.

5.2 Experimental design

Table 5.1 shows the experimental design for the adult C57BL/6J mice that were placed in DD and subjected to light pulses at different points of their subjective night. We used both Aschoff Type 1 and 2 protocols to deliver the light pulses (Jud et al., 2005). For the Aschoff type 1 protocol, the pulsed group received light pulses by having their cages moved into a light environment, then moved back 30 minutes later. The non-pulsed group received sham pulses by moving the cages but not placing them in a light environment and moving the cages once more 30 minutes later. For the Aschoff type 2 protocol the cages were not moved and the lights

	C57BL/6J pulsed group (DD – n=6 males and 6 females; LD – n=6 males and 3 females; LL – n=7 males and 4 females)	**C57BL/6J non-pulsed group (DD – n=5 males; LD – n=2 males and 3 females; LL – n=5 males)**
P0	Lactation (DD, LD, LL)	Lactation (DD, LD, LL)
P21	All animals placed in LD Animals were weaned and individually housed between P21 and P23.	All animals placed in LD Animals were weaned and individually housed between P21 and P23.
P23	Locomotor activity in LD	Locomotor activity in LD
P43	Locomotor activity in DD	Locomotor activity in DD
P44	CT 16 light pulse (Aschoff type 2 protocol) – lights turned on for 30 minutes, cages not moved	Sham pulse at CT16 (cages were not moved and lights were not turned on).
P56	CT16 light pulse (Aschoff type 1 protocol) – cages moved to a cabinet with lights on for 30 minutes then placed back in original cabinet.	At CT16, cages moved but no light pulse was given. Cages were moved again 30 minutes later.
P71	CT22 light pulse (Aschoff type 1 protocol) – cages moved to a cabinet with lights on for 30 minutes then placed back in original cabinet.	At CT22, cages moved but no light pulse was given. Cages were moved again 30 minutes later.
P91	CT16 light pulse (Aschoff type 1 protocol) – cages moved to a cabinet with lights on for 30 minutes then placed back in original cabinet. One hour after the end of the light pulse, animals were culled in complete darkness and brains were collected.	At CT16, cages moved but no light pulse was given. Cages were moved again 30 minutes later. One hour later, animals were culled in complete darkness and brains were collected.

Table 5.1: Experimental design for the C57BL/6J mice used to determine the effects of early light environment on the response of the circadian system to light pulses. Mean light intensity was 330µW/cm^2 provided by LED light. DD – constant darkness; LD – 12:12 hour light-dark cycle; LL – constant light; CT – circadian time; LED - light-emitting diode.

were switched on (pulsed group) or kept off (non-pulsed group).
We were not able to perform the pulsed and non-pulsed experiments at the same time due to constraints on space and equipment. We therefore performed the pulsed experiment first and the non-pulsed experiment second. There were some differences in locomotor activity behaviour between the first and second experiment, which may have been due to small changes in the living environment that we did not control for such as the amount of bedding or minor changes in temperature (within the confines of our temperature range as set out in the Materials and Methods). However, as we found no differences in the effects of early light environment on locomotor activity behaviour in either experiment, we used the pulsed animals to analyse the locomotor activity behaviour as there were more mice in each group in this experiment. The non-pulsed animals were used to determine whether their locomotor activity behaviour shifted after sham pulses.
At the end of the experiment mice were subjected to a light pulse or a sham pulse at CT16 and were culled 1 hour after the end of the light pulse in complete darkness. Their eyes were removed before transferring them to a light environment where the brains were removed and processed for immunoreactivity against cFOS, pERK and PER2.
Statistical analysis was carried out by means of an ANOVA of general linear models using SYSTAT software.
For all experiments the independent variables were light during lactation (DD, LD or LL) and pulsed or non-pulsed when analysing the size of phase shift or the immunohistochemistry. For the locomotor activity behaviour the dependent variables were period, percentage of variance, amplitude, power content of the first harmonic, duration of alpha, percentage of total duration, amount of activity during alpha, percentage of total activity, phase of entrainment (in LD only) and size of phase shift (after a light pulse or sham pulse).
For the immunohistochemistry the dependent variables were number of cFOS-positive cells, density of pERK staining and number of PER2-positive cells.

5.3.1 Effects of early light environment on the locomotor activity behaviour of adult C57BL/6J mice in LD, DD and in response to light pulses

A total of 32 male and female mice were born in DD, LD or LL and kept in these conditions until P21 when they were all placed in LD. They were then weaned between P21 and P23, housed individually and their locomotor activity behaviour was monitored under LD followed by DD. When the mice were in DD, light pulses were administered at CT16 and CT22 and the size of phase shift was measured. Figure 5.2 shows representative actograms of mice from both the non-pulsed and pulsed groups throughout the experiment.

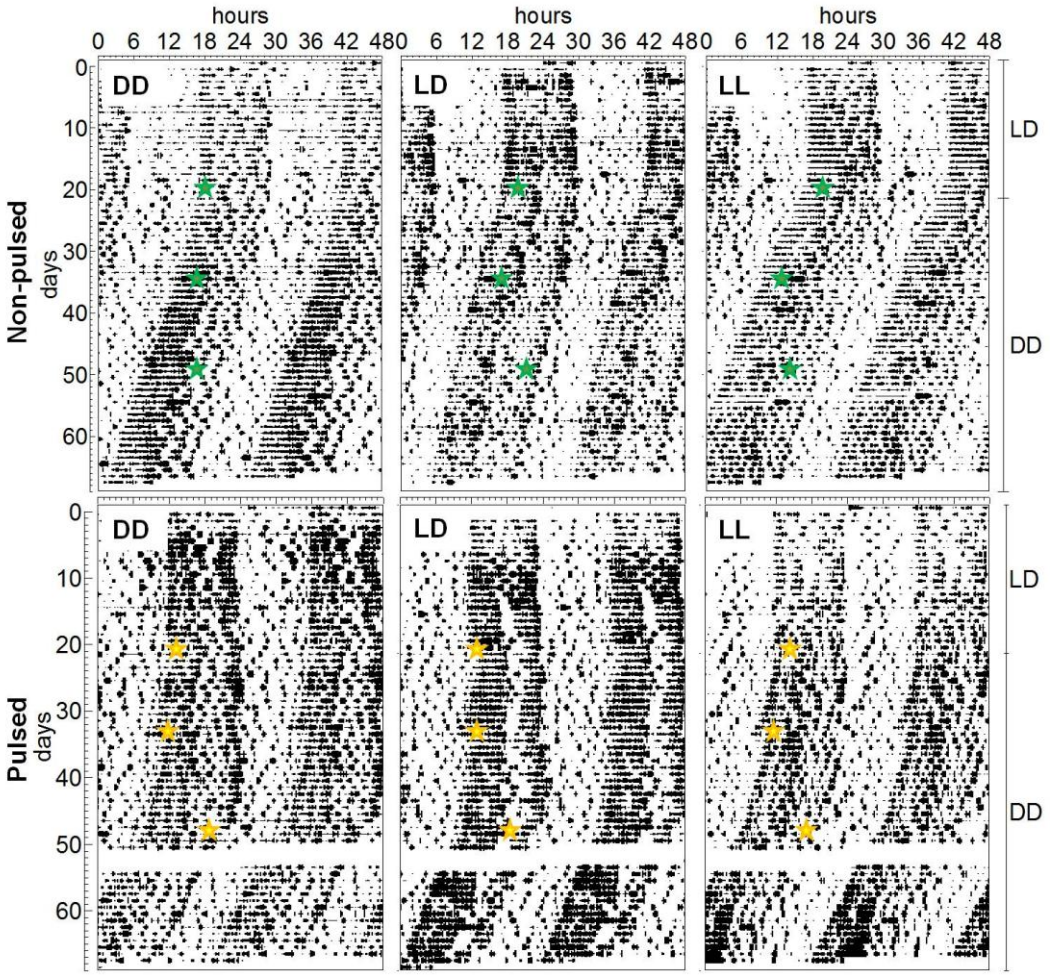

Figure 5.2: Effects of early light environment on the behavioural response to light pulses. Representative actograms of mice raised in DD, LD or LL from P0 to P21 then transferred to LD. Day 0 represents the first day their behaviour was monitored. Their locomotor activity was monitored in LD before they were transferred to DD and a light pulse (yellow star) or sham pulse (green star) was administered at CT16. Their activity was monitored for 2 weeks before another light pulse or sham pulse was administered at CT16. Their activity was again monitored for 2 weeks before administering a light pulse or sham pulse at CT22. Their activity was monitored for 2 weeks before administering another light pulse at CT16 and culling the mice one hour after the light pulse. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light; CT – circadian time.

		Period (hours)	% variance	Amplitude	Duration of alpha (hours)	Amount of activity during alpha (total counts)
LD stage	DD	24±0.00	32.54±8.88	16.57±5.38	12.35±0.28	1663.98±532.39
	LD	24±0.00	34.37±8.42	17.01±5.17	12.08±0.28	1712.35±555.53
	LL	24±0.00	31.19±4.94	14.09±4.65	12.20±0.34	1367.67±537.30
DD Stage 1	DD	23.74±0.13	31.94±9.50	18.95±16.79	14.15±1.10	1877.84±600.81
	LD	23.74±0.21	37.85±11.06	18.94±7.49	13.89±1.13	2217.93±825.88
	LL	23.91±0.16	29.86±5.44	14.02±4.46	13.67±1.08	1728.32±554.52
DD Stage 2	DD	23.82±0.20	25.22±6.80	19.88±16.26	14.23±1.10	1764.53±704.57
	LD	23.82±0.16	30.68±8.66*	22.33±16.39	13.54±1.35	1941.38±860.22
	LL	23.87±0.13	22.06±3.84	12.44±3.38	13.88±1.49	1349.70±474.39
DD Stage 3	DD	23.51±0.14	30.42±10.85	23.63±19.15	12.98±2.26	2691.93±2038.14
	LD	23.45±0.16	35.48±9.68	26.31±15.89	12.78±1.96	2447.44±1449.18
	LL	23.61±0.21	28.83±7.39	15.44±5.06	12.93±1.71	1475.97±464.07

Table 5.2: Effects of early light environment on the locomotor activity behaviour of C57BL/6J mice. Mice were raised in DD, LD or LL from P0 to P21 then placed in LD at P21. After two weeks in LD the mice were placed in DD and a light pulse was administered at CT16. Locomotor activity behaviour was analysed in DD for two weeks (DD Stage 1) before administering a second light pulse at CT16. Locomotor activity behaviour was analysed for two weeks after this light pulse (DD Stage 2) before a light pulse was administered at CT22. Locomotor activity was analysed for two weeks after this light pulse (DD Stage 3) before the animals received a final light pulse at CT16 and were culled one hour after the end of the light pulse. Data is presented as mean ± standard deviation. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light; CT – circadian time. *$p<0.05$ vs. LL.

Table 5.2 shows a summary of the locomotor activity behaviour analysis results in LD and in DD after each light pulse. In the LD stage, we found no significant differences in locomotor activity behaviour due to early light environment.

We then placed the mice in DD and the next day administered a 30-minute light pulse (or a sham pulse) at CT16 and measured the size of phase shift in response to this light pulse. We found that all mice that had received a light pulse showed a phase delay which was significantly longer than the delay seen in mice that had received a sham pulse ($p<0.01$; Figure 5.3). However there was no difference in the size of phase delay due to early light environment in either the mice that had received a light pulse or a sham pulse. Indeed, after a light pulse, the average phase delay was -0.67±0.32 hours in mice raised in DD, -0.69±0.51 hours in mice raised in LD and -0.91±0.43 hours in mice raised in LL. Meanwhile, in mice that received a sham pulse, the average phase delay was -0.06±0.13 hours in mice raised in DD, -0.15±0.33 hours in mice raised in LD and -0.09±0.13 hours in mice raised in LL. We noted that after the light pulse the size of phase shift was small, due to the fact that the mice had not been dark-

adapted for long. Therefore we decided to dark adapt the mice before administering a second light pulse at CT16, as this has previously been shown to increase the size of phase delay (Refinetti, 2003).

We first monitored locomotor activity behaviour in DD and found no differences due to early light environment in any of the variables that we measured (Table 5.2). After two weeks in DD we administered the second light pulse at CT16. Compared to this first light pulse we administered, the phase delay was significantly longer after the second CT16 light pulse ($p<0.01$; Figure 5.3). The mice that had received the sham pulse had significantly shorter delays than those that received a light pulse ($p<0.01$; Figure 5.3) but we saw no difference in the size of phase shift due to early light environment in either the mice that had received a light pulse or a sham pulse. In mice that had received a light pulse, the average phase delay was -3.32±0.50 hours in mice raised in DD, -2.84±0.43 hours in mice raised in LD and -2.68±0.70 hours in mice raised in LL. Meanwhile, after the sham pulse, there was no phase shift in the mice raised in DD or LL and the average phase delay in mice raised in LD was -0.08±0.19 hours.

We then analysed the locomotor activity behaviour after the second CT16 light pulse and found that mice raised in LD had a significantly higher percentage of variance than mice raised in LL ($p<0.05$; Table 5.2). Apart from that, we saw no difference in the locomotor activity behaviour due to early light environment.

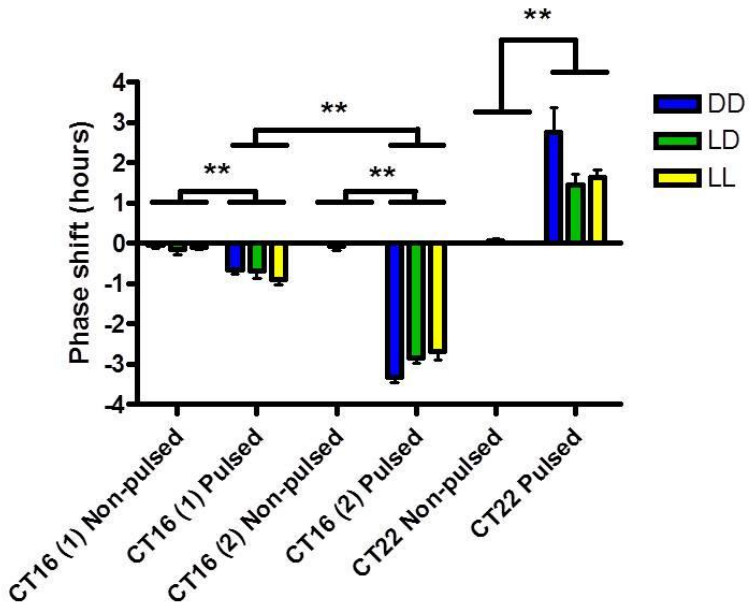

Figure 5.3: Effects of early light environment on the size of phase shift after CT16 and CT22 light pulses. Mice were raised in DD LD or LL from P0 to P21 then placed in LD for two weeks. They were then placed in DD and given a light pulse at CT16 – CT16(1) – followed by two weeks in DD. They were then given a second light pulse at CT16 – CT16(2) – followed by a further two weeks in DD. They were then given a light pulse at CT22 followed by two more weeks in DD. The non-pulsed mice received a sham pulse at the same circadian time as the pulsed mice received a light pulse. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light; CT – circadian time. ** $p<0.01$

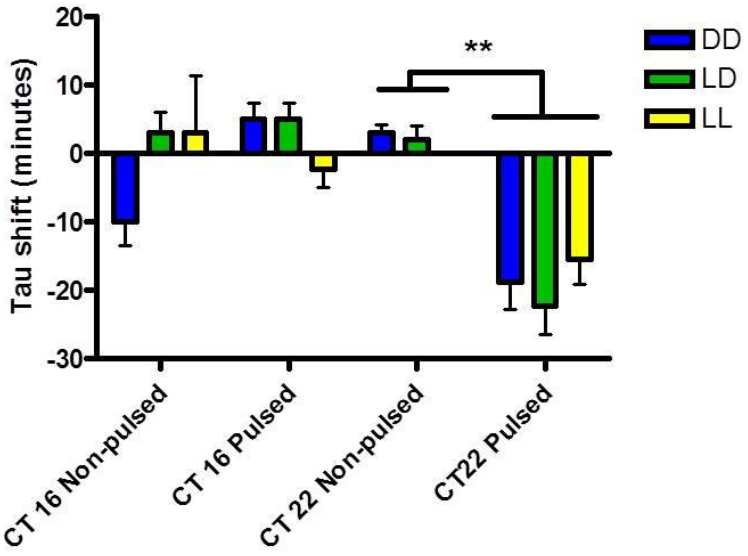

Figure 5.4: Effects of early light environment on the tau shift after light pulses at CT16 and CT22. C57BL/6J mice were raised in DD, LD or LL from P0 to P21 then placed in LD for two weeks. They were then placed in DD and given a light pulse the next day at CT16. Locomotor activity behaviour was monitored for two weeks before a second light pulse was given at CT16. Locomotor activity behaviour was monitored and the difference in tau before and after the CT16 light pulse was calculated in both mice that received the light pulse and mice that received the sham pulse. After a CT22 light pulse locomotor activity behaviour was monitored and the difference in tau before and after the CT22 light pulse was calculated in both mice that received a light pulse and mice that received a sham pulse. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light; CT – circadian time. **p<0.01

After a further two weeks in DD we administered a light pulse at CT22, triggering a phase advance which was significantly longer in the mice that had received a light pulse compared to the mice that received a sham pulse ($p<0.01$; Figure5.3). However we saw no difference in the size of phase advance due to early light environment in either the mice that had received a light pulse or a sham pulse. Indeed, in the mice that received a light pulse, the average phase advance was 2.77±1.97 hours in the mice raised in DD, 1.46±0.78 hours in the mice raised in LD and 1.62±0.69 hours in the mice raised in LL. In the mice that received a sham pulse there was no shift in the mice raised in DD or LL, and an average phase advance of 0.06±0.13 hours in the mice raised in LD.

We then monitored the locomotor activity behaviour in DD after the CT22 light pulse and found no difference in behaviour due to early light environment in any of the variables we measured. We did find that in the mice that received a light pulse, there was a significant shortening of tau after the CT22 light pulse, which we did not see in the mice that received a sham pulse or in the mice that received a CT16 light pulse or sham pulse ($p<0.01$; Figure 5.4), but no differences in the tau shift due to early light environment.

5.3.2 Effects of early light environment on photic activation in the SCN

A total of 47 male and female mice were raised in DD, LD or LL from P0 to P21 then all placed in LD at P21 and weaned between P21 and P23. They were then housed individually and their locomotor activity behaviour was monitored in LD, followed by DD and in response to light pulses at CT16 and CT22.

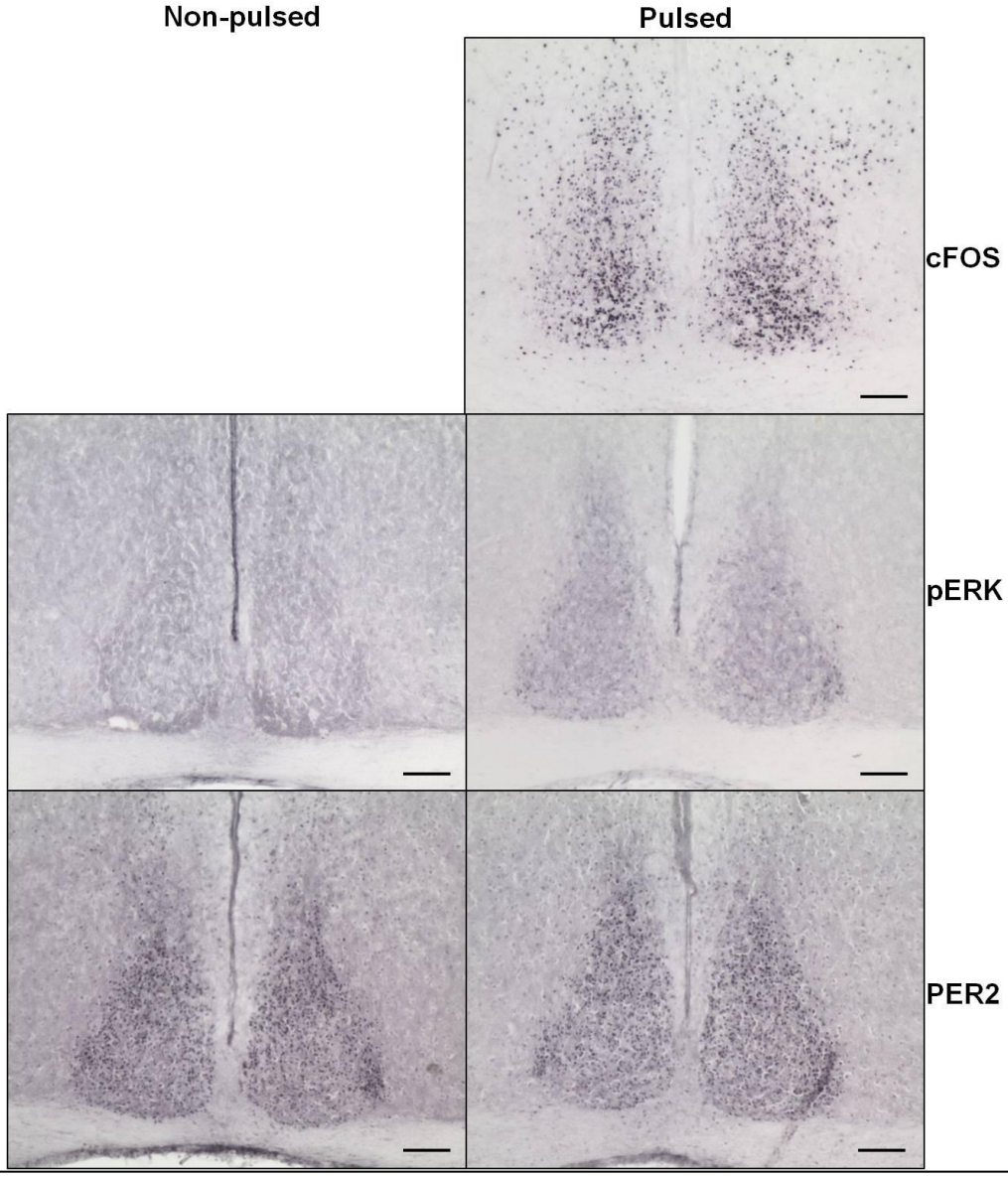

Figure 5.5: Effects of early light environment on cFOS, pERK and PER2 expression in the SCN after a CT16 light pulse. Representative photomicrographs of SCN staining in C57BL/6J mice after a CT16 light pulse (Pulsed) or a sham pulse (Non-pulsed). After lactation, the mice were kept in LD for two weeks before being transferred to DD and receiving light pulses or sham pulses at CT16 and CT22. At the end of the experiment a 30-minute light pulse was administered at CT16 and the mice were culled one hour after the end of the light pulse. Their brains were then processed using immunohistochemistry. DD – constant darkness; LD – 12:12 hour light-dark cycles; CT – circadian time; SCN – suprachiasmatic nuclei. Scale bar: 100µm.

We wanted to look at the activation of the SCN after a light pulse at CT16. We therefore administered a 30-minute light pulse at CT16, culled the mice one hour after the end of the light pulse and processed the brains for immunoreactivity against cFOS, pERK and PER2.
We were unable to look at cFOS expression in the mice that received a sham pulse due to problems with the antibody. However, previous work within our lab has shown that early light environment does not affect baseline cFOS expression in the SCN (Brooks et al., 2011). After a light pulse we observed nuclear cFOS staining throughout the SCN. However we saw no difference in the amount of cFOS-positive nuclei due to early light environment (Figure 5.5, Table 5.3).
We then measured the density of pERK immunostaining and, as expected, we found that the density of staining was significantly higher in mice that had received a light pulse compared to mice that had received a sham pulse ($p<0.01$; Figure 5.5, Table 5.3) but there were no differences in the density of staining due to early light environment in either group (Figure 5.5, Table 5.3).
Finally we counted the number of PER2-positive nuclei and found that the number of PER2-positive nuclei did not increase after a light pulse at CT16 compared to the mice that received a sham pulse and was not affected by early light environment (Figure 5.5, Table 5.3).

	Non-pulsed			**Pulsed**		
	DD	**LD**	**LL**	**DD**	**LD**	**LL**
Number of cFOS-positive nuclei/mm^2	N/A	N/A	N/A	2511.63 ± 500.61	2370.80 ± 350.59	2298.17 ± 426.30
pERK optical density (a.u.)	7.84 ± 2.05	6.02 ± 0.93	5.22 ± 2.00	20.40 ± 7.16**	19.89 ± 4.37**	21.60 ± 8.21**
Number of PER2-positive nuclei/mm^2	2129.51 ± 775.52	2457.51 ± 579.74	2384.94 ± 551.10	2429.11 ± 487.56	2348.44 ± 377.61	2262.39 ± 545.36

Table 5.3: Effects of early light environment on cFOS, pERK and PER2 expression in the SCN of C57BL/6J mice after a light pulse at CT16. Mice were raised in DD, LD or LL from P0 to P21 then placed in LD at P21. They were kept in LD for two weeks then transferred to DD where they were given light pulses or sham pulses at CT16 and CT22. At the end of the experiment they were given a 30-minute light pulse or sham pulse at CT16, culled one hour after the end of the light pulse and their brains were processed for immunoreactivity against cFOS, pERK and PER2. Data expressed as mean ± standard deviation. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light; CT – circadian time; SCN – suprachiasmatic nucleus. **$p<0.01$ vs. non-pulsed.

5.4 Discussion

The aim of this project was to explore how early light environment would affect the responses of C57BL/6J mice to light stimuli. We examined both behavioural and molecular responses to a light pulse and found no differences due to early light environment in locomotor activity behaviour or in the photic signalling pathway after light pulses.

5.4.1 Locomotor activity behaviour

Due to constraints on space and equipment, it was necessary to perform the locomotor activity behaviour experiment in two parts, separating the pulsed and the non-pulsed group. Although not ideal, we hoped that we would find similar responses in the two groups, perhaps allowing us to combine the data. We did find similar responses due to early light environment in the pulsed and non-pulsed groups; however we found differences in the amplitude of locomotor activity behaviour between the pulsed and non-pulsed groups. We are unsure as to why we saw differences in amplitude but as the two experiments were performed at different times it is possible that it was due to a change in their living environment which we have not controlled for as we did not expect them to affect changes in behaviour due to early light environment, for example the amount of bedding or minor changes in temperature between the two experiments. Since we saw no differences in the effects of early light environment on locomotor activity behaviour between the pulsed and non-pulsed groups, we chose to use locomotor activity behaviour of our pulsed group as there were higher numbers of mice in each group (9-12 mice per group) compared to the non-pulsed experiment (5 per group). The non-pulsed mice were used to ensure there were no phase shifts after sham pulses and to measure changes in tau after sham pulses.

We first monitored locomotor activity in LD and found no differences in locomotor activity behaviour due to early light environment. This data does not match what has previously been published (Smith and Canal, 2009) but is in agreement with my previous experiment (see Chapter 3). We believe that the differences between my data and the published data may be due to the type and intensity of light used during the experiments as the published data used fluorescent light at 57$\mu W/cm^2$ while my experiments used LED light at 330$\mu W/cm^2$.

We next placed the mice in DD and gave them a light pulse the next night at CT16 (Aschoff Type 2 protocol). As expected from light pulses during the early night (Daan and Pittendrigh, 1976a), we saw phase delays of just under an hour on average, with almost no phase shift in the mice that received a sham pulse. We saw no differences in the size of phase shift due to early light environment. However, since the size of the phase delay was quite small we were unsure of whether we were missing a small effect of early light environment. It has previously been shown that the size of phase shift after a light pulse increased the longer mice were kept in DD (dark adaptation) (Refinetti, 2001, 2003, 2007). We therefore decided to keep our mice in DD for two weeks then repeat the light pulse at CT16 (Aschoff Type 1 protocol). After this second light pulse we obtained much longer phase delays (two to three hours on average), with almost no phase shift in the mice that received a sham pulse. We saw no difference in the size of phase delay due to early light environment. Our previous data had shown that in LL, mice

raised in DD had a significantly longer tau than mice raised in LD and LL, implying that mice raised in DD were more sensitive to light. We therefore hypothesised that mice raised in DD would have longer phase delays after a light pulse at CT16 than mice raised in LD or LL. This did not occur in our mice, although there did seem to be slightly longer delays in the DD-raised mice compared to the LL-raised mice. The results imply that the response of mice to light pulses was unaffected by early light environment. However it has been shown that the mechanisms involved in phase shifting after a CT16 light pulse and a CT22 light pulse are different (Yan and Silver, 2002) with *Per2* being involved in phase delays and *Per1* involved in phase advances (Albrecht et al., 2001; Yan and Silver, 2002, 2004). It is therefore possible that early light environment only affects one pathway and not another. To test this we kept the mice in DD for a further two weeks before giving them a light pulse at CT22 (Aschoff Type1I protocol). As expected, we saw phase advances of two to three hours on average (Daan and Pittendrigh, 1976a), with almost no phase shift in the mice that received sham pulses. However we saw no differences in the size of the phase advance due to early light environment, although the DD-raised mice seemed to have longer shifts than the LL-raised mice. It therefore seems that early light environment does not affect the mechanisms involved in either phase delays or phase advances. However we only tested the response of mice to light pulses at two times during the subjective night, which may have led us to miss any effects early light environment has on the full phase response curve. Indeed, it has been suggested that the shape of the phase response curve may predict the lengthening of tau when mice are placed in LL (Pendergast et al., 2010). Using *Per1, Per2* or *Per3* knock-out mice, Pendergast et al. (2010) found that mice that had longer delay portions of their phase response curve than advance portions had longer taus in LL. We could hypothesise that in our experiment, mice raised in DD would have longer delay portions than advance portions of their phase response curves than mice raised in LD or LL. Such differences would not have been detected in the current study as we studied the response to light pulses in the middle of the delay portion (CT16) and the advance portion (CT22). Further work is needed to confirm this hypothesis. It is also possible that we missed an effect of early light environment due to the fact that we monitored locomotor activity behaviour using infra-red beams, which tracks not only voluntary movement but also picks up behaviours such as grooming, feeding etc. (Jud et al., 2005) which risks making the recordings more noisy. Wheel-running on the other hand only picks up voluntary behaviour and such experiments usually obtain much cleaner onsets of activity than activity obtained using infra-red. Had we used wheel-running, we may have seen a difference due to early light environment in the size of phase shift. Finally there is evidence that the size of phase shift after a light pulse can reach maximum levels after a saturating light pulse (Foster et al., 1991). If the light intensity used for our light pulses was saturating for all three groups, we may not have seen any differences due to early light environment. Thus, it would be interesting to administer lower, non-saturating light pulses to ascertain whether early light environment can affect phase shifting.

After each light pulses we noted changes in tau. After the light pulse at CT16 we noted that the tau lengthened, although this was not significant. After the CT22 light pulse we noted a significant shortening of tau. These changes in tau have previously been documented and are referred to as "after-effects" (Pittendrigh and Daan, 1976a; Refinetti, 2001). We saw no

differences in the after-effects of the light pulse due to early light environment, indicating that each group responded to the light pulses in a similar way.
Finally, we monitored locomotor activity behaviour throughout the time that mice were in DD and, in general, found no differences in locomotor activity behaviour due to early light environment. The only exception was after the Aschoff Type 1 CT16 light pulse, when mice raised in LD had a significantly higher percentage of variance than mice raised in LL. This effect was lost after the CT22 light pulse. As we only found this one difference in DD which was subsequently lost after the next light pulse we can assume that early light environment did not cause any major changes to locomotor activity behaviour in DD. Previous work in CD1 mice has shown no significant differences in locomotor activity behaviour due to early light environment when they were placed in DD as adults. Furthermore there was no significant effect on the size of the phase delay after a light pulse given at CT15 in these mice (Canal-Corretger et al., 2001b). These results are similar to what we have shown here. In C57BL/6J mice there is no published work on locomotor activity behaviour in DD. In Wistar rats it has been shown that being raised in LL reduced the size of the phase delay after a CT15 light pulse compared to rats raised in DD (Canal-Corretger et al., 2000) indicating that the photic response can be affected by early light environment, but that species and/or strain may affect how changes due to early light environment manifest themselves. However, as Wistar rats are albino it is possible that the effects of early light environment on phase shifting may have been as a result of changes at the retinal level.
On the other hand, our results may strengthen the argument that the light information reaching the SCN is the same regardless of the light environment in which they are raised. Our previous findings that early light environment affects tau length when adult mice are placed in LL seems to indicate that light may be processed differently, so it will be important to produce a complete phase response curve as a first step towards fully understanding whether and how early light environment alters photic processing in the SCN.

5.4.2 The photic signalling pathway

After light pulses during the subjective night, various intracellular signalling pathways are activated in SCN neurons (see Figure 5.1) (Zhu et al., 2012). We wanted to see whether early light environment affected various components of the photic signalling pathway. We focussed on the effects of a light pulse at CT16 as this has been more widely studied than the effects of a light pulse at CT22. The first component of the photic signalling pathway that we were interested in was pERK. ERK, part of the MAPK superfamily, has been shown to be phosphorylated after a light pulse at CT16 (Obrietan et al., 1998). Furthermore it seems that ERK plays an important role in phase shifting after light pulses, as disruption of ERK phosphorylation prevents phase shifting after a light pulse during the early night (Butcher et al., 2002; Hainich et al., 2006). As expected, after a light pulse at CT16 we saw a significant increase in the amount of pERK immunostaining in the SCN. However we saw no difference in baseline pERK expression or in the amount of pERK after a light pulse due to early light environment. It therefore seems that this part of the photic signalling pathway is not affected by early light environment.

The next component of the photic signalling pathway that we were interested in was activation of cFOS by light, which occurs downstream from the MAPK signalling pathway (Obrietan et al., 1999; Zhu et al., 2012). Increases in cFOS expression have been noted after light pulses given during the early or late night (Colwell and Foster, 1992) and it is generally used as a marker for activation of the SCN by light. Due to problems with the antibody we were unable to measure cFOS expression in the SCN of non-pulsed mice. However we have previously published work on cFOS expression in the SCN of mice that received a light pulse at CT16 using the Aschoff Type 2 protocol (Brooks et al., 2011). In that study we found a significant increase in cFOS expression after a light pulse, with no difference in baseline cFOS expression due to early light environment. We can therefore assume that in the present study we would find no differences in baseline cFOS expression and a significant increase in cFOS expression after the light pulse. Although we have already published work on activation of cFOS after a CT16 light pulse, we decided to look at cFOS expression in this study because the published work used an Aschoff Type 2 protocol (Brooks et al., 2011). Here we saw significantly longer shifts after the Aschoff Type 1 protocol compared to after the Aschoff Type 2 protocol. We wondered whether we would see differences in cFOS expression after the Aschoff Type 1 protocol that we had not caught in our published work. However, we did not see any differences in cFOS expression due to early light environment after a light pulse at CT16 using the Aschoff Type 1 protocol. It therefore seems that activation of this part of the photic signalling pathway is also not affected by early light environment.

Finally, we were interested in the expression of PER2 after a light pulse at CT16. *Per2* has previously been shown to be upregulated after a light pulse (Zylka et al., 1998). Furthermore it seems that *Per2* plays a crucial role in mediating phase delays, as mice that have the *Per2* gene knocked out were not able to shift after a light pulse given during the early night (Albrecht et al., 2001). We counted the number of PER2-positive cells 90 minutes after the start of a 30-minute light pulse given at CT16. We found no significant difference in the number of PER2-positive cells in mice that had received a light pulse compared to mice that had received a sham pulse. Furthermore there was no difference in the number of PER2-positive cells due to early light environment in either the pulsed or non-pulsed groups. Previous work has shown that *mPer2* mRNA levels are already moderately high at CT16 and that they significantly increase 90 minutes and two hours after a light pulse (close to the time that we measure PER2 protein expression) (Yan and Okamura, 2002; Yan and Silver, 2002). PER2 protein expression is increased 4 hours after the beginning of the light pulse (Yan and Silver, 2004). So it is possible that we saw no difference in the amount of PER2 protein expression because we took our samples too early to see the rise in protein expression. Indeed, Yan and Silver (2004) found high numbers of PER2 protein at CT18 (close to our sampling time) in both their pulsed (at CT16) and non-pulsed groups. The differences between the pulsed and non-pulsed groups became apparent from CT20 onwards (Yan and Silver, 2004). Therefore to determine any effects of a light pulse on PER2 protein expression we would need to sample at a later CT. If we were to sample at this later CT it would also be interesting to take into account differences between the core and shell SCN when counting PER2-positive nuclei. Indeed, it has been shown that phase delays are associated with increases in PER2 protein expression only in the shell (Yan and Silver, 2004). It is therefore possible that we would see differential effects of

early light environment on PER2 expression in the core and the shell. We saw no differences in PER2 expression due to early light environment, which may indicate that PER2 expression is not altered by early light environment but may also be due to sampling at the wrong time. Further work will be needed to obtain definitive results on how PER2 is affected by early light environment.

Our data does not show any changes in the photic signalling pathway after a light pulse administered at CT16. We did not look at the effects of a light pulse given at CT22.The photic signalling pathway involved in a phase advance after a light pulse during the late night is slightly different to that of a light pulse given in the early night. pERK and cFOS are upregulated after a light pulse given during the late night (Colwell and Foster, 1992; Obrietan et al., 1998), but the phase advance seems to be mediated by *Per1* and not *Per2*. Indeed it has been shown that there is no upregulation of *Per2* after a light pulse given during the late night, while *Per1* is upregulated (Albrecht et al., 1997). Furthermore, *Per1* knockout mice do not phase advance after a light pulse given during the late night (Albrecht et al., 2001). Increases in PER1 protein expression in the shell SCN after a late-night light pulse have also been associated with the phase advance, much like PER2 expression after an early night light pulse was associated with phase delays (Yan and Silver, 2004). Therefore it is possible that we have missed an effect of early light environment on the photic signalling pathway by not looking at the SCN after a light pulse at CT22. However, as we saw no differences in the size of phase advance after a CT22 light pulse, we can hypothesise that the photic signalling pathway mediating phase advances is also unaffected. It can also be argued that we cannot rule out that some elements of the photic signalling pathway that we have not examined have been altered by early light environment. This is indeed true, but we feel that we have looked at sufficient stages of the photic signalling pathway to have a good idea that early light environment does not affect this part of the photic pathway in mice.

5.4.3 Conclusions

We have not seen any effects of early light environment on the response to light pulses in mice, either when looking at behaviour or at elements of the photic signalling pathway. Therefore it is possible that the effects of early light environment on the locomotor activity behaviour of mice in LL (see Chapter 3) are due to changes in the output pathways in the SCN. Indeed, it has previously been shown that early light environment affected arginine vasopressin (AVP) (a key SCN output) expression in the SCN with mice raised in LL having significantly lower levels of AVP expression compared to mice raised in LD and DD (Smith and Canal, 2009), indicating that perhaps the output signals coming from the SCN were altered by early light environment. In the same study it was found that vasoactive intestinal polypeptide (VIP) levels in the SCN were also reduced in mice raised in LL compared to mice raised in LD and DD. VIP is thought to be involved in photic entrainment and synchronisation of cells within the SCN (Vosko et al., 2007). Perhaps early light environment affects how cells are synchronised with each other. This study has allowed us to rule out some components of the photic response of the circadian system as being affected by early light environment, but leaves open further questions, such as whether

we would see differences in a complete phase response curve or whether we missed a role for PER2 in the effects of early light environment on the photic response of the circadian system.

6. Effects of early light environment on the locomotor activity behaviour and PER2::LUC rhythms of mPer2::luc mice

6.1 Introduction

Early light environment has been shown to alter locomotor activity behaviour as well as neuropeptide, astrocyte and tyrosine hydroxylase (TH) expression in the hypothalamus of adult mice (Canal-Corretger et al., 2001b; Canal et al., 2009; Smith and Canal, 2009; Brooks et al., 2011). Furthermore, my work has shown that early light environment does not seem to affect retinal function or structure in C57BL/6J mice (see Chapter 3) implying that the changes we see in physiology and behaviour may not be due to changes in photic input but perhaps in how the suprachiasmatic nucleus (SCN) interprets photic information. Furthermore, the size of phase shift in response to light pulses at circadian time 16 (CT16) and CT22 was not affected by early light environment, and components of the signalling pathway involved in the immediate cellular photic response (cFOS, pERK and PER2) were not affected by early light environment after a CT16 light pulse (see Chapter 5).

We wanted to explore next whether the molecular clock was affected by early light environment. Circadian rhythms are driven by a molecular network of autoregulatory transcriptional-translational feedback loops, consisting of core clock genes such as *Per1, Per2, Cry1, Cry2, Bmal1, Clock* etc. (Ko and Takahashi, 2006). Of these clock genes it has been shown that the circadian rhythms of *mPer2* mRNA and mPER2 protein expression are extremely robust making this gene of particular interest in the study of circadian rhythms (Yoo et al., 2004). The mPer2::luc mouse was therefore developed by the Takahashi group allowing for real-time recording of PER2::LUC expression in tissue culture, with robust PER2::LUC rhythms found in the SCN as well as in peripheral tissue (Yoo et al., 2004). Furthermore, the locomotor activity behaviour of mPer2::luc mice was assessed in 12:12 hour light-dark cycles (LD), constant darkness (DD) and in response to a 6-hour light pulse at CT16, and was found to be no different than that of wild type mice (129SvEv x C57BL/6J) (Yoo et al., 2004).Of interest to our work is the finding that PER2 is upregulated after light pulses during the early night (Yan and Silver, 2002, 2004), indicating that it is responsive to light and leading us to wonder whether it could also be affected by light during postnatal development.

Therefore the aim of these experiments was to determine how early light environment affects clock gene expression, in particular *Per2*, using the mPer2::luc mouse strain. We therefore assessed the effects of early light environment on 1) locomotor activity behaviour of mPer2::luc mice when they were placed in different light conditions; 2) PER2::LUC expression in the SCN after being housed in LD and after exposure to LL and 3) PER2::LUC expression in peripheral tissues.

6.2 Experimental design

Table 6.1 shows the experimental design for the mPer2::luc mice used to determine how their locomotor activity behaviour is affected by early light environment in different light conditions. At the end of the experiment the mice were placed in constant light (LL) and after their locomotor activity was monitored they were culled and their SCN were harvested and cultured to determine whether early light environment affects SCN PER2::LUC rhythms in mice after prolonged exposure to LL.

To determine how early light environment affects the baseline PER2::LUC rhythms, a separate experiment was conducted where mice were raised in DD, LD or LL from birth (postnatal day 0 - P0) to P21, then placed in LD. Between P50 and 6 months of age, mice were culled and tissue culture was performed on the SCN, heart, lung, liver and spleen at *Zeitgeber* time 4 (ZT4). Statistical analysis was carried out by means of an ANOVA of general linear models using SYSTAT software.

For all experiments the independent variable was lactation (DD, LD or LL). When analysing locomotor activity behaviour, the dependent variables were period, percentage of variance, amplitude, power content of the first harmonic, duration of alpha, area under the curve, percentage of total area and phase of entrainment (if analysing the data of mice in LD). When analysing the tissue culture the dependent variables were period, amplitude, phase, damping rate and rhythmicity index.

	mPer2::luc mice (DD – n=8 males and 3 females; LD – n=7 males and 3 females; LL – n=8 males and 3 females)
P0	Lactation (DD, LD, LL)
P21	All animals placed in LD Animals were weaned and individually housed between P21 and P24
P24	Locomotor activity in LD
P45	Locomotor activity in DD
P59	CT16 light pulse (Aschoff type 1 protocol) – cages moved to a cabinet with lights on for 30 minutes then placed back in original cabinet.
P78	Locomotor activity in LD
P92	Locomotor activity in LL
P113-129	Animals culled in LL at 10:00 h (all but one animal arrhythmic)

Table 6.1: Experimental design for the mPer2::luc mice used to determine a locomotor activity behaviour profile in various light conditions. Mean light intensity was 330µW/cm^2 provided by LED light. DD – constant darkness; LD – 12:12 hour light-dark cycle; LL – constant light; CT – circadian time; LED - light-emitting diode.

6.3.1 Effects of early light environment on the locomotor activity behaviour of mPer2::luc mice

A total of 32 male and female mPer2::luc mice were born in DD, LD or LL and kept in these conditions until P21 when they were all placed in LD. They were weaned between P21 and P24, housed individually and their locomotor activity behaviour was monitored in different light conditions. We started off looking at their locomotor activity in LD. We then placed them in DD and gave them a light pulse at CT16 when they had been in DD for 2 weeks. We monitored their locomotor activity in DD after the light pulse for 2 weeks then placed them back in LD. After

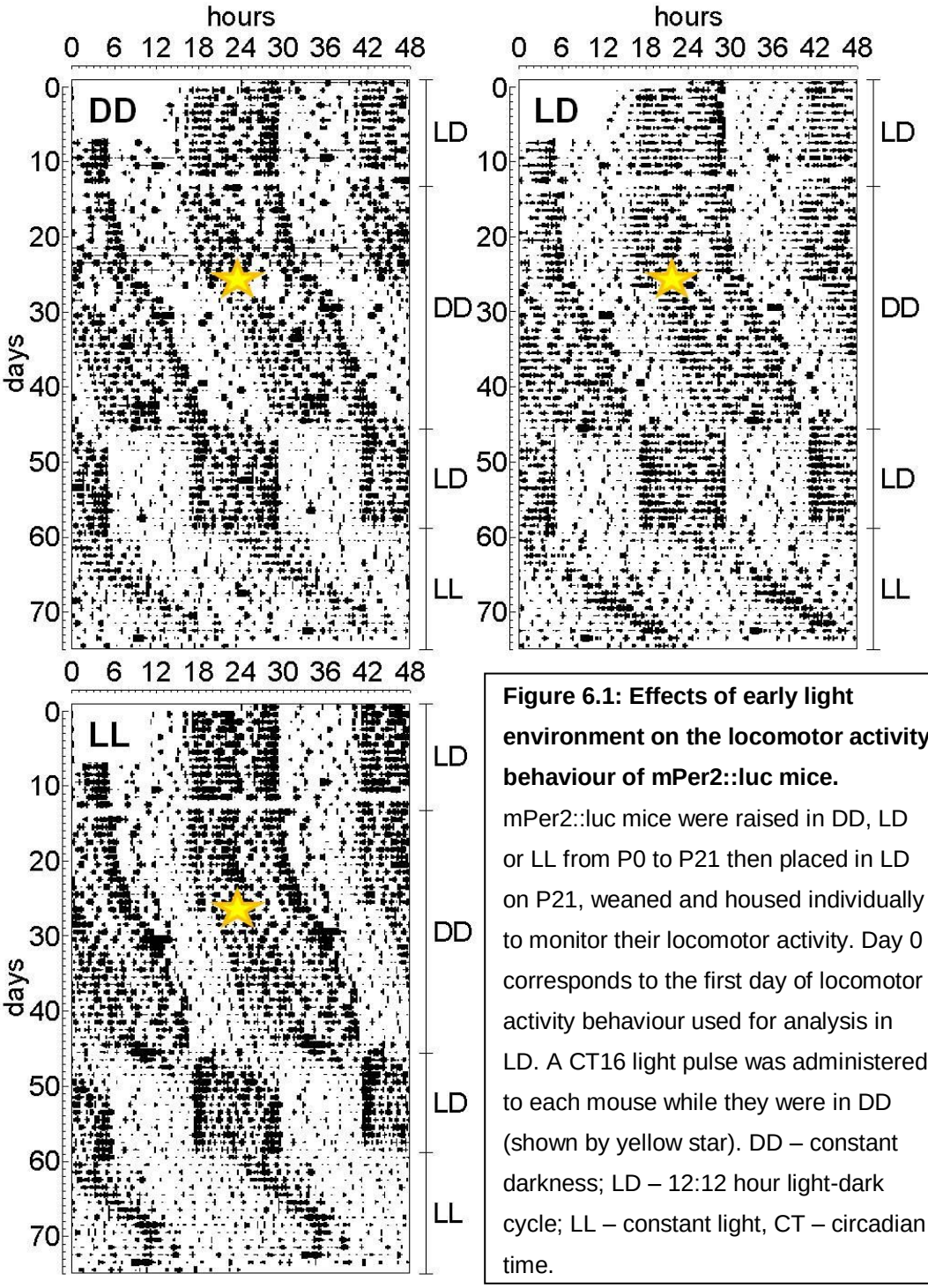

Figure 6.1: Effects of early light environment on the locomotor activity behaviour of mPer2::luc mice. mPer2::luc mice were raised in DD, LD or LL from P0 to P21 then placed in LD on P21, weaned and housed individually to monitor their locomotor activity. Day 0 corresponds to the first day of locomotor activity behaviour used for analysis in LD. A CT16 light pulse was administered to each mouse while they were in DD (shown by yellow star). DD – constant darkness; LD – 12:12 hour light-dark cycle; LL – constant light, CT – circadian time.

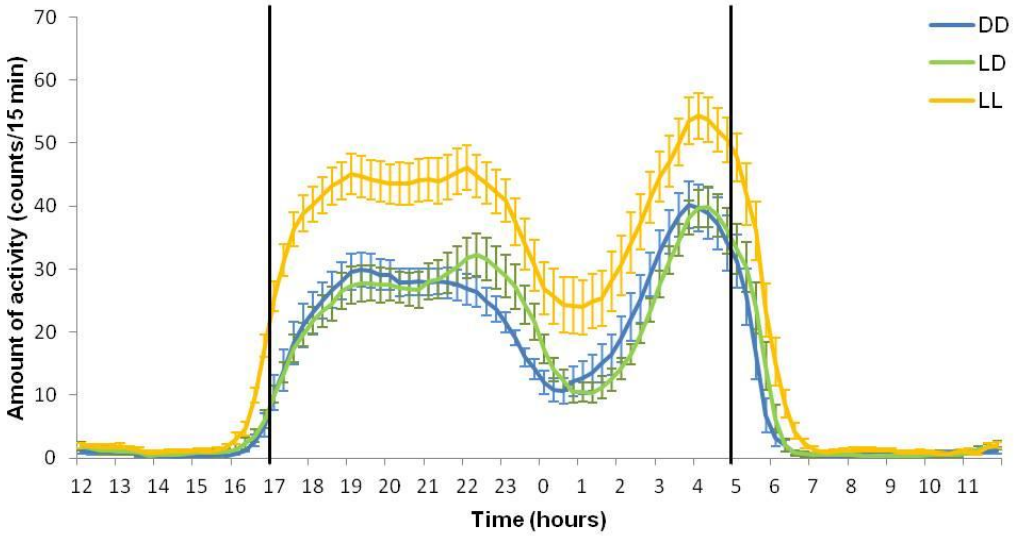

Figure 6.2: Effects of early light environment on the amount of activity in LD. Average activity in LD over 24 hours of mPer2::luc mice raised in DD, LD or LL from P0 to P21 then placed in LD and housed individually. Black vertical lines indicate the time when the lights turned off (17:00h) and back on (05:00h). Mice raised in LL had a significantly higher amplitude and amount of activity than mice raised in DD and LD. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light.

2 weeks in LD we placed them in LL and their locomotor activity was monitored for at least 2 weeks. Figure 6.1 shows representative actograms of mice from each group over the course of the experiment.

In the first stage of LD we found that mice raised in LL had a significantly higher amplitude of locomotor activity than mice raised in DD ($p<0.01$) and LD ($p<0.05$) (Figure 6.2). Indeed, mice raised in LL had average amplitudes of 17.50±4.49, while mice raised in DD and LD had average amplitudes of 13.07±2.42 and 13.81±1.98 respectively. Mice raised in LL also had a significantly higher amount of activity during their active phase (alpha) compared to mice raised in DD and LD ($p<0.01$, Figure 6.2). Indeed, the total amount of activity during alpha in mice raised in LL was 2046.49±460.50 counts while mice raised in DD and LD had a total amount of activity during alpha of 1223.58±312.56 counts and 1276.89±312.346 counts respectively. We found no differences in any other variable we measured in LD.

We then placed the mice in DD and monitored their locomotor activity. Table 6.2 shows a summary of the changes we saw due to early light environment in DD either before or after the CT16 light pulse. We found that the period was significantly longer after the light pulse ($p<0.01$; Table 6.2) but there were no differences due to early light environment. When we looked at the amplitude of the locomotor activity rhythm in DD we found no difference due to early light environment before the CT16 light pulse, but we found that after the light pulse, the amplitude of locomotor activity behaviour was significantly higher in mice raised in LL compared to mice raised in LD ($p<0.01$; Table 6.2). Finally we found that the amount of activity during alpha was significantly higher in mice raised in LL compared to mice raised in DD and LD ($p<0.01$; Table 6.2) both before and after the light pulse. We also measured the size of phase delay after the

	Before CT16 light pulse			After CT16 light pulse		
Light during lactation	**DD**	**LD**	**LL**	**DD**	**LD**	**LL**
Period (hours)	24.06 ± 0.19	24.17 ± 0.13	24.12 ± 0.17	24.25 ± 0.18$^{\#}$	24.38 ± 0.12$^{\#}$	24.33 ± 0.15$^{\#}$
Amplitude	12.57 ± 2.27	10.31 ± 3.15	15.33 ± 4.17	12.32 ± 3.74	10.56 ± 2.88^{++}	14.76 ± 6.97
Amount of activity during alpha (total counts)	1405.19 ± 254.52	1441.60 ± 458.34	2004.75 ± 513.74**	1497.41 ± 327.20	1527.13 ± 272.62	2014.21 ± 704.47**

Table 6.2: Effects of early light environment on the locomotor activity behaviour of mPer2::luc mice in DD before and after a light pulse at CT16. Mice were born in DD, LD or LL and raised in these conditions until P21. They were then transferred to LD and their locomotor activity behaviour was monitored. The table above shows details of changes occurring when the mice were kept in DD, before and after a light pulse was administered at CT16. Data presented as mean ± standard deviation. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light; CT – circadian time. # $p<0.01$ vs. before CT16 light pulse; ** $p<0.01$ vs. LD and DD; $^{++}p<0.01$ vs. LL.

CT16 light pulse and found no differences due to early light environment. Indeed, mice raised in DD had an average delay of -3.63±0.86 hours, mice raised in LD had an average delay of -3.50±1.00 hours and mice raised in LL had an average delay of -3.26±0.70 hours.

When we placed the mice back in LD, we found that the amplitude of locomotor activity behaviour was significantly higher in mice raised in LL compared to mice raised in LD ($p<0.05$; Figure 6.1), similarly to what we found in the first LD stage. Indeed, the average amplitude of locomotor activity behaviour was 20.06±5.75 in mice raised in DD, 16.01±2.30 in mice raised in LD and 24.01±8.82 in mice raised in LL. We found no other differences in locomotor activity behaviour in LD due to early light environment.

Finally we wanted to look at the locomotor activity behaviour of the mice when they were in LL. We found no differences in the tau length due to early light environment (Figure 6.3A). Mice raised in DD had an average tau of 25.41±0.38 hours, mice raised in LD had an average tau of 25.31±0.19 hours and mice raised in LL had an average tau of 25.20±0.29 hours. However, we noted that almost all the mice became arrhythmic after some time in LL, so we counted how many days mice remained rhythmic in LL. We found that mice raised in DD were not able to remain rhythmic for as long as mice raised in LL ($p<0.05$; Figure 6.3B). Indeed, the average number of days mice raised in DD were able to remain rhythmic in LL was 10.60±5.84 days, while mice raised in LD were able to remain rhythmic for 14.43±4.35 days and mice raised in LL were able to remain rhythmic for 17.22±5.47 days. Furthermore, we found that the percentage of variance was significantly higher in mice raised in LL compared to mice raised in DD ($p<0.01$; Figure 6.3C) and the amplitude of locomotor activity behaviour was significantly higher in mice raised LL compared to mice raised in DD ($p<0.01$) and LD ($p<0.05$) (Figure 6.3D). The average

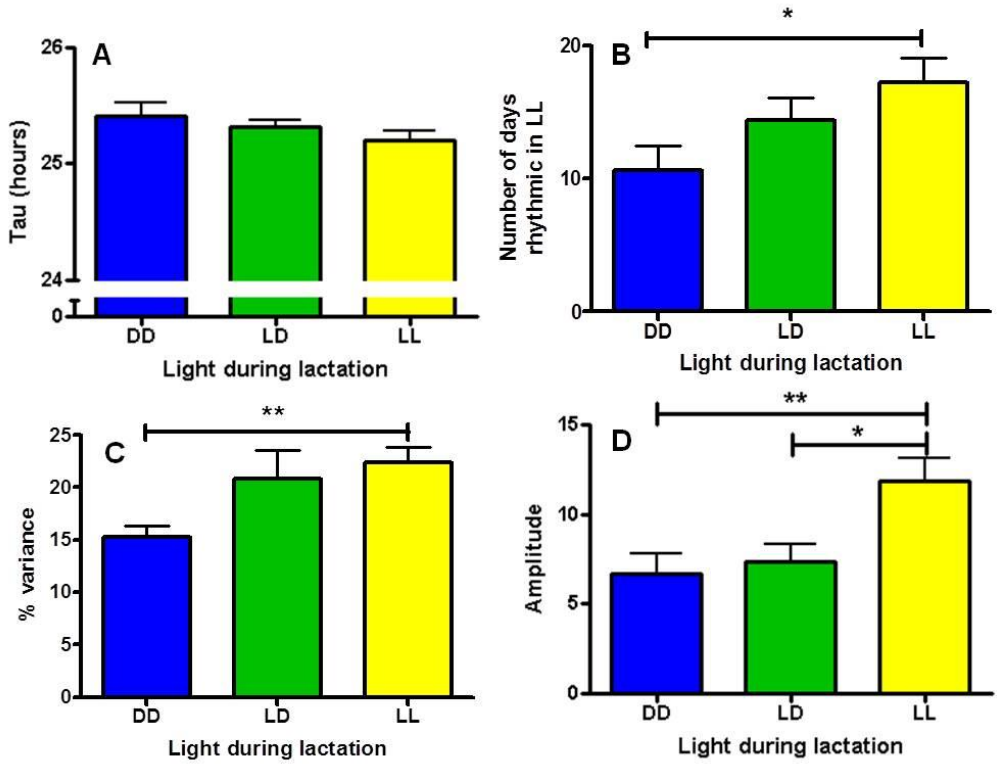

Figure 6.3: Effects of early light environment on locomotor activity behaviour of mPer2::luc mice in LL. Tau (**A**), number of days rhythmic in LL (**B**), percentage of variance (**C**) and amplitude (**D**) in LL of mPer2::luc mice raised in DD, LD or LL from P0 to P21 before being placed in LD, followed by DD, then LD once more, before finally being placed in LL. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light. *p<0.05; ** p<0.01.

percentage of variance in mice raised in DD was 15.28±3.06, 20.83±8.21 in mice raised in LD and 22.43±4.83 in mice raised in LL. The average amplitude of locomotor activity behaviour in mice raised in DD was 6.68±3.50, 7.38±2.85 in mice raised in LD and 11.85±4.25 in mice raised in LL. We also found a trend for mice raised in LL to have a higher power content of the first harmonic than mice raised in DD (p=0.051). Indeed, the average power content of the first harmonic was 5.28±2.62 in mice raised in DD, 7.96±4.78 in mice raised in LD and 9.70±3.79 in mice raised in LL.

6.3.2 Effects of early light environment on PER2::LUC rhythms in the SCN of adult mPer2::luc mice

A total of 33 mPer2::luc males and females were raised in DD, LD or LL from P0 to P21 then placed in LD, weaned and group housed according to sex. They were kept in LD from P21 until they were used for tissue culture from P50 onwards.

We found that the amplitude of PER2::LUC rhythms in culture was significantly lower in mice raised in DD compared to mice raised in LD (p<0.01) and LL (p<0.05) (Table 6.3; Figure 6.4). We found no differences in any other variable measured in the SCN.

	Period (hours)	Phase (hours)	Amplitude	Damping rate (days)	Rhythmicity index
DD	24.71±0.45	-2.28±0.66	27.41±13.21	5.59±0.91	0.99±0.01
LD	24.53±0.21	-2.44±0.46	50.03±17.70**	6.50±0.41	0.99±0.01
LL	24.50±0.52	-2.55±0.53	47.77±19.91*	6.61±1.37	0.99±0.01

Table 6.3: Effects of early light environment on PER2::LUC expression in the SCN. Mice were raised in DD, LD or LL from P0 to P21 then housed in LD from P21 onwards. Tissue culture was performed at ZT4 and phase was calculated relative to ZT12. Data presented as mean ± standard deviation. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light. *p<0.05 compared to DD; **p<0.01 compared to DD

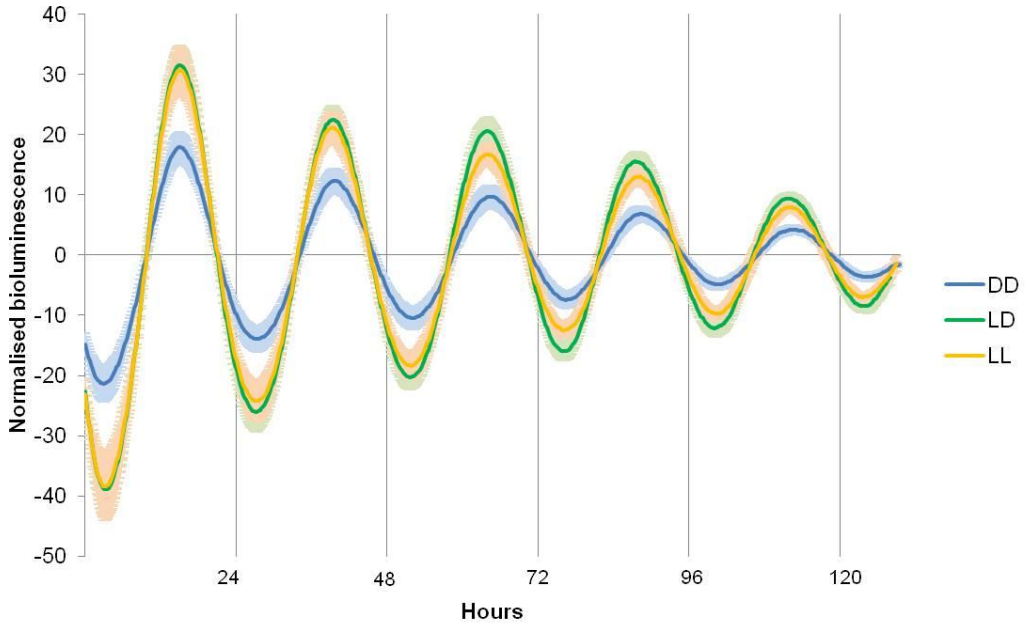

Figure 6.4: Effects of early light environment on PER2::LUC expression in the SCN. Rhythms of PER2::LUC in the SCN of mPer2::luc mice raised in DD (n=10), LD (n=12) or LL (n=11) from P0 to P21 then placed in LD. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light

6.3.3 Effects of early light environment on PER2::LUC rhythms in the SCN of adult mPer2::luc mice after prolonged exposure to LL

We wanted to see how PER2::LUC rhythms were affected by being kept in constant light for a prolonged period, and whether there were any differences due to early light environment. mPer2::luc mice that had previously been used to monitor locomotor activity behaviour (see Table 6.1) were kept in LL until they were culled and their SCN was placed into tissue culture. All but one mouse used to study PER2::LUC rhythms after LL had become behaviourally arrhythmic by the time tissue culture was performed. The only rhythmic mouse was raised in LL and had similar PER2::LUC rhythms to other mice raised in LL. The rhythmicity index was significantly reduced compared to the mice that were used for the baseline experiment ($p<0.01$), indicating that the quality of the PER2::LUC rhythms was reduced in the mice that had been in

	Period (hours)	Phase (hours)	Amplitude	Damping rate (days)	Rhythmicity index
DD	24.29±0.50	-5.34±2.02	21.71±8.10	6.31±2.52	0.72±0.29
LD	24.63±0.66	-5.72±2.81	19.63±15.59	7.20±2.94	0.55±0.37
LL	24.72±0.81	-6.00±1.65	17.51±11.04	7.21±1.90	0.67±0.30

Table 6.4: Effects of early light environment on PER2::LUC expression in the SCN after prolonged exposure to LL. Mice were raised in DD, LD or LL from P0 to P21, placed in LD at P21 then kept in DD, followed by LD then finally followed by LL. After at least two weeks in LL the mice were used for tissue culture at 10:00h. All mice were arrhythmic by the time of tissue culture. Phase was calculated relative to 17:00h. Data presented as mean ± standard deviation.DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light.

LL. In order to be consistent in including rhythmic data, we applied a goodness of fit measure to the waveforms (see Materials and Methods Chapter 2) and only waveforms with a goodness of fit higher than 0.8 were used for the analysis. Thus, of the 32 mice that were culled, 16 had useable data for analysis (n=6 in DD group, n=5 in LD and LL groups).

We found a significant reduction in the amplitude of PER2::LUC rhythms in the mice that had been in LL compared to the mice used for the baseline line experiment ($p<0.01$; Table 6.4) and a significant change in phase, with mice that had been in LL showing a peak in oscillation significantly earlier than the mice that were used for the baseline experiment ($p<0.01$; Table 6.4). We found no differences due to early light environment in PER2::LUC rhythms in the SCN of mPer2::luc mice that had been in LL for at least 3 weeks (Table 6.4; Figure 6.5).

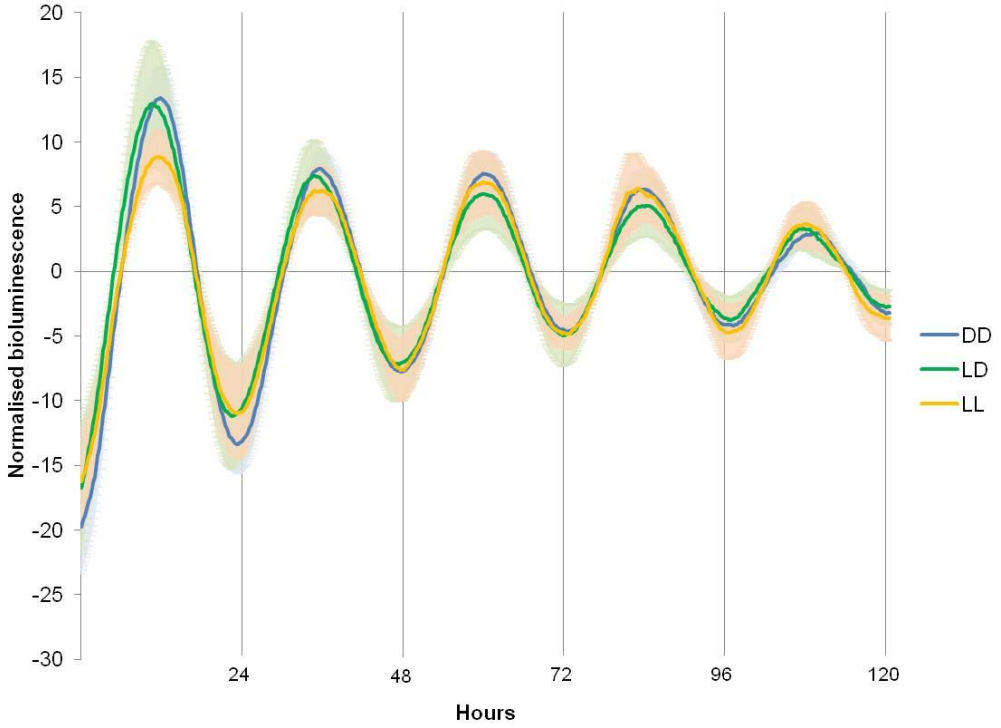

Figure 6.5 Effects of early light environment on PER2::LUC expression in the SCN after prolonged exposure to LL. mPer2::luc mice were raised in DD (n=6), LD (n=5) or LL (n=5) from P0 to P21, placed in LD at P21 then later on in life kept in LL before taking the SCN. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light.

6.3.4 Effects of early light environment on PER2::LUC rhythms in peripheral tissue

mPer2::luc male and female mice were born in DD, LD or LL and kept in these light conditions until P21 when they were placed in LD and housed according to sex. After P50 they were culled and their peripheral tissue (heart, lung, liver and spleen) was taken for tissue culture.
We found that PER2::LUC rhythms in the heart had a significantly lower amplitude in mice raised in DD compared to mice raised in LL ($p<0.05$, Figure 6.6A, Table 6.5). We found no other differences in PER2::LUC rhythms in the heart due to early light environment.
In contrast to this, we found in the lung that mice raised in DD had a significantly higher amplitude of PER2::LUC rhythms than mice raised in LL ($p<0.05$, Figure 6.6B, Table 6.5). We found no other differences in PER2::LUC rhythms in the lung due to early light environment.
In the spleen, we found that the peak of the PER2::LUC rhythms occurred significantly earlier in mice raised in LL compared to mice raised in LD ($p<0.05$, Figure 6.6C, Table 6.5). We found no other differences in PER2::LUC rhythms in the liver due to early light environment.
In the liver, we found no differences in PER2::LUC rhythms due to early light environment (Figure 6.6D, Table 6.5).

		Period (hours)	Phase (hours)	Amplitude	Damping rate (days)	Rhythmicity index
Heart	**DD**	22.79±1.29	5.02±0.99	3.76±0.96*	5.10±1.43	0.91±0.05
	LD	22.80±0.98	5.59±1.36	5.09±1.19	5.56±1.81	0.95±0.04
	LL	22.44±0.85	5.97±1.30	5.22±1.03	5.69±1.41	0.94±0.04
Lung	**DD**	23.92±0.81	5.11±1.99	7.28±3.75*	5.59±1.01	0.94±0.06
	LD	24.26±0.69	6.27±1.07	3.93±1.71	5.86±0.76	0.94±0.05
	LL	24.18±0.78	6.11±1.57	3.92±1.40	5.62±1.01	0.95±0.02
Liver	**DD**	22.51±2.56	2.52±1.77	6.56±3.19	5.94±1.83	0.68±0.26
	LD	22.50±1.54	2.95±1.76	7.23±4.17	5.11±2.32	0.72±0.18
	LL	22.82±1.87	2.94±0.87	7.30±4.13	6.55±2.70	0.69±0.16
Spleen	**DD**	24.62±0.94	4.16±1.80	3.54±1.11	6.72±2.13	0.85±0.05
	LD	24.67±0.80	5.42±1.24*	4.87±1.49	6.37±1.70	0.89±0.06
	LL	24.62±1.43	3.63±1.70	4.61±2.22	5.77±1.20	0.90±0.05

Table 6.5: Effects of early light environment on PER2::LUC expression in peripheral tissue. Mice were raised in DD, LD or LL from P0 to P21, then placed in LD from P21 onwards. Tissue culture was performed at ZT4 and phase was calculated in relation to ZT12. Data presented as mean ± standard deviation. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light. *$p<0.05$ compared to LL

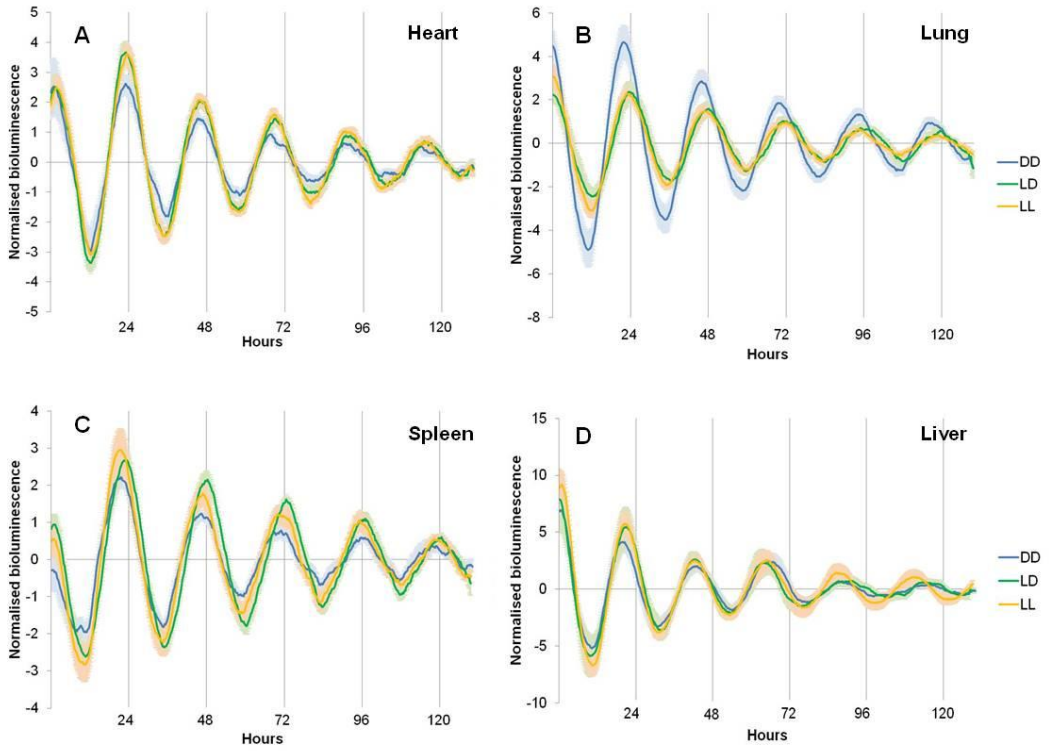

Figure 6.6: Effects of early light environment on PER2::LUC expression in peripheral tissue. mPer2::luc mice were raised in DD, LD or LL from P0 to P21 then housed in LD and used between P50 and 6 months of age. **A:** PER2::LUC rhythms in the heart (n=8 in each group). **B:** PER2::LUC rhythms in the lung (DD – n=12; LD – n=6; LL – n=10). **C:** PER2::LUC rhythms in the spleen (DD – n=11; LD – n=13; LL – n=10). **D:** PER2::LUC rhythms in the liver (DD – n=9; LD – n=6; LL – n=12). DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light.

6.4 Discussion

The aim of these experiments was to determine the effects of light during postnatal development on clock gene expression. In particular, we focussed on the *Per2* clock gene and used transgenic mPer2::luc mice for these experiments. We assessed the effects of early light environment on: 1) locomotor activity behaviour, 2) PER2::LUC expression in the SCN and 3) PER2::LUC expression in peripheral tissues.

6.4.1 Early light environment affects the locomotor activity behaviour of mPer2::luc mice

mPer2::luc mice have a knock-in mutation whereby the *Luciferase* gene is fused to the terminal exon of the endogenous *mPer2* locus which results in the creation of the mPER2::LUC fusion protein (Yoo et al., 2004). Locomotor activity behaviour of these mice was tested when they were developed and no differences in entrainment to an LD cycle or activity in DD were found compared to their wild-type counterparts (129svEv X C57BL/6J) confirming that the mPER2::LUC fusion protein was functional in vivo (Yoo et al., 2004).
Here we wanted to determine whether the locomotor activity rhythms of mPer2::luc mice were affected by early light environment and, if so, whether the effects were similar to those observed in C57BL/6J mice in our lab. First we monitored locomotor activity behaviour in LD and found that mice raised in LL had stronger locomotor activity rhythms than mice raised in LD and DD, as shown by the increased amplitude of locomotor activity in mice raised in LL. We also found that mice raised in LL were more active than mice raised in DD and LD. These results are in contrast to what we found in C57BL/6J mice housed in similar lighting conditions throughout life (330$\mu W/cm^2$ provided by LED light), where we found no differences in locomotor activity behaviour due to early light environment when the mice were in LD as adults (see Chapters 3 and 5). However the activity of mPer2::luc mice in LD is similar to the findings in C57BL/6J mice housed in a lower intensity and different type of light (57$\mu W/cm^2$ provided by fluorescent light) where mice raised in LL were also found to have stronger rhythms and were more active than mice raised in DD and LD, as shown by changes in percentage of variance, amplitude and amount of activity during the active phase (Smith and Canal, 2009). When we take the results from the two strains and the two light conditions together, it seems that the type and intensity of light can affect locomotor activity behaviour and whether we see differences due to early light environment, a subject that has already been broached in Chapter 4. Here, the fact that mPer2::luc mice and C57BL/6J mice housed under the same light conditions responded differently implies that they may not cope with different types and intensities of light in the same way. Furthermore as the behaviour of mPer2::luc mice housed under high intensity LED light is similar to that of C57BL/6J mice housed under lower intensity fluorescent light, it seems that mPer2::luc mice may be able to cope with higher intensity LED light better than C57BL/6J mice.
We next placed the mPer2::luc mice in DD and monitored their activity for 2 weeks before administering a 30-minute light pulse at CT16 and monitoring their locomotor activity behaviour for a further two weeks after the light pulse. We found no differences in the size of the phase delay after the CT16 light pulse due to early light environment. The size of phase shift was similar to what has previously been measured in mPer2::luc mice (Yoo et al., 2004) but slightly

longer than what we have measured in C57BL/6J mice (about three and a half hours in mPer2::luc mice compared to two and a half to three hours in C57BL/6J mice - see Chapter 5). The size of phase shift after a light pulse is dependent on factors such as the duration of the light pulse, the light intensity of the pulse, whether cages are moved to administer the light pulse and the amount of time mice have been in DD before the light pulse was given (Refinetti, 2001, 2003; Jud et al., 2005). We did not perform the light pulses for the two strains at the same time, but we did try to keep the protocol as consistent as possible between the experiments ensuring that the duration and intensity of light were not altered between the two experiments. In both experiments, the cages were moved to give the light pulse so it is possible that the amount of movement was different between the two strains as we did not control for the amount of movement allowed when transporting cages to the light environment. One difference between the protocol used to administer light pulses to the C57BL/6J mice and the mPer2::luc mice was the amount of dark adaptation the two strains underwent. Previous work has shown that after dark adaptation, the size of phase shift after a light pulse was increased (Refinetti, 2001, 2003). In this study, mPer2::luc mice were in DD for 14 uninterrupted days before the light pulse was administered. In contrast to this, the C57BL/6J mice were placed in DD, subjected to an initial light pulse the next night, then kept in DD for 12 days before another light pulse at CT16 was administered. The difference between the two protocols may explain why mPer2::luc mice have slightly longer phase delays compared to the C57BL/6J mice. However it is important not to discount the possibility that strain has affected the size of phase shift. It seems likely that a combination of all of these factors may have led to the difference in the size of phase shift we see in this experiment. Importantly, no effect of early light environment was seen in the size of the phase shift after a light pulse at CT16 in mPer2::luc mice, similar to what I have shown in C57BL/6J mice (see Chapter 5). Previous work in CD1 mice has also found no difference in the size of phase shift after a light pulse at CT15 (Canal-Corretger et al., 2001b). However, it has been shown that Wistar rats raised in LL had significantly shorter phase delays after a light pulse at CT15 compared to rats raised in DD (Canal-Corretger et al., 2000) which seems to indicate that rats raised in LL have a more stable clock than rats raised in DD (Abraham et al., 2010).

In the mPer2::luc mice, we found that the light pulse caused a significant lengthening of tau in all groups of mice, but no effect of early light environment on the amount of lengthening. Light pulses have previously been shown to affect tau (defined as an "after-effect" of the light pulse), with light pulses during the early night causing a lengthening of tau, as we have seen in this experiment, and light pulses during the late night causing a shortening of tau (Pittendrigh and Daan, 1976a). In DD, we also found that mice raised in LL had significantly more activity during their active phase than mice raised in LD and DD, both before and after the light pulse. Furthermore, mice raised in LL had significantly higher amplitudes of locomotor activity behaviour than mice raised in LD, but only after the light pulse. Although the data in DD was less robust than what we saw when the mice were in LD, the data shows that mice raised in LL had stronger rhythms than mice raised in LD and DD when they were housed in DD. There is no published data on the effects of early light environment on the locomotor activity behaviour of C57BL/6J or mPer2::luc mice in DD, but my work has shown no effect on the behaviour of C57BL/6J mice when they were housed in DD (see Chapter 5). Previous work in CD1 mice has

found no difference in locomotor activity behaviour due to early light environment when adult mice were placed in DD (Canal-Corretger et al., 2001b).
In LL we found no differences in tau length due to early light environment, although mice raised in DD seemed to have a longer tau than mice raised in LD and LL. This result is not exactly the same as what I have previously shown in C57BL/6J mice (see Chapter 3) and what has been published in CD1 mice (Canal-Corretger et al., 2001b) where mice raised in DD had a significantly longer tau than mice raised in LD and LL. However, we found that mice raised in DD became arrhythmic faster than mice raised in LL. Furthermore, we found that mice raised in LL had significantly stronger rhythms than mice raised in DD and LD, as shown by an increased percentage of variance and amplitude of locomotor activity behaviour. Previous work in Wistar rats also failed to show changes in tau in LL due to early light environment because rats raised in LD and DD became arrhythmic in LL, but similarly to what we found in mPer2::luc mice, it was shown that rats raised in LL were able to remain rhythmic in LL, in contrast to rats raised in DD and LD that became arrhythmic in LL (Cambras et al., 1998). Furthermore, rats raised in LL also had stronger locomotor activity rhythms than rats raised in DD and LD when they were in LL as adults (Canal-Corretger et al., 2003a).
Although our locomotor activity behaviour findings in mPer2::luc mice are not exactly the same as what was found in C57BL/6J mice, our results show that in both strains, mice raised in LL had stronger rhythms than mice raised in DD and LD. We therefore believe that mPer2::luc mice can be used as a model for the exploring the effects of early light environment on PER2::LUC rhythms in tissue culture.

6.4.2 Early light environment affects PER2::LUC rhythms in SCN tissue culture

The next aim of these experiments was to determine how early light environment affects the rhythm of PER2::LUC protein expression in the SCN. mPer2::luc mice were raised in DD, LD or LL from P0 to P21 then housed in LD from P21 onwards. They were taken for tissue culture from P50 at ZT4.
We found that early light environment affected the amplitude of PER2::LUC rhythms, with mice raised in DD having a significantly lower amplitude rhythm than mice raised in LD or LL. We found no effects of early light environment on period, phase, damping rate or rhythmicity index. It is hypothesised that the amplitude of PER2::LUC rhythms in the whole SCN, such as we measure it, reflects the coupling strength of individual neurons within the SCN (Abraham et al., 2010) and that a reduced amplitude could reflect weaker coupling between cells. If this is the case it is possible that mice raised in DD may have reduced coupling compared to mice raised in LD and LL. Higher amplitude PER2::LUC rhythms have been associated with more robust circadian rhythms (Abraham et al., 2010), which may reflect the stronger locomotor activity rhythms of the mice raised in LD and LL compared to the mice raised in DD in our study. Of particular interest in our work is locomotor activity behaviour in LL, where we find that mice raised in DD became arrhythmic in LL faster than mice raised in LD and LL and showed weaker circadian rhythms of locomotor activity. Constant light has been shown to reduce synchrony between mammalian neurons (Ohta et al., 2005). If coupling was already weaker in the mice raised in DD then it is feasible that LL would be more disruptive to mice raised in DD than mice

raised in LD or LL. However we must consider how LL would affect mice during development. Indeed it has been shown that during postnatal development (at P20), LL also caused loss of synchrony between mammalian neurons, although individual neurons did oscillate (Ohta et al., 2006). If these mice were kept in LL for a further 4 weeks, 67% of the mice were not able to develop circadian rhythms, while most mice raised in LD until P20 then transferred to LL for four weeks were able to maintain circadian rhythms over the four weeks (Ohta et al., 2006). Thus it seems that during development, LL prevented SCN neurons from synchronising with each other, but did not prevent the cells from having their own circadian rhythms. Previous work has shown that rats raised in LL then kept in LL after weaning did eventually develop circadian rhythms (Cambras et al., 1998). However, the same study showed that rats that were raised in DD then transferred to LL after weaning were not able to maintain circadian rhythms in LL. It therefore seems that rats raised in LL had a delayed development of overall circadian rhythms in the absence of external time cues. In contrast to this rats raised in DD did develop circadian rhythms, but it seems that because they were not exposed to light during development, they were unable to cope with constant light after weaning.

In our study, mice were raised in DD, LD or LL from P0 to P21 then transferred to LD from P21 onwards. We can hypothesise that during postnatal development, mice raised in DD and LD developed synchronised circadian rhythms within the SCN, while mice raised in LL had individual cells expressing circadian rhythms, but no overt circadian rhythm in the SCN as a whole. When LL-raised mice were transferred to LD, SCN cells rapidly synchronised and the mice became entrained to LD, just as mice raised in DD did. However, because the mice raised in DD had never been exposed to light, they were not able to cope with the light information as well as the mice raised in LD and LL. Evidence for this can be found in the weaker locomotor activity rhythms in these mice, as well as the reduced amplitude of PER2::LUC rhythms which may be evidence of reduced coupling between cells.

It is important to consider what could be mediating coupling between SCN cells and whether it could be affected by early light environment. Vasoactive intestinal polypeptide (VIP) has been shown to play an important role in synchronising SCN cells (Maywood et al., 2006b; Brown et al., 2007; Hughes et al., 2008). Indeed, applying the adenylate cyclase inhibitor N-(Cis-2-phenyl-cyclopentyl) azacyclotridecan-2-imine-hydrochloride (MDL), which interferes with VIP-mediated coupling, to SCN slices resulted in a reduction in coupling strength in the SCN (Abraham et al., 2010). Furthermore, transgenic mice that lack the VIP receptor $VPAC_2$ (*vipr2*$^{-/-}$) have been shown to have a reduced amplitude of PER2::LUC rhythms compared to wild type mice (Hughes et al., 2011). The period of PER2::LUC rhythms in these mice was not significantly different from wild types, but it was shown that the phase of PER2::LUC rhythms in *vipr2*$^{-/-}$ mice was reset by the procedure, while the phase in wild type mice was associated with a particular CT (close to CT12). They surmised that VIP plays an important role in the maintenance of robust SCN function (Hughes et al., 2011). In our experiments we saw a reduction in amplitude in the mice raised in DD but no effect of early light environment on phase. Indeed, the phase in our experiments was consistent with what has been published on phase of PER2::LUC rhythms when the procedure is performed during the day (slightly advanced compared to ZT12) (Yoshikawa et al., 2005; Guilding et al., 2010; Hughes et al., 2011). It is possible that early light environment affects the levels of VIP expression, leading to

alterations in the amplitude of PER2::LUC rhythms. Furthermore it is possible that we don't see any effect on phase because the changes due to early light environment are more subtle and therefore can only be visualised in the changes in amplitude. Previous work in our lab has shown that VIP expression in the SCN was affected by early light environment, with mice raised in LL having reduced expression compared to mice raised in DD and LD (Smith and Canal, 2009). This is surprising as evidence suggests that reduced levels of VIP would be associated with reduced synchronisation between SCN cells, which we believe was occurring in the mice raised in DD, not the mice raised in LL. The above mentioned study looked at VIP expression at two time points but found no differences in VIP levels due to time of day. Circadian variation in VIP expression has been documented (Dardente et al., 2004) so it is possible that early light environment affects overall circadian oscillations of VIP and that we would see different results if we looked at VIP expression over 24 hours. It would also be interesting to look at the effects of early light environment on the levels of $VPAC_2$ receptor in the SCN as this may alter how effective VIP can be in the SCN.

Synaptic transmission also plays an important role in synchronisation between SCN neurons. Indeed, when tetrotodoxin (TTX), which blocks voltage-gated sodium channels thus preventing the generation of action potentials, is applied to SCN slices, the synchrony between cells is reduced, as well as the amplitude of individual cell oscillations (Honma et al., 2000; Yamaguchi et al., 2003). Finally, gap junctions could also be involved in the synchronisation of SCN neurons as it has been shown that cells can still fire in synchrony even when calcium mediated synaptic transmission is inhibited and that electrical coupling mediates this synchronisation (Bouskila, 1993; Jiang et al., 1997).

The reduction in amplitude of PER2::LUC rhythms that we see in our study could be due to a loss of synchrony between the cells, a loss of amplitude of oscillations of individual cells or a combination of both. To test this it will be important to measure PER2::LUC rhythms in individual cells. If the amplitude of individual cells is also reduced, this could indicate an effect of early light environment on core clock mechanisms such as the autoregulatory transcription-translation feedback loop which dictates the period of the clock. Visualising PER2::LUC expression in individual SCN neurons would allow us to determine whether individual neurons have altered amplitudes or periods due to early light environment.

Coupling strength has been associated with a tissue's ability to entrain to different T-cycles. The SCN, where cellular coupling is strong, is not able to entrain to extreme T-cycles such as 20 hour or 28 hour cycles. However, the lung, which has low amplitude oscillations and therefore low coupling strength, is able to entrain to these extreme T-cycles (Gibbs et al., 2009; Abraham et al., 2010). This allows peripheral tissues with low coupling strength to entrain to weaker *Zeitgebers*. This is essential as peripheral tissues receive time-of-day signals indirectly from the SCN. Meanwhile, the SCN must be able to ignore weak signals and only entrain to strong signals such as light, to ensure proper synchronisation to the external light environment. An interesting example of this can be found in recent work exploring the role of body temperature rhythms in synchronising peripheral tissues. When peripheral tissues were placed in culture, they were able to synchronise to changes in temperature within the physiological range (36-38.5°C), while the SCN could not. However, if TTX was applied to SCN tissue in culture, thus inhibiting synaptic transmission between SCN cells, SCN tissue was able to entrain to changes

in temperature in a similar way to peripheral tissue (Buhr et al., 2010). This study demonstrates a possible way that peripheral tissues may synchronise to the SCN, and indicates the importance of strong coupling within the SCN to maintain entrainment solely to the external environment.

Coupling strength in the SCN can be reduced by applying the adenylate cyclase inhibitor MDL which results in the SCN being able to entrain to 20-hour T-cycles (Abraham et al., 2010). It would be interesting to see whether early light environment alters the ability to entrain to different T-cycles. Previous work using Wistar rats has found that rats raised in LL seem to be able to entrain more easily to 22 to 25 hour T-cycles than rats raised in LD and DD, implying that rats raised in LL may have reduced coupling strength (Canal-Corretger et al., 2003a). However, I have established that in experiments determining the effects of early light environment on the photic response of the circadian system, the use of albino animals can lead to results which may be due to changes in the SCN or in the retina, and the effects of retinal damage cannot be separated from the effects in the SCN (see Chapter 3). Furthermore, it will be interesting to extend the above-mentioned experiment by using more extreme cycles such as 20- and 28- hour T-cycles.

The period and phase of PER2::LUC rhythms that we measured were within the levels measured in previous experiments (Yoo et al., 2004; Yoshikawa et al., 2005; Guilding et al., 2010; Hughes et al., 2011; Loh et al., 2011; Pendergast et al., 2012). We saw no effect of early light environment on the period or phase, indicating that the fundamental circadian clock function was unaffected by early light environment. Furthermore, we saw no effect of early light environment on the damping rate or rhythmicity index indicating that the stability of the PER2::LUC rhythms over time was not affected by early light environment. Thus it seems that early light environment does not affect the overall function of the clock, but perhaps only affects the communication between individual clock cells.

6.4.3 Effects of early light environment on PER2::LUC rhythms in LL

As we are particularly interested in the effects of early light environment on the circadian system when adult mice are in LL, we wanted to look at PER2::LUC rhythms of mice that were housed in LL. We had previously monitored their locomotor activity behaviour and by the time they were used for tissue culture all mice but one were behaviourally arrhythmic. The only rhythmic mouse left had PER2::LUC rhythms which were not different from other mice within the group (LL-reared group). We found that PER2::LUC rhythms from mice that had been in LL were not as robust as the baseline oscillations we had previously measured. In fact, using a goodness of fit calculation, we found only 16 of the 32 mice available had rhythms with a goodness of fit above 0.8 (DD: n=6; LD: n=5; LL: n=5), but no effect of early light environment on the goodness of fit. The reduced robustness in rhythms was illustrated by a reduction in the rhythmicity index compared to the baseline experiment, indicating that the rhythms were more unstable. However we saw no difference in the damping rate between the two experiments or due to early light environment, indicating that the stability of PER2::LUC oscillations over time was unaffected by early light environment or by long-term exposure to LL. Our initial hypothesis for this experiment was that similar to what we had seen in vivo, we would see a lengthening of the period of

PER2::LUC rhythms when the mice were taken from LL. However we saw no difference in period compared to the baseline experiment and no effect of early light environment on the period. Furthermore when we measured the phase we found that it was significantly different to what we had measured in the baseline experiment (mice taken from LL had peak PER2::LUC expression at 13:00h (ZT6 if animals were in LD) while the peak was between ZT9 and ZT10 for the baseline experiment). Previous work has shown that when mice were placed in LL for as little as 5 days, the clock was reset by the tissue culture procedure (Yoshikawa et al., 2005) perhaps due to LL reducing coupling between mammalian neurons (Ohta et al., 2005), which resulted in the rhythms being sufficiently damped to be completely reset (Yoshikawa et al., 2005). It is therefore highly likely that as our mice were in LL for at least 3 weeks their clocks were reset by the tissue culture procedure. Furthermore, in mice exposed to LL for 50 days, PER2 protein levels were elevated and constitutively expressed (Muñoz et al., 2005). In our mice, it is therefore possible that in vivo, PER2 protein expression was not rhythmic and that any rhythms we saw in vitro were a direct result of resetting by the procedure.

We saw no difference in phase due to early light environment suggesting that early light environment does not affect how the SCN tissue is reset by the tissue culture procedure. The amplitude of PER2::LUC rhythms was significantly reduced in these mice compared to the baseline experiment, but we found no difference in the amplitude due to early light environment. This is in contrast to what we found during the baseline experiment, where mice raised in DD had a significantly lower amplitude of PER2::LUC rhythms compared to mice raised in LD and LL. It is possible that this is due to the clock being reset by the tissue culture procedure. It may also be due to the lower sample numbers in the LL experiment (as a result of the large number of mice that had no or poor PER2::LUC rhythms) (Baseline experiment: DD - n=10; LD - n=12; LL - n=11; LL experiment: DD - n=6; LD - n=5; LL - n=5). In general we found that PER2::LUC oscillations in the LL experiment were less robust than in the baseline experiment and this may be due to the effects of LL on the SCN. Previous studies have shown that constant light can affect the synchrony between SCN neurons, resulting in weaker oscillations (Ohta et al., 2005) and we have seen a similar effect in our mice. As they were all arrhythmic by the time we performed tissue culture we may have been unable to distinguish any differences in PER2::LUC rhythms due to early light environment. If we had performed tissue culture while the mice were still rhythmic, we may have seen differences in PER2::LUC rhythms due to early light environment. It may also be interesting to attempt to mimic LL chemically in tissue culture. Recent work has suggested that the use of sorbitol to activate c-Jun N-terminal kinases (JNK), a member of the MAPK family, could be used as a useful tool for mimicking LL in tissue culture (Yoshitane et al., 2012) as they may play a role in controlling the oscillation speed and phase response of the clock. It may also be possible to mimic the glutamate signals occurring in response to constant light by placing cultures in a low concentration of glutamate. Finally, recent work has suggested that long-term application of a VPAC2 agonist may be able to mimic LL as such a treatment causes a lengthening of tau in both locomotor activity behaviour and tissue culture (Pantazopoulos et al., 2010). The authors of this paper suggest that VIP-VPAC2 may play a role in coupling strength. This is of particular interest to our work as we believe that early light environment may affect coupling strength in the SCN of adult mice, and that VIP may be

involved in these changes because we have previously shown a significant decrease in VIP expression in the SCN of LL-reared mice (Smith and Canal, 2009).

6.4.4 Effects of early light environment on PER2::LUC rhythms in peripheral tissue

The final aim of our experiment was to determine whether early light environment affects PER2::LUC rhythms in peripheral tissue. We have previously shown that TH and cFOS expression in many areas of the hypothalamus was affected by early light environment, suggesting that the effects of early light environment are more far-reaching than we had initially thought and that dopamine may play an important role in communicating these alterations from the SCN to other brain areas (Brooks et al., 2011).

We measured PER2::LUC rhythms in the heart, lung, liver and spleen. We found similar period and phase measurements as what has previously been published (Yoo et al., 2004; Pendergast et al., 2012) in all peripheral tissues. Compared to the SCN tissue, the phases of all peripheral tissues we measured were delayed by a few hours, which is similar to what is documented in vivo (Zylka et al., 1998).

We found that early light environment affected PER2::LUC rhythms in the heart, lung and spleen, but not in the liver. In the heart, we found that mice raised in DD had significantly reduced amplitudes of PER2::LUC rhythms compared to mice raised in LD and LL. Circadian rhythms in the heart are associated with changes in heart rate and blood pressure (for review see Hastings et al., 2003). Furthermore, it has been shown that rotating shift workers were more likely to suffer death due to ischemic heart disease than the rest of the population (Fujino et al., 2006). A reduced amplitude of PER2::LUC expression in the heart could therefore indicate alterations in circadian heart function which could lead to heart problems.

In the lung, we found that it was the mice raised in LL who had reduced amplitudes of PER2::LUC rhythms compared to mice raised in DD and LD, an opposite response to what has been shown in the SCN and the heart. In humans, alterations in circadian rhythms in the lung may affect the severity of airway inflammation in asthma patients (Gibbs et al., 2009), and glucocorticoids may be involved in this. Previous work has shown that PER2 expression in the lung is responsive to glucocorticoids, and the cells which oscillate in the lung (the murine Clara cells) are coexpressed with glucocorticoid receptors (Gibbs et al., 2009). Previous work within our lab has shown that early light environment affects the physiology of many areas of the hypothalamus, including areas which are associated with the stress response (Brooks et al., 2011). It is possible that early light environment induces a differential stress response leading to altered levels of glucocorticoids being released. This may lead to alterations in activation of glucocorticoid receptors in the lung during development which may lead to changes in PER2 expression. However, it has been suggested that glucocorticoids may be a general circadian signal for all peripheral tissues, and other tissues such as the liver and heart, have been shown to respond to application of the glucocorticoid hormone analogue dexamethasone (Balsalobre et al., 2000). As we found different responses to early light environment in the heart, lung and liver, it seems that whatever is causing the differences in PER2::LUC rhythms due to early light environment may be more complicated and more work will be needed to elucidate how early light environment affects peripheral tissue.

In the spleen, we found that the peak of PER2::LUC expression occurred significantly earlier in mice raised in LL compared to mice raised in LD, implying that perhaps the synchrony of spleen oscillations to external environment was affected by light during postnatal development. Circadian rhythms in the spleen are involved with changes in the immune response (Silver et al., 2012), so alterations in the circadian response of the spleen due to early light environment may affect the immune response in our mice.

Interestingly we found no differences due to early light environment in PER2::LUC rhythms in the liver, which is involved in food processing and energy homeostasis (Damiola et al., 2000). It has been shown that the liver can be uncoupled from the SCN using restricted feeding, so it seems that food is quite a strong *Zeitgeber* for the liver (Damiola et al., 2000). As our mice were fed ad libitum during postnatal development, it is possible that early light environment has therefore not had the same effect on the liver as on other peripheral tissues.

We have shown that early light environment affects peripheral tissues in different ways. This may be a reflection of how different peripheral tissues receive signals about the time of day from the SCN and highlights the importance of further study on communication between the SCN and peripheral tissues. Furthermore, we have shown that although light does not directly affect peripheral tissues, circadian rhythms in these tissues are still affected by early light environment, which could lead to changes in the cardiovascular, pulmonary and immune systems.

6.4.5 Conclusions

In these experiments we have shown that early light environment affects the locomotor activity behaviour of mPer2::luc mice in a similar way to what we have previously found in C57BL/6J mice (see chapter 3 and [2]). Furthermore we have shown that early light environment affects PER2::LUC rhythms in the SCN, with mice raised in DD showing lower amplitude oscillations than mice raised in LD and LL. This result may indicate that early light environment affects coupling strength in the SCN, leading to weaker coupling in mice raised in DD. As LL has been shown to desynchronise neurons in both adult and developing mice (Ohta et al., 2005, 2006), we speculate that the absence of light signals during development has led to mice raised in DD being less able to cope with light signals as adults than mice raised in LD and LL.

Cellular coupling in the SCN may be mediated by neuropeptides, changes in synaptic strength or gap junctions so this result may indicate changes in neuronal signalling in the SCN due to early light environment.

Finally we showed that early light environment also affects PER2::LUC rhythms in peripheral tissues, leading to possible changes in the cardiovascular, pulmonary and immune systems. This finding opens up new questions such as why different peripheral tissues respond differently to early light environment, and whether this could be due to how the SCN communicates with each tissue. Furthermore, babies that are born prematurely are sometimes kept in constant light conditions (Ohta et al., 2006) and these findings propose that these babies may be at risk of developing alterations in the cardiovascular, pulmonary and immune systems. Further research on the effects of light during postnatal development is therefore crucial to answer these questions.

7. Discussion

The aim of this project was to determine how early light environment affects the photic response of the circadian system in adult mice. The circadian system consists of an input pathway (the retina and retinohypothalamic tract - RHT), the central pacemaker, and outputs, such as locomotor activity behaviour. Each component of the circadian system was studied in turn to get an overview of how the photic response is altered by early light environment.

7.1 Effects of early light environment on the retinas of CD1 mice

Light is the most powerful *Zeitgeber* to affect the circadian system and the main pathway for light information reaching the suprachiasmatic nucleus (SCN) is via the retina. The basic experimental design in this project was to raise mice in constant darkness (DD), normal 12:12 hour light-dark cycles (LD) or constant light (LL) from birth (postnatal day 0 - P0) to P21, then house them all in LD before studying various aspects of the photic response of the circadian system. As it has been previously documented that LL can cause retinal damage in albino mice (LaVail et al., 1987a), we wanted to see whether early light environment could affect retinal function or structure in albino (CD1) and pigmented (C57BL/6J) mice. We found that in CD1 mice, being raised in LL caused a significant decrease in retinal function and significant damage to the retinal structure compared to mice raised in DD and LD. Interestingly, in a separate experiment, we noted that the amount of retinal damage that CD1 mice suffered may be affected by the light environment after P21. Indeed, the CD1 mice that suffered severe retinal damage had been raised in LL from P0 to P21, and then transferred to LD, with an LED (light-emitting diode) light source throughout life. Meanwhile, CD1 mice raised in LL provided by LED light from P0 to P21 then transferred to LD provided by higher intensity fluorescent light did not suffer severe retinal damage, but instead showed altered retinal structure which may indicate changes in the efficiency of photic transduction in the retina. Thus it seems that the light environment into which CD1 mice were transferred after weaning affected the structure and function of the retina. The damaging effects of LL during postnatal development have previously been questioned as studies have shown that Wistar rats raised in LL up until P21 did not suffer retinal damage but rats raised in LL until P35 did, implying that retinal damage occurred between P21 and P35 (Harada et al., 1998, 2000). Our data seems to indicate that exposure to fluorescent light after P21 may rescue the retina from light-induced retinal damage, even if it is high intensity fluorescent light, as in our experiments. The main difference between LED and fluorescent light is their spectral irradiance curves (see Introduction). It is possible that the two light sources emit more or less light at wavelengths which may cause significant damage to the retina. In particular, we have noted that LED light emits much higher levels of melanopic lux (m-lux) than fluorescent light. During postnatal development, the number of melanopsin-expressing retinal ganglion cells (RGCs) increases, before being pruned back later on (González-Menéndez et al., 2010a). This increase did not occur in CD1 mice raised in LL, but the number of melanopsin-expressing RGCs could be rescued if mice were placed back in LD by P11 (González-Menéndez et al., 2010b). However, they did not look to see whether the number of

melanopsin-expressing RGCs could be rescued after P21. Perhaps what we see in the CD1 mice placed in fluorescent light from P21 onwards reflects a rescue of the retina from light-induced retinal damage. Even in CD1 mice placed in fluorescent light after weaning, we still saw an alteration in the structure of the retina if they were raised in LL, so it seems that the effects of LL on the retinas of CD1 mice were not fully reversible. Our main finding is that CD1 mice are susceptible to light-induced retinal damage during postnatal development, and that exposure to LED light after weaning seems to result in more serious effects than fluorescent light. Therefore, CD1 mice may not be a good model for studying how early light environment affects the rest of the circadian system, as it would not be possible to separate retinal effects from other effects.

7.2 Effects of early light environment on the retinas of C57BL/6J mice

In C57BL/6J mice housed under LED light throughout life (a light regime that caused severe damage in CD1 mice) we found that early light environment did not seem to affect the retinal function or structure. Pigmented rats and mice are protected from light-induced retinal damage as the pigment in their eyes absorbs any escaping light and prevents light from reaching the retina from anywhere other than the pupil. As the pupil can constrict to as much as 0.1mm, this provides ample protection from light-induced retinal damage (Rapp and Williams, 1980). C57BL/6J mice have previously been shown to have altered locomotor activity behaviour, as well as altered levels of neuropeptide, glial fibrillary acidic protein (GFAP), tyrosine hydroxylase (TH) and cFOS expression in the SCN and in other areas of the hypothalamus (Smith and Canal, 2009; Brooks et al., 2011). As we found no obvious alterations in retinal function or structure due to early light environment, we can conclude that early light environment may affect other parts of the circadian system, such as the SCN itself.

However, we cannot rule out that being raised in different light conditions during postnatal development does not affect retinal development. The development of the photosensitivity of the retina occurs postnatally, and seems to occur in two broad stages. At birth some intrinsically photosensitive retinal ganglion cells (ipRGCs) are already responsive to light (Hannibal and Fahrenkrug, 2004), and further research has shown that these are M1 ipRGCs expressing Opn4S (Hughes et al., 2012). From P10 onwards, rods and cones become photosensitive, as well as M2 ipRGCs expressing Opn4L (Hughes et al., 2012). The effect of light during postnatal development has been examined in C3H/He mice exposed to LD or LL and no difference was found in the number of M1 or M2 melanopsin-expressing cells during postnatal development regardless of whether mice were raised in LL or LD (González-Menéndez et al., 2010a, 2010b). Although this result implies that in pigmented mice, early light environment does not seem to affect the development of ipRGCs, we cannot rule out other changes in the development of the retina. Perhaps the organisation of the retina has been affected, altering how light information is received and interpreted. After a light pulse, we saw no difference in the amount of cFOS, pERK or PER2 expression in the SCN due to early light environment implying that the photic information reaching the SCN is the same but we cannot rule out more subtle changes which may not be quantifiable using immunohistochemistry. It is also possible that the communication between the retina and the brain is altered as a result of changes in, for example, how many ipRGC axons have developed and synapsed onto the SCN and other brain areas. This could be

measured by labelling the RHT using cholera toxin subunit β injected into the eye which allows for the visualisation of ipRGC innervation in the brain. In this way, SCN innervation by ipRGC could be quantified (for example of the method, see King et al., 2003).
One of the experimental paradigms used during this project was exposing adult mice to LL for prolonged durations to explore the effects of LL on locomotor activity behaviour. As LL has been shown to induce retinal damage (Noell et al., 1966; Rapp and Williams, 1979), it was important to determine whether the retinas of C57BL/6J mice would be affected by prolonged exposure to LL. We found that although there was a general reduction in retinal function after exposure to LL, this was not dependent on whether mice had been reared under DD, LD or LL. Thus any effects of early light environment we may observe when mice are placed in LL as adults are probably not a result of changes in the retina but instead may reflect how the circadian system responds to being exposed to LL.
Although we cannot exclude discreet effects of early light environment on the retinas of C57BL/6J mice, our data indicates that the gross retinal structure and function seems to be unaffected by early light environment, making this strain a good model for studying how early light environment affects the photic response of the circadian system.

7.3 Effects of early light environment on neuropeptide and astrocyte expression in the SCN of CD1 mice

One of the initial observations made at the beginning of this project was that CD1 mice and C57BL/6J mice had altered responses to exposure to LD, with CD1 mice raised in LL having a lower amplitude of locomotor activity behaviour compared to mice raised in DD and LD while C57BL/6J mice raised in LL had a higher amplitude of locomotor activity behaviour compared to mice raised in DD and LD (Canal-Corretger et al., 2001b; Smith and Canal, 2009). It has also been shown that these C57BL/6J mice raised in LL had reduced arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) expression in the SCN and reduced AVP expression in the paraventricular nucleus (PVN) (Smith and Canal, 2009). We wanted to know whether, in CD1 mice, neuropeptide expression would reflect the locomotor activity behaviour findings and show opposite results to what was shown in C57BL/6J mice. Interestingly, we found that neuropeptide expression seemed to be dependent either on the level of light-induced retinal damage or on the amount of melanopic lux (m-lux) the retina detects. Indeed, CD1 mice raised in LL who suffered severe light-induced retinal damage as a result of being exposed to LED light throughout life (and therefore were exposed to high levels of m-lux throughout life) had significantly higher levels of VIP and more AVP-positive cells in the SCN than mice raised in LD and DD. CD1 mice that only suffered small changes to the structure of the retina as a result of being exposed to LED light during postnatal development and then being transferred to fluorescent light (and therefore receiving lower levels of m-lux) did not have any significant changes in VIP and AVP levels due to early light environment. As previously published, C57BL/6J mice exposed to low intensity fluorescent light throughout life (and therefore not suffering retinal damage and being exposed to the lowest levels of m-lux), had higher levels of AVP and VIP expression if they were raised in DD compared to LD or LL. The amount of m-lux a light source emits could feasibly affect neuropeptide expression in the SCN as a light source

emitting high levels of m-lux (for example LED light) would activate ipRGCs to a greater extent. As ipRGCs innervate the SCN, this would result in higher levels of innervation under LED light for example. VIP is the neuropeptide which is involved in receiving photic information from the retina and transferring this photic information to the rest of the SCN (Vosko et al., 2007) so alterations in the photic signals from the retina during postnatal development may be visible in altered VIP expression in the SCN of adult mice. Alterations in AVP expression may be further evidence of altered communication in the SCN (Abrahamson and Moore, 2001). Furthermore, retinal damage may result in reduced ipRGC innervation to the SCN. We saw a reduced pupil response in the CD1 mice raised in LL compared to the mice raised in LD if these mice were housed under LED light throughout life. The pupil response is a measure of the non-photic visual response and can give an indication of the integrity of ipRGCs (Lucas et al., 2001, 2003). A reduced pupil response would imply damage to the ipRGCs, which may lead to reduced innervation to the SCN as well. Our results reveal a susceptibility of neuropeptide expression in the SCN to retinal damage and type and intensity of light, which should be taken into account when designing experiments.

In the same experiment, we found no difference in GFAP expression in the SCN of CD1 mice housed under LED throughout life, in contrast to what was published in C57BL/6J mice housed under low intensity fluorescent light throughout life (Canal et al., 2009). This may indicate that at high light intensities, GFAP expression was saturated and therefore subtle differences in GFAP expression due to early light environment were not visible. It is also possible that the light-induced retinal damage suffered by the mice raised in LL altered the levels of GFAP in the SCN as it has previously been shown that GFAP expression in the SCN can be reduced by enucleation (Lavialle et al., 2001).

7.4 Effects of early light environment on locomotor activity behaviour in LL

One of the key effects of early light environment on locomotor activity behaviour is the finding that rats raised in LL were able to remain rhythmic when placed in LL as adults while rats raised in DD and LD became arrhythmic in LL (Cambras et al., 1998). Mice, who are less susceptible to becoming arrhythmic when exposed to LL as adults, have been shown to have longer taus in LL if they were raised in DD compared to if they were raised in LL (Canal-Corretger et al., 2001b). Aschoff's rule states that in constant conditions the higher the light intensity the longer the tau (Aschoff, 1960) which leads us to hypothesise that the mice raised in DD may have been more sensitive to light than the mice raised in LL. However this study was performed in albino mice, which may mean that the difference in sensitivity was due to the fact that mice raised in LL had damaged retinas. Therefore, we wanted to confirm that we would also see such changes in behaviour in LL in C57BL/6J mice, which did not seem to suffer retinal damage. We showed that at all light intensities tested, C57BL/6J mice raised in DD had significantly longer taus than mice raised in LL. As these mice appeared to have intact retinas, we hypothesised that the way photic information is processed in the SCN was altered by early light environment, leading to mice raised in DD interpreting light as being at a higher intensity than mice raised in LL.

It is, however, important to consider the possibility that other brain areas which may play a role in tau lengthening in LL have also been affected by early light environment. For example the intergeniculate leaflet (IGL) receives direct photic input from ipRGCs (Pickard, 1985). It has been shown that ablation of the IGL reduced the tau lengthening which normally occurs in LL (Pickard et al., 1987), so it seems that the IGL may be involved in the behavioural response to exposure to LL. It is possible that early light environment may affect the development of the IGL, which may lead to the alterations in tau lengthening due to early light environment. Our results, where mice raised in DD had longer taus than mice raised in LL, would imply that being raised in LL somehow reduces the efficacy of the IGL. Alternatively IGL lesions may be associated with a reduction in the amount of locomotor activity behaviour (Janik and Mrosovsky, 1994) which may itself mediate the reduction of tau lengthening which occurs after IGL lesions (Pickard et al., 1987). In our data we did not see alterations in the amount of activity during the active phase due to early light environment when mice were placed in LL, which would seem to indicate that if there are any effects of early light environment on the IGL, they would be relatively minor.

7.5 The contribution of species, strain and type of light to the locomotor activity behaviour

We also monitored locomotor activity behaviour in LD in C57BL/6J mice on two occasions during this project and saw no difference in behaviour due to early light environment. This is in contrast to what has previously been published where C57BL/6J mice raised in LL had a higher amplitude of locomotor activity and more stable rhythms than mice raised in LD and DD (Smith and Canal, 2009). One of the differences between the published work and my experiments was the type and intensity of light used. The published work used white fluorescent light with a mean intensity of 57$\mu W/cm^2$ while my experiments used white LED light with a mean intensity of 330$\mu W/cm^2$. The light intensity itself may be sufficient to explain the differences between the two experiments, as high intensity light may saturate the circadian system which may lead to us being unable to see differences due to early light environment. Indeed, it has been shown that the size of phase shift after a light pulse reaches a maximum level at saturating light intensities (Foster et al., 1991), which may mean the circadian system as a whole can be saturated by light. The size of the phase shift can also be affected by the wavelength of light used. The largest phase shifts occur at wavelengths close to 500nm, the wavelength to which melanopsin is sensitive (480nm) (Takahashi et al., 1984; Boulos, 1995). As the wavelength was shortened, the sensitivity to light pulses reduced gradually, but as the wavelength was lengthened, the sensitivity dropped suddenly (Takahashi et al., 1984). In general, the sensitivity to green, blue and white light pulses was pretty similar, but much reduced for red light pulses, which rodents are much less sensitive to. In our case it is possible that exposure to a light source with a high m-lux level resulted in an altered effect of early light environment on locomotor activity behaviour in LD compared to mice exposed to fluorescent light.

Similarly, when we monitored locomotor activity behaviour in DD, we saw no difference in any variable we measured due to early light environment in the C57BL/6J mice, a result which is in contrast to what has been shown in Wistar rats where rats raised in DD had a higher power

content of the first harmonic compared to rats raised in LL (Canal-Corretger et al., 2001a). The difference in result may be due to the differences in the type and intensity of light used, as previously discussed, or they may be a result of species differences.

During this project we also used mPer2::luc mice raised on a C57BL/6J background to visualise real time PER2::LUC protein expression in vitro. When this strain of mouse was first developed, their locomotor activity behaviour was tested to ensure that the fusion of the *Luciferase* gene to *Per2* would not affect behaviour and found no differences in the ability to entrain or to shift after a light pulse at CT16 (Yoo et al., 2004). As we had already obtained different locomotor activity behaviour results depending on the type of light used, we wanted to determine how early light environment affected locomotor activity behaviour in mPer2::luc mice to ensure they were a suitable model for exploring the effects of early light environment on the photic response of the circadian system. In LD, we found that mPer2::luc mice raised in LL had a significantly higher amplitude of locomotor activity and higher amount of activity during their active phase than mice raised in DD and LD. We also found that in DD, mice raised in LL had stronger rhythms than mice raised in LD or LL. However, we found no difference in the size of phase shift after a light pulse at CT16 due to early light environment. In LL, we found that mice raised in DD became arrhythmic faster than mice raised in LL and that mice raised in LL had overall stronger rhythms than mice raised in DD and LD. We were able to conclude from this data that mPer2::luc mice were a suitable model for studying the effects of early light environment on PER2::LUC rhythms in vitro.

Of particular note when comparing the effects of early light environment across studies is that different species and strains seemed to produce different results. In the earliest studies using Wistar rats it was shown that only rats raised in LL were able to maintain circadian rhythms when placed in LL as adults, rats raised in DD and LD became arrhythmic (Cambras et al., 1997, 1998). Furthermore, in DD, rats raised in DD had a significantly higher percentage of variance compared to rats raised in LL (Canal-Corretger et al., 2001a) and in LD, rats raised in DD had a higher power content of the first harmonic than rats raised in LL (Canal-Corretger et al., 2003a). Finally, rats raised in LL seemed to be able to entrain to T-cycles easier than rats raised in DD (Canal-Corretger et al., 2003a), implying that the coupling strength within the SCN of rats raised in LL was weaker than that of those raised in DD (Abraham et al., 2010). These results indicate that although rats raised in LL could cope better with being in LL as adults, rats raised in DD seemed to have overall stronger circadian rhythms than rats raised in LL. Similarly, in CD1 mice, being raised in LL resulted in mice having a shorter tau when placed in LL as adults compared to mice raised in DD (Canal-Corretger et al., 2001b). Furthermore, in LD, mice raised in DD had a higher amplitude of locomotor activity behaviour than mice raised in LL (Canal-Corretger et al., 2001b). These results seem to imply that in LL, CD1 mice raised in DD were detecting a higher light intensity than mice raised in LL, and that in LD, mice raised in DD had stronger rhythms than mice raised in LL.

In C57BL/6J mice housed under low intensity fluorescent light it was shown that mice raised in DD had significantly lower amplitudes of locomotor activity compared to mice raised in LL (Smith and Canal, 2009) implying that in this case, it was the mice raised in LL that had the stronger rhythms. Furthermore, in mice housed under high intensity LED light, mice raised in DD had significantly longer taus when placed in LL compared to mice raised in LL, implying that

they perceived a higher light intensity than mice raised in LL. However, we have established in C57BL/6J mice that retinal function and structure did not seem to be affected by early light environment so we believe that early light environment affects how the SCN interprets photic information. In mice raised under high intensity LED light we saw no other differences in locomotor activity behaviour due to early light environment in LD, DD or after light pulses. Finally in mPer2::Luc mice we found mice raised in DD became arrhythmic in LL faster than mice raised in LL and that mice raised in LL had overall stronger rhythms, implying that they could cope with LL better than mice raised in DD. Furthermore, in LD and DD, mice raised in LL were also shown to have stronger rhythms compared to mice raised in DD.

Therefore, in all species and strains studied, animals raised in LL seemed to cope better with being in LL as adults than animals raised in DD. However there is a stark contrast in behaviour between albino and pigmented animals. Indeed, in albino animals (Wistar rats and CD1 mice) in LD and DD, the animals raised in DD seemed to have the stronger rhythms. Conversely, in pigmented animals (C57BL/6J mice and mPer2::luc mice) in LD and DD, the animals raised in LL seemed to have the strongest rhythms. These differences between albino and pigmented animals may be due to the fact that albino animals raised in LL suffered retinal damage. It is possible that as a result, the behaviour of these animals was altered in LD and DD, leading to the animals raised in DD having stronger rhythms.

It is also worth mentioning that C57BL/6J mice (and therefore mPer2::luc mice raised on a C57BL/6J background) do not appear to produce the pineal hormone melatonin, which has been shown to inhibit neuronal firing in the SCN during the night and cause phase advances when administered at dusk (Goto et al., 1989; Liu et al., 1997). On the other hand rats do produce melatonin (Tamarkin et al., 1985) so it is possible that melatonin affects behaviour in LD and DD, leading to rats raised in DD having stronger rhythms than rats raised in LL, while C57BL/6J mice and mPer2::luc mice raised in DD have weaker rhythms than mice raised in LL. In this case, it would seem that melatonin does not affect how different species and strains respond to being exposed to LL as adults, as the response is similar regardless of whether the animal produces melatonin or not.

Finally, when comparing the locomotor activity behaviour of C57BL/6J mice and mPer2::luc mice housed under high intensity LED light, it was noted that mPer2::luc mice seemed to be much more affected by early light environment than C57BL/6J mice. This is surprising as these mPer2::luc mice were raised on a C57BL/6J background and therefore the only difference between the two strains should have been the fusion of the *Luciferase* gene onto the *mPer2* gene. Previous work has shown that this fusion resulted in no alterations in entrainment or the ability to respond to light pulses (Yoo et al., 2004). However, here, we have shown that these mice seemed to be more sensitive to the effects of early light environment. The results of the mPer2::luc mice in high intensity LED light reflected those seen in C57BL/6J mice in low intensity fluorescent light. As previously discussed, the apparent lack of effects due to early light environment (except in LL) in C57BL/6J housed under high intensity LED light may be due to a saturation of light signals reaching the SCN. In this case, it is possible that mPer2::luc mice have a higher saturation threshold, leading to the visualisation of the effects of early light environment at higher light intensities.

The important thing to note is that across all species and strains so far studied, early light environment has a robust effect on locomotor activity behaviour in LL: being raised in LL allows animals to cope better with exposure to LL as adults.

7.6 Effects of early light environment on the response to light pulses

As we hypothesised that early light environment may affect how the SCN interprets photic information, we wanted to test how the pacemaker responded to light administered at different times of day. As expected, a light pulse administered at circadian time 16 (CT16) resulted in a phase delay while a light pulse administered at CT22 resulted in a phase advance. However we found no difference in the size of the phase shift due to early light environment, implying that the phasic response to light was unaffected by early light environment. However, until the effects of early light environment are determined for the full phase response curve, it is not possible to know whether the phasic response to light is actually affected or not. Indeed, the shape of the phase response curve may itself be affected by early light environment, and this has been hypothesised to be associated with the amount of tau lengthening in LL (Pendergast et al., 2010). It will be interesting to see whether early light environment affects the shape of the phase response curve. Although we did not see any differences in the size of phase shift after light pulses at CT16 and CT22, we wanted to see whether the intracellular photic signalling pathway was affected by early light environment. We measured pERK, cFOS and PER2 expression in the SCN after a light pulse at CT16 and saw significant increases in pERK and cFOS expression after a light pulse as expected. However there were no differences in the amount of pERK or cFOS due to early light environment indicating that the early intracellular photic signalling pathway was unaffected by early light environment. We did not see an increase in PER2 expression after a light pulse at CT16, probably because we took samples before PER2 protein expression increases. Furthermore we saw no difference in the level of PER2 protein in the SCN due to early light environment. We chose to measure PER2 levels as it is a core clock gene which responds to light pulses and therefore may give us an idea of whether the molecular clock is affected by early light environment.

7.7 Effects of early light environment on PER2::LUC expression in the SCN

Although we saw no differences in PER2 protein expression after a CT16 light pulse, we hoped that we may see differences by measuring real-time PER2::LUC protein expression in vitro as it has previously been shown that real time clock gene monitoring provides a more accurate and sensitive technique for measuring changes in the SCN (Hughes et al., 2008). When we did this, we found that the amplitude of PER2::LUC rhythms was significantly reduced in mice raised in DD compared to mice raised in LD and LL. We saw no differences in period, phase, damping rate or rhythmicity index. We measured PER2::LUC levels in whole SCN tissue, meaning that we saw a sum of what was occurring in individual cells.

7.7.1 A possible role for neuronal coupling in the SCN

Throughout this project we have noted that some changes in behaviour and neuropeptide expression due to early light environment are not robust and are altered depending on the type or intensity of light. However, a consistent and robust effect that we have noted is that in LL as adults, animals raised in LL have more stable locomotor activity behaviour than mice raised in DD. This is shown either by animals raised in DD and LD being unable to remain rhythmic in LL while mice raised in LL remain rhythmic, or by mice raised in DD having significantly longer taus in LL as adults than mice raised in LL.

To explain the general function of the circadian system, Diez-Noguera proposed a model in which oscillating units within the SCN synchronise, with light reducing the level of synchrony (Diez-Noguera, 1994). In LL, the level of synchrony is reduced to such a level that animals become behaviourally arrhythmic. Later work showed that animals raised in LL were able to remain rhythmic in LL as adults and therefore may have a higher level of synchrony (Cambras et al., 1998). The circadian system has been described as individual oscillating neurons that form networks and synchronise their oscillations to each other, a phenomenon known as coupling. These networks form part of larger networks which together produce the oscillations of the whole SCN, ultimately resulting in the synchronised oscillations of both nuclei (Vosko et al., 2007). Coupling strength may be associated with entrainment in vivo and the amplitude of PER2::LUC rhythms in vitro (Abraham et al., 2010). Indeed, stronger coupling is associated with a higher amplitude of PER2::LUC rhythms and a reduced entrainment range. Interestingly, we found in our experiments that mice raised in DD had significantly lower amplitudes of PER2::LUC rhythms than mice raised in LD or LL. This result implies that the strength of coupling between SCN neurons is reduced in mice raised in DD. This matches the hypothesis formulated in the model put forward by Diez-Noguera (1994) where the level synchronisation between oscillatory units reflects the ability of a mouse to remain rhythmic in LL, with mice with a lower level of synchronisation being more likely to become arrhythmic in LL or having a longer tau.

Early light environment has previously been shown to affect synchronisation within the SCN. Indeed, rats raised under T22 cycles did not show desynchronisation between locomotor activity rhythms and temperature rhythms, while rats raised under T24 then transferred to T22 did (Anglès-Pujolràs et al., 2007). The desynchronisation between locomotor activity rhythms and temperature rhythms may reflect a desynchronisation between ventrolateral and dorsomedial SCN, in which case being raised under a T22 cycle may have resulted in stronger synchronisation between these two SCN compartments compared to rats raised under T24. This study lends support to our hypothesis that early light environment may affect coupling strength in the SCN.

Synchronisation between SCN neurons can be mediated by different mechanisms that may all act together to produce overall SCN rhythms. Synaptic transmission (Honma et al., 2000; Yamaguchi et al., 2003), VIP (Maywood et al., 2006b; Brown et al., 2007; Hughes et al., 2008) and gap junctions (Bouskila, 1993; Jiang et al., 1997) have all been associated with coupling between SCN neurons. VIP is of particular interest to us as previous work within our lab has shown that early light environment alters VIP expression in the SCN (Smith and Canal, 2009).

Furthermore, it has been shown that interfering with VIP-mediated coupling by applying the adenylate cyclase inhibitor N-(Cis-2-phenyl-cyclopentyl) azacyclotridecan-2-imine-hydrochloride (MDL) resulted in a reduction in coupling strength in the SCN (Abraham et al., 2010). In addition, mice lacking the receptor for VIP, VPAC2 (*vipr2*$^{-/-}$ mice) also had a reduced amplitude and altered phase of PER2::LUC rhythms compared to wild-type mice (Hughes et al., 2011). Alterations in VIP expression due to early light environment may therefore indicate that early light environment affects VIP-mediated coupling. However we have seen that early light environment affects VIP expression differently depending on the type and intensity of light. It is still possible that VIP plays a key role in the effects of early light environment on the level of synchronisation between neurons as we have not explored the effects of early light environment on its receptor VPAC2. As knocking out the VPAC 2 receptor results in a reduction in PER2::LUC oscillations (Hughes et al., 2011), similar to what we see in the mice raised in DD, it is possible that early light environment has a consistent effect on VPAC2 expression in the SCN.

It is also important to consider whether early light environment may affect other coupling mechanisms such as synaptic transmission and gap junctions. Alterations in synaptic transmission could lead to changes in coupling strength as application of tetrotodoxin (TTX) desynchronises SCN neurons (Yamaguchi et al., 2003). Furthermore, as GABA is the main SCN neurotransmitter it is possible that alterations in GABA signalling affect coupling in the SCN (Liu and Reppert, 2000). The role of gap junctions in coupling is not well understood and seems to be thought of as less important than that of synaptic transmission and neuropeptides (Welsh et al., 2010). However it is possible that gap junctions are affected by early light environment and contribute to the effects of early light environment on coupling strength.

7.7.2 A possible role for astrocytes

In his model, Diez-Noguera (1994) noted that light reducing the level of synchrony may not itself explain how increasing the light intensity may increase tau length, as described by Aschoff's rule (Aschoff, 1960). He therefore introduced the concept of "neutral elements" that would act to stabilise the system. In his paper, the neutral elements were proposed to be astrocytes (Diez-Noguera, 1994). Astrocytes have indeed been shown to play a role in regulating locomotor activity behaviour in LL. A study using GFAP-mutant mice showed that they were able to entrain to LD and had normal responses to light pulses in DD. However, compared to wild-type C57BL/6J mice, they had longer taus in LL or became arrhythmic (Moriya et al., 2000). This response to LL was very similar to what we saw in C57BL/6J mice raised in DD compared to those raised in LL and led to the hypothesis that early light environment would affect GFAP expression in the SCN. This was indeed shown to be the case, with mice raised in LL having higher numbers of GFAP-positive cells than mice raised in DD, accompanied by reduced GFAP expression compared to mice raised in DD (Canal et al., 2009). A decrease in the number of GFAP-positive cells in the mice raised in DD could be compared to the GFAP-mutant mice previously mentioned (Moriya et al., 2000) and this result matches the mutant mouse data very nicely as mice raised in DD have longer taus than mice raised in LD and LL. However, in the current project, we measured GFAP expression in the SCN of CD1 mice housed under LED

light throughout life and found no differences due to early light environment. We may not have seen differences because the retinas of the CD1 mice raised in LL were damaged. Indeed it has previously been shown in hamsters that enucleation reduces GFAP expression in the SCN compared to hamsters raised in DD and LL (Lavialle et al., 2001) and we believe that in a similar way, light-induced retinal damage may have altered GFAP levels and masked the effects of early light environment. It is however possible that, similarly to what we saw with AVP and VIP expression, GFAP expression was also affected by the type and intensity of light.

7.7.3 A possible role for individual SCN neurons

We have focused so far on the possibility that alterations of the amplitude of PER2::LUC rhythms in the SCN are a result of changes in coupling strength. However, it is possible that a reduction of the amplitude of PER2::LUC rhythms is due to an overall reduction in the amplitude of oscillation in each individual cell (Yamaguchi et al., 2003; Maywood et al., 2007) This often seems to occur in parallel with a loss of synchrony between neurons and VIP may not only play a role in coupling between neurons but also the maintenance of synchrony within individual neurons (Aton et al., 2005). It is also possible that the speed of the molecular clock has been affected by early light environment. Indeed, it has previously been shown that in mice exposed to LL for 50 days, *mPer2* gene expression remained rhythmic, but mPER2 protein expression was elevated and non-rhythmic (Muñoz et al., 2005). It was hypothesised that LL may be preventing degradation of mPER2, which would normally occur in DD, and that this may have mediated the lengthening of tau seen during exposure to LL. It is possible that being raised in LL could result in an increased ability to degrade PER2 compared to mice raised in DD, which would lead to mice raised in LL having a shorter tau in LL as adults compared to mice raised in DD. The speed of the clock can be altered by applying protein synthesis inhibitors such as cycloheximide (CHX) (Feldman, 1967) and it may be possible to test whether the speed of the clock is affected by early light environment by applying CHX and measuring any altered responses due to early light environment.

Some evidence seems to indicate that early light environment may affect the oscillations of individual neurons. Mice raised in long photoperiods had broader waveforms than mice raised in short photoperiods, and this was mediated by alterations in the waveform and period of individual neurons (Ciarleglio et al., 2011).

My data seems to indicate that it is more likely that alterations in tau length are due to desynchrony among cells, as documented by Ohta et al. (2005). In this case it is more likely that altered coupling strength would mediate the changes in tau length we saw due to early light environment as the mice with reduced coupling strength (i.e. those raised in DD) would become desynchronised faster in LL than those with stronger coupling.

However, it will be important to prove this. This can be done by imaging PER2::LUC expression in individual cells. If coupling strength is affected by early light environment, this will be demonstrated by individual cells being slightly out of phase from each other. It will also be possible to measure whether it is the amplitude of PER2::LUC rhythms in each individual cell which is affected.

7.8 Criteria for a robust mechanism mediating the effects of early light environment

We have focused on the alterations in behaviour in LL due to early light environment, as these are the most robust. However, in some cases, we have seen changes in behaviour in DD and LD, particularly in the mPer2::luc mice in our experiments and in C57BL/6J mice housed under a different type and intensity of light in previous work (Smith and Canal, 2009). In LD and DD, any changes that we have noted during this project have implied that mice raised in LL have stronger rhythms than mice raised in DD and LD. However, previous work seems to indicate that these changes may be dependent on species, strain and/or type of light. It is possible that these changes are weaker and may be affected by signals downstream from the SCN. Indeed, it has been argued that output rhythms may not necessarily reflect pacemaker function, as output rhythms can be directly affected by stress, for example (Meerlo et al., 1997). In the case of LL, it is possible that the effects of early light environment on the tonic response of the SCN are so strong that they are not affected by downstream signals, or it is possible that LL suppresses the downstream signals themselves, resulting in behaviour which is mainly mediated by alterations in the SCN.

We believe that the mechanisms which are affected by early light environment must follow a few basic criteria. The first is that it should be a mechanism which is robustly affected by early light environment, regardless of the type and intensity of light. This is important as we have shown that early light environment affects locomotor activity behaviour in LL in the same way regardless of species, strain, sex, type and intensity of light. We believe it is unlikely that the same behaviour would be mediated by different mechanisms depending on what type of light the animals are housed under for example.

As we have seen different responses to light which we believe may be a result of using different types of light at different intensities, we must consider whether it is possible that we would see differences in PER2::LUC rhythms if our mPer2::luc mice were housed in a different light environment, such as fluorescent light. As we have previously noted, a consistent effect of early light environment, regardless of species, strain, type or intensity of light is that mice raised in LL have more stable/stronger rhythms than mice raised in DD when they are placed in LL as adults. We have shown that the PER2::LUC rhythms of mice raised in DD have a significantly reduced amplitude to those of mice raised in LD and LL. If, as we believe, the reduced amplitude mediates the behaviour we see in LL, then we would expect the effects of early light environment on PER2::LUC rhythms to be robust across different light types and intensities. However, it is possible that this is not the central mechanism involved in mediating this behaviour, in which case it is possible that we would not be able to replicate the changes in PER2::LUC rhythms in animals raised in different types of light. We believe that the mechanism central to the changes caused by early light environment would be robust under different types of light. Using different types of light during development would be an interesting way to verify the robustness of this central mechanism.

The other important criterion is that the mechanism should be directly responsive to light, as our effects are a result of alterations in light environment. We did not see any effects of early light environment on the size of the phase shift after light pulses administered at CT16 or CT22, which implies that the phasic response to light is unaffected by early light environment. On the

other hand, we have shown alterations due to early light environment in the tonic response to light, so we believe that the mechanism which is altered by early light environment would also be involved in the tonic response to light. VIP is a good candidate for this, but we have seen differential effects of VIP expression depending on the type and intensity of light, which would seem to indicate that the VIP response is not robust. However we have not explored all aspects of VIP function in the SCN, such as expression of its receptor VPAC2. GFAP may also be part of the mechanism and it will be important to clarify whether the alterations of GFAP expression by early light environment are robust. As proposed by the model put forward by Diez-Noguera (1994), it is possible that VIP and GFAP work in concert, with VIP affecting coupling strength and GFAP stabilising the coupling mechanisms. In this case it is possible that the roles of VIP and GFAP alter depending on the type and intensity of light but that the overall outcome remains the same. More work is needed to find the robust mechanism which is altered by early light environment and that mediates alterations in PER2::LUC rhythms in the SCN.

7.9 Effects of early light environment on PER2::LUC expression in peripheral tissue

As we have previously shown that early light environment also affects TH and cFOS expression in other areas of the brain (Brooks et al., 2011), we wanted to see whether PER2::LUC rhythms in peripheral tissue would also be affected. Interestingly, we found that different tissues are affected in different ways. The amplitude of PER2::LUC expression in the heart was significantly lower in the mice raised in DD compared to the mice raised in LD and LL, while in the lung, the amplitude of PER2::LUC expression was significantly higher in the mice raised in DD compared to the mice raised in LD and LL. In the spleen, the phase of PER2::LUC expression was significantly earlier in mice raised in LL compared to mice raised in LD. Interestingly, we found no alterations in PER2::LUC rhythms in the liver due to early light environment. These findings indicate that the effects of early light environment are far reaching and have long term consequences on physiological functions such as the immune response, lung and cardiac function. However, it seems that metabolism is less affected and this may be due to the fact that our mice were all fed ad libitum. It seems that the liver responds more to food availability than light environment, a finding which has been demonstrated in other studies where the liver was shown to be able to oscillate in antiphase to the SCN by providing food only during the day (Damiola et al., 2000). In other peripheral tissues, the differential responses to early light environment are probably a manifestation of the different ways that peripheral tissues are entrained to the external environment via the SCN. Not much is known about the signals used by the SCN to entrain peripheral tissues and our data implies that depending on the function of the tissue, the signal will be different. It is also possible that each tissue interprets the information differently, resulting in differential PER2::LUC expression to suit its function. What this data indicates is that there is still much to learn on the extent of the effects of early light environment and how the SCN entrains peripheral tissues.

7.10 Conclusion

The purpose of this project was to determine how early light environment affects the photic response of the circadian system. All photic information reaches the SCN via the retina, and our first aim was to determine whether early light environment affected the retina. In CD1 mice, an albino strain, we found that being raised in LL caused severe damage to the retinal structure and a reduction in retinal function. However, in C57BL/6J mice, early light environment did not seem to affect the structure or function of the retina, making this strain a good model for further studies on how early light environment affects the photic response of the circadian system. As early light environment seems to affect the behaviour of rats and mice particularly when they are in LL as adults, we next confirmed that in C57BL/6J mice, being raised in DD resulted in mice with a longer tau in LL as adults than mice raised in LL indicating that the effects of early light environment on locomotor activity behaviour in LL also occur in this strain.

Previous work has shown that neuropeptide and GFAP expression is affected by early light environment in C57BL/6J mice (Canal et al., 2009; Smith and Canal, 2009). We wanted to know whether this would also occur in CD1 mice that show different behavioural responses to early light environment than C57BL/6J mice. We found that the effects of early light environment on neuropeptide and GFAP expression also seem to depend on the type and intensity of light in which the animals are raised. Furthermore it seems that the damaging effects of being raised in LL on the retinas of CD1 mice can be reduced if they are transferred to fluorescent light after weaning. What these results indicate is that the effects of early light environment in CD1 mice are unpredictable and seem to be highly affected by type and intensity of light, as well as retinal damage. This data also provides a warning for work in C57BL/6J mice that the type and intensity of light may also affect the data, so care should be taken when designing experiments.

Next we wanted to explore whether early light environment would affect the ability to shift after light pulses. We found no differences in the size of the phase shift after light pulses given at CT16 and CT22, indicating that early light environment does not seem to affect the phasic response of the circadian system. Furthermore, in the SCN, we found no differences in the amount of cFOS, pERK or PER2 expression after a light pulse, indicating that this part of the photic signalling pathway does not seem to be affected by early light environment.

Although we saw no differences in PER2 expression after a light pulse at CT16, we wanted to look at real-time expression of PER2::LUC in vitro. We found mice raised in DD had a significantly reduced amplitude of PER2::LUC rhythms compared to mice raised in LD and LL. We believe that this reduction in amplitude reflects a reduction in coupling strength between SCN neurons in mice raised in DD and that this would explain the differences in behaviour that we saw due to early light environment. In particular, a reduction in coupling would result in a faster loss of synchrony between SCN neurons in LL compared to animals with stronger coupling. Further work is needed to confirm that the alterations in PER2::LUC amplitude are indeed a result of reduced coupling.

Finally we have shown that early light environment affects PER2::LUC rhythms in peripheral tissue indicating that the effects of early light environment reach far and wide.

Our results have shown that early light environment affects principally the tonic response of the circadian system. We believe that this is mediated by altered coupling between SCN neurons.

As we have seen that the effects of early light environment on behaviour in LL occur across species and strains, we believe that this could also occur in humans. Babies born prematurely are sometimes placed in incubators with the lights on all the time, which may reflect exposing rats and mice to LL during postnatal development. Our data seems to indicate that being exposed to LL during postnatal development results in animals that can cope better with exposure to extreme light environment such as LL. However we cannot say whether this would be the same in humans, who are diurnal. We believe that in humans, early light environment may affect the ability to perform shift work or the amount of time it takes to recover from jet lag. Furthermore, our findings that early light environment affects peripheral tissue put forward the alarming possibility that in humans, early light environment may affect cardiac, immune or lung function. In particular, it has been shown that rotating shift workers are more likely to die from ischemic heart disease (Fujino et al., 2006) and we are concerned that this may be exacerbated in people who were born prematurely and kept in incubators with the lights on constantly. It will be important to continue this research to establish whether this could actually be the case in humans as, in our 24-hour society, such information may help people to adjust their lifestyles to ensure a longer and healthier life.

7.11 Future directions

Our principal finding was that early light environment affects locomotor activity behaviour when animals are placed in LL as adults. Specifically, animals raised in DD have longer taus or become arrhythmic compared to animals raised in LL, implying that animals raised in DD are more sensitive to light than animals raised in LL. This alteration in behaviour occurs both in rats and mice, in various strains and in both males and females. Furthermore, it is not dependent on retinal damage as we have shown that C57BL/6J mice, a pigmented strain, show no gross alterations in retinal function or structure due to early light environment, while CD1 mice, an albino strain do, yet their behaviour in LL is similar. The next step in these experiments should be to elucidate the mechanisms which mediate the changes in behaviour in LL in adults due to early light environment.

In this project we have shown that the amplitude of PER2::LUC expression is reduced in mPer2::luc mice raised in DD compared to mice raised in LL and we surmised that this may be due to a reduced synchrony between SCN neurons in mice raised in DD (Abraham et al., 2010). We believe that reduced neuronal synchrony would explain the differences in locomotor activity behaviour that we see when adult mice are placed in LL. To confirm that neuronal synchrony in the SCN has been affected by early light environment, PER2::LUC expression can be monitored in individual SCN cells.

Reduced cellular synchrony in the SCN can also have other effects on overall locomotor activity behaviour such as an alteration in the ability to entrain to T-cycles and differences in the rate of recovery from jetlag (Abraham et al., 2010). We would hypothesise that mice raised in DD would be able to entrain to more extreme T-cycles and would recover from jetlag faster than mice raised in LL as a reduction in SCN neuronal synchrony would make the clock more flexible to change.

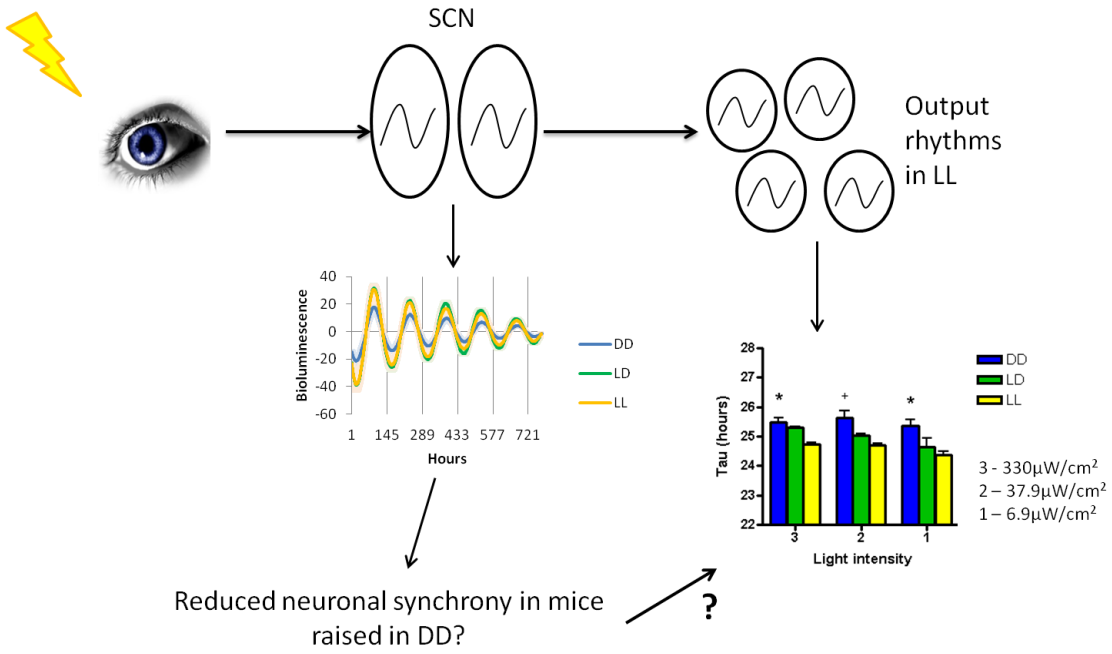

Figure 7.1: Schematic diagram of the key effects of early light environment on the photic response of the circadian system. Light is detected by the retina and reaches the SCN via the retinohypothalamic tract. We have shown no major effects of early light environment on retinal structure or function in C57BL/6J mice. In rats and mice, albino and pigmented, males and females, we have shown that animals raised in DD have longer taus or become arrhythmic compared to mice raised in LL. We have also shown that the amplitude of PER2::LUC expression is reduced in mPer2::luc mice raised in DD which we believe may be a result of reduced neuronal synchronisation in the SCN. We hypothesise that this may mediate the changes in behaviour in LL that we see due to early light environment. SCN – suprachiasmatic nucleus; DD – constant darkness, LD – 12:12 hour light-dark cycles; LL – constant light; RHT – retinohypothalamic tract.

There is evidence that VIP may be involved in neuronal synchronisation in the SCN (Maywood et al., 2006b; Brown et al., 2007; Hughes et al., 2008, 2011). As VIP is the neuropeptide involved in receiving photic information and synchronising the SCN to the external light environment (Abrahamson and Moore, 2001), it seems like an ideal candidate for a mechanism mediating the alterations in neuronal synchrony due to early light environment. Furthermore, it has previously been shown that early light environment may affect VIP expression in the SCN (Smith and Canal, 2009), but quantification of *Vip* mRNA has not yet been performed and this would provide good evidence of whether VIP is involved in the alteration of SCN neuronal synchronisation by early light environment. This can be done by using quantitative polymerase chain reaction (qPCR). A role for VIP in alterations due to early light environment could be confirmed behaviourally by raising VIP knockout mice (VIP/PHI$^{-/-}$ mice for example) in DD, LD or LL. If VIP is involved in the effects of early light environment on locomotor activity behaviour, you would expect to see no differences in behaviour due to early light environment in VIP knockout mice. It would also be interesting to quantify levels of the VIP receptor VPAC2 using qPCR as there is evidence that the role of VIP in SCN neuronal synchronisation may be via the VPAC2 receptor (Hughes et al., 2011).

Astrocytes may also play a role in neuronal synchronisation (Diez-Noguera, 1994) so quantifying GFAP in the SCN using qPCR would show us whether it is also involved in the effects of early light environment on SCN neuronal synchronisation.

By running these experiments, we would be able to test whether early light environment affects neuronal synchronisation and whether this is mediated by altered levels of VIP and/or GFAP in the SCN. We believe that altered neuronal synchronisation due to early light environment is a good candidate for a mechanism mediating the changes we see in locomotor activity behaviour in LL in adult mice and rats and that these experiments would confirm this.

Appendix 1: Effects of early light environment on $P75^{NTR}$ levels in the retina

A1.1 Introduction

In both rats and mice, the light environment in which they are raised has been shown to affect the physiology of the suprachiasmatic nucleus (SCN) and other areas of the hypothalamus, as well as affecting locomotor activity behaviour (Canal-Corretger et al., 2000, 2001b; Smith and Canal, 2009; Brooks et al., 2011). An important factor to consider in the experimental design of these studies is what effect raising animals in constant light (LL) would have on their retinas, and whether this could contribute to the changes we see in their behaviour and in the hypothalamus. In Chapter 3, I demonstrated that the light environment in which C57BL/6J mice (a pigmented strain) were raised did not affect retinal structure and function using electroretinography (ERG), pupillometry (PLR) and histology. However, CD1 mice (an albino strain) raised in LL had significantly damaged retinal structure and reduced retinal function compared to CD1 mice raised in constant darkness (DD) or normal 12:12 hour light-dark cycles (LD). Interestingly, the CD1 mice raised in LL were still able to entrain to LD and free-ran in DD with similar periods to the mice raised in DD and LD.

Similar findings have been found in neonatal rats treated with monosodium glutamate (MSG), which targets primarily retinal ganglion cells in the retina. As adults, these rats were still able to entrain to LD and free-ran with the same period as rats that were not treated with MSG. The levels of the low-affinity neurotrophin receptor $P75^{NTR}$ were measured in the suprachiasmatic nucleus (SCN) of these rats and it was found that there was a reduction in $P75^{NTR}$ levels in rats treated with MSG compared to controls (Beaulé and Amir, 2001). The levels of $P75^{NTR}$ in the SCN may reflect the damage to retinal ganglion cells caused by MSG, as a subset of these retinal ganglion cells (the intrinsically photosensitive retinal ganglion cells – ipRGCs) synapse directly onto the SCN (Moore and Lenn, 1972) and are responsible for sending information to the SCN about external light environment (Berson et al., 2002).

$P75^{NTR}$ also plays a role in light-induced retinal degeneration. It has been shown that rats that suffered light-induced retinal degeneration due to being kept in LL from postnatal day 2 (P2) to P35 had increased levels of $P75^{NTR}$ in the Müller glial cells of the retina compared to controls. The same study found that blocking $P75^{NTR}$ expression or the complete removal of $P75^{NTR}$ prevented light-induced retinal degeneration (Harada et al., 2000).

The aim of this study was to evaluate how the levels of $P75^{NTR}$ were affected in mice raised in DD, LD or LL from P0 to P21 then placed in LD. We hypothesized that the levels of $P75^{NTR}$ in the SCN may be reduced in CD1 mice raised in LL as they may have reduced ganglion cell innervations to the SCN. However, we were unable to test this hypothesis as we were unable to optimise the anti-$P75^{NTR}$ antibody to stain brain sections. Therefore we focused on measuring $P75^{NTR}$ levels in the retina using western blots. As the CD1 mice raised in LL underwent light-induced retinal degeneration, we expected to see increased levels of $P75^{NTR}$ in the retinas of these mice compared to those raised in DD and LD. Furthermore, as the C57BL/6J mice raised

in LL did not undergo light-induced retinal degeneration, it is possible that they had reduced levels of $P75^{NTR}$ levels which aided in the survival of the photoreceptors in this group of mice.

A1.2 Materials and methods

A1.2.1 Experimental design

	C57BL/6J mice (DD – n=7 males; LD – n=5 males and 2 females; LL – n=7 males)	**CD1 mice (n=7 males in each group)**
P0	Lactation (DD, LD, LL)	Lactation (DD, LD, LL)
P21	All animals placed in LD Animals were weaned and individually housed between P21 and P25.	All animals placed in LD Animals were weaned and individually housed between P21 and P25.
P25	Locomotor activity in LD	Locomotor activity in LD
P35-55	ERG (P35-55)	ERG (P35-55)
P66	P66: Mice culled at ZT14, brains and eyes collected	P66: Mice culled at ZT14, brains and eyes collected

Table A1.1: Experimental design for the mice used to determine the effects of early light environment on the levels of P75NTR in the retina. Mean light intensity was 330µW/cm^2 provided by LED light. DD – constant darkness; LD – 12:12 hour light-dark cycles; LL – constant light; ERG – electroretinography; ZT – *Zeitgeber* time; LED - light-emitting diode.

A1.2.2 Western blots

Western blots were used to determine the levels of the low-affinity neurotrophin receptor $P75^{NTR}$ in the retina. One eye from each mouse studied was removed and frozen on dry ice. Retinal samples were homogenised in ice-cold RIPA buffer (Cell Signaling Technology, Beverly, MA, US) containing 1mM PMSF (Sigma-Aldrich).

Samples containing 200µg of retinal protein were resolved by 12.5% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for one hour in Tris-buffered saline (TBS) containing 0.05% Tween and 5% skim milk (Sigma-Aldrich). The membranes were then incubated with polyclonal anti-P75NTR made in rabbit (1:10000 in TBS containing 0.05% Tween and 5% skim milk; Millipore, Watford, UK) at 4°C. After primary antibody incubation the membranes were washed 4 times in TBS containing 0.05% Tween then incubated in a horseradish peroxidase (HRP)-linked secondary antibody (anti-rabbit IgG HRP, 1:1000; Cell Signaling Technology) made in TBS containing 0.05% Tween for 1 hour. The membranes were washed 3 times in TBS containing 0.05% Tween and detected by chemiluminescence (LumiGLO, Cell Signaling Technology) according to the manufacturer's instructions.

To verify the loading, membranes were treated with rabbit polyclonal to beta-tubulin (HRP) (1:1000; Abcam, Cambridge, UK) for one hour, washed 3 times in TBS then detected by chemiluminescence.
Data was quantified with densitometric analysis using ImageJ and normalised to the tubulin loading control.

A1.2.3 Statistical analysis

Statistical analysis was performed by means of an ANOVA of general linear models using SYSTAT (Version 10, SPSS Inc., Chicago, IL, USA). Independent variables were strain (C57BL/6J or CD1) and light during lactation (DD, LD or LL). The dependent variable was density of $P75^{NTR}$ expression.

A1.3 Results

A total of 42 mice were used for this study, 21 C57BL/6J and 21 CD1 mice raised in DD, LD or LL from P0 to P21. At P21 they were all placed in LD and their locomotor activity was monitored to ensure that they were entrained to the light-dark cycle. Their retinal function was then assessed using ERG (see Chapter 3 for results) before they were culled and their brains and eyes were removed. One eye was frozen directly on dry ice and homogenised for western blotting.
We found that C57BL/6J mice had significantly higher $P75^{NTR}$ expression than CD1 mice ($p<0.001$). However, we found no differences in the density of $P75^{NTR}$ signal due to early light environment in either strain and no interactions between strain and light during lactation (Figure A1.1).

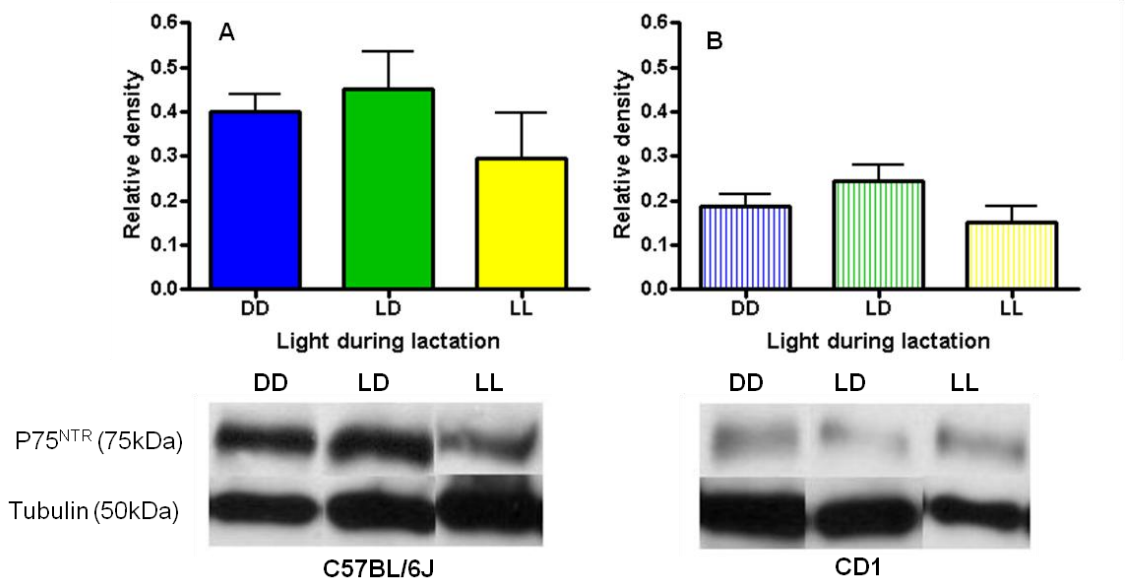

Figure A1.1: Effects of early light environment on $P75^{NTR}$ expression in the retina. Density of $P75^{NTR}$ staining in the retinas of C57BL/6J mice (**A** – DD n=5, LD n=5, LL n=3) and CD1 mice (**B** – DD n=6, LD n=5, LL n=5) relative to the tubulin loading control. DD – constant darkness; LD – 12:12 hour light-dark cycle, LL – constant light

A1.4 Discussion

The aim of this study was to determine whether $P75^{NTR}$ levels in the retina are affected by the light environment in which C57BL/6J and CD1 mice are raised. Previous work has shown that as a result of light-induced retinal degeneration, the levels of $P75^{NTR}$ in the retina were increased. Conversely the blockade or complete absence of $P75^{NTR}$ prevented light-induced retinal degeneration (Harada et al., 2000).

We found no differences in the levels of $P75^{NTR}$ in the retina due to early light environment in either the CD1 mice or the C57BL/6J mice. Our hypothesis was that the CD1 mice raised in LL would have increased levels of $P75^{NTR}$ to mirror the damage seen in their retinas (see Chapter 3). We found in this study that this was not the case. This may be due to differences in the experimental design between our experiment and the work done by Harada *et al.* (2000) in which they found increased $P75^{NTR}$ levels in the retinas of Wistar rats that had been in LL from P2 to P35. In rats that were in LL from P2 till P21 then placed in LD from P21 to P35 (a similar protocol to our study) they found comparable levels of $P75^{NTR}$ to controls (Harada et al., 2000). It is possible that as our protocol was similar to this (kept in LL until P21 then placed in LD) we were also seeing normal levels of $P75^{NTR}$. However, along with normal levels of $P75^{NTR}$, the study found that being kept in LL from P2 to P21 did not cause retinal damage, and that the retinal damage occurred after P21 (Harada et al., 1998, 2000). This does not agree with my work as I have found significant retinal damage in albino mice raised in LL from P0 to P21 then kept in LD provided by LED light as adults (see Chapter 3). The divergence between my work and this study may be due to species differences – it is possible that light-induced retinal degeneration could occur later in rats than mice. It may also be due to the type of light used. In their studies, Harada et al. housed their rats in light intensities ranging from 100-200 lux and triggered light-induced retinal degeneration using light intensities ranging from 1200-2000 using Tungsten lamps (Harada et al., 1996, 1998, 2000). In our studies, the light intensity was always 2000 photopic lux provided by LED light, regardless of whether the mice were in LD or LL. It is likely that the difference in light intensities used explains the higher levels of damage seen in our mice compared to the rats in their studies.

As we found no differences in the levels of $P75^{NTR}$ in the retinas of our CD1 mice due to early light environment we can hypothesise that the levels of $P75^{NTR}$ are only elevated during ongoing retinal damage and normalise when animals are placed back into LD.

In C57BL/6J mice we hypothesised that mice raised in LL would have reduced levels of $P75^{NTR}$ which could contribute to their ability to be protected from light-induced retinal damage. We found no differences in the level of $P75^{NTR}$ in the retinas of C57BL/6J mice due to early light environment. As mentioned when discussing the CD1 mice, the experimental design in this study was slightly different to the published work so our results may be due to a normalisation of $P75^{NTR}$ levels in the retina after being placed back in LD. On the other hand the retinas of C57BL/6J mice may be protected from light-induced retinal damage in some other way. Indeed, the pigment in the eyes of C57BL/6J has been shown to give protection against the damaging effects of light. Thanks to the pigment light can only reach the retina through the pupil. Furthermore, in bright light the pupil has been shown to constrict to a diameter of 0.1mm

meaning only a small amount of light can reach the retina therefore preventing light-induced retinal damage (Rapp and Williams, 1980; Lyubarsky et al., 2004).

We found that C57BL/6J mice had significantly higher levels of $P75^{NTR}$ in their retinas compared to CD1 mice, which may be attributed to strain differences.

In the SCN, $P75^{NTR}$ expression has been shown to be reduced in neonatal rats treated with MSG (Beaulé and Amir, 2001). As $P75^{NTR}$ has been shown to have no involvement in photic entrainment (Beaulé and Amir, 2001, 2002) it was hypothesised that this reduction in expression could be linked to a reduction in the amount of retinal ganglion cells innervating the SCN, as MSG targets mainly retinal ganglion cells (Beaulé and Amir, 2001). We were unable to test whether CD1 mice raised in LL would have reduced $P75^{NTR}$ expression in the SCN. However I have previously shown that the pupil response of CD1 mice raised in LL was significantly reduced compared to those raised in LD (see Chapter 3), indicating a reduction in the non-photic visual response in these mice. The non-photic visual response is partly mediated by intrinsically photosensitive retinal ganglion cells (ipRGCs) so it is possible that the reduction in response could be due to a reduction in the number of ipRGCs innervating the olivary pretectal nucleus (OPN), the area of the brain that mediates the pupil light reflex (Trejo and Cicerone, 1984; Clarke and Ikeda, 1985). In the same way we could hypothesise that the number of ipRGCs innervating the SCN of CD1 mice raised in LL may also be reduced. Further experiments testing the levels of $P75^{NTR}$ in the SCN would be required to test this hypothesis. We can conclude from this study that $P75^{NTR}$ levels in the retina are not affected by early light environment but may be affected by the strain of mouse used.

Appendix 2: Published article

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ORIGINAL ARTICLE

Differential hypothalamic tyrosine hydroxylase distribution and activation by light in adult mice reared under different light conditions during the suckling period

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Abstract In mammals, early light experience during a critical period within the first 3 weeks of postnatal development has long-lasting effects on circadian locomotor activity behaviour and neuropeptide expression in the suprachiasmatic nucleus (SCN) of the hypothalamus, site of the principal pacemaker. Dopamine is thought to be involved in the modulation of photic input within the SCN and in tadpoles, the expression of tyrosine hydroxylase (TH), a rate-limiting enzyme in the synthesis of dopamine, in the SCN is altered by previous light history. We thus hypothesised that dopaminergic neurons may be important for the development of the adapted responses to light that we have previously observed. To test this, we raised mice in either constant darkness, 12:12 h light–dark cycles or constant light during the first 3 weeks after birth, and later examined the expression of TH and FOS in the hypothalamus of these mice as adults, both in the dark and after exposure to a light pulse. We found that early light experience affects TH and FOS expression, both baseline levels and in response to a light pulse, in brain areas which are directly connected to the SCN, and are associated with the circadian control of neuroendocrine function. Therefore, our results suggest that the long-lasting alterations induced by early light environment on several hypothalamic nuclei may be relayed through the SCN, and that TH-expressing cells may play a role in conveying/establishing these alterations. These data suggest a role of early light experience in the regulation of future hormonal homeostasis and circadian behaviour.

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Keywords Dopamine · Circadian · Mouse · Development · Early life experience

Abbreviations

ADP	Anterodorsal preoptic nucleus
ARC	Arcuate nucleus
AVP	Arginine vasopressin
CT	Circadian time
DD	Constant dark
DMH	Dorsomedial hypothalamic nucleus
FOS+	cFOS-positive cells
LD	Light–dark
LL	Constant light
PE	Periventricular hypothalamic nucleus
PVN	Paraventricular hypothalamic nucleus
SCN	Suprachiasmatic nucleus
SPZ	Subparaventricular zone of the hypothalamus
TH	Tyrosine hydroxylase
TH+	TH-positive cells
TH/FOS+	TH- and cFOS-positive cells
VIP	Vasointestinal polypeptide
ZI	Zona incerta

Introduction

In mammals, daily, circadian rhythms in physiology and behaviour are controlled by the suprachiasmatic nucleus (SCN) of the hypothalamus, considered the principal pacemaker (Moore 1982). Previous studies have shown that light experienced during the first postnatal weeks has long-lasting effects on the animals' behaviour. Specifically, it affects the ability of rats to display a circadian rhythm of

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locomotor activity when exposed to a continuous light (LL) environment (Canal-Corretger et al. 2001b), the behavioural phase shifts of adult rats and mice after a light pulse, and the stability and amplitude of the locomotor activity rhythm under light–dark cycles (LD) and constant darkness (DD) (Canal-Corretger et al. 2001a, b). These effects were observed to be a consequence of the light experienced during a critical period of days between the second and third postnatal weeks, and not when the animals were exposed to different environments during the first postnatal week or after weaning (Canal-Corretger et al. 2001b). Moreover, the long-term differences in behaviour depended only on the light environment in which the pup had been reared, and not on the rhythmicity of its mother (Cambras et al. 1997); and persisted after the animals had been enucleated (Canal-Corretger et al. 2003). In addition, we have recently shown reduced expression of two critical circadian neuropeptides, arginine vasopressin (AVP) and vasointestinal polypeptide (VIP), in the SCN of adult mice reared under LL during the first three postnatal weeks, compared to those reared in LD or DD (Smith and Canal 2009). These results suggest that postnatal light experience may affect circadian clock function and clock output. However, the exact neural mechanisms behind the long-term effects of early light experience and its consequences are still unclear.

In the SCN, dopamine is thought to be involved in the modulation of neural circadian pacemakers and their responses to photic inputs (Novak and Nunez 1998). Tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, has been shown to be present in neurons in and around the SCN of rats (Battaglia et al. 1995; Jaeger et al. 1983), hamsters (Vincent 1988; Novak and Nunez 1998), sheep (Tillet et al. 1989, 1994), shrew (Karasawa et al. 1994), the common marmoset (Karasawa et al. 2007) and mice (Ruggiero et al. 1984). In addition, TH-positive (TH+) cells may play a role in the developing SCN. In Siberian hamsters, the SCN receives innervations by TH neurons from the day of birth up until postnatal day 20 (P20), with very few TH+ neurons present in the adult SCN (Duffield et al. 1999). In Syrian hamsters, TH+ immunoreactivity was detected in the SCN from P5 to P20, but not in the adult hamster (Strother et al. 1998). In rats, the SCN receives transient innervation by TH+ neurons and fibres during development, first appearing on P2, increasing to a peak on P10 and dropping by puberty (Ugrumov et al. 1994; Battaglia et al. 1995; Beltramo et al. 1994). The importance of the first two postnatal weeks for TH development coincides with the critical time for the maturation and differentiation of many SCN anatomical and functional features, such as synapses (Laemle et al. 1991), glial cells (Moore 1991), entraining pathways such as the retinohypothalamic and geniculohypothalamic tract (Moore 1991), efferent pathways of VIP neurons (Laemle 1988) and firing rate (Morin 1994). Consequently, TH+ fibres have been suggested to have the capacity for synaptic transmission in the developing SCN (Ugrumov et al. 1994), together with a role in the integration of photic and non-photic inputs via regulation of activity of VIP-expressing neurons (Battaglia et al. 1995). Furthermore, a recent paper in frog tadpoles showed that when these were exposed to bright light, the number of TH+ neurons (used as a marker for dopaminergic neurons in this study) in their SCN increased, allowing them to adapt more rapidly to subsequent exposure to light (Dulcis and Spitzer 2008). Consequently, in mice, dopamine may also play a role in the programming effects of early light experience on the circadian system.

We thus hypothesised that neurons expressing tyrosine hydroxylase may be important for the development of different responses to light in mice reared under different light environments during the postnatal period. The first aim of the present study was to investigate the expression of TH+ neurons in the hypothalamus of adult mice reared under different light conditions during the first three postnatal weeks. The second aim was to test the role of TH+ neurons in the adapted response of the adult to photic stimuli by examining the expression of c-FOS in TH+ cells after the mice had received a light pulse in the dark.

Materials and methods

Animals

All experimental procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Throughout the experiment, mice were kept under a controlled ambient temperature of 21°C with water and food (B&K Universal, Hull, UK) available ad libitum. Cages were changed every fortnight at random times during the day. Infrared goggles were used in the routine maintenance of the animals in the dark.

A total of six pregnant female C57BL/6J mice raised in our colony were kept under a 12:12 h light–dark cycle (lights on at 6:00 h, lights off at 18:00 h, LD) until the day of delivery. From the day of birth (postnatal day 0, P0), the litters were kept in either LD cycles (LD group), constant darkness (DD group) or constant light (LL group). At cage floor level, the mean light intensity was 302.97 $\mu W/cm^2$. On weaning day (P21), male pups from all groups ($n = 8$ LD group, $n = 8$ DD group and $n = 10$ LL group) were moved to individual cages and kept under LD cycles until the end of the experiment. At P35, lights were switched off at 18 h and the animals remained in DD until killed at P36.

Immunohistochemistry

On the day when they were killed (P36), a 30-min light pulse (light intensity 302.97 μW/cm^2) was administered at Circadian Time 16 (CT16; 4 h after the onset of activity; light-pulsed mice) to half the mice of all groups, the other half remained in the dark (non-pulsed mice). All mice were then culled 1 h later by cervical dislocation in complete darkness with the aid of infrared goggles and the eyes were removed to prevent any light reaching the SCN. Brains were carefully removed and placed in fixative (4% paraformaldehyde; Sigma, Poole, UK) for 2 days at 4°C. The brains were then transferred to 30% sucrose (Sigma) for cryoprotection before being rapidly frozen and stored at −80°C until cut. Coronal sections of 30 μm were cut using a sledge microtome (Bright Instruments Ltd., Huntingdon, UK). Sections from the following regions were collected (Fig. 1): Anterodorsal preoptic nucleus (ADP; 0.38 mm anterior to Bregma to 0.34 mm posterior to Bregma), SCN (0.22–0.82 mm posterior to Bregma), paraventricular hypothalamic nucleus (PVN 0.58–1.22 mm posterior to Bregma), periventricular hypothalamic nucleus (PE 0.58–1.22 mm posterior to Bregma), subparaventricular zone of the hypothalamus (SPZ 0.70–0.94 mm posterior to Bregma), zona incerta (ZI 1.22–1.94 mm posterior to Bregma), dorsomedial hypothalamic nucleus (DMH 1.46–1.94 mm posterior to Bregma) and arcuate nucleus (ARC 1.22–2.06 mm posterior to Bregma) according to the mouse brain atlas (Paxinos and Franklin 2004).

Immunoreactivity to TH (1:10,000; rabbit polyclonal, Sigma) and c-FOS (1:8,000, chicken polyclonal, Sigma) was detected using standard techniques as described by Smith and Canal (2009). Briefly, separate series of tissue sections were processed for TH, c-FOS or TH and c-FOS. For the double-labelling, sections stained first with c-FOS were then stained with TH. The sections were incubated in primary antibody at 4°C for 48 h, followed by a biotinylated goat anti-rabbit or goat anti-chicken secondary antibody (both 90 min; 1:400; Vector Laboratories, Inc., Burlingame, CA, USA), then ABC solution (90 min; 1:200; Vector Laboratories). TH staining was visualised using diaminobenzidine (DAB substrate kit, Vector Laboratories) and c-FOS staining was visualised using diaminobenzidine intensified with a nickel solution as a chromogen.

Control sections were processed as described above but without the primary antibody, which resulted in no staining.

Immunoreactivity analysis

Sections were examined using a Leica DME microscope (Leica Microsystems, Wetzlar, Germany), and the number of TH-positive (TH+), c-FOS-positive (FOS+) and TH/c-FOS-positive (TH/FOS+) cells was manually counted by two researchers blind to the experimental groups. In the ADP, PE, SPZ, PVN, ZI, DMH and SCN, the positive cells in both the left and right regions were counted, and the average per section obtained. For the ARC, only one count per section, including all the cells in the nucleus, was obtained. Sections used in this analysis (minimum of 3 sections per brain region) were taken, equally, from each single animal of all experimental groups. The total numbers of TH+, FOS+ and TH/FOS+ cells were divided by the total area analysed for each region to reflect the density of immunoreactive cells/mm^2. An average density of immunoreactive cells in any given region was obtained for each individual mouse before calculating a group average.

Statistical analysis

Statistical analysis was carried out by means of an ANOVA of general linear models, using SYSTAT (version 10, SPSS, Inc., Chicago, IL, USA) software. The independent variables were light conditions during lactation (LD, DD or LL), light pulse (pulsed or non-pulsed), and brain region (ADP, SCN, PVN, PE, SPZ, ZI, DMH and ARC). The dependent variables were number of TH+, FOS+ and TH/FOS+ cells per mm^2. When a statistically significant difference was encountered ($p < 0.05$), a Bonferroni post hoc test was applied. Results are expressed as average ± standard deviation unless otherwise stated.

Results

TH+ cells were identified as having brown staining that filled the cell soma and projections. TH+ cell bodies were noted in the ADP, PVN, PE, SPZ, ZI, DMH and ARC, but not in the SCN. FOS+ cells were identified as having dark, blue-black nuclear staining, and were noted in all brain areas studied. Finally, TH/FOS+ cells were identified as having a dark, blue-black nucleus surrounded by brown cytoplasmic staining.

Anterodorsal preoptic nucleus

We found a high density of TH+ cells in the ADP, which depended on the postnatal light environment ($p < 0.01$). Specifically, DD-reared mice had a significantly higher number of TH+ cells than LD-reared mice (Table 1; Fig. 2). We found no significant effect of a light pulse on the number of TH+ cells ($p > 0.05$, Table 1). The postnatal light environment also had a significant effect on the number of FOS+ cells in the ADP ($p < 0.05$). The post hoc analysis revealed that LL-reared mice displayed a higher

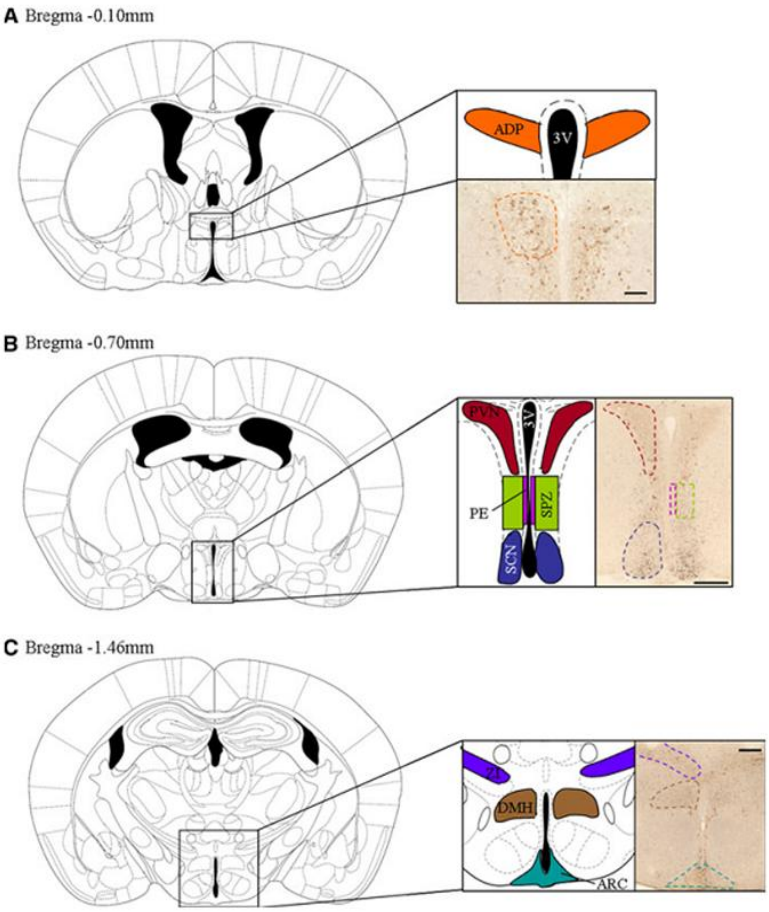

Fig. 1 Schematic drawing of serial coronal sections from the anterior to the posterior of the mouse hypothalamus, with insets of photomicrographs of double TH and FOS staining. **a** Anterodorsal preoptic nucleus (ADP) and third ventricle (3V), **b** suprachiasmatic nucleus (SCN), periventricular hypothalamic nucleus (PE), subparaventricular zone of the hypothalamus (SPZ) and paraventricular hypothalamic nucleus (PVN), and **c** zona incerta (ZI), dorsomedial hypothalamic nucleus (DMH) and arcuate nucleus (ARC). Schematic sections adapted from Paxinos and Franklin (2004). *Scale bar* on photomicrographs 100 μm

number of FOS+ cells than DD- ($p < 0.01$) and LD-reared mice ($p < 0.05$, Table 2; Fig. 2). The light pulse also exerted a significant effect on the density of FOS+ cells, with higher values in the pulsed animals compared to the non-pulsed animals ($p < 0.01$), independently of the postnatal light conditions the animal had been raised in (Table 2; Fig. 2). No significant effect of the postnatal light environment was found on the number of TH/FOS+ cells in the ADP ($p > 0.05$, Table 3; Fig. 2). However, we found that the density of TH/FOS+ cells increased in all groups after a light pulse ($p < 0.01$, Table 3; Fig. 2).

Periventricular hypothalamic nucleus

We found moderately dense TH+ cells in the PE, which were not affected by the postnatal light environment, or by the light pulse treatment ($p > 0.05$, Table 1). Regarding the number of FOS+ cells, we found that it did not depend on postnatal light environment ($p > 0.05$, Table 2), but was significantly increased after a light pulse ($p < 0.01$, Table 2). We did not find a significant effect of either postnatal light environment or light pulse on the number of TH/FOS+ cells ($p > 0.05$, Table 3).

Subparaventricular zone of the hypothalamus

We found moderately dense TH+ cells in the SPZ. There was no significant effect of either postnatal light environment or light pulse on the number of TH+ cells in the SPZ ($p > 0.05$, Table 1). We did, however, find a significant effect of postnatal light environment on the number of FOS+ cells in the SPZ ($p < 0.01$, Table 2). Specifically, LL-reared mice had significantly higher levels of FOS+ cells than LD-reared mice ($p < 0.01$, Table 2; Fig. 3). We also found that the light pulse significantly increased the number of FOS+ cells in all groups of mice ($p < 0.01$, Table 2; Fig. 3). Interestingly, postnatal light environment significantly affected the number of TH/FOS+ cells in the

Table 1 Number of TH+ cells per mm^2 in mice reared in constant darkness (DD, $n = 8$), 12:12 h light–dark cycles (LD, $n = 8$) or constant light (LL, $n = 10$)

	DD		LD		LL	
	Non-pulsed	Pulsed	Non-pulsed	Pulsed	Non-pulsed	Pulsed
ADP	49.0 ± 6.3[a**]	40.2 ± 7.5[a**]	32.1 ± 5.0	30.9 ± 6.4	36.6 ± 8.9	39.2 ± 4.9
PE	17.6 ± 10.1	28.1 ± 14.7	12.9 ± 5.4	24.0 ± 10.1	17.1 ± 9.3	16.2 ± 7.0
SPZ	24.9 ± 5.1	19.9 ± 2.7	24.4 ± 6.1	20.0 ± 5.9	24.1 ± 6.8	25.5 ± 3.8
PVN	26.5 ± 3.9[a*,b*]	29.9 ± 5.7[a*,b*]	22.6 ± 5.1	19.7 ± 6.6	21.7 ± 7.1	20.8 ± 5.7
ZI	74.7 ± 4.0[a**]	81.1 ± 17.2[a**]	34.8 ± 13.8	32.5 ± 14.5	72.2 ± 14.5[a**]	79.2 ± 12.2[a**]
DMH	23.7 ± 8.0[b#]	23.5 ± 2.3[b#]	18.0 ± 4.2	21.6 ± 4.5	17.4 ± 1.7	16.9 ± 6.1
ARC	84.3 ± 29.1	111.8 ± 4.9	80.6 ± 17.6	67.7 ± 24.8	85.6 ± 21.2	88.3 ± 5.8

Data presented as mean ± standard deviation

* $p < 0.05$; ** $p < 0.01$; # $p = 0.057$

[a] Versus LD

[b] Versus LL

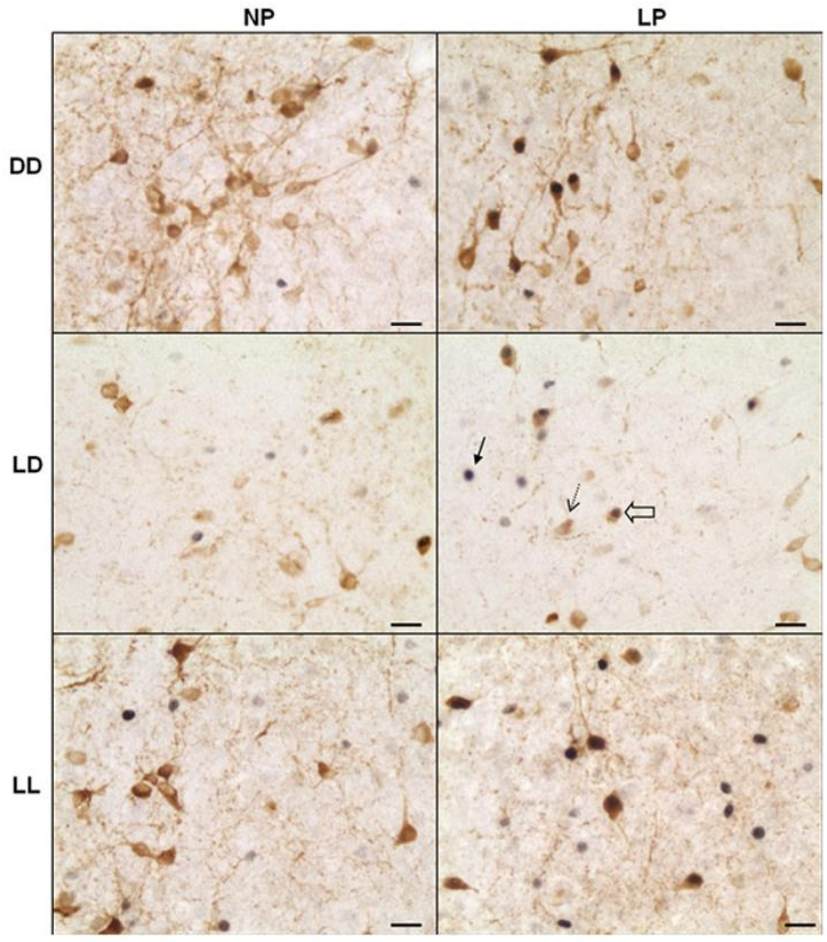

Fig. 2 Bright field photomicrographs of the hypothalamus at the level of the anterodorsal preoptic nucleus (ADP). TH+ cells can be identified as cells with cytoplasmic staining (*dashed arrow*). FOS+ cells can be identified as cells with nuclear staining (*black arrow*). TH/FOS+ cells can be identified as having both nuclear and cytoplasmic staining (*open arrow*). *Scale bar* 20 μm. Constant darkness (DD); light–dark (LD); constant light (LL); light-pulsed (LP); non-pulsed (NP)

Table 2 Number of FOS+ cells per mm^2 in mice reared in constant darkness (DD, $n = 8$), 12:12 h light–dark cycles (LD, $n = 8$) or constant light (LL, $n = 10$)

	DD		LD		LL	
	Non-pulsed	Pulsed	Non-pulsed	Pulsed	Non-pulsed	Pulsed
ADP	20.9 ± 7.6^{b**}	40.7 ± 10.7^{b**,c**}	33.2 ± 12.5	40.1 ± 5.3^{c**}	39.8 ± 3.0^{a*}	55.9 ± 9.6^{a*,c**}
PE	48.9 ± 20.1	127.9 ± 28.7^{c**}	28.8 ± 4.0	122.9 ± 25.9^{c**}	50.7 ± 24.9	114.2 ± 27.7^{c**}
SPZ	114.3 ± 41.8	173.9 ± 74.4^{c**}	54.0 ± 10.6	103.6 ± 30.4^{c**}	135.1 ± 49.7^{a**}	233.1 ± 46.0^{a**,c**}
PVN	46.9 ± 11.2	52.2 ± 6.9	34.7 ± 6.4	42.4 ± 12.3	59.9 ± 9.4^{a**}	63.3 ± 13.8^{a**}
ZI	34.7 ± 12.6	34.0 ± 12.6	34.3 ± 2.9	34.9 ± 11.9	38.8 ± 17.1	38.0 ± 9.3
DMH	35.1 ± 11.0	77.4 ± 2.2^{c**}	33.8 ± 9.0	71.9 ± 4.1^{c**}	41.3 ± 5.0	80.0 ± 4.7^{c**}
ARC	32.4 ± 1.5	35.1 ± 21.9	31.4 ± 7.3	47.5 ± 17.3	41.6 ± 13.6	29.9 ± 9.2
SCN	116.9 ± 40.8	493.0 ± 17.1^{c**}	79.6 ± 22.5	502.7 ± 72.3^{c**}	117.2 ± 16.9	500.0 ± 54.0^{c**}

Data presented as mean ± standard deviation

* $p < 0.05$; ** $p < 0.01$

[a] Versus LD

[b] Versus LL

[c] Versus non-pulsed

Table 3 Number of TH/FOS+ cells per mm^2 in mice reared in constant darkness (DD, $n = 8$), 12:12 h light–dark cycles (LD, $n = 8$) or constant light (LL, $n = 10$)

	DD		LD		LL	
	Non-pulsed	Pulsed	Non-pulsed	Pulsed	Non-pulsed	Pulsed
ADP	4.0 ± 2.7	17.8 ± 9.5^{c**}	5.5 ± 4.3	11.8 ± 1.4^{c**}	6.1 ± 2.1	13.6 ± 3.8^{c**}
PE	1.4 ± 1.7	3.9 ± 2.7	1.2 ± 1.6	0.2 ± 0.4	2.7 ± 2.6	2.7 ± 1.8
SPZ	1.1 ± 0.8	1.6 ± 0.3	0.7 ± 0.3	1.0 ± 0.8	2.1 ± 1.6^{a*}	2.5 ± 1.9^{a*}
PVN	1.2 ± 0.9	2.4 ± 1.8	0.3 ± 0.0	0.5 ± 0.5	3.0 ± 2.0^{a**}	2.7 ± 1.8^{a**}
ZI	1.6 ± 1.2	1.8 ± 1.6	1.7 ± 0.9	1.0 ± 0.5	1.4 ± 1.1	2.2 ± 1.8
DMH	1.9 ± 1.3	9.3 ± 2.2^{a**,b**}	1.5 ± 0.7	1.5 ± 0.5	2.6 ± 1.4	2.8 ± 2.2
ARC	1.4 ± 1.0	0.6 ± 0.3	0.8 ± 0.7	0.7 ± 0.8	0.4 ± 0.6	1.2 ± 1.4

Data presented as mean ± standard deviation

* $p < 0.05$; ** $p < 0.01$

[a] Versus LD

[b] Versus LL

[c] Versus non-pulsed

SPZ ($p < 0.05$), with LL-reared mice presenting a higher number of TH/FOS+ cells than LD-reared mice ($p < 0.05$, Table 3; Fig. 3). No differences in the number of TH/FOS+ cells were found between the pulsed and non-pulsed animals ($p > 0.05$, Table 3).

Paraventricular hypothalamic nucleus

We found sparse TH+ cells in the PVN, with staining mainly restricted to its borders. We found a significant effect of postnatal light environment on the number of TH+ cells in the PVN ($p < 0.05$). Post hoc analysis revealed that DD-reared mice had significantly higher number of TH+ cells than LD- and LL-reared mice ($p < 0.05$, Table 1; Fig. 4). No significant effect of the light pulse on the number of TH+ cells in the PVN was found ($p > 0.05$, Table 1). Interestingly, we found a significant effect of postnatal light environment on both the number of FOS+ and TH/FOS+ cells in the PVN ($p < 0.01$), with higher FOS+ and TH/FOS+ cell numbers in LL- than LD-reared mice ($p < 0.01$, Tables 2, 3; Fig. 4). We found no significant effect of the light pulse on the number of either FOS+ or TH/FOS+ cells in the PVN ($p > 0.05$, Tables 2, 3).

Zona incerta

TH+ cells throughout the ZI were extremely dense. We found a significant effect of postnatal light environment on

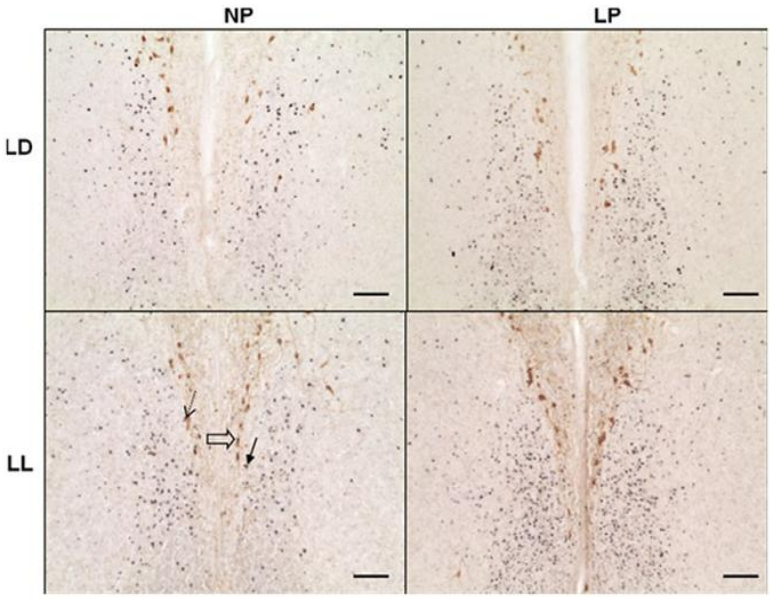

Fig. 3 Bright field photomicrographs of the hypothalamus at the level of the subparaventricular zone of the hypothalamus (SPZ). TH+ cells can be identified as cells with cytoplasmic staining (*dashed arrow*). FOS+ cells can be identified as cells with nuclear staining (*black arrow*). TH/FOS+ cells can be identified as having both nuclear and cytoplasmic staining (*open arrow*). Example sections were taken from LD- and LL-reared groups. *Scale bar* 100 µm. Light–dark (LD); constant light (LL); light-pulsed (LP); non-pulsed (NP)

the number of TH+ cells in the ZI ($p < 0.001$). Specifically, DD- and LL-reared mice had a significantly higher number of TH+ cells than LD-reared mice ($p < 0.01$, Table 1). We found no effect of the light pulse on the number of TH+ cells in the ZI ($p > 0.05$, Table 1). Furthermore, we found no significant effect of either postnatal light environment or light pulse on the number of FOS+ and TH/FOS+ cells in the ZI ($p > 0.05$, Tables 2; 3).

Dorsomedial hypothalamic nucleus

We found dense TH+ cells in the DMH, which were not affected by the light pulse ($p > 0.05$, Table 1). There was, however, a slight tendency for DD-reared mice to show a higher number of TH+ cells than LL-reared mice ($p = 0.057$). We did not find a significant effect of postnatal light environment on the number of FOS+ cells in the DMH ($p > 0.05$, Table 2). The number of FOS+ cells was affected by the light pulse, with a significant increase in the number of FOS+ cells after the light pulse ($p < 0.01$, Table 2; Fig. 5). Interestingly, we found a highly significant interaction between postnatal light environment and light pulse in the number of TH/FOS+ cells ($p < 0.001$): in the non-pulsed animals, there were no significant differences in the number of TH/FOS+ cells due to postnatal light environment, however, the light pulse induced a significant increase in the number of TH/FOS+ cells in the DD-reared mice, which as a consequence presented higher numbers of TH/FOS+ cells than the LD- and LL-reared mice ($p < 0.01$, Table 3; Fig. 5).

Arcuate nucleus

We found no changes in the number of TH+, FOS+ and TH/FOS+ cells due to postnatal light environment or in response to the light pulse in the ARC ($p > 0.05$, Tables 1, 2, 3).

Suprachiasmatic nucleus

There were no TH+ cell bodies detected in the SCN, and therefore we only counted the number of FOS+ cells in this area. As expected, the number of FOS+ cells significantly increased after a light pulse ($p < 0.01$, Table 2; Fig. 6), but interestingly, we found no differences between LD-, DD- and LL-reared mice, which showed a similar number of FOS+ cells ($p > 0.05$, Table 2).

Discussion

Our findings confirm previous observations about the presence of TH+ cells in many areas of the hypothalamus, including ADP, PE, SPZ, PVN, ZI and DMH (Ruggiero et al. 1984). A summary of the key results is shown in Table 4.

Anterodorsal preoptic nucleus

In the present study, we found that DD-reared mice showed higher numbers of TH+ cells than control LD-reared mice.

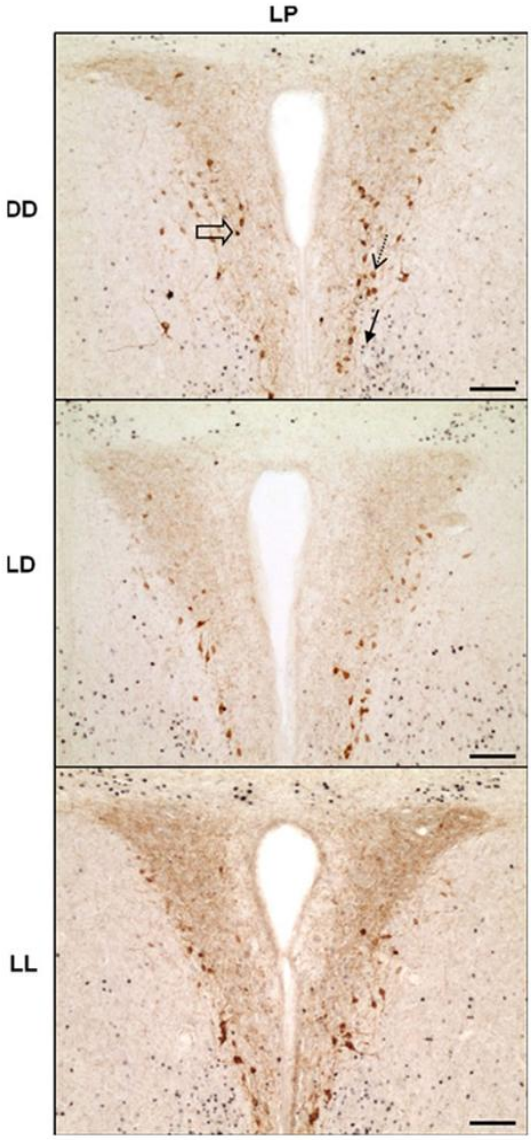

Fig. 4 Bright field photomicrographs of the hypothalamus at the level of the paraventricular hypothalamic nucleus (PVN). TH+ cells can be identified as cells with cytoplasmic staining (*dashed arrow*). FOS+ cells can be identified as cells with nuclear staining (*black arrow*). TH/FOS+ cells can be identified as having both nuclear and cytoplasmic staining (*open arrow*). Example sections were taken from DD-, LD- and LL-reared groups that received a light pulse. *Scale bar* 100 µm. Constant darkness (DD); light–dark (LD); constant light (LL); light-pulsed (LP)

Moreover, all light-pulsed mice showed a similar number of activated TH+ (TH/FOS+) cells, independently of the group, which was higher than non-pulsed animals. Since the number of TH+ cells was initially higher in DD-reared compared to LD-reared mice, this suggests that the light pulse produced less TH+ cell activation in DD-reared animals. Although the role of ADP in mice is not fully understood, ADP has been associated with hypothalamic responses to stress, including reaction to avoidance stimuli, swim stress and to hot and cold environments (Veening et al. 2004). Both cold and warm ambient temperatures increase the number of FOS+ cells in the preoptic area including ADP (Veening et al. 2004; Kiyohara et al. 1995; Baffi and Palkovits 2000). Interestingly, the increase in FOS expression after acute cold stimulation decreases in animals that were raised in the cold during postnatal development (P0–P14) (Miyata et al. 1998), suggesting that the responses of ADP to environmental stimuli may be influenced by early life experience. This hypothesis agrees with our finding that the activation of TH+ cells in DD-reared mice after a light-pulse was weaker compared to LD-reared mice. It is thus possible that ADP responds to different types of environmental stress, including temperature and light alterations, and that this response is programmed by early life experience. Our results also suggest that TH-expressing cells may play an important role in these adapted responses to the environment.

Another finding in the present experiment is that the overall FOS expression increased in the ADP after a light pulse. Interestingly, FOS expression was particularly elevated in LL-reared mice compared to DD- and LD-reared mice, independently of whether they had received a light pulse or not. First, this result indicates a rapid response of ADP to an acute light stimulus (within an hour). Although there are no known direct projections between the retina and ADP, photic information may reach the ADP indirectly via the SCN, since afferent projections from the SCN into the ADP have been described (Watts 1991). Secondly, this result indicates that LL-reared mice have an overall higher number of activated cells in the ADP. Because of the previously mentioned correlation between FOS activation in the ADP and a response to a variety of stressful stimuli (Veening et al. 2004; Kiyohara et al. 1995; Baffi and Palkovits 2000; Miyata et al. 1998), further experiments are required in order to establish whether LL-reared mice have higher overall levels of stress.

Periventricular hypothalamic nucleus

We found a significant increase in FOS+ cells in the PE after a light pulse. Similarly to ADP, there is no evidence of direct projections from the retina to the PE (Hannibal and Fahrenkrug 2004; Hattar et al. 2002). However, VIP outputs from the retinorecipient core SCN have been shown to project to the PE (Horvath 1997; Mahoney et al.

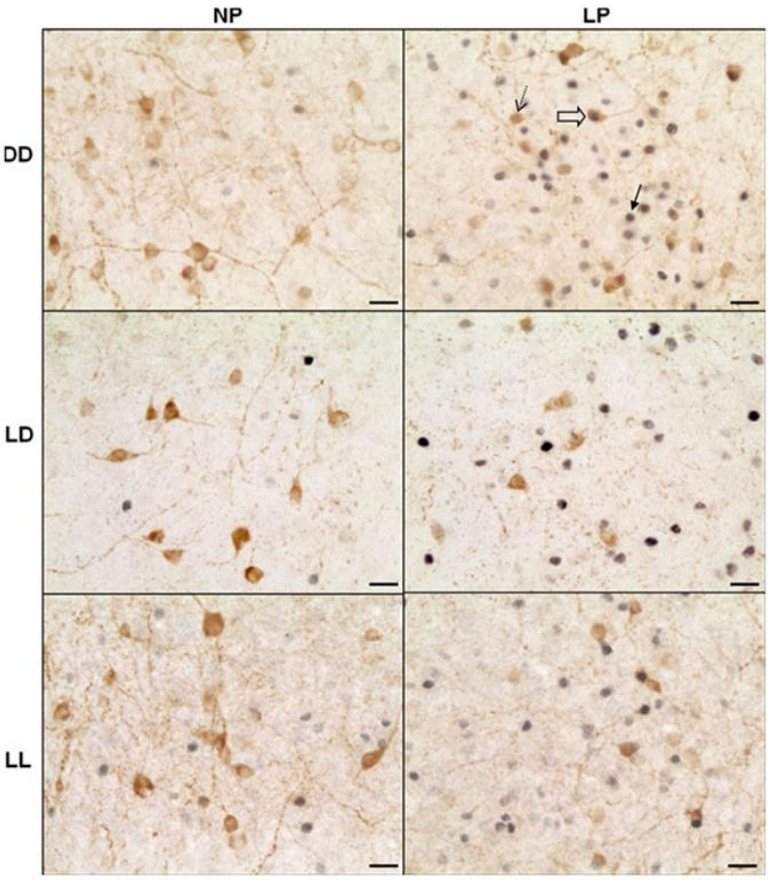

Fig. 5 Bright field photomicrographs of the hypothalamus at the level of the dorsomedial hypothalamic nucleus (DMH). TH+ cells can be identified as cells with cytoplasmic staining (*dashed arrow*). FOS+ cells can be identified as cells with nuclear staining (*black arrow*). TH/FOS+ cells can be identified as having both nuclear and cytoplasmic staining (*open arrow*). *Scale bar* 20 µm. Constant darkness (DD); light–dark (LD); constant light (LL); light-pulsed (LP); non-pulsed (NP)

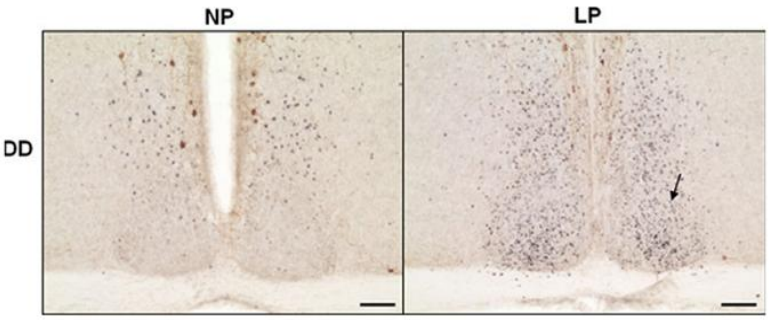

Fig. 6 Bright field photomicrographs of the hypothalamus at the level of the suprachiasmatic nucleus (SCN). Only FOS+ cells were identified in the SCN (*black arrow*). Example sections were taken from the DD-reared group. *Scale bar* 100 µm. Constant darkness (DD); light-pulsed (LP); non-pulsed (NP)

2007). Therefore, photic information from the retina could rapidly reach the PE via the SCN.

Interestingly, we found no significant differences in the number of TH+ cells or activated TH+ cells in the PE in response to the light pulse. These results suggest that TH+ cells in the PE are not involved in the responsiveness of this brain area to light stimuli, which could also explain the finding that the number of TH+ and TH/FOS+ cells in the PE was not influenced by postnatal light experience.

Subparaventricular zone of the hypothalamus

Exposure of mice to a light pulse in the dark induced a significant increase in FOS+ cells in the SPZ. This is not

Table 4 Summary of significant results, indicating, for a given antibody (TH, FOS or TH/FOS), which brain areas show significantly different cell density between the experimental groups (DD, mice raised in constant darkness; LD, mice raised under 12:12 h light–dark cycles, and LL, mice raised in constant light)

	TH		FOS		TH/FOS	
	Lactation	Pulse	Lactation	Pulse	Lactation	Pulse
ADP	DD > LD	n.s.	LL > DD,LD	LP > NP	n.s.	LP > NP
PE	n.s.	n.s.	n.s.	LP > NP	n.s.	n.s.
SPZ	n.s.	n.s.	LL > LD	LP > NP	LL > LD	n.s.
PVN	DD > LD, LL	n.s.	LL > LD	n.s.	LL > LD	n.s.
ZI	LD < DD, LL	n.s.	n.s.	n.s.	n.s.	n.s.
DMH	DD > LL	n.s.	n.s.	LP > NP	In LP, DD > LD, LL In NP, n.s.	
ARC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
SCN	N/A	N/A	n.s.	LP > NP	N/A	N/A

ADP anterodorsal preoptic nucleus, *PE* periventricular hypothalamic nucleus, *SPZ* subparaventricular zone of the hypothalamus, *PVN* paraventricular hypothalamic nucleus, *ZI* zona incerta, *DMH* dorsomedial hypothalamic nucleus, *ARC* arcuate nucleus, *SCN* suprachiasmatic nucleus, *LP* light-pulsed, *NP* non-pulsed, *N/A* not applicable, *n.s.* not significant

surprising, as photic information can easily reach the SPZ either through direct projections from the retina (Hannibal and Fahrenkrug 2004), and/or from the numerous projections from the SCN, through AVP, VIP and angiotensin fibres (Watts et al. 1987; Abrahamson and Moore 2001; Mahoney et al. 2007).

We also found that postnatal light experience affected both the number of FOS+ and of activated TH+ cells in the SPZ, with LL-reared mice showing an increased number of cells than LD-reared mice. Therefore, cells in the SPZ of LL-reared mice seem to be more activated than those in control mice regardless of whether they had received a light pulse or not.

Paraventricular hypothalamic nucleus

Despite the direct projections from the eye into the PVN (Abrahamson and Moore 2001), no effect of a light pulse on the expression of TH+, FOS+ or TH/FOS+ cells was observed. This result suggests that the PVN does not display an acute response to light. Nevertheless, the PVN seems to be still capable of being influenced by external light information, as indicated by the significant effect of postnatal light experience on the number of TH+, FOS+ and TH/FOS+ cells in the PVN found in the present experiment. These results suggest that PVN development may be programmed by postnatal light experience. This hypothesis is supported by our previous finding that AVP expression in the PVN is increased in DD- compared to LL- and LD-reared mice (Smith and Canal 2009).

FOS expression in the PVN has been found to increase after exposure to cold ambient temperatures (Baffi and Palkovits 2000; Kiyohara et al. 1995), and after swim and restraint stress (Cullinan et al. 1995), which suggests that FOS expression in the PVN may be used as a marker of stress response. Interestingly, and similarly to ADP, stress-induced FOS expression in the PVN appears to be influenced by postnatal experience (Miyata et al. 1998), indicating that the stress response in the PVN is susceptible to programming by early life experience. In the present experiment, we found that both the number of FOS+ cells and the number of TH/FOS+ cells were increased in LL- compared to LD-reared mice. There is thus a possibility that early light experience may affect the baseline response of the PVN to stress, and that this effect is mediated, at least in part, by TH+ cells. This hypothesis is supported by the finding that the glucocorticoid receptor is expressed in TH+ neurons in the PVN (Ceccatelli et al. 1989), and hence TH+ neurons may be directly involved in the regulation of the stress response in the PVN.

Zona incerta

We found that the number of TH+ cells in the ZI was significantly higher in DD- and LL-reared mice compared to control LD-reared mice. This is an interesting result, since it seems to point towards a strong influence of continuous versus cycled light environment on the development of the ZI. Cholera toxin injections into the eye have demonstrated a few fibres projecting into the ZI (Abrahamson and Moore 2001). This indicates that the ZI may receive photic information directly from the retina, which could explain the long-term effects of postnatal light experience we have here observed. The consequences of this long-term change are difficult to predict, since not much is known on this brain region, but they may affect a wide range of behaviours, since it has been suggested that the ZI may act as a sensory link

to arousal, attention, locomotion and visceral function (Mitrofanis 2005).

Dorsomedial hypothalamic nucleus

In the DMH, we found a significant increase in the number of FOS+ cells after a light pulse, indicating that the DMH responds rapidly to an acute light stimulus. No direct projections from the eye have been described in the DMH, but a moderate number of fibres from the SCN (Watts et al. 1987), PVN (Watts et al. 1987) and SPZ (Abrahamson and Moore 2001) have been found, which could indirectly provide the DMH with photic information.

TH+ cells in the DMH may be influenced by postnatal light environment, as indicated by the finding that DD-reared mice had a tendency towards higher levels of TH+ cells than LL-reared mice. Interestingly, we found that the number of TH/FOS+ cells in the dark was similar in all groups, but after a light pulse, the number of activated TH+ cells increased to a significantly higher level in the DD-reared mice, compared to LD- and LL-reared mice. Therefore, it seems that TH+ cells in the DMH of DD-reared mice are particularly sensitive to an acute light stimulus. These findings suggest that TH + cells in the DMH may be susceptible to programming by early life experience. Moreover, FOS expression in the DMH has been shown to increase after exposure to stress-induced hyperthermia (Veening et al. 2004), cold stress (Baffi and Palkovits 2000) and swim and restraint stress (Cullinan et al. 1995). These data seem to point towards an effect of postnatal light experience on the stress response.

Arcuate nucleus

No significant differences due to postnatal light experience or due to a light pulse were found in the number of TH+, FOS+ or TH/FOS+ cells in the ARC. It is thus possible that the ARC is not programmed by early light experience. Moreover, because of the critical role played by ARC on the regulation of food intake (Blevins and Baskin 2010), our results suggest that the control of feeding behaviour may not be highly dependent on either acute or postnatal light environments.

Suprachiasmatic nucleus

As expected, we found a significant increase in the number of FOS+ cells after a light pulse, in agreement with previous literature (Colwell and Foster 1992). This occurred regardless of the light environment in which the mice were raised. The expression of FOS is one of the first events to occur in the SCN after a light pulse, and therefore it is used as an indicator of photic input reaching the SCN. These results suggest that the light information coming from the retina into the SCN is similar between the groups.

No TH+ cell bodies were found in the SCN during the course of this experiment. The distribution of TH+ cells in the adult SCN appears to be species-dependent: very few or no cell bodies were previously found in the mouse SCN (Ruggiero et al. 1984), while few diffusely distributed TH+ cells were found in the marmoset SCN (Karasawa et al. 2007), very few cell bodies and sparse fibers were described in the Siberian hamster SCN (Duffield et al. 1999) and only fibres have been reported in the SCN of adult rats (van den Pol and Tsujimoto 1985; Ugrumov et al. 1994).

During postnatal development, between P1 and P20 in Siberian hamsters (Duffield et al. 1999), and around P10 in rats (Battaglia et al. 1995; Ugrumov et al. 1994), there is a transient expression of TH in the SCN. Dopaminergic cues have been found to mimic the effect of light in the perinatal SCN (Viswanathan et al. 1994; Weaver et al. 1995), and it is thought that TH fibres may have the capacity for synaptic transmission during development (Ugrumov et al. 1994). Glutamate is the principal neurotransmitter within the retinohypothalamic tract (RHT), conveying photic cues from the retina to the SCN (Ebling 1996).The RHT terminates in the ventrolateral region of the SCN, an area which is rich in VIP+ neurons (Antle and Silver 2005). VIP plays a critical role in photic synchronisation of the SCN to the environment (Abrahamson and Moore 2001; Vosko et al. 2007). Moreover, TH varicosities have been shown to make contact with VIP + neurons in the SCN (Mahoney et al. 2007), and it is thus thought that TH+ cells are involved in the integration of photic inputs to the SCN. Interestingly, we have found VIP expression in the SCN to depend on postnatal light experience (Smith and Canal 2009). It is thus tempting to speculate that glutamate input to the SCN during development could initiate a cascade of events leading to altered neurotransmitter expression in VIP+ and TH+ cells, affecting synaptic function, and consequently, long-term SCN cell communication and function.

Summary and conclusions

The aim of this study was to examine the expression of TH+ neurons in the hypothalamus of adult mice reared under different light conditions during the first three postnatal weeks. Since all mice were synchronised to normal 24 h LD cycles for 2 weeks before they were killed, we believe that the differences observed between the groups reflect a long-lasting effect of early light experience.

Here we found that, compared to control LD-reared mice, DD-reared mice had increased numbers of TH+ cells in the ADP, PVN and ZI. We also found differences

between LL- and LD-reared mice in the ADP, SPZ and PVN, but in contrast to DD-reared mice, these changes were not in the number of TH+ cells, but in the total number of activated cells (FOS+) and TH+ cell activation (TH/FOS+). These results are interesting, since they suggest different mechanisms for the long-term effects of DD and LL environments on the hypothalamus. While LL-reared adult mice have similar numbers of TH+ cells as control LD-reared mice, their cells seem to be in an enhanced activated state; whereas DD-reared mice have a constitutively higher number of TH+ cells than LD-reared mice. In germane to the current study, Dulcis and Spitzer found that, after they were exposed to light, there was an increased number of TH+ cells in the SCN of tadpoles raised in a dark environment. This was due to the expression of an additional type of neurotransmitter (in this case, dopamine) in pre-existing neurons (Dulcis and Spitzer 2008). Further experiments in the mouse examining specific dopamine expression and release in these brain regions is required to clarify the role of this neurotransmitter in the long-term effects we have observed.

In our study, the only brain region that showed a differential response to a light pulse between the experimental and control animals was the DMH. This region controls a wide range of circadian responses, including corticosterone secretion, feeding and sleep-wake cycles (Saper et al. 2005), and this is partly through TH+ neurons (Lookingland and Moore 2005). On the one hand, these findings point towards a possible programming effect of early light experience on the stress response, and this is supported by the significant effects of early light environment we observed in the ADP and PVN, regions which are also involved in the stress response (Veening et al. 2004; Kiss et al. 2008; Arancibia et al. 2000; Baffi and Palkovits 2000; Cullinan et al. 1995). In addition, the alterations we observed in the DMH, ZI and SPZ, regions that are implicated in the regulation of locomotor activity and sleep (Lookingland and Moore 2005), could explain the behavioural differences previously observed between the LD, LL and DD animals, which include differences in the levels of activity, strength and amplitude of the locomotor activity rhythm under LD cycles, and free-running period in LL (Smith and Canal 2009; Canal et al. 2009; Canal-Corretger et al. 2001a).

It is also worth noting that not all the brain areas that were affected both by early light experience and the light pulse (ADP, SPZ, PVN, DMH) have direct projections from the retina (ADP and DMH do not). What is common between these brain areas, though, is that they all receive projections from the SCN (Watts et al. 1987; Watts 1991; Abrahamson and Moore 2001). This result, coupled to the finding that the differences between DD- and LL-reared mice versus LD-reared mice are always in the same direction (DD > LD in TH staining, and LL > LD in FOS staining) independently of the brain region studied, suggest that SCN input, rather than direct retinal afferents, is mediating the long-lasting effects of early light experience.

Early light experience has long-term consequences at the behavioural level, which include how the animal responds to environmental light stimuli later in life, and also at the SCN level. In the present study we provide evidence of long-lasting alterations in other hypothalamic nuclei, and of a possible involvement of the SCN in these changes. Moreover, here for the first time we demonstrate that, similarly to amphibians, TH+ neurons in the mouse seem to play an important role in communicating these long-term alterations from the SCN to other hypothalamic nuclei. The ultimate consequences of these long-term alterations are still unknown, but the wide range of hypothalamic nuclei affected by early light experience suggests important effects on the regulation of neuroendocrine functions and behaviour.

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Appendix 3: Further details on statistical analyses

	Test used	F-value and df	P-value	Page number
Section 3.3.1 Figure 3.1A	Two-way ANOVA – light during lactation (DD, LD or LL) and light intensity. Between-group (lactation) and within-group (intensity) experimental design.	Lactation: $F_{2,153}$=0.346 Intensity: $F_{8,153}$=82.780 Lactation*Intensity: $F_{16,153}$=0.470	Lactation: p=0.708 Intensity: p<0.001 Lactation*Intensity: p=0.958	59
Section 3.3.1 Figure 3.1B	Two-way ANOVA –light during lactation (DD, LD or LL) and light intensity. Between-group (lactation) and within-group (intensity) experimental design.	Lactation: $F_{2,153}$=2.453 Intensity: $F_{8,153}$=65.523 Lactation*Intensity: $F_{16,153}$=0.520	Lactation: p=0.089 Intensity: p<0.001 Lactation*Intensity: p=0.934	59
Section 3.3.1 Figure 3.1C	Two-way ANOVA – light during lactation (DD, LD or LL) and time. Between-group (lactation) and within group (time) experimental design.	Lactation: $F_{2,578}$=24.658 Time: $F_{35,578}$=19.054 Lactation*Time: $F_{70,578}$=0.121	Lactation: p<0.001 Time: p<0.001 Lactation*Time: p=1.000	59
Section 3.3.2 Figure 3.2A	Two-way ANOVA – light during lactation (DD, LD or LL) and light intensity – followed by a one-way ANOVA –	Lactation: $F_{2,159}$=21.097 Intensity: $F_{8,159}$=51.178 Lactation*Intensity: $F_{16,159}$=5.087 At 3.1$\log_{10}\mu W/cm^2$: $F_{2,17}$=11.990	Lactation: p<0.001 Intensity: p<0.001 Lactation*Intensity: p<0.001 At 3.1$\log_{10}\mu W/cm^2$: p=0.001 At 2.1$\log_{10}\mu W/cm^2$:	60

	light during lactation - for each light intensity. Between-group (lactation) and within group (intensity) experimental design.	At 2.1log_{10}µW/cm^2: $F_{2,16}$=4.267 At 1.1log_{10}µW/cm^2: $F_{2,18}$=3.119 At 0.1log_{10}µW/cm^2: $F_{2,18}$=5.027 At -0.9log_{10}µW/cm^2: $F_{2,18}$=0.020 At -1.9log_{10}µW/cm^2: $F_{2,18}$=0.452 At -2.9log_{10}µW/cm^2: $F_{2,18}$=1.146 At -3.9log_{10}µW/cm^2: $F_{2.18}$=2.167 At -4.9log_{10}µW/cm^2: $F_{2,18}$=1.759	p=0.033 At 1.1log_{10}µW/cm^2: p=0.069 At 0.1log_{10}µW/cm^2: p=0.018 At -0.9log_{10}µW/cm^2: p=0.981 At -1.9log_{10}µW/cm^2: p=0.643 At -2.9log_{10}µW/cm^2: p=0.340 At -3.9log_{10}µW/cm^2: p=0.143 At -4.9log_{10}µW/cm^2: p=0.201	
Section 3.3.2 Figure 3.2B	Two-way ANOVA – light during lactation (DD, LD or LL) and light intensity – followed by a one-way ANOVA – light during lactation - for each light intensity. Between-group (lactation) and within-group (intensity) experimental design.	Lactation: $F_{2,159}$=42.392 Intensity: $F_{8,159}$=47.578 Lactation*Intensity: $F_{16,159}$=3.978 At 3.1log_{10}µW/cm^2: $F_{2,17}$=11.458 At 2.1log_{10}µW/cm^2: $F_{2,16}$=6.780 At 1.1log_{10}µW/cm^2: $F_{2,18}$=8.726 At 0.1log_{10}µW/cm^2: $F_{2,18}$=6.811 At -0.9log_{10}µW/cm^2: $F_{2,18}$=4.822 At -1.9log_{10}µW/cm^2: $F_{2,18}$=1.634 At -2.9log_{10}µW/cm^2: $F_{2,18}$=2.512 At -3.9log_{10}µW/cm^2: $F_{2.18}$=0.159 At -4.9log_{10}µW/cm^2: $F_{2,18}$=0.070	Lactation: p<0.001 Intensity: p<0.001 Lactation*Intensity: p<0.001 At 3.1log_{10}µW/cm^2: p=0.001 At 2.1log_{10}µW/cm^2: p=0.007 At 1.1log_{10}µW/cm^2: p=0.002 At 0.1log_{10}µW/cm^2: p=0.006 At -0.9log_{10}µW/cm^2: p=0.021 At -1.9log_{10}µW/cm^2: p=0.223 At -2.9log_{10}µW/cm^2: p=0.109 At -3.9log_{10}µW/cm^2: p=0.854 At -4.9log_{10}µW/cm^2: p=0.933	60
Section 3.3.2	Two-way ANOVA	Lactation:	Lactation: p<0.001	60

Figure 3.2C	– light during lactation (DD, LD or LL) and time – followed by a one-way ANOVA – light during lactation – for each time point. Between-group (lactation) and within-group (time) experimental design.	$F_{2,538}$=770.096 Time: $F_{35,538}$=2.750 Lactation*Time: $F_{70,538}$=1.369 At 0.56 minutes: $F_{2,16}$=0.879 At 0.11 minutes: $F_{2,15}$=8.283 At 1.67 minutes: $F_{2,15}$=7.562 At 2.22 minutes: $F_{2,15}$=10.958 At 2.78 minutes: $F_{2,16}$=10.572 At 3.33 minutes: $F_{2,16}$=16.801 At 3.89 minutes: $F_{2,16}$=19.790 At 4.44 minutes: $F_{2,16}$=19.232 At 5 minutes: $F_{2,16}$=15.905 At 5.56 minutes: $F_{2,16}$=18.017 At 6.11 minutes: $F_{2,16}$=17.656 At 6.67 minutes: $F_{2,16}$=27.157 At 7.22 minutes: $F_{2,16}$=24.812 At 7.78 minutes: $F_{2,16}$=30.210 At 8.33 minutes: $F_{2,16}$=14.753 At 8.89 minutes: $F_{2,16}$=26.777 At 9.44 minutes: $F_{2,16}$=24.140 At 10 minutes: $F_{2,16}$=24.191 At 10.56 minutes: $F_{2,16}$=31.344	Time: p<0.001 Lactation*Time: p=0.031 At 0.56 minutes: p=0.434 At 0.11 minutes: p=0.004 At 1.67 minutes: p=0.005 At 2.22 minutes: p=0.001 At 2.78 minutes: p=0.001 At 3.33 minutes: p<0.001 At 3.89 minutes: p<0.001 At 4.44 minutes: p<0.001 At 5 minutes: p<0.001 At 5.56 minutes: p<0.001 At 6.11 minutes: p<0.001 At 6.67 minutes: p<0.001 At 7.22 minutes: p<0.001 At 7.78 minutes: p<0.001 At 8.33 minutes: p<0.001 At 8.89 minutes: p<0.001 At 9.44 minutes: p<0.001 At 10 minutes: p<0.001 At 10.56 minutes: p<0.001 At 11.11 minutes: p<0.001	

		At 11.11 minutes: $F_{2,16}$=33.137 At 11.67 minutes: $F_{2.16}$=28.273 At 12.22 minutes: $F_{2,15}$=29.826 At 12.78 minutes: $F_{2.15}$=18.164 At 13.33 minutes: $F_{2,15}$=14.534 At 13.89 minutes: $F_{2,15}$=21.639 At 14.44 minutes: $F_{2,14}$=24.449 At 15 minutes: $F_{2,14}$=36.270 At 15.56 minutes: $F_{2,14}$=28.631 At 16.11 minutes: $F_{2,14}$=50.302 At 16.67 minutes: $F_{2,14}$=26.686 At 17.22 minutes: $F_{2,14}$=38.077 At 17.78 minutes: $F_{2,13}$=33.891 At 18.33 minutes: $F_{2,12}$=19.562 At 18.89 minutes: $F_{2.12}$=31.428 At 19.44 minutes: $F_{2,12}$=31.335 At 20 minutes: $F_{2,12}$=29.323	At 11.67 minutes: p<0.001 At 12.22 minutes: p<0.001 At 12.78 minutes: p<0.001 At 13.33 minutes: p<0.001 At 13.89 minutes: p<0.001 At 14.44 minutes: p<0.001 At 15 minutes: p<0.001 At 15.56 minutes: p<0.001 At 16.11 minutes: p<0.001 At 16.67 minutes: p<0.001 At 17.22 minutes: p<0.001 At 17.78 minutes: p<0.001 At 18.33 minutes: p<0.001 At 18.89 minutes: p<0.001 At 19.44 minutes: p<0.001 At 20 minutes: p<0.001	
Section 3.3.3 Figure 3.3A	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,12}$=0.064	p=0.938	61
Section 3.3.4	One-way ANOVA	$F_{2,10}$=7.651	P=0.010	61

Figure 3.3B	– light during lactation (DD, LD or LL). Between-group experimental design.			
Section 3.3.5 Figure 3.4A	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,6}$=3.749	P=0.088	63
Section 3.3.6 Figure 3.4B	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,10}$=11.202	P=0.003	63
Section 3.3.7 Figure 3.5	Two-way ANOVA – light during lactation (DD, LD or LL) and light intensity – followed by a one-way ANOVA – light during lactation – for each light intensity. Between-group (lactation) and within-group (intensity) experimental design.	Lactation: $F_{2,33}$=32.465 Intensity: $F_{3,33}$=40.916 Lactation*Intensity: $F_{6,33}$=4.425 At 330µW/cm^2: $F_{2,9}$=13.049 At 37.9µW/cm^2: $F_{2,6}$=5.621 At 6.9µW/cm^2: $F_{2,9}$=6.921 In DD: $F_{2,10}$=0.862 For mice raised in DD: $F_{3,14}$=17.755 For mice raised in LD: $F_{3,6}$=7.115 For mice raised in LL: $F_{3,14}$=20.716	Lactation: p<0.001 Intensity: p<0.001 Lactation*Intensity: p=0.002 At 330µW/cm^2: p=0.002 At 37.9µW/cm^2: p=0.042 At 6.9µW/cm^2: p=0.015 In DD: p=0.862 For mice raised in DD: p<0.001 For mice raised in LD: p=0.021 For mice raised in LL: p<0.001	64
Section 3.3.8 Figure 3.6A	Two-way ANOVA – light during lactation (DD, LD or LL) and light intensity.	Lactation: $F_{2,90}$=1.587 Intensity: $F_{8,16}$=178.087 Lactation*Intensity: $F_{16,90}$=0.439	Lactation: p=0.210 Intensity: p<0.001 Lactation*Intensity: p=0.968	66

	Between-group (lactation) and within-group (intensity) experimental design.			
Section 3.3.8 Figure 3.6B	Two-way ANOVA – light during lactation (DD, LD or LL) and light intensity – followed by a one-way ANOVA – light during lactation – for each intensity. Between-group (lactation) and within-group (intensity) experimental design.	Lactation: $F_{2,90}$=12.965 Intensity: $F_{8,90}$=216.414 Lactation*Intensity: $F_{16,90}$=1.038 At 3.1log_{10}µW/cm^2: $F_{2,10}$=6.746 At 2.1log_{10}µW/cm^2: $F_{2,10}$=1.763 At 1.1log_{10}µW/cm^2: $F_{2,10}$=4.290 At 0.1log_{10}µW/cm^2: $F_{2,10}$=1.783 At -0.9log_{10}µW/cm^2: $F_{2,10}$=1.215 At -1.9log_{10}µW/cm^2: $F_{2,10}$=0.201 At -2.9log_{10}µW/cm^2: $F_{2,10}$=2.630 At -3.9log_{10}µW/cm^2: $F_{2.10}$=0.770 At -4.9log_{10}µW/cm^2: $F_{2,10}$=0.730	Lactation: p<0.000 Intensity: p<0.000 Lactation*Intensity: p=0.426 At 3.1log_{10}µW/cm^2: p=0.014 At 2.1log_{10}µW/cm^2: p=0.221 At 1.1log_{10}µW/cm^2: p=0.045 At 0.1log_{10}µW/cm^2: p=0.218 At -0.9log_{10}µW/cm^2: p=0.337 At -1.9log_{10}µW/cm^2: p=0.821 At -2.9log_{10}µW/cm^2: p=0.121 At -3.9log_{10}µW/cm^2: p=0.489 At -4.9log_{10}µW/cm^2: p=0.506	66
Section 3.3.8 Figure 3.6C	Two-way ANOVA – light during lactation (DD, LD or LL) and time. Between-group (lactation) and within-group (time) experimental design.	Lactation: $F_{2,360}$=1.768 Time: $F_{35,360}$=15.130 Lactation*Time: $F_{70,360}$=0.297	Lactation: p=0.172 Time: p<0.001 Lactation*Time: p=1.000	66
Section 3.3.8 Figure 3.6D	One-way ANOVA – light during lactation (DD, LD	$F_{2,10}$=0.814	p=0.471	66

	or LL). Between-group experimental design.			
Section 3.3.8 Figure 3.6E	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,10}$=0.297	p=0.749	66
Section 4.3.2 Figure 4.1	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,11}$=8.825	p=0.005	79
Section 4.3.2 Figure 4.2A	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,12}$=2.704	p=0.107	80
Section 4.3.2 Figure 4.2B	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,13}$=6.393	p=0.012	80
Section 4.3.2 Figure 4.3A	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,13}$=1.099	p=0.362	81
Section 4.3.2 Figure 4.3B	One-way ANOVA – light during lactation (DD, LD	$F_{2,13}$=0.116	p=0.892	81

	or LL). Between-group experimental design.			
Section 4.3.2 Figure 4.4A	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,11}$=0.049	p=0.952	82
Section 4.3.2 Figure 4.4B	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,11}$=0.021	p=0.979	82
Section 4.3.3 Figure 4.5	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,11}$=1.031	p=0.389	83
Section 4.3.3 Figure 4.6A	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,11}$=1.640	p=0.238	84
Section 4.3.3 Figure 4.6B	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,11}$=1.621	p=0.242	84
Section 4.3.3 Figure 4.7A	One-way ANOVA – light during lactation (DD, LD	$F_{2,11}$=1.031	p=0.389	85

	or LL). Between-group experimental design.			
Section 4.3.3 Figure 4.7B	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,11}$=1.276	p=0.318	85
Section 4.3.4	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,12}$=10.287	p=0.002	86
Section 5.3.1 Table 5.2 – LD stage	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	Period: No variation % variance: $F_{2,29}$=0.433 Amplitude: $F_{2,29}$=1.014 Duration of alpha: $F_{2,29}$=2.142 Amount of activity during alpha: $F_{2,29}$=1.262	Period: No variation % variance: p=0.653 Amplitude: p=0.375 Duration of alpha: p=0.136 Amount of activity during alpha: p=0.298	100
Section 5.3.1 Table 5.2 – DD stage 1	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	Period: $F_{2,29}$=3.819 % variance: $F_{2,29}$=2.136 Amplitude: $F_{2,29}$=0.679 Duration of alpha: $F_{2,29}$=0.562 Amount of activity during alpha: $F_{2,29}$=0.854	Period: p=0.034 % variance: p=0.136 Amplitude: p=0.515 Duration of alpha: p=0.576 Amount of activity during alpha: p=0.436	100
Section 5.3.1 Table 5.2 – DD stage 2	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental	Period: $F_{2,29}$=0.326 % variance: $F_{2,29}$=4.233 Amplitude: $F_{2,29}$=1.547 Duration of alpha: $F_{2,29}$=0.495	Period: p=0.724 % variance: p=0.024 Amplitude: p=0.230 Duration of alpha: p=0.495 Amount of activity	100

	design.	Amount of activity during alpha: $F_{2,29}$,1.663	during alpha: p=0.207	
Section 5.3.1 Table 5.2 – DD stage 3	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	Period : $F_{2,29}$=2.407 % variance: $F_{2,29}$=1.307 Amplitude: $F_{2,29}$=1.534 Duration of alpha: $F_{2,29}$=0.033 Amount of activity during alpha: $F_{2,29}$=1.526	Period: p=0.108 % variance: p=0.286 Amplitude: p=0.233 Duration of alpha: p=0.968 Amount of activity during alpha: p=0.234	100
Section 5.3.1 Figure 5.3	Three-way ANOVA – light during lactation (DD, LD or LL), pulse (yes or no) and type of pulse (CT16(1), CT16(2) or CT22) Between-group (lactation and pulse) and within-group (type) experimental design.	Lactation: $F_{2,122}$=0.300 Pulse: $F_{1,122}$=17.653 Type: $F_{2,122}$=120.057 Lactation*Pulse: $F_{2,122}$=0.395 Lactation*Type: $F_{4,122}$=1.703 Pulse*Type: $F_{2,122}$=114.948 Light*Pulse*Type: $F_{4,122}$=2.038	Lactation: p=0.561 Pulse: p<0.001 Type: p<0.001 Lactation*Pulse: p=0.675 Lactation*Type: p=0.154 Pulse*Type: p<0.001 Lactation*Pulse*Type: p=0.093	101
Section 5.3.1 Figure 5.4	Three-way ANOVA – light during lactation (DD, LD or LL), pulse (yes or no) and type of pulse (CT16, or CT22) – followed by a two-way ANOVA – lactation and pulse Between-group (lactation and pulse) and within-group (type)	Lactation: $F_{2,82}$=0.308 Pulse: $F_{1,82}$=13.114 Type: $F_{1,82}$=16.150 Lactation*Pulse: $F_{2,82}$=1.180 Lactation*Type: $F_{2,82}$=1.252 Pulse*Type: $F_{1,82}$=28.411 Lactation*Pulse*Type: $F_{2,82}$=2.923 At CT16: Lactation: $F_{2,41}$=1.545 Pulse: $F_{1,41}$=1.686 Lactation*Pulse:	Lactation: p=0.736 Pulse: p=0.001 Type: p<0.001 Lactation*Pulse: p=0.313 Lactation*Type: p=0.291 Pulse*Type: p<0.001 Lactation*Pulse*Type: p=0.059 At CT16: Lactation: p=0.225 Pulse: p=0.201 Lactation*Pulse: p=0.026	102

	experimental design.	$F_{2,41}$=3.991 At CT22: Lactation: $F_{2,41}$=0.195 Pulse: $F_{1,41}$=35.339 Lactation*Pulse: $F_{2,41}$=0.571	At CT22: Lactation: p=0.824 Pulse: p<0.001 Lactation*Pulse: p=0.570	
Section 5.3.2 Table 5.3	Two-way ANOVA – lactation (DD, LD or LL) and pulse (yes or no) – except for cFOS-positive cells where a one-way ANOVA – lactation – was performed. Between-group experimental design.	cFOS-positive cells: Lactation: $F_{2,12}$=0.421 pERK optical density: Lactation: $F_{2,41}$=0.135 Pulse: $F_{1,41}$=59.458 Lactation*Pulse: $F_{2,41}$=0.377 PER2-positive cells: Lactation: $F_{2,41}$=0.184 Pulse: $F_{1,41}$=0.018 Lactation*Pulse: $F_{2,41}$=0.699	cFOS-positive cells: p=0.666 pERK optical density: Lactation: p=0.874 Pulse: p<0.001 Lactation*Pulse: p=0.688 PER2-positive cells: Lactation: p=0.833 Pulse: p=0.893 Lactation*Pulse: p=0.503	104
Section 6.3.1 Figure 6.2	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	Amplitude: $F_{2,29}$=8.767 Amount of activity during alpha: $F_{2,29}$=16.801	Amplitude: p=0.001 Amount of activity during alpha: p<0.001	116
Section 6.3.1 Phase shift after CT16 light pulse	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	$F_{2,29}$=0.551	p=0.582	117
Section 6.3.1 Table 6.2	Two-way ANOVA – light during lactation (DD, LD or LL) and before or after CT16 light pulse – followed by one-way ANOVA – lactation.	Period: Lactation: $F_{2,58}$=2.967 Before vs. After: $F_{1,58}$=26.832 Lactation*Before vs. After: $F_{1,58}$=0.022 Amplitude: Lactation: $F_{2,57}$=3.791 Before vs. After:	Period: Lactation: p=0.059 Before vs. After: p<0.001 Lactation*Before vs. After: p=0.978 Lactation: p=0.028 Before vs. After: p=0.300	117

	Between-group (lactation) and within-group (before or after pulse) experimental design.	$F_{1,57}$=1.096 Lactation*Before vs. After: $F_{2,57}$=0.292 Area under the curve: Lactation: $F_{2,57}$=10.327 Before vs. After: $F_{1,57}$=0.298 Lactation*Before vs. After: $F_{2.57}$=0.056 Before CT16 light pulse: Amplitude: $F_{2,28}$=0.329 Area under the curve: $F_{2,29}$=3.956 After CT16 light pulse: Amplitude: $F_{2,28}$=5.802 Area under the curve: $F_{2,28}$=6.820	Lactation*Before vs. After: p=0.748 Area under the curve: Lactation: p<0.001 Before vs. After: p=0.587 Lactation*Before vs. After: p=0.946 Before CT16 light pulse: Amplitude: p=0.329 Area under the curve: p=0.030 After CT16 light pulse: Amplitude: p=0.008 Area under the curve: p=0.004	
Section 6.3.1 Locomotor activity in LD after DD	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	Amplitude: $F_{2,29}$=4.839	Amplitude: p=0.015	117
Section 6.3.1 Figure 6.3	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	Tau: $F_{2,28}$=1.341 Days until arrhythmic: $F_{2,23}$=3.664 % variance: $F_{2,27}$=4.666 Amplitude: $F_{2,28}$=6.778 Power content of the first harmonic: $F_{2,26}$=3.254	Tau: p=0.278 Days until arrhythmic: p=0.042 % variance: p=0.018 Amplitude: p=0.004 Power content of the first harmonic: p=0.055	118
Section 6.3.2 Table 6.3 Figure 6.4	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	Period: $F_{2,19}$=0.838 Phase: $F_{2,18}$=2.918 Amplitude: $F_{2,19}$=6.193 Damping rate: $F_{2,19}$=2.812 Rhythmicity index: $F_{2,19}$=0.387	Period: p=0.448 Phase: p=0.080 Amplitude: p=0.008 Damping rate: p=0.085 Rhythmicity index: p=0.685	119
Section 6.3.3	One-way ANOVA	Period: $F_{2,13}$=0.678	Period: p=0.525	120

Table 6.4 Figure 6.5	– light during lactation (DD, LD or LL). Between-group experimental design.	Phase: $F_{2,13}$=0.127 Amplitude: $F_{2,13}$=0.175 Damping rate: $F_{2,13}$=0.243 Rhythmicity index: $F_{2,13}$=0.964	Phase: p=0.882 Amplitude: p=0.841 Damping rate: p=0.788 Rhythmicity index: p=0.407	
Section 6.3.3 Comparison of baseline vs. LL tissue culture	One-way ANOVA – difference between baseline and LL experiment. Between-group experimental design.	Amplitude: $F_{1,43}$=20.905 Phase: $F_{1,43}$=68.256 Rhythmicity index: $F_{1,43}$=41.087	Amplitude: p<0.001 Phase: p<0.001 Rhythmicity index: p<0.001	119
Section 6.3.4 Table 6.5 Heart	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	Period: $F_{2,25}$=0.346 Phase: $F_{2,25}$=1.345 Amplitude: $F_{2,21}$=4.593 Damping rate: $F_{2,25}$=0.729 Rhythmicity index: $F_{2,25}$=1.773	Period: p=0.711 Phase: p=0.279 Amplitude: p=0.022 Damping rate: p=0.492 Rhythmicity index: p=0.191	121
Section 6.3.4 Table 6.5 Lung	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	Period: $F_{2,26}$=0.519 Phase: $F_{2,26}$=1.445 Amplitude: $F_{2,25}$=5.165 Damping rate: $F_{2,26}$=0.191 Rhythmicity index: $F_{2,26}$=0.283	Period: p=0.601 Phase: p=0.254 Amplitude: p=0.013 Damping rate: p=0.827 Rhythmicity index: p=0.756	121
Section 6.3.4 Table 6.5 Liver	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	Period: $F_{2,25}$=0.083 Phase: $F_{2,25}$=0.263 Amplitude: $F_{2,25}$=0.869 Damping rate: $F_{2,25}$=1.155 Rhythmicity index: $F_{2,25}$=0.121	Period: p=0.921 Phase: p=0.771 Amplitude: p=0.432 Damping rate: p=0.331 Rhythmicity index: p=0.887	121
Section 6.3.4 Table 6.5 Spleen	One-way ANOVA – light during lactation (DD, LD or LL). Between-group experimental design.	Period: $F_{2,31}$=0.010 Phase: $F_{2,30}$=4.006 Amplitude: $F_{2,31}$=2.116 Damping rate: $F_{2,31}$=0.797 Rhythmicity index: $F_{2,31}$=2.804	Period: p=0.990 Phase: p=0.029 Amplitude: p=0.138 Damping rate: p=0.460 Rhythmicity index: p=0.076	121

Section A1.3 Figure A1.1	Two-way ANOVA – light during lactation (DD, LD or LL) and strain (C57BL/6J or CD1). Between-group experimental design.	Lactation: $F_{2,21}$=2.729 Strain: $F_{1,21}$=19.570 Lactation*Strain: $F_{2,21}$=0.272	Lactation: p=0.088 Strain: p<0.001 Lactation*Strain: p=0.765	153

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