Circadian rhythmicity and light sensitivity of the zebrafish brain

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I, Helen Anna Moore, confirm that the work presented in this thesis is my own.

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Abstract

Light is important for entraining circadian rhythms, which regulate a wide range of biological processes. Zebrafish have directly light responsive tissues (Whitmore et al 2000) and are thus a useful vertebrate model for circadian rhythmicity and light sensitivity. Recent studies show the pineal regulates locomotor rhythms (Li et al 2012). However, there are many unresolved questions concerning the neurobiological basis of the zebrafish clock, such as whether neuronal pacemakers, which drive rhythms in other tissues, are present throughout the brain.

In this study, *per3-luc* zebrafish confirm that both central and peripheral tissues are directly light sensitive and have endogenous circadian rhythmicity. Chromogenic *in situ* hybridization reveals localised expression of several core zebrafish clock genes, a rhythmic gene, *per3*, and two light responsive genes, *cry1a* and *per2*. Adult brain nuclei with expression include the suprachiasmatic nucleus, periventricular grey zone of the optic tectum, and granular cells of the rhombencephalon. Pilot experiments using high-resolution spatial recording of *per3-luc* brain slices show some of these regions can display robust rhythmicity in DD. Some of the cells expressing clock genes are neurons, and therefore neurons were further investigated.

C-fos, a marker for neuronal activity in mammalian photoreceptors, is upregulated in at least four different responses to light in zebrafish, in different brain nuclei. This suggests the brain contains several types of photosensitive cells, which respond to different lighting conditions.

Zebrafish larvae exhibit developmental changes in spatial circadian gene expression of *per3* and light induction of *c-fos*.

Finally, the photopigment group of opsins were investigated for their potential role in light entrainment. *Exorh* was prominent solely in the pineal. *Rgr1* was found in numerous nuclei, many of which had shown expression of *cry1a*, *per2* and *per3*.

Overall, this thesis shows that the zebrafish brain is not uniformly light sensitive. Localised regions in the zebrafish brain with strong rhythmicity and light sensitivity are neuronal pacemaker candidates.

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Overview

Circadian rhythmicity and light sensitivity of the zebrafish brain

Glossary of Terms

^{35}S-ATP, ^{35}sulphur-labeled adenosine 5'-(gamma-thiotriphosphate);
A, anterior thalamic nucleus;
AB TL, AB tupfel long fin;
ANOVA, analysis of variance;
AP, alkaline phosphatase;
APN, accessory pretectal nucleus;
AS, antisense;
ATN, anterior tuberal nucleus;
BCIP, 4-chloro-3-indolyl phosphate;
bp, base pair;
BSA, bovine serum albumin;
CCe$_{gra}$; corpus cerebellum;
CCe$_{mo}$l; corpus cerebellum;
CCG, clock controlled gene;
cDNA, complementary DNA;
C-fos, cellular proto-oncogene fos;
CIL, central nucleus of the inferior lobe;
CISH, chromogenic *in situ* hybridisation;
Clock* gene,* circadian locomotor output kaput;
CM, corpus mammilare;
CO, optic chiasm;
CP, central posterior thalamic nucleus;
CPN, central pretectal nucleus;
Cry, cryptochrome;
CT, circadian time;
Ct, cycle time;
Cv, commissural nucleus of V;
cyc, cyclops;
D, dorsal telencephalic area;
DAO, dorsal accessory optic nuclei;
DAPI, 4',6-diamidino-2-phenylindole;
Dc, central zone of D;
DD, constant darkness;
Dd; dorsal zone of D;
DIG, digoxigenin;
DIL, diffuse nucleus of the inferior lobe;
DL, dark-light cycle;
Dl, lateral zone of D;
Dm, medial zone of D;
DNA, deoxyribonucleic acid;
dNTP, deoxyribonucleotide triphosphate;
DP, dorsal posterior thalamic nucleus;
Dp, posterior zone of D;
dpf; days post fertilisation;
DTN, dorsal tegmental nucleus;
DTT, dithiothreitol;
dUTP, deoxyuridine triphosphate;
E, efficiency value;
ECL, external cellular layer;
EDTA, ethylenediaminetetraacetic acid;
EG, eminentia granularis;
EN, entopeduncular nucleus;
EW, Edinger-Westphal nucleus;
Exorh, extra-ocular rod-opsin or exo-rhodopsin;
FCS, foetal calf serum;
flh, floating head;
GFAP, glial fibrillary acidic protein;
GL; glomerular layer;
Ha, ventral and dorsal habenula;
Hc, caudal zone of periventricular hypothalamus;
hcrt, hypocretin;
hcrtr, hypocretin receptor;
Hd, dorsal zone of periventricular hypothalamus;
HLH, helix-loop-helix;
hpf; hours post fertilisation;
HuC/D, human neuronal protein;
Hv, ventral zone of periventricular hypothalamus;
Hyb, hybridisation solution;
ICL, internal cellular layer of olfactory bulbs;
IF, immunofluorescence;
IL, inferior lobe;
IPD, imaging photon detector;
ISH, *in situ* hybridisation;
L15, Leibovitz L-15 medium;
LB, lysogeny broth;
LC, Locus coeruleus;
LCa$_{gra}$; lobus caudalis cerebelli;
LCa$_{mol}$; lobus caudalis cerebelli;
LD, light-dark cycle;
LH, lateral hypothalamic nucleus;
LL, constant light;
LLF, lateral longitudinal fascicle;
Luc, luciferase;
Lws, long-wave-sensitive opsin;
MAB, maleic acid buffer;
mbl, masterblind;
MeOH, methanol;
MMEP, multimicroelectrode plate arrays;
MNV, mesencephalic nucleus of trigeminal nucleus;
mRNA, messenger RNA;
NBT, 5-bromo- nitro blue tetrazolium chloride;
NI, nucleus isthmus;
NIII, Oculomotor nucleus;
NIn, nucleus interpeduncularis;
NLL, nucleus of lateral lemniscus;

NLV, nucleus lateralis valvulae;
Nmlf, nucleus of the medial longitudinal fascicle;
Novo, novel opsin;
NS, not significant;
NT, nucleus taeniae;
OB, olfactory bulb;
OCT, optimal cutting temperature compound;
OligoDT, deoxy-thymine nucleotides;
Opn1, cone opsin;
Opn2, rod opsin;
Opn3, encephalopsin/panopsin;
Opn4, melanopsin;
Opn4m, mammalian related melanopsin;
Opn4x, non-mammalian related melanopsin;
Opn5, neuropsin;
OT, optic tract;
P/S, penicillin/streptomycin;
PACAP, pituitary adenylate cyclase–activating protein;
PB, phosphate buffer;
PBS, phosphate buffered saline;
PBT, phosphate buffered saline with tween;
PCN, paracommissural nucleus;
PCR, polymerase chain reaction;
Per, period;
PFA, paraformaldehyde;
PGa, preglomerular anterior nuclei;
PGl, preglomerular lateral nuclei;
PGm, medial preglomerular nucleus;
PGZ, periventricular grey zone of optic tectum;
PI, propidium iodide;
PL, perilemniscal nucleus;
PO, posterior pretectal nucleus;
POD, peroxidase;
PPa, parvocellular preoptic nucleus, anterior part;
PPd, dorsal part of the pretectal diencephalic cluster;
Ppp, parvocellular preoptic nucleus, posterior part;
PPv, periventricular pretectal nucleus, ventral part;
PS, superficial pretectal nuclei;
PTN, posterior tuberal nucleus;
PTU, phenylthiourea;
PVO, paraventricular organ;
qPCR, quantitative PCR;
rd/rd cl, rodless coneless mice;
Rgr, retinal g protein-coupled receptor;
Rh, rhodopsin;
RHT, retinohypothalamic tract;
RNA, ribonucleic acid;
RNase, ribonuclease;
ROR, retinoic acid-related orphan receptor;
rpL13, ribosomal protein L13;
RRE, retinoic acid-related orphan receptor response elements;
Rrh, peropsin/ retinal pigment epithelium-derived rhodopsin homolog;
RT-PCR, real-time PCR;
RV, rhombencephalic ventricle;
S, sense;
SCN, suprachiasmatic nucleus;
SD, standard deviation;
SEM, standard error of the mean;
SGN, secondary gustatory nucleus;
SR, superior raphe nucleus;
SRF, superior reticular formation;
SSC, saline sodium citrate;
Sws, short-wave-sensitive opsin;
TAE, Tris base, acetic acid and EDTA buffer;
Tef, thyroid embryonic factor*;*
TelV, telencephalic ventricle;
TeO, optic tectum;
TIM, timeless;
TL, torus longitudinalis;
TLa, torus lateralis;
Tmt, teleost multiple tissue opsin;
Tpp, periventricular nucleus of the posterior tuberculum;
TS, torus semicircularis;
UV, ultraviolet;
V, Ventral telencephalic area;
Va, vertebrate ancient opsin;
Val, valvula cerebelli;
Val_{gra}, granular layer of lateral division of valvula cerebelli;
Val_{mol}; molecular layer of lateral division of valvula cerebelli;
Vam_{gra}; granular layer of medial division of valvula cerebelli;
Vam_{mol}; molecular layer of medial division of valvula cerebelli;
VAO, ventral accessory optic nuclei;
Vc, central nuclei of V;
Vd, dorsal nucleus of V;
Vl, lateral nuclei of V;
VL, ventrolateral thalamic nucleus;
VM, ventromedial thalamic nucleus;
Vp, postcommissural nucleus of V;
Vs, supracommissural nucleus of V;
Vv, ventral nucleus of V;
WISH, whole mount *in situ* hybridisation;
Wt, wild type;
ZL, zona limitans;
ZT, Zeitgeber time.

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Chapter 1

Introduction

This general introduction is written to give an overview of the field, more specific introductions can be found at the beginning of each results chapter (Chapters 3-6).

1.1 Circadian Biology

Biological timekeeping, by an internal clock, is universal to all living organisms, from bacteria to mammals. When adapting to an environment with regular daily and seasonal cycles, anticipating the timing of these cycles can provide organisms with a vital mechanism of survival. Arguably the change between day and night in a 24-hour period is one of the most dramatic regular environmental changes that many organisms have adapted to anticipate.

The field of circadian biology strives to understand how organisms adapt their behaviour and physiology to a 24-hour cycle. The term 'circadian' was coined by Franz Halberg in 1959 from the Latin *circa* meaning 'around' and *dies* meaning 'day', and is used to describe daily rhythms that persist with a periodicity of 'around about a day' when in constant conditions.

There is a high degree of conservation between the genetic and biochemical basis of the circadian timing systems between organisms from flies to humans, strengthening the theory that clocks have evolved universally as an adaptive mechanism. Circadian systems are able to regulate a wide variety of physiological and behavioural processes including metabolism, sleep, hormone regulation, thermoregulation, and cognitive function. Synchronisation to the environment is an essential feature of clocks and better enables organisms to remain healthy; disruption to these internal rhythms, characterized by dampened oscillations or internal desynchronisation, can result in diseases or disorders.

This introductory chapter aims to demonstrate how scientists are beginning to have a deeper understanding of how circadian time is regulated in many different species. However, this understanding remains incomplete. Therefore, this section will continue on to outline some outstanding questions in a teleost vertebrate clock model, the zebrafish. The goal of this project will be to further characterise the regulation of circadian timing in the zebrafish brain.

1.1.1 The foundation of circadian biology

The study of circadian biology is relatively young compared to most other areas in physiology. Some of the major questions for circadian biology began to be framed in the 1950s, when many diverse experiments were being described at a time before unifying principles had brought the

field together. In 1959 a symposium held at Cold Spring Harbor gathered many researchers in the developing field to agree upon the foundational principles of circadian biology.

Colin Pittendrigh, who is considered one of the founders of circadian biology, gave a seminal talk at this symposia outlining the organisation of the circadian system (Pittendrigh 1960). He used the definition of a circadian rhythm as a rhythm with a free-running period that is an approximation to the period of the earth's rotation. He then outlined many empirical generalisations about circadian rhythms, most of which hold true today.

The crucial difference between a circadian and a diurnal rhythm is that circadian rhythms persist in constant conditions, reflecting an endogenous oscillator, 'clock', that is not simply responding to environment changes. However, a few exogenous stimuli can reset, 'entrain', this clock to the external environment; the dominant ones used in most experimental work are light and temperature. These external synchronising stimuli have been termed 'Zeitgeber' from the German word '*time giver'*. When a clock is disconnected and no longer entrained by the external environment it will persist in producing rhythmic output at its own, innate pace, termed 'free-running'. The phase of a free-running circadian rhythm can be shifted by brief exposure to Zeitgebers.

Another key property of a circadian rhythm is that it must be temperature compensated so the rate at which it runs will remain fixed at different temperatures. Many chemical processes are liable to speed up at higher temperature and slow down at cooler ones. In biology, the Q_{10} rule states that for every 10°C rise in temperature a metabolic rate will double. However, an endogenous oscillator must be insensitive to this in order to reliably keep time (Pittendrigh 1954).

Initially in the 1960s, circadian biology was seen as a diffuse time-measuring process throughout the entire organism. However, Pittendrigh proposed that there was a distinct "central oscillatory pacemaker" that exerts control over timing in peripheral tissues, driving rhythmicity throughout the organism. There are three key properties underlying a 'core clock': an endogenous self-sustaining oscillator, which generates near-24-hour rhythmic outputs, and can be entrained by external stimuli (Figure 1). The search then began to determine where this core clock would be found, with the light-sensitive retina and brain being key starting points.

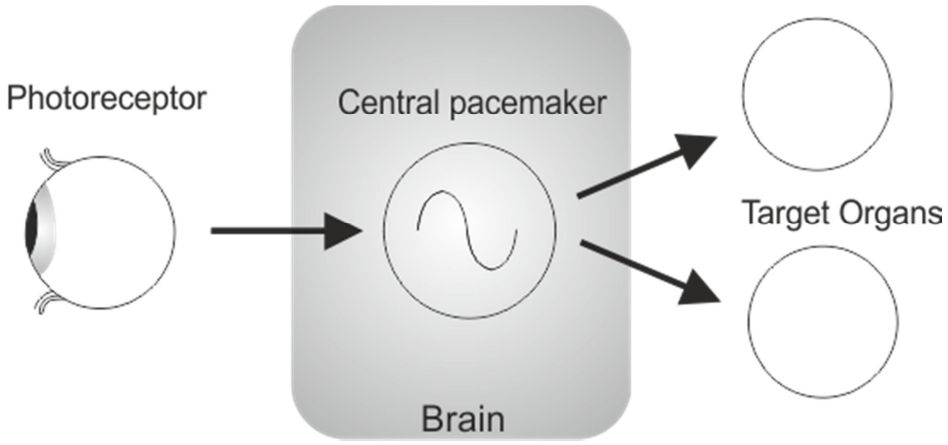


Figure 1 A core clock

This schematic shows the three key properties underlying the classic 'core clock' model. 1. A photoreceptor or other sensory organ that can detect changes to the external environment and signal to a central pacemaker. 2. A central pacemaker that has an endogenous self-sustaining oscillator, which has a near-24-hour rhythm. 3. Target organs that receive rhythmicity from the central pacemaker.

1.1.2 The search for neuronal pacemakers

For regions of the brain to generate biological rhythms in other areas of the body they must have certain characteristics. These regions, 'neuronal pacemakers', fulfil certain criteria. Firstly, they must be able to generate robust endogenous rhythms in constant conditions. They must also produce a signal to drive rhythms in other areas of the body. The pacemaker must also be able to be reset by exogenous environmental cues, and be temperature compensated (Mendoza & Challet 2009). When a neuronal pacemaker is removed the timing of the activities driven by the pacemaker, such as changes in core body temperature, will become arrhythmic. This rhythm must be retrievable by the re-transplantation of the pacemaker, which should carry its endogenous periodicity with it.

The endogenous rhythmic signals produced from a neuronal pacemaker can be observed in a number of ways, for example by measuring the cyclic expression of core clock gene transcripts (as will be further explored in section 1.14), neuronal activity, neuropeptide levels, hormonal levels, or energy metabolism (Herzog & Schwartz 2002). One particular hormone, melatonin, is

often released from the pineal in a circadian manner, high at night and low in the day, and is thought to convey circadian timing throughout the body.

Initially invertebrates were a popular model for investigating the organisation of the circadian timing system in the brain due the viability of brain transplantation and the relative simplicity of their nervous systems, with few, identifiable neurons. This section will describe two pacemakers investigated in invertebrates to demonstrate how circadian biologists have approached the search for neuronal pacemakers. The discussion will then focus on the vertebrate system with the discovery of the mammalian suprachiasmatic nucleus (SCN).

1.1.2.1 Optic lobes in the cockroach

Some of the first evidence of a neuronal circadian pacemaker was found by Pittendrigh using decapitated and surgically ablated cockroaches, *Leucophaea maderae.* Experiments showed that the optic lobes in the brain were necessary for functional, robust, circadian rhythms of locomotor activity (Nishiitsutsuji-Uwo & Pittendrigh 1968). Further work on the cockroach showed that the optic lobes are able to fulfil all the criteria for a neuronal pacemaker.

Most importantly for its role as a neuronal pacemaker, the neurons in the optic lobes of the cockroaches have an endogenous firing pattern that is self-sustaining and rhythmic in constant conditions (Colwell & Page 1990). Also, cockroaches are able to entrain to Zeitgebers, such as light dark (LD) and temperature cycles. Indeed, pulses of low temperature placed directly on the optic lobes are able to phase delay locomotor rhythmicity (Page 1981).

The optic lobes are able, and sufficient, to drive rhythms in locomotion: these rhythms are abolished when the optic tracts are severed, but reappear with the same free-running period when neural connections between the optic lobe and the midbrain regenerate after 3-5 weeks (Page 1983). When the optic lobes are surgically removed the locomotor activity remains arrhythmic indefinitely. Furthermore, transplanting optic lobes of individuals with vastly different free-running periods showed that the optic lobes can carry endogenous rhythms from the donor to the host (Page 1982). Therefore, the optic lobes in the cockroach are able to fulfil all the criteria of a neuronal pacemaker.

1.1.2.2 Silkmoth

The silkmoth was also a useful invertebrate model early on for understanding neuronal pacemakers. Like the cockroach, silkmoths are amenable to removing and re-implanting the brain, and although the implanted brain does not form new neural connections it is able to secrete hormones. A detailed study using the silkmoth shows that not only does their brain contain a pacemaker that synchronises eclosion rhythmicity with the environmental LD cycle, but the brain is also directly light sensitive (Truman & Riddiford 1970).

The eclosion behaviour, which describes adult silkmoths shedding their pupal skin, is under circadian control. Eclosion is gated by the environmental light dark cycle and will persist in free-running conditions, however ablation of the brain abolishes this gating and the moths emerge irrespective of time of day or night. Removal of the eyes and other tissues has no effect on eclosion. Whereas, implanting a brain into a brain-ablated animal restores entrainment and free-running behaviour. As the brain in these experiments was implanted into the abdomen, it can be deduced that the brain drives the rhythmicity of eclosion gating, and it does this by producing hormonal signals.

It was also demonstrated that the silkmoth brain contains the necessary photoreceptors to synchronise the eclosion rhythm with the environmental light cycle. Surgically putting the brain in the posterior end of the body resulted in photosensitivity moving with the brain to that region.

Using different species of silkmoth with different phases of eclosion timing for the transplantation experiments, the researchers were able to show the brain was sufficient to only convey timing information. The donor brain did not affect the individual emergence behaviour of the host, but did affect the timing of the behaviour. Thus the silkmoth brain or a region within it yet to be identified, fulfils all the criteria for a neuronal pacemaker.

1.1.2.3 Discovery of the mammalian SCN as the core clock

There are now numerous examples of neuronal pacemakers found in many species. The most intensively researched is the SCN in mammals. The SCN is part of the hypothalamus and located above the optic chiasm (Figure 11). The SCN in mammals has been referred to as the ‘core clock’, ‘master clock’, ‘master pacemaker’, ‘central clock’ and many other derivative terms.

The initial findings that hinted at the importance of the mammalian hypothalamus in circadian rhythmicity were produced by one of the founders of chronobiology, Curtis Richter (Richter 1978). Richter monitored the circadian rhythms of locomotor, feeding, and drinking behaviour in rats that had surgically ablated body regions including the pineal, pituitary, gonads, adrenals and hypothalamus. The lesion studies narrowed the search to show that the frontal part of the hypothalamus was essential for body rhythms, without it, although the rats would still exhibit feeding, drinking and locomotor behaviours, they were arrhythmic.

When the SCN in the hypothalamus is removed from rats, the circadian rhythmicity in locomotor and drinking behaviour is totally abolished (Stephan & Zucker 1972). In the same year it was shown that cutting the optic nerve before the optic chiasm abolishes adrenal coritcosterone rhythms, but a lesion after the optic chiasm does not destroy rhythms (Moore & Eichler 1972). This provided clues that a direct neural tract from the retina to the SCN was important for relaying light information to the SCN, which was soon identified using autoradiographic tracing and named the retinohypothalamic tract (Hendrickson, Wagoner, & Cowan 1972; Moore & Lenn 1972). However, it was still not yet known whether the SCN was sufficient to drive rhythms in other regions, or whether other regions were also required and if their contribution was abolished the SCN alone would not prevent arrhythmicity.

Electrophysiological *in vivo* studies showed the SCN, like other neuronal pacemakers, has a robust endogenous circadian firing pattern, high in the day and low at night (Inouye & Kawamura 1979). Neurons in brain regions outside the SCN were firing with an inverse rhythmic phase to those within the SCN. When the neural connections around the SCN were severed, the SCN continued to be rhythmic; however the other brain regions lost their rhythmicity. This showed how the endogenous rhythmic firing pattern in the SCN could drive patterns of firing in of other brain regions.

Other evidence to the strong rhythmicity in the SCN was provided by analysing the metabolic activity *in vivo* of the SCN (Schwartz, Davidsen, & Smith 1980). The SCN was more active during the light, non-active period, than the dark period of rats on a 12:12LD cycle, and this rhythmicity persisted in constant darkness. No other brain region has been shown to exhibit such a strong circadian rhythmicity in metabolic activity (Foster & Kreitzman 2005).

Perhaps the most definitive evidence that the mammalian SCN was the central pacemaker driving circadian synchronisation came from experiments performed using the *tau* mutant hamster (Ralph et al. 1990). The *tau* hamster has a shorter free running period of approximately 20 hours, which can be quantified by wheel running behaviour in constant darkness. The SCN from the *tau* hamster was surgically removed and replaced with the SCN from the wild type. The mutant hamster returned to having a normal period of 24 hours, whereas a wild type hamster containing the *tau* SCN had a period of 20 hours. This elegant experiment shows the SCN is necessary and sufficient to dictate period and phase timing to control locomotor rhythms.

More recent experiments have tried to describe how the SCN is able to maintain strong endogenous oscillations. The individual neurons are coupled, using vasoactive intestinal polypeptide (VIP) as a signal to synchronise rhythmic periodicity with each other (Aton & Herzog 2005). The intercellular coupling of SCN neurons has been modelled to show that even small decreases of the VIP-producing cells in the SCN can have dramatic effects of the period and amplitude of daily rhythms, which may be the scenario in aging (Vasalou, Herzog, & Henson 2009).

1.1.3 Multiple neuronal pacemakers

Many species have been found to have more than one neuronal pacemaker (Figure 2). In particular, these core clocks have been found in the pineal, deep brain photoreceptors and, in some instances, the retina (Underwood & Carolina 2009). Identifying these neuronal pacemakers and understanding how they interact to regulate circadian timing has been an important feature of circadian research.

Section 1.1.6 will detail further how our understanding of multiple clocks is increasing in the mammalian system. However, the focus of this project will be to further our understanding of the circadian timing system in the zebrafish vertebrate clock model. Therefore, this section will use a few examples to detail what is known about networks of neuronal pacemakers in other model species, such as birds, teleost, and invertebrates. This section here will emphasise how important it is to find regions within the brain that have robust endogenous rhythms, in order to determine the regulation of the circadian timing system.

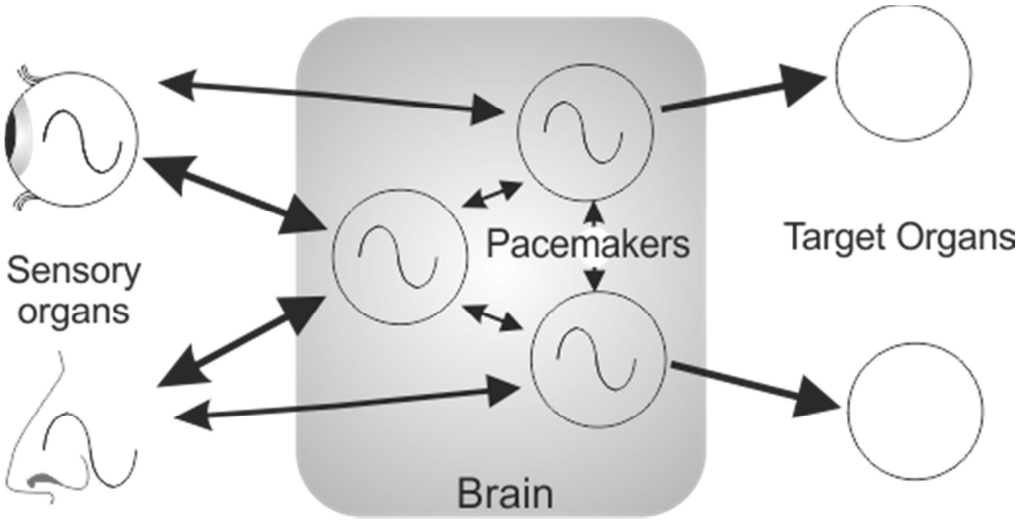


Figure 2 Circadian timing systems can have multiple neuronal pacemakers

Many species have been shown to have multiple neuronal pacemakers; these can receive input from various sensory organs and interact together to drive rhythmicity in peripheral tissues. Neuronal pacemakers can also feedback to the sensory organs to drive rhythms in sensitivity in these tissues.

1.1.3.1 Avian neuronal pacemakers

Circadian systems in birds are complex with multiple photoreceptors and multiple pacemakers, and wide variation between different species (Underwood, Steele, & Zivkovic 2001). The systems often represent a network of neuronal pacemakers that interact with each other to regulate circadian timing. Two avian clock models with different systems, outlined here, are the house sparrow and Japanese quail.

The pineal is a neuronal pacemaker in the house sparrow, *Passer domesticus*. Pinealectomised sparrows exhibit arrhythmic body temperature and locomotor activity in constant conditions, and transplantation of the pineal shows it can carry timing information to a recipient sparrow (Zimmerman & Menaker 1979). However, the circadian timing system in sparrows has a further complexity: pinealectomised sparrows can still entrain to a light dark cycle. Entrainment to LD cycles can also persist after removal of the eyes, although the threshold for entrainment by light usually increases, which demonstrates that eyes do input into the circadian system (McMillan, Keatts, & Menaker 1975). The SCN is located in the hypothalamus of birds, and when removed from sparrows these birds can no longer entrain to a light dark cycle. However, although the SCN in sparrows remains rhythmic shortly after the removal of pineal or eyes, it will become

arrhythmic, and therefore the SCN is not considered as a true pacemaker in the sparrow, but part of the photosensitivity of the circadian regulation system (Underwood et al. 2001). This highlights the importance of a robust endogenous rhythm as a criterion in the identification of neuronal pacemakers.

In the Japanese quail, unlike the sparrow, the pineal is not considered a neuronal pacemaker as it is not endogenously rhythmic. Instead, the central circadian pacemakers are found in each eye. The eyes produce robust rhythms of melatonin secretion both *in vivo* and *in vitro*, and this can be entrained by light dark cyles. Removal of the eyes produces arrhythmia in locomotor activity and thermoregulation in constant darkness (DD) (Underwood 1994). Patching each eye alternately for twelve hours in constant light (LL) can cause the melatonin rhythm in each eye to go out of phase with the other (Steele et al. 2003). However, under normal conditions these bilaterally located pacemakers are able to couple and produce hormonal and neural outputs to regulate the circadian system throughout the animal.

1.1.3.2 Teleost neuronal pacemakers

The organisation of the circadian system has not been as well as characterised in teleosts as in other species. Much historical research in teleost has focussed on the pineal and its role in producing melatonin and the subsequent seasonal timing of reproduction. This has led some researchers to conclude that the pineal can act as a neuronal pacemaker.

A potential neuronal pacemaker has been described in the pike, *Esox lucius*. Photoreceptor cells in the pineal of the pike appear to fulfil all the necessary criteria to be considered a neuronal pacemaker (Bolliet et al. 1997). Cultures of these cells are able to entrain to a LD cycle and have a free-running period of up to 27 hours in DD, as measured by melatonin secretion. The variability of the free-running period between cultures was very low suggesting that the cells either have a very similar endogenous oscillation and/or are strongly coupled, a characteristic of the mammalian SCN. However, the effect of pinealectomy on the pike has not yet been investigated, nor whether transplantation of pineals can carry the donor endogenous rhythms. Further investigation is required for a more complete understanding of the role of the pineal in the circadian timing system in the pike.

The role of the pineal has also been investigated in goldfish, *Carassius auratus* (Kezuka 1992). Under LD the circulating plasma melatonin levels peak during the middle of the dark period. In DD, this rhythmicity persists in a circadian manner for 3 days before dampening but remaining high. In LL there was no rhythmicity and levels remained low. Pinealectomy abolishes the high levels of melatonin at mid-dark, whereas removal of the eyes has little effect. However, melatonin rhythms still persisted in pinealectomised goldfish, albeit at a very low-level and a different phase. This shows the importance of the pineal in secreting rhythms of circulating melatonin, but suggests other pacemakers are also important in regulating circadian outputs.

1.1.3.3 Invertebrate neuronal pacemakers

Invertebrates often have multiple pacemaker structures in their brain. One of the most widely studied invertebrate circadian timing systems is in the fruit fly *Drosophila melanogaster*, this will be further discussed in section 1.1.7. This section will look at experiments on the marine snail, *Aplysia californica,* to demonstrate how evidence can arise for multiple pacemakers.

A neuronal pacemaker was pinpointed to the basal retinal neurons in *Aplysia* (Jacklet 1969), these are interneurons in the optic nerves found close to the retinal photoreceptors. The basal retinal neurons fire action potentials to signal rhythmic information to other areas of the brain and drive rhythms in locomotion. In culture the firing pattern of these interneurons endogenously oscillates, free-runs in constant conditions and can be synchronised to LD cycles. Removal of the eyes reduces the amplitude of locomotor activity, however it did not stop the free-running locomotor rhythms in all specimens tested. In addition, photoreception is not exclusive to the eyes, with eyeless *Aplysia* responding to photic input (Lickey et al. 1977). Furthermore, in culture, the two eyes will eventually become out of phase with each other. Although the eyes are able to fulfil the criteria of a neuronal pacemaker, it is likely an additional oscillatory pacemaker structure exists outside of the eyes, and a network of multiple pacemakers exist to drive locomotor rhythmicity (Jordan, Lickey, & Hiaasen 1985; Lickey et al. 1977), however these have yet to be identified.

1.1.4 Clock genes

In the 1960s when the scientific principles for circadian rhythms were being elucidated, little was known about the genetic basis for clocks. Since then there have been numerous molecular

discoveries in many species identifying key clock genes, those which produce proteins that are fundamental for the generation and co-ordination of cellular rhythms.

Mutagenesis screens revealed genes that alter or abolish circadian rhythms, mainly circadian period, in mutants. The first clock phenotype, *period*, was described in *Drosophila* in 1972 (Konopka & Benzer 1971). In the 1990s there was a rapid number of discoveries, including the first mammalian circadian gene, *Clock (Circadian Locomotor Output Cycles Kaput),* which was identified by Takahashi and colleagues (Antoch et al. 1997). The identification of these numerous genes showed a considerable conservation between different species. This homology allowed genes identified in one system to be rapidly isolated in another.

The characterisation of the genes and protein products brought about a model of time-delayed negative transcription and translation feedback loops. In the mammalian system, the main negative feedback loop (Figure 3) involves the positive transcription regulators CLOCK and BMAL, which heterodimerize and bind to E-boxes (CACGTG) in the promoter sequences of the negative transcription regulators, *period* (*per*) and *cryptochrome* (*cry*). This initiates the transcription of *per* and *cry* genes, and their protein products accumulate in the cytoplasm. PER and CRY reach a critical number and translocate back into the nucleus where they repress the CLOCK:BMAL heterodimer from forming and stop their own transcription.

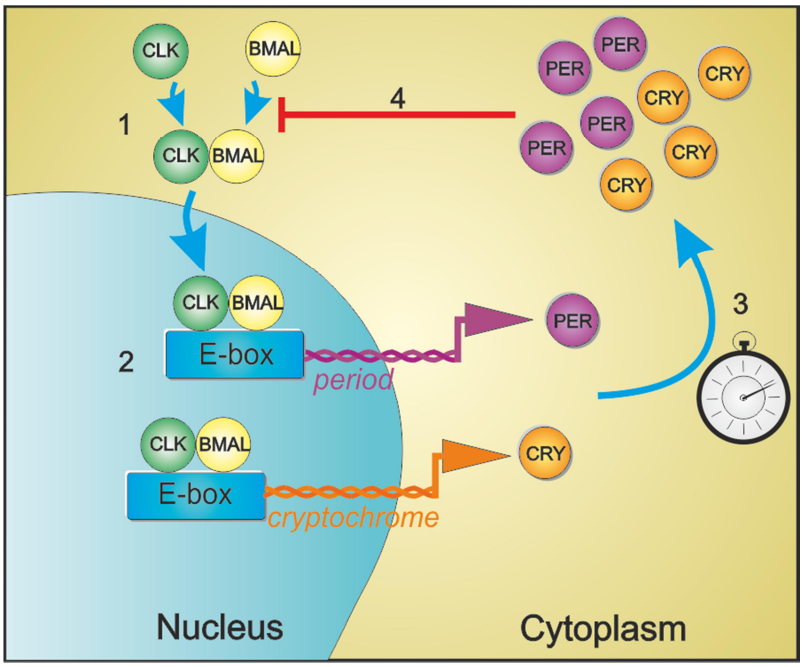


Figure 3 The circadian molecular clock

This is a simple schematic of one of the main loops of the circadian molecular clock. **1.** CLOCK and BMAL form a heterodimer and enter the nucleus. **2.** This heterodimer binds to E-boxes to activate the synthesis of PER and CRY. **3.** PER and CRY are negative transcriptional regulators, they accumulate in the cytoplasm causing a time-delay. This time-delay determines the period length of the cycle, approximately 24 hours. **4.** Once phosphorylated and reaching an optimal concentration the PER and CRY proteins enter the nucleus and prevent CLOCK and BMAL dimerizing, thus stopping their own activation.

There are also multiple feedback loops to maintain robustness to the system, preventing fluctuations. In the mammalian system a secondary loop involves CLOCK:BMAL upregulating *Rev-erb* and *Ror* genes, whose protein products affect the transcription of *bmal*.

Clock genes are able to regulate the timing of other genes, named clock-controlled genes or circadian output genes. These genes are dependent on circadian oscillations to time their expression to specific phase of the day or night. Approximately 8-10% of the mammalian genome is thought to be temporally controlled by the circadian clock (Lowrey & Takahashi 2004). Often, these genes are key rate limiting enzymes in metabolic processes, such as the cytochrome P450 liver enzymes (Rutter, Reick, & McKnight 2002), but a wide range of biological processes appear to be under clock control.

The elucidation of the molecular clock has advanced the understanding of circadian regulation and provided a tool to study the inner workings of circadian rhythms within cells and tissues.

1.1.5 Peripheral clocks

The identification of clock genes led to a renewed investigation of circadian oscillations in peripheral tissues. Investigating the expression of *period* in the thorax and abdominal tissues of *Drosophila* led to the discovery that circadian oscillators are present throughout the body (Plautz et al. 1997) (Figure 4). This was soon followed by the finding that oscillations in mammalian *per1* and *per2* clock genes could be induced in cultured rat fibroblasts with a high concentration exposure to serum (Balsalobre, Damiola, & Schibler 1998). In 2000, it was shown that the liver, lung, and skeletal muscle from a *per1-luciferase* rat will continue to express oscillations in clock genes in culture for several days, albeit with the free-running rhythms from these peripheral tissues dampening much faster than those from the SCN (Yamazaki et al. 2000).

The finding that peripheral tissues could contain endogenous circadian oscillations echoed results from many experiments that were carried out before the discovery of the mammalian SCN and then largely ignored. For example, it was known in the 1970s that corticosteroid secretion from rodent adrenal glands remains rhythmic in culture (Andrews 1971).

A key difference between peripheral clocks and pacemakers is the ability to drive rhythms. Transplantation of a pacemaker tissue will set the phase and the period of rhythms in the host organism to that of the donor pacemaker tissue, whilst the transplantation of peripheral clocks will not affect the rhythmicity of the host organism. This has led many mammalian circadian biologists viewing the SCN as a pacemaker at ‘the top of the hierarchy’ as a ‘master’ clock instructing ‘slave’ peripheral oscillators (Yamazaki et al. 2000).

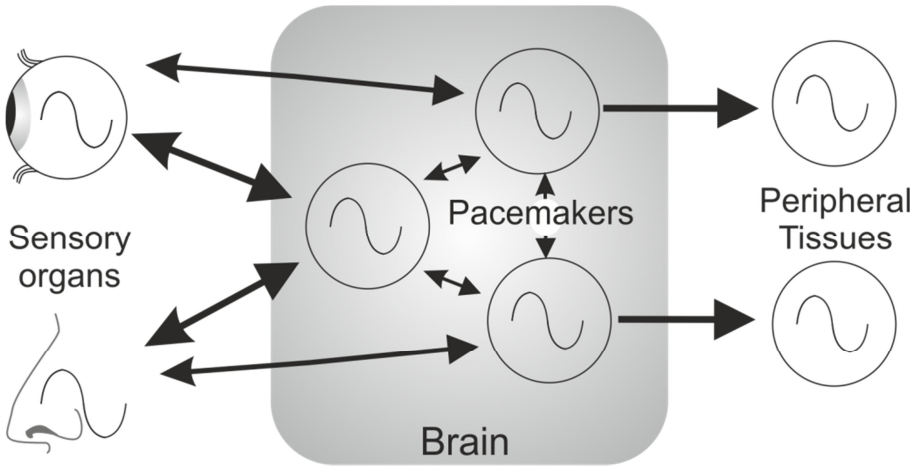


Figure 4 Peripheral clocks

Oscillations in clock genes can be found in peripheral tissues; however cell cultures of peripheral tissues demonstrate rapid dampening. Neuronal pacemakers drive rhythmicity in these tissues by synchronising the cellular clock rhythms.

1.1.6 Another neuronal pacemaker in the mammal

The olfactory bulb (OB) has been shown to be another discrete neuronal pacemaker outside of the SCN in mammals. The OB is able to fulfil all the criteria of a core clock: it can be entrained by exogenous stimuli, it has an endogenous self-sustaining rhythm, and it is able to drive rhythms in other tissues.

A key experiment showing the OB as a neuronal pacemaker showed that brain regions outside of the SCN were able to produce robust rhythms in *per1* expression. This initial work using *per1-luciferase* rat cultures showed that the OB, out of thirteen other brain areas examined, was able to remain rhythmic *in vitro* (Abe et al. 2002). The OB maintains a strong rhythmicity of *per1* expression even 21 days after the SCN has been ablated and behavioural rhythms are absent (Granados-Fuentes, Prolo, et al. 2004). This shows that the OB is able to maintain endogenous rhythmicity independently without connections to the SCN. The circadian period of *per1* rhythmicity in the OB was also temperature compensated (Granados-Fuentes, Saxena, et al. 2004).

The OB can be entrained by environmental temperature cycles. However, the OB could only entrain to LD cycles when connected to the SCN (Abraham et al. 2005). As temperature cycles

are influenced by the SCN *in vivo*, and the SCN response to light can be modulated by the OB sensing olfactory signals (Amir et al. 1999), it is likely that the SCN and OB are able to feedback to each other and affect the generation of rhythms.

Finally, it was shown that the OB was able to drive rhythms in the piriform cortex in mice (Granados-Fuentes, Tseng, & Herzog 2006). The piriform cortex was no longer able to produce circadian rhythms in odour-evoked c-Fos expression when the OB was ablated. Moreover, the connection between the tissues is lateralized: when the left olfactory bulb was removed the rhythm in the left piriform cortex was abolished, but the rhythm in the right piriform cortex persisted unchanged.

The mammalian circadian system must now be viewed as a network with multiple oscillators. It has been suggested that due to the focus on clock outputs there may be more neuronal pacemakers in mammals that have been overlooked (Herzog 2007). This opens up an interesting research area to understand the function of a more networked circadian timing system.

1.1.7 A decentralised clock model

The fruit fly *Drosophila melanogaster* is a popular model for understanding the molecular and genetic bases of circadian regulation. Numerous central pacemakers have been identified including lateral and dorsal neuron populations. These are directly light-sensitive, are able to oscillate in culture, and drive numerous output rhythms, including locomotor activity and eclosion (Herzog 2007). Transplantation of the brain of a short-period mutant fly into an arrhythmic *per1* null fly can establish locomotor rhythmicity with a short-period to the host (Handler & Konopka 1979).

However, studies of the interaction of peripheral clocks with the pacemaker structures in *Drosophila* are revealing that many of peripheral tissues have autonomous circadian oscillators (Figure 5). Many peripheral tissues are light-sensitive (Plautz et al. 1997), entraining directly to environmental cues and over-riding signals from the pacemakers (Giebultowicz 2001). Furthermore, the circadian gene expression in these peripheral cells can oscillate with the same

or earlier phase as those found in the pacemaker structure, which would suggest they are not 'following the lead' from pacemakers.

Therefore, the current model for the circadian system in *Drosophila* is described as decentralised (Figure 5), with peripheral tissues autonomously regulating local clock function and not requiring a 'master' neuronal pacemaker in order to be rhythmic.

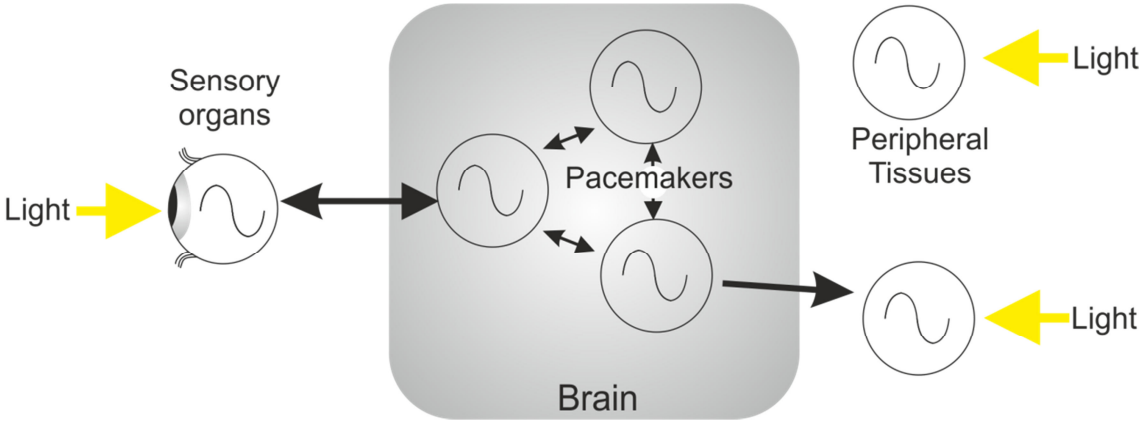


Figure 5 A decentralised circadian timing system

Decentralised clocks are now being described in some models such as *Drosophila*. Whilst neuronal pacemakers are able to drive rhythmicity in some peripheral tissues, other peripheral tissues can be entrained directly by light and other Zeitgebers, and this direct entrainment can promote independence and override signals from pacemakers.

1.1.8 The changing definition of a 'clock'

The clock is a useful metaphor for describing the endogenous daily cycles in biological timekeeping. The complexity of 'the clock' is becoming increasingly more apparent. Circadian timing systems often incorporate a network of multiple oscillators as well as numerous inputs, outputs and feedback loops (Figure 5). The central pacemaker's ability to have robust endogenous oscillations that can drive rhythms in other regions maintains their distinction from peripheral oscillators. However, with peripheral oscillators able to function autonomously, a 'hierarchy' in the system is becoming harder to define.

The next section will explore the circadian timing system in the zebrafish, which will be the focus of this project. Whilst there is evidence to suggest that this fish is decentralised like the *Drosophila*, very little is known about the neuronal regulation of the zebrafish circadian timing system.

1.2 Zebrafish

Zebrafish, *Danio Rerio,* are found in the wild in the freshwater steams and slow-moving waters in South and East Asia. Zebrafish are one of 20,000 fish species in the teleost group. This group includes most living fish today and although the zebrafish cannot entirely represent this vast and diverse group it has many uses as a vertebrate model. George Streisinger pioneered the zebrafish model in the 1950s and since then the zebrafish has become the second most commonly used animal model in the UK, only second to mice.

Initially, the zebrafish was a popular developmental model due to its rapid *ex vivo* development (Figure 6). However, zebrafish have many other advantages including their amenability to genetic manipulation and the large numbers that can be grown quickly and inexpensively. Zebrafish are now commonly used to study many other aspects of biology, including cancer, toxicology, and regeneration.

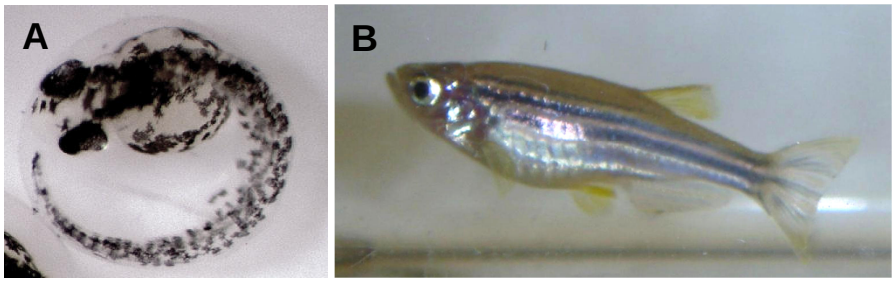


Figure 6 *Danio Rerio*

Zebrafish are a popular developmental model due to their rapid *ex vivo* development and transparency. **A)** 72 hours post fertilisation (hpf) unhatched larvae in chorion. **B)** Adult wild type (AB) 6 months.

1.2.1 Zebrafish as a clock model

Zebrafish are an excellent vertebrate model for understanding circadian rhythmicity and light sensitivity. This section will outline what is currently known about the circadian system in zebrafish, which only started to be investigated in 1996 (Cahill 1996). Comparisons will be made to other teleosts, which are also starting to be investigated.

Early work revealed that circadian oscillations of clock genes are found in the brain, pineal, eyes and peripheral tissues (Whitmore et al. 1998). Further investigation revealed that the circadian oscillators in their peripheral tissues can be directly reset by light (Whitmore, Foulkes, & Sassone-Corsi 2000). In this study, wild type zebrafish were dissected, from the same group of fish at the same time, and their hearts and kidneys cultured in LD and DL cycles in 'side-by-side' incubators. The cultured tissues rapidly entrained to the LD cycle they were exposed to. Consequently, these tissues must contain the photopigments and signalling pathways essential for resetting their circadian oscillators. These experiments were the first to reveal light-responsive peripheral clocks in a vertebrate model, showing the similarity between zebrafish and *Drosophila*, which were shown to have light-responsive peripheral clocks only a few years earlier (Plautz et al. 1997).

Later experiments using *per3-luciferase* zebrafish revealed other peripheral organs, including the gall bladder and spleen, have robust free-running rhythms in culture and can be directly entrained to the light dark cycle (Kaneko, Hernandez-Borsetti, & Cahill 2006). Embryonic cell lines were also able to retain this light responsive characteristic (Whitmore et al. 2000), leading to the hypothesis that all cells and tissues in the zebrafish have clocks that are directly entrainable by light.

Single cell imaging of a zebrafish cell line revealed that even after several months in constant conditions, zebrafish cells can continue to oscillate, albeit at different periods and out of phase with each other (Carr & Whitmore 2005). A short 15-minute light pulse was sufficient to rapidly reset the population rhythms, leading the authors to suggest that, as light plays a dominant role, there may have been little selective pressure to create a molecular clock in the zebrafish with precise and robust free-running periods.

There are other Zeitgebers that can influence the phase of the zebrafish clock, such as temperature and feeding. As light is thought to be the dominant Zeitgeber, and as most of the zebrafish body is light-sensitive, this is the primary starting point for manipulating the clock. It will likely be beyond the scope of this project to investigate other Zeitgebers, and instead their timing and intensity will be kept constant.

Having autonomous light-sensitive peripheral clocks means the zebrafish may represent a decentralised system, like the *Drosophila* clock model, which does not require a core clock for synchronisation. However, it is not known whether all the cells in the zebrafish are truly independent or whether they 'follow the lead' of pacemaker structures as part of a circadian pacemaker hierarchy. Indeed, recent investigation into the pineal by Li et al (2012), which will be explained in detail further on, suggests that neuronal pacemakers do exist in the zebrafish and can be used to regulate activity rhythms in peripheral tissues.

1.2.1.1 Teleost clock genes

Zebrafish *clock* was the first teleost circadian gene cloned (Whitmore et al. 1998), followed a few years later by *period* (Delaunay et al. 2000). So far in the zebrafish, there have been three *clock*, three *bmal*, four *period (per)*, and six *cryptochrome (cry)* genes cloned (Hirayama 2003; Vallone & Gondi 2004). The zebrafish seems to share many of the same clock genes as many other species including mammals. However, there are multiple replications of genes, which may represent the complex fine-tuning of the zebrafish circadian timing system to produce subtle levels of control and regulation.

Zebrafish share the same c*lock, bmal, per* and *cry* loop, described in Figure 3, as mammals and other species. However, some of the clock genes, c*ry1a* and *per2,* are directly light responsive clock genes (Figure 7). Light is able to upregulate these repressor transcription factors, and in constant light the oscillations of the molecular clock "stops". Zebrafish also have a 'stabilising loop' with REV-ERBα and RORa fine-tuning the rhythmic oscillations of CLOCK and BMAL (Vatine et al. 2011).

More recently, research has begun analysing the molecular bases of clocks in a wide range of other teleosts. The goldfish, *Carassius auratus*, is in the cypriniform order like zebrafish and therefore closely related to zebrafish. Goldfish express *per1-3* and *cry1-3* and these are 90% homologous to the zebrafish clock genes and are rhythmic in central and peripheral organs on a LD cycle (Velarde et al. 2009).

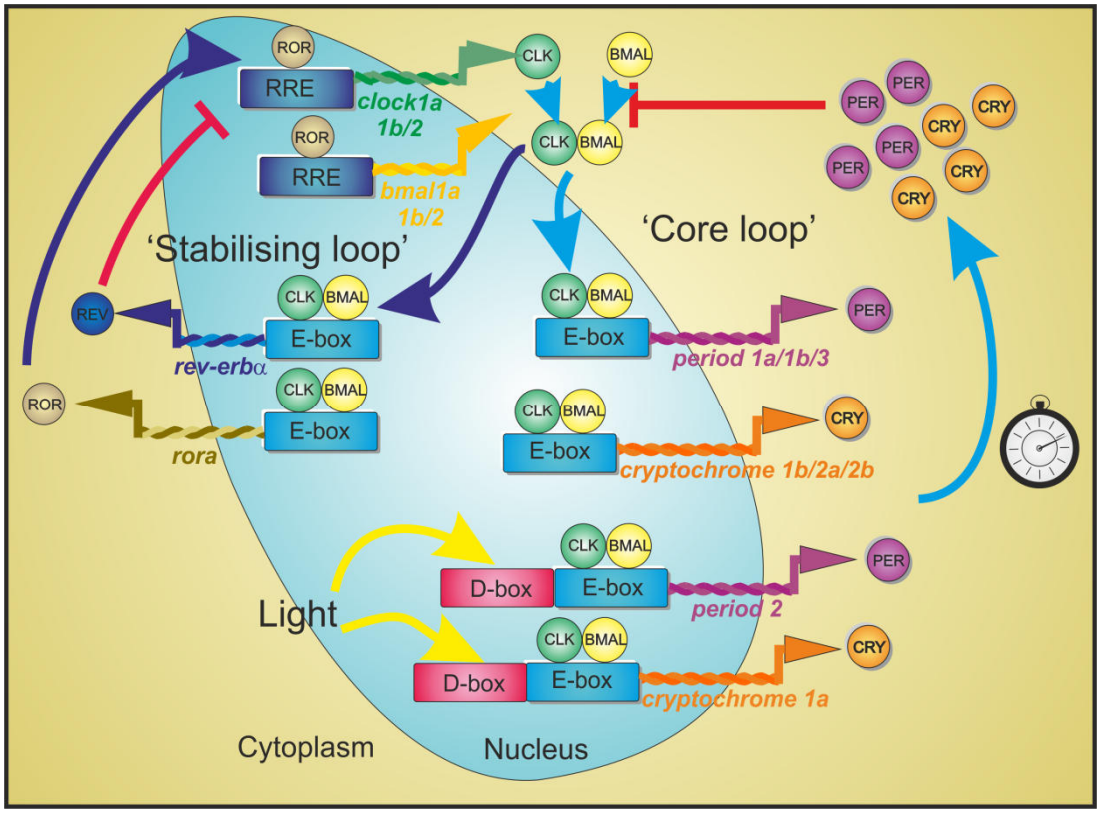


Figure 7 The zebrafish molecular clock

The zebrafish has multiple replications of circadian genes. Zebrafish share the same core molecular loop with c*lock, bmal, per* and *cry*, as mammals, and a stabilising loop with *rev-erbα* and *rora*. CLOCK and BMAL form a heterodimer and enter the nucleus to bind to E-boxes. In the core loop these activate the transcription of *period1a/1b/3* and *cryptochrome1b/2a/2b*, which accumulate in the cytoplasm and stop the CLOCK:BMAL complex forming. In the stabilising loop the CLOCK:BMAL heterodimer binds to E-boxes to activate the transcription of *rev-erbα* and *rora*. These compete to bind to retinoic acid-related orphan receptor response elements (RRE) in the promoters of *clock1a/1b/2* and *bmal1a/1b/2*. ROR activates transcription, whereas REV-ERBα represses the transcription, of the *clock* and *bmal* genes. Additionally, light can regulate the expression of *period2* and *cryptorchrome1a,* which both contain D-box sequences in their promoter that allows light to directly activate their expression.

The first examination of the clock gene *per* in a marine fish was done in golden rabbitfish, *Siganus guttatus*, (Park et al. 2007). The PER protein in this reef fish is highly homologous to PER1 in zebrafish, and its expression in LD is also similar. In the rabbitfish *per* mRNA was found in both neural and peripheral tissues, and was rhythmic in cultured whole brain and pineal gland under LD. Another marine fish, the European sea bass, *Dicentrarchus labrax*, also expresses *per1* throughout many peripheral and central organs (Sánchez, Madrid, & Sánchez-Vázquez 2010). The common coral reef fish, the threespot wrasse, *Halichoeres trimaculatus*, has circadian expression of *per1*, *per2*, *bmal1* and *cry1* mRNA (Hur et al. 2012). The rhythmicity of these genes corresponded with melatonin release, locomotor activity and night-time burying behaviour.

The flatfish Senegalese sole, *Solea senegalensis,* is a nocturnal fish that has recently been investigated for expression of circadian genes *per1*, *per2, per3* and c*lock* (Martín-Robles et al. 2011, 2012). The genes were expressed in all the tissues tested, which included the brain and liver. The phase of *per3* on a LD cycle is similar to that in zebrafish, and will be discussed in more detail in chapter 3. However, the phases of *per1* and *clock* are opposite to the expression seen in zebrafish, which may explain the nocturnal nature of this fish.

A key part of this project will be to investigate the expression of clock genes in the zebrafish in different tissues and under different conditions. Monitoring the expression of clock genes will be vital to describe the nature of molecular oscillations, for example, whether tissues have robust circadian oscillations, or are directly light sensitive.

1.2.1.2 Circadian output rhythms in zebrafish

There are many outputs of the circadian clock that have been recognised in the zebrafish, which are summarised in Figure 8. One of the first observations of a circadian output in the zebrafish was an increase in the secretion of the hormone melatonin from the retina and pineal during the night (Cahill 1996).

Studies have also shown a link between the circadian clock and cell cycle rhythms; the zebrafish cell cycle is regulated by light with phases appearing to be under circadian control. Mitosis increases at around 6am in zebrafish embryos and cell lines, with S-phase occurring in

the early night (Tamai et al. 2004). This mechanism is thought to have evolved to protect replicating DNA from the harsh UV rays during the middle of the day.

During the night larval zebrafish lose visual sensitivity, this has been suggested to be due to the zebrafish utilizing its energy efficiently (Emran et al. 2010). Adult zebrafish also have a reduced sensitivity to light in their rod and cone photoreceptors in the early morning before lights on. This rhythm persists in constant conditions and is considered to be driven by an endogenous clock (Li & Dowling 1998). During the day zebrafish produce more interphotoreceptor retinoid-binding protein (IRBP), which helps the retina adapt to environmental lighting being involved in the regeneration of active photopigments (Rajendran et al. 1996).

Zebrafish are diurnal animals, like humans, preferring to sleep during the night. Many of the key components in sleep regulation in mammals are conserved in zebrafish, including many neural regions and response to hypnotic drugs. Zebrafish alternate between bursts of activity and inactivity during the day and night, with more frequent and longer bouts of inactivity at night. On a 14:10LD wild type zebrafish will spend 60-70% of the night in these inactive sleep bouts. The periods of inactivity can be as short as 4s during the day but are typically 45s during the night. Zebrafish can be observed sleeping in these periods of inactivity by a drooping caudal fin and a higher threshold to arousal stimuli. Constant light, even at moderate a level, is able to bypass normal homeostatic sleep regulation and suppress sleep and circadian activity rhythms in zebrafish (Yokogawa et al. 2007). This complements the finding that constant light can ‘stop’ the molecular clock (Tamai, Young, & Whitmore 2007).

The rhythmic change in the pattern of neuronal firing is one characteristic output of a neuronal pacemaker. It has not yet been demonstrated whether neuronal firing is under circadian regulation in any region of the zebrafish brain, and this project will begin the investigations into this. Can a neuronal pacemaker be identified in the zebrafish brain by its neuronal firing characteristics?

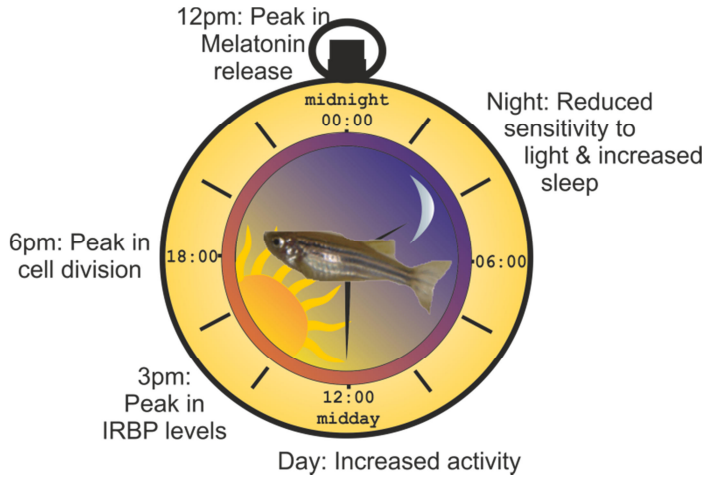


Figure 8 Outputs of the Zebrafish Clock

There are many parts of the zebrafish physiology which are under regulation by the circadian clock. This schematic summarizes a few of them.
[IRBP, interphotoreceptor retinoid-binding protein]

1.2.1.3 Circadian outputs in other teleosts

Studies investigating circadian outputs in other teleosts have mostly focussed on locomotor and feeding activity. These studies have revealed that whilst some teleost species are diurnal, like the zebrafish and pike, and some are nocturnal, like the Senegalese sole, many others exhibit both diurnal and nocturnal behaviours. The locomotor and feeding activity rhythms in these teleosts are usually governed by food availability, thermal preference and predator-prey relationships.

The European sea bass is mostly diurnal during the summer and nocturnal during winter, although there are individual differences within populations studied (Sánchez-Vázquez et al. 1998). The sharpsnout seabream, *Diplodus puntazzo*, has a mixture of both diurnal and nocturnal locomotor activity rhythms, which were observed spontaneously switching during the experimental trials (Vera, Madrid, & Sánchez-Vázquez 2006). Individuals within a population of Nile tilapia, *Oreochromis niloticus*, also exhibit arrhythmic behaviour alongside both diurnal and nocturnal locomotor activity patterns on an LD cycle (Vera et al. 2009).

1.3 The organisation of the circadian system in the zebrafish brain

As the zebrafish has directly light responsive tissues, it has been hypothesised that the teleost circadian system is highly decentralized similar to that found in *Drosophila*. A large focus of circadian research on zebrafish has been dedicated to these peripheral tissues, and very little on whether there are neuronal pacemakers. Recently, however, research by Li *et al* (2012) has shown that the zebrafish pineal can act as a neuronal pacemaker, suggesting that the zebrafish system may also have a network of multioscillators, with pacemakers that are yet to be identified.

Firstly, this section will briefly outline what is known about the zebrafish brain, and our current limited understanding of the neural regulation of the circadian system. It will then describe the techniques and strategies that could be employed to gain a greater understanding of the circadian system with in the zebrafish brain.

1.3.1 The zebrafish brain

The general organisation of the zebrafish central nervous system bears great resemblance to that of other vertebrates, with the same basic subdivisions that are found in higher vertebrates including mammals (Bally-Cuif & Vernier 2010). These subdivisions are show in Figure 9 and will be briefly outlined, using details from the zebrafish neuroanatomical atlas (Wullimann, Rupp, & Reichert 1996).

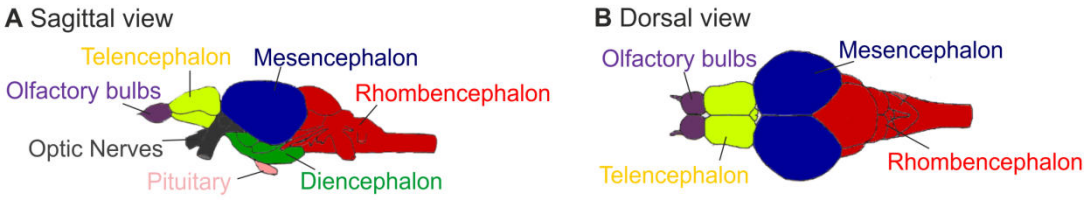


Figure 9 The main subdivisions of the zebrafish brain

The presencephalon, or forebrain, is comprised of olfactory bulbs, the telencephalon and the diencephalon. Histologically, the telencephalon can be further subdivided into the pallium, also referred to as the dorsal telencephalic area, and the subpallium, or ventral telencephalic area. The diencephalon contains the epithalamus, thalamus, hypothalamus, pretectum, and optic

nerves. The pineal protrudes dorsally as part as the epithalamus. The pituitary gland is attached under the hypothalamus by the delicate infundibular stalk.

The mesencephalon, or midbrain, can be separated into the tectum, tegmentum and isthmus. The optic tectum is homolog to the mammalian superior colliculu; it is a multisensory integrated region that receives visual inputs. Like other teleosts, the zebrafish tectum is in several layers composed with different cells. One of the most distinct layers, the periventricular grey zone (PGZ) layer, is densely packed with piriform neurons with small cell bodies.

The rhombencephalon, or hindbrain, contains the cerebellum, medulla, vagal lobe, and numerous cranial nerves.

1.3.1.1 The zebrafish visual system

The zebrafish visual system contains a direct retino-thalamofugal tract and an indirect retino-tecto-thalamofugal system, both of which terminate in the ventral telencephalic area (Bally-Cuif & Vernier 2010). In the direct retino-thalamofugal tract, stimulus from the retina directly activates neurons in the thalamic nuclei, these neurons project to the medial zone of the dorsal telencephalic area (Dm) which projects to dorsal regions in the ventral telencephalic area (Vd). In the indirect route, light stimulus through the retina activates neurons in the PGZ of the optic tectum, which then activates the preglomerular nucleus (PG). The PG activates the Dm, which projects to the Vd. Numerous other brain nuclei have also been reported to have retinofugal projections in the teleost, including the lateral hypothalamus (LH), parovocellular preoptic nucleus (PP), SCN, torus longitudinalis (TL), and lateral division of the valvula cerebelli (Val) (Northcutt & Wullimann 1987).

1.3.1.2 Neurogenesis

The zebrafish is becoming a popular model for regeneration and the zebrafish brain has a large capacity for neurogenesis during adulthood (Adolf et al. 2006; Zupanc, Hinsch, & Gage 2005). Proliferation zones are located throughout the brain in specific regions, including the OB, dorsal telencephalon (D), preoptic area, dorsal zone of the periventricular hypothalamus (Hd), TL, vagal lobe (X), medulla oblongata, and the cerebellum.

In terms of finding a neuronal pacemaker, the high plasticity of the zebrafish brain can be problematic for monitoring the effects of long-term lesion studies. The lesions soon heal and

prevent study of the effect of the outputs on a putative circadian pacemaker. However, the brains ability to regenerate and form new neural connections may make transplantation experiments possible, and if a prospective neuronal pacemaker were to be found, it could be tested to see whether it was able to drive rhythmicity in a host.

1.3.2 Zebrafish neuronal pacemakers

The zebrafish circadian system has been described as a decentralised system with all cells capable of being entrained by light. However, the brain has never before been examined in detail. This next section will describe some of the circadian characteristics of putative neuronal pacemakers in the zebrafish: the pineal, retina, and SCN. There could be other sites of neuronal pacemakers, and this section will be followed by a description of how these areas could be located.

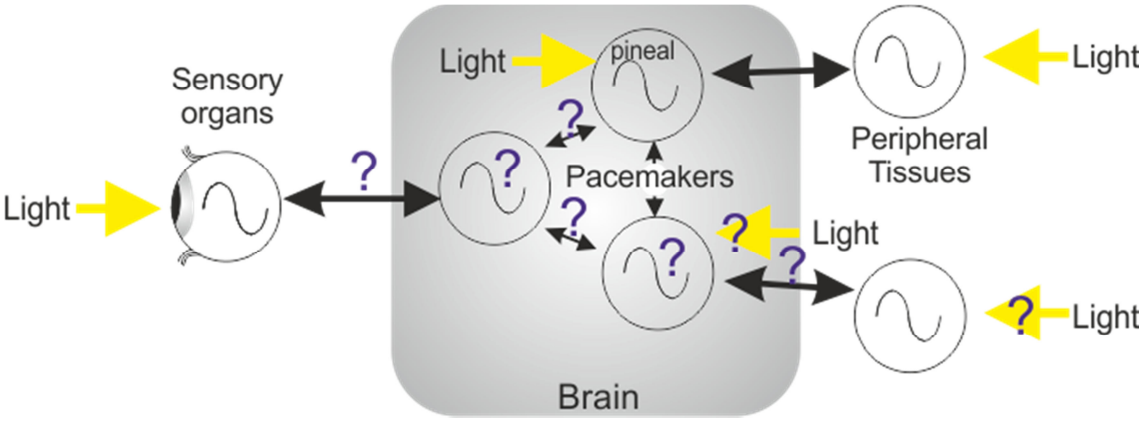


Figure 10 The unknowns in the zebrafish circadian timing system

There are many unknown factors in the zebrafish circadian timing system. Light has been shown to directly set the timing of molecular clocks in peripheral tissues; however whether this happens to all cells is unknown. The pineal has been identified as a neuronal pacemaker, although other pacemakers may exist. The pineal is directly light sensitive, however whether other neuronal pacemakers are, or whether they rely on signals from light sensitive tissues, remains to be elucidated. The mechanism of how neuronal pacemakers interact with the different peripheral tissues, with each other, and with sensory organs such as the eyes also remains to be established. This project will seek to explore a few of these unknowns.

1.3.2.1 The zebrafish pineal is a neuronal pacemaker

As described earlier, the photosensitive cells in the pike pineal have demonstrated to be autonomous circadian oscillators, containing all the necessary criteria to be considered a neuronal pacemaker (Bolliet et al. 1997). Evidence has also been accumulating that these pineal cells also regulate circadian rhythms in the zebrafish.

Cultures of the zebrafish pineal directly entrain to light (Cahill 1996), and produce a strong endogenous rhythm of melatonin release. In the zebrafish, melatonin can act as a sleep-promoting agent, and regulate circadian rhythmicity of sleep in constant conditions in aged zebrafish (Zhdanova, Yu, & Lopez-Patino 2008). Aged zebrafish (4-5 years) have a reduced expression of core clock genes, lower production of melatonin, reduced sleep-time, and a reduction in the daily amplitude of locomotor activity. Regular night time administration of melatonin to these aged zebrafish, in constant dim light conditions, was able to promote sleep and restore a circadian locomotor rhythm.

A recent study provided more firm evidence that the zebrafish pineal is a neuronal pacemaker (Li et al. 2012). By selectively ablating the photosensitive cells in the pineal, the researchers showed that circadian rhythms in locomotor activity were abolished, and the circadian rhythms in retinal photoreceptor sensitivity were reduced. The visual sensitivity of the pineal-cell-ablated zebrafish was unaltered in LD conditions, however the rhythms were dampened in DD, suggesting that retinal photoreceptors are able to entrain to light, but do not have a strong endogenous oscillator and instead receive driving signals from the pineal in constant conditions.

As the zebrafish pineal is directly entrainable by light, has strong endogenous oscillations in constant conditions, and is able to entrain rhythms in other tissues, it is likely that the pineal is a central neuronal pacemaker in the zebrafish. Transplantation experiments using clock mutant zebrafish will be crucial to the final evidence to show the pineal can carry the donor's endogenous rhythmicity into a host.

1.3.2.2 The retina is unlikely to be a pacemaker

The zebrafish retina is directly light sensitive, however it is unlikely to be a pacemaker as it does not appear to have a strong endogenous rhythm, nor able to drive rhythms in other tissues.

Like the pineal, the retina has been shown to produce melatonin in the zebrafish. Contrastingly however, in constant conditions the retinal release of melatonin rapidly dampens in culture conditions (Cahill 1996). It is important to note though, retina is notoriously difficult to maintain in culture, and so one cannot exclude cell death as adding to this rapid dampening.

A functional retina was not required for rhythms in cell division, or for normal clock gene expression, in the blind mutant zebrafish *lakritz* (Dickmeis et al. 2007). Additionally, there is evidence to indicate that the eyes are not important for circadian entrainment in the developing embryo: in the gastrulation stage where the eyes have not yet developed, there is an acute light response in the induction of *per2* expression (Dekens & Whitmore 2008; Tamai et al. 2004). Therefore, the retina is unlikely to be an autonomous driver for circadian rhythms.

1.3.2.3 The role of the SCN in zebrafish

There is a region of the zebrafish brain that has been anatomically defined as the SCN (Figure 11). However, whether the SCN has the same regulatory role in circadian biology as it does in mammals has not been established.

In fact, there is evidence to suggest that the SCN may not have a central role in the circadian clock in zebrafish as it does in mammal. The period and phase of pineal rhythms of melatonin are normal in the *cyclops* (*cyc*) mutant zebrafish, which lack ventral brain structures including the SCN (Noche et al. 2011). However, the amplitude of the melatonin rhythm is reduced in *cyc*, and this raises the possibility that the SCN, or another ventral brain structure, is contributing to the regulation of rhythmicity in the pineal.

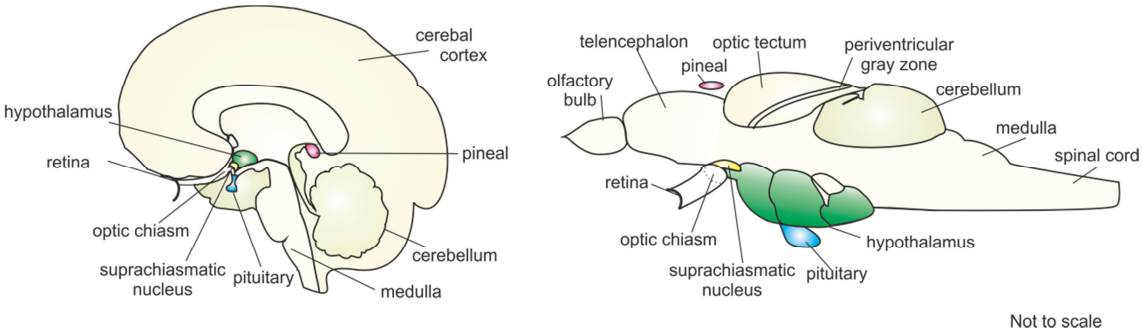


Figure 11 The anatomical location of the suprachiasmatic nucleus (SCN) in the human and zebrafish brain

The SCN is viewed as the master pacemaker in the mammalian brain. However, the role of the SCN in regulating circadian rhythms in the zebrafish has not yet been elucidated.

1.3.3 Techniques to study circadian regulation

A major goal for research on circadian rhythmicity has been elucidating the anatomical localisation of neuronal pacemakers. Many of the neuronal pacemakers in different species have been found by starting with the photoreceptive input into the circadian system and tracing the path to the pacemaker (Menaker, Takahashi, & Eskin 1978). In the zebrafish this poses a challenge, as many of the organs and cells studied appear to be directly photoreceptive. Another historical approach has been to create lesions in different regions of the brain, and determine whether any have an effect on output rhythms. Lesion experiments, however, can take a long time with thousands of repetitions required. One of the aims of this project is to narrow down the search for a neuronal pacemaker by characterising the rhythmicity and light sensitivity of different regions in the zebrafish brain, by use of gene expression analysis. If any regions show robust rhythmicity of clock genes and light sensitivity both *in vivo* and *in vitro,* these regions are good candidates to be neuronal pacemakers. This section will outline some of the more novel techniques that will be used in this project.

1.3.3.1 Bioluminescent reporters

High resolution, real-time recordings of the activity of the molecular clock can be achieved using transgenic animals that employ luciferase-gene promoter constructs as a reporter of circadian gene expression profiles. The promoter of a clock gene drives the expression of the *luciferase* (*luc*) gene, an enzyme that in the presence of the substrate luciferin will produce light. The

intensity of bioluminescence correlates with the clock gene's expression. Bioluminescence can be measured temporally by bioscintillation counters as frequently as every hour. The luciferase protein has a rapid turnover allowing it to accurately report gene expression in real-time. The half-life of luciferase in a stably transfected a mammalian cell line is approximately 3 hours (Thompson, Hayes, & Lloyd 1991).

Bioluminescent reporters have been used to study circadian biology in a wide range of models, including cyanobacteria (Mackey et al. 2007), *Arabidopsis* (Millar et al. 1995), and rat (Yamaguchi et al. 2000). The technique was invaluable in the first demonstration of peripheral circadian oscillators, using a *per-luc* Drosophila (Plautz et al. 1997).To monitor clock function in an automated fashion in zebrafish, a *per3-luc* transgenic zebrafish line was created by Greg Cahill (Kaneko & Cahill, 2005; Kaneko et al, 2006). This allows the recording of *per3* rhythms in long-term tissue cultures.

Recent technology advancements now allow the bioluminescence from these cultures to be imaged spatially. This can provide information of where the gene is being expressed and how its expression affects neighbouring regions.

1.3.3.2 Zebrafish mutants

One advantage of using zebrafish as a model organism is the vast amount of mutants that have been characterised. In general, most of the mutations are recessive, and cause the larvae to be unable to feed independently so they will die within a week of age, when the nutrients from the yolk sac are depleted. However, they provide us with a system that is lacking specific tissues or brain regions that can investigate the importance of these regions in controlling circadian rhythms of clock gene expression during embryonic development.

Masterblind (*mbl*) are eyeless mutant zebrafish produced from a mutation screening (Heisenberg et al. 1996). They are lacking most of their anterior neural structures, including olfactory placodes and the telencephalon, and have a larger pineal in the posterior forebrain. This recessive mutation is lethal and homozygous fish die after a few days. This eyeless mutant can be investigated to show how necessary or not the eyes are for clock gene expression during development.

The recessive mutation *floating head* (*flh*) was first identified in commercially bought fish that had undergone a spontaneous mutation (Talbot et al. 1995). *Flh* fish have disrupted notochord formation in embryonic development, preventing them from swimming. The *flh* mutant embryos can be identified by a shortened tail (Figure 12 red arrows) and they do not survive older than a week. *Flh* mutant embryos have a decreased production and differentiation of photoreceptors and projection neurons in the pineal (Masai et al. 1997). This mutant can be studied to determine the necessity and potential role of the pineal on embryonic clock gene expression.

A *mbl/flh* carrier can be produced from crossing *flh* and *mbl* carriers. *Mbl/flh* larvae do not develop a pineal, akin to the *flh* fish, and have no eyes (Masai et al. 1997)(Figure 12). Monitoring *mbl/flh* larvae can help reveal the role of eyes and pineal in generating circadian rhythms during embryonic development. A bioluminescent *mbl/flh* fish can also be created to view circadian gene rhythms at a high resolution.

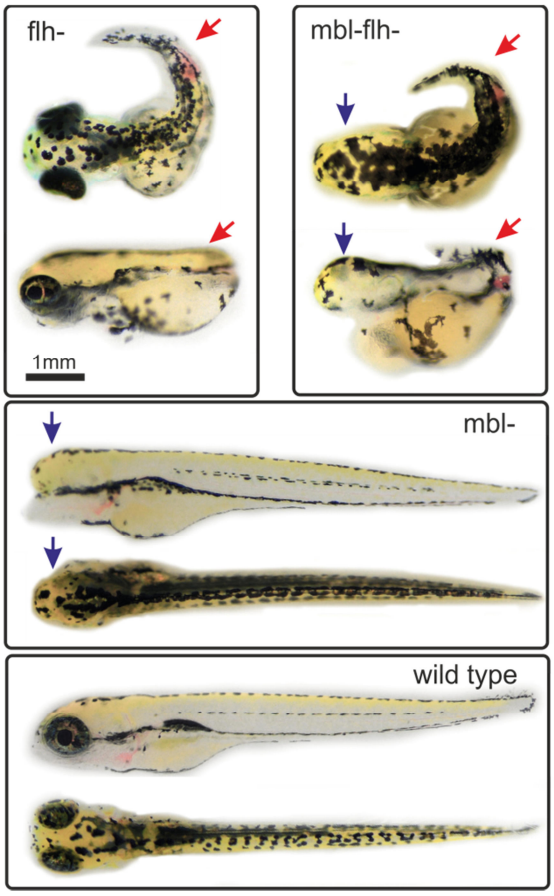


Figure 12 Zebrafish mutant larvae

Dorsal and sagittal views of 6 dpf *flh*, *mbl*, *mbl/flh* and wild type larvae. Red arrows indicate the shortened body length characteristic of *flh* mutants; blue arrows indicate the lack of eyes in the *mbl* mutants.

1.3.4 Photoreception

Light is the strongest Zeitgeber for resetting the circadian clock in the zebrafish, and it has been shown to do this in all cells and tissues tested so far. However, the photoreceptors and phototransduction signals that detect light in the zebrafish have yet to be fully evaluated. There are several candidates, and it is likely that there will be more than one involved and perhaps even tissue-specific photopigments, which play specialised roles.

Opsins are one group of light-sensitive receptors that are important in both visual and non-visual photodetection in various species. As there are over 40 different opsin genes found in the zebrafish genome, they may represent a way for the different zebrafish tissues to detect light (Wayne Davis, personal communication). However which ones are expressed and may have a role in phototransduction has yet to be fully elucidated. A part of this project will be to evaluate the expression of some of these opsins in the zebrafish brain.

1.4 Project Aims

The zebrafish circadian system has previously been considered as highly decentralised with most cells and tissues including the brain, containing directly light-responsive circadian oscillators. However, with recent studies strongly suggesting that the pineal is a neuronal pacemaker it is likely that a network of multioscillators exist in the zebrafish as they do in many other species. The goal of this project is to further investigate the circadian system in the zebrafish brain and determine the potential for the zebrafish to contain other discrete neuronal pacemaker structures.

This project intends to address the following questions:

- Are there any localised regions of the brain that display robust circadian rhythmicity *in vivo* and *in vitro*?
- Is the whole brain equally light-responsive, in which all cells are directly light-responsive, or are there localised regions of enhanced light sensitivity?
- Are clock genes expressed in neurons? Can the light entrainment of the clock be observed in specific neural regions?
- Are the classical photosensitive structures, the eyes and pineal, important in entraining circadian rhythms during zebrafish development?
- Finally, do opsins play a role in entraining the biological clock in the zebrafish brain?

Chapter 2

General Methods

This material and methods chapter outlines the general methods used throughout the thesis. Where any experiments have deviated from these the details can be found in the methods section within each results chapter (Chapters 3-6).

2.1 Animals

Zebrafish (*Danio rerio*) were bred and kept within the Fish Facility at University College London (UCL). All zebrafish were monitored and fed twice daily a diet of brine shrimp and dry food in the morning and afternoon. The zebrafish were maintained on a 14:10 hour light/dark cycle for optimization of breeding, or for short experiments kept in DD in a dark cabinet within the facility. The light intensity was approximately 800µW/cm$^{2.}$ Embryos were collected shortly after breeding began at lights on, 9am. The wild type strain of zebrafish used was AB Tupfel long fin (AB TL).

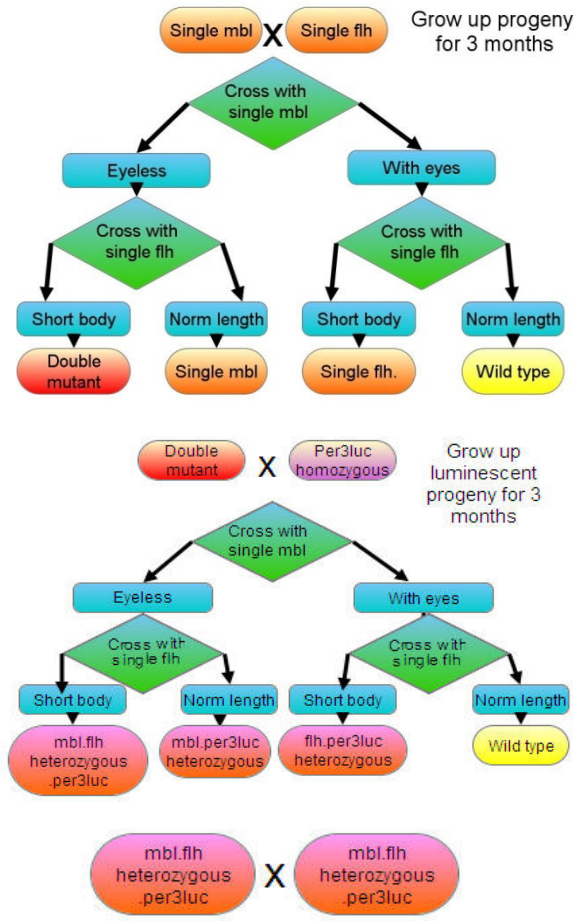
Experiments were designed in accordance with the three R's principle of using animals in scientific research: replacement, reduction, and refinement. Experiments were performed under the Animals (Scientific Procedures) Act, 1986.

2.1.1 Mutant zebrafish

Masterblind (*mbl*) and *floating head* (*flh*) single mutant carrier fish are bred in the UCL fish facility. To create *mbl-flh-per3-luc* embryos, adult single carriers of the *mbl* mutation were crossed with carriers of *flh* mutation; the offspring were raised, and screened for those that carried both mutations (Figure 13). These double mutant carriers were crossed with *per3-luc* fish to create luminescent double mutant carriers. Once identified, the double mutant carriers were crossed to produce luminescent offspring that expressed *mbl*, *flh*, *mbl/flh*, and wild type phenotypes. Offspring expressing the *mbl* or *flh* phenotype do not survive past one week of age.

2.1.2 Dissection

Adult zebrafish were killed by rapid immersion into chilled water. Their organs were removed whilst in submersion in autoclaved PBS. For a review of the zebrafish anatomy, see Menke et al (2011). The zebrafish brain was removed from the skull, and the pituitary gland was removed separately. With care the pineal, olfactory bulbs and optic nerves could remain attached, although they were removed in some preparations when specified. When the brain was separated into parts, the optic tectum, which is classified as part of the mesencephalon, was detached from rest of the mesencephalon, which was kept with the diencephalon (Figure 14).



1. Single *mbl* x single *flh*

	M	**m**		**F**	**F**
M	MM	Mm	**F**	FF	FF
M	MM	Mm	**f**	Ff	Ff

Approx 25% will be double mutant

2. Double mutant x *per3-luc* homozygous.

	M	**m**		**F**	**f**
M	MM	Mm	**F**	FF	Ff
M	MM	Mm	**F**	FF	Ff

All luminescent (heterozygous).
Approx 25% will be double mutant

3. *mbl/flh/per3-luc* x *mbl/flh/per3-luc*

	M	**m**		**F**	**f**
M	MM	Mm	**F**	FF	Ff
m	Mm	mm	**f**	Ff	ff

75% will be luminescent
Approx 25% *mbl/flh*, 25% *mbl*, 25% *flh*, 25% wt.

Figure 13 Creating *mbl/flh/per3-luc* embryos

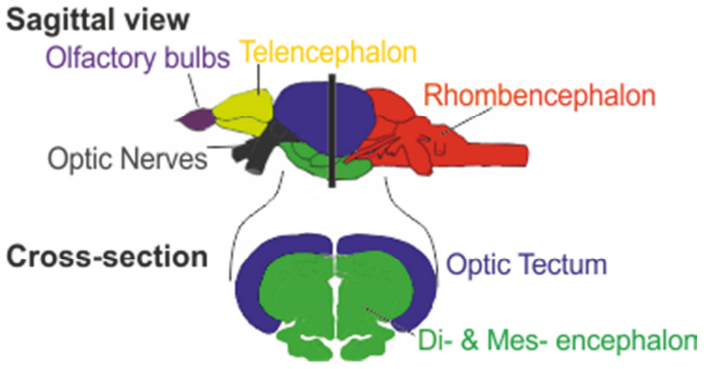


Figure 14 Zebrafish brain regions

2.2 Bioluminescent monitoring

Bioluminescent monitoring is an efficient method to study the expression levels of a gene of interest in real-time and at high resolution. In this project the *per3-luc* transgenic zebrafish used was developed by Greg Cahill (Kaneko & Cahill 2005) to monitor circadian rhythms. Luciferase from the firefly produces bioluminescence when in the presence of the substrate luciferin. In the *per3-luc* transgenic the *period3* (*per3*) promoter drives the expression of *luciferase* (*luc*). The intensity of bioluminescence produced correlates with *per3* expression. The timing/phase of the luminescent rhythm corresponds to the expression profile of the endogenous gene.

Using transgenic fish with luciferase as a reporter for gene expression has many advantages: fewer fish are required to monitor expression levels, more time points are able to be taken over a 24-hour period, and data from several days can be recorded. This is in line with the "refinement" of experimental practise to "reduce" animal usage.

2.2.1 Non-spatial bioluminescent recording

Total bioluminescence from *per3-luc* transgenic zebrafish was monitored using a Topcount NXT scintillation counter (2-detector model, Packard). The Topcount is kept at 27°C in a dark room to ensure temperature stability and specific light dark conditions.

Zebrafish embryos were placed into 96-well plates containing 0.5 mM luciferin in egg water (Westerfield 2000). Tissues were placed in wells containing Leibovitz's L-15 no phenol red (L15) medium (Invitrogen at Life Technologies Ltd UK), supplemented with 15% foetal calf serum (FCS, Biochrom AG), penicillin (100U/ml), streptomycin (100µg/ml) and gentamicin (50µg/ml) (Invitrogen at Life Technologies Ltd UK). The plates were stacked into the scintillation counter, and intercalated with transparent plates to ensure uniform illumination. Each well was counted over 10s at hourly intervals. Embryos will survive in the sealed plates for up to 6 days, when they are taken out and raised in the fish facility. Cultured tissues produced strong luminescent rhythms for at least 14 days without contamination.

2.2.1.1 Analysis

Bioluminescence data from the Topcount were imported into Excel (Microsoft) by the Import and Analysis macro (Steve Kay, Scripps Institute). Wells where the larvae had died or tissue had been contaminated were excluded from analysis.

For the larvae experiments, repeated experiments meant that measurements were taken at irregular intervals, and therefore it was necessary to standardise the time periods for comparability between different samples and sample groups. Simple interpolation was used to achieve this, via the *splinefun* function (http://bitly.com/Sfse6r) from the statistics package for R (http://www.r-project.org/). The first derivative points of the signals gave the time of the peak and troughs throughout the recording, from which the period length and phase shifts were measured.

For the tissue experiments, extraction of period and phase timing these values were standardised with *splinefun* as above, but for this data, excessive noise in the signal, especially around shifts between LD and DD cycles, made accurate identification of periods infeasible. Measurements were smoothed according to a kernel regression using the *npreg* (http://bitly.com/VlENLI) regression from the *np* package for R (http://www.r-project.org), with a bandwidth chosen by expectation maximization. Finally, period and phase shifts were calculated using first derivatives as above.

2.2.2 Spatial bioluminescent imaging

Spatial bioluminescence was recorded using an Imaging Photon Detector (IPD) (Sciencewares). This has a photon detector (Photek Inc. UK) and a CCD camera (Dage-MTI, IN) mounted on an inverted microscope (Zeiss), with a heated stage set at 28°C. Image 32 (Photek, UK) was used to record and analyse the data.

2.2.2.1 Reducing pigmentation

One of the challenges encountered when imaging zebrafish larvae was the formation of pigmented melanophores on the skin. Phenythiourea (PTU) was used to reduce pigmentation to produce clearer bioluminescent spatial images. PTU can be toxic and therefore a range of concentrations were tested from 0.0014% (75μM) (Karlsson, 2001), to 0.003% (Westerfield

2000). PTU (75μM) was added directly into the embryo water containing the larvae at 24hpf and prevented pigmentation for the full duration of time that it was in contact with the larvae.

2.3 In situ hybridisation

2.3.1 Description of assay

In situ hybridisation (ISH) allows for the spatial visualisation of gene expression in fixed tissue. A riboprobe is hybridised to the sample and used to detect the expression of mRNA. A ribroprobe is a 500-1000bp reverse complement ("antisense") sequence to the mRNA of interest that has been tagged with a hapten such as digoxigenin (DIG). The DIG-probe can be detected on fixed, frozen sectioned samples using chromogenic ISH (CISH).

In CISH, the riboprobe is hybridised to the sample, and detected using an anti-DIG secondary antibody that is conjugated to alkaline phosphatase (AP). When the AP substrate, vector NBT/BCIP, is applied, any area that has bound riboprobe will turn purple against a pink background. This can be viewed and imaged under a microscope (Figure 15A). CISH shows regional mRNA expression, and in this project will be used to localise expression in brain regions.

CISH can produce detailed spatial gene expression patterns on fixed, frozen section tissue. However, they are time-consuming and produce low sample numbers. Whole mount ISH (WISH) can be used when the tissue is transparent and small enough that the probe can easily penetrate. For instance, zebrafish larvae are typically only 4mm long at day 5 of development. WISH uses the same DIG-probe and AP detection technique as CISH (Figure 15B). The signal resolution in WISH is low, but higher sample numbers can be produced.

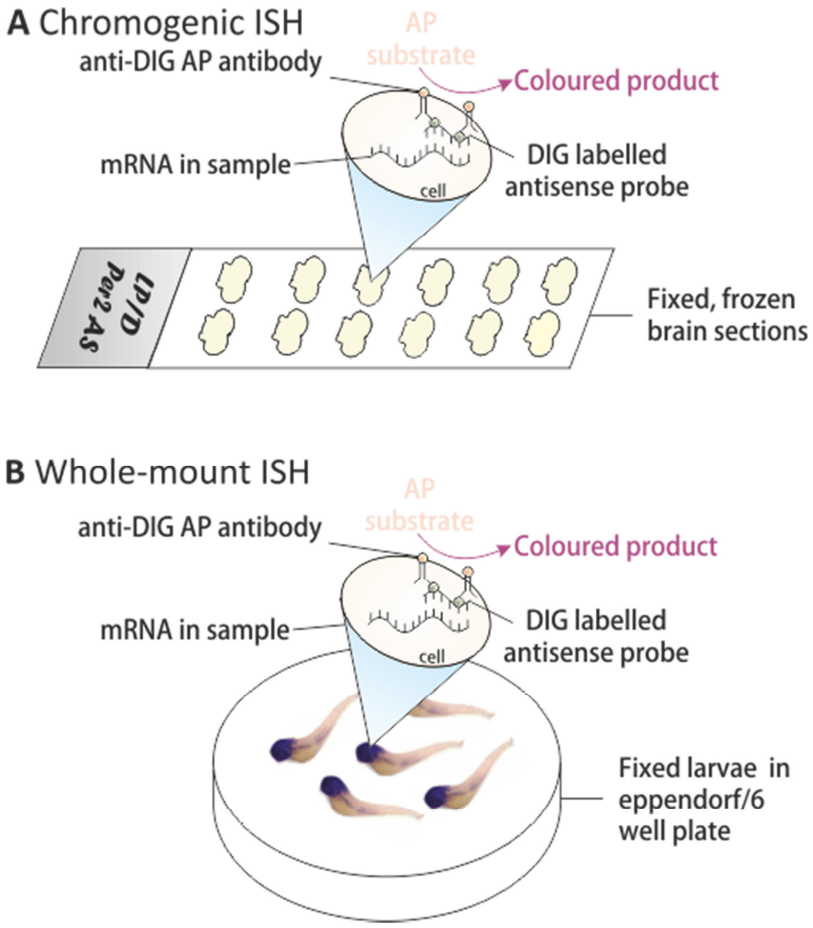


Figure 15 Schematic of different *in situ* hybridization (ISH) techniques

ISH is used to detect mRNA in a sample by using a DIG-labelled antisense probe, and a secondary anti-DIG AP antibody. In the presence of the substrate of AP, NBT/BCIP, cells containing the target mRNA will turn a purple colour. **A)** Chromogenic ISH can be used to detect mRNA spatially in fixed, frozen zebrafish brain sections. **B)** Whole mount ISH is used to detect mRNA in small transparent tissues, and in this case zebrafish larvae.

[AP, alkaline phosphatase; BCIP, 4-chloro-3-indolyl phosphate; DIG, digoxigenin; NBT, 5-bromo- nitro blue tetrazolium chloride]

2.3.2 Probe design

Probes were made between 500-1000bp long in both the “antisense” (AS), and “sense” (S) direction. The AS probe hybridises to the mRNA in the sample and the S probe acts as a control for background non-specific binding. In some genes the AS mRNA is also produced as part of the regulatory framework around transcription (Katayama et al. 2005). In this case the S probe would be an inappropriate control and instead a random mRNA sequence can be generated. However, AS expression is quite rare and so for this project the S probe was chosen to be used as a control in the first instance.

The sequence for the probes was amplified by PCR from RNA from PAC2 zebrafish embryonic cells or zebrafish embryos. The sequence was subcloned into a plasmid, generally pGEM®-Teasy (Promega Ltd, Southampton UK). The plasmid containing the probe sequence was amplified in a maxi prep. For creating the hapten labelled probe, the plasmid was linearised using restriction enzymes and then the riboprobe would be synthesised using RNA polymerases with DIG-labelled nucleotides.

2.3.3 Sample preparation

For CISH experiments brains dissected from adult zebrafish, or whole larvae, were fixed for 2 hours in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB). The samples were washed in phosphate buffered saline (PBS) and cryopreserved overnight at 4°C in 30% sucrose in 0.1M PB. The tissues were embedded in Tissue-Tek® optimal cutting temperature (OCT) compound (Sakura) and stored at -80°C. 10μm thick sections were cryosectioned at -18°C onto Superfrost Plus microscope slides (VWR) and either used immediately at RT or stored at -80°C for up to six months.

For WISH, embryos were fixed in 4% PFA in an eppendorf tube overnight at 4˚C. The samples were washed with PBS and stored in 100% methanol (MeOH) for 15 min at RT followed by –20˚C for a minimum of two hours and maximum of one year.

2.3.4 Chromogenic *In Situ* Hybridisation assay

A standard CISH protocol was used. Briefly, slides were brought to room temperature (RT) and fixed with 4% PFA in 0.1M phosphate buffer (PB) for 10 min, washed with PBS three times for 3

min each, and treated for 5 min with 1μg/ml proteinase K in 50mM Tris-HCl (ph7.5) and 6mM EDTA. Samples were then re-fixed in 4% PFA in 0.1M PB for 5 min, washed with PBS three times for 3 min each, and acetylated in triethanolamine, glacial hydrochloric acid, and acetic anhydride solution for 10 min to reduce nonspecific RNA probe binding.

Three more 5 min washes with PBS followed, and then slides were pre-hybridised in a 1 hour incubation with hybridisation solution [50% formamide, 5X saline sodium citrate buffer (SSC) (0.75M NaCl, 0.075M trisodium citrate, pH7.0), 5X Denharts (0.5g Ficoll (Type 400, Pharmacia) 0.5g polyvinvyl pyrrolidone (Sigma, UK), 0.5g BSA (Fraltion V, Sigma), autoclaved water up to 50ml), 250μg/ml Bakers yeast tRNA, and 500μg/ml salmon sperm DNA].

Samples were left to hybridise with the probe overnight at 72°C in hybridisation solution containing the DIG-labelled mRNA probe, with a coverslip to prevent evaporation of the solution. Slides either had AS or control S probes. The following day they were washed once in 5XSSC for 5 min, and two 40 min washes in 0.2X SSC (30mM NaCl, 3mM trisodium citrate, pH7.0) at 72°C. Slides were then brought to room temperature in 0.2XSSC for 5 min, followed by 5 min in buffer 1 (0.1M Tris pH7.5, 0.15M NaCl). Non-specific binding sites were blocked using 10% goat serum (Sigma, UK) in buffer 1, and incubated overnight at room temperature with anti-DIG-alkaline phosphatase (anti-DIG-AP) with 1% goat serum in buffer 1.

On the final day the slides were washed in buffer 1 and incubated with the AP substrate, 5-bromo- nitro blue tetrazolium chloride (NBT) and 4chloro-3-indolyl phosphate (BCIP) in buffer 3 (0.1M Tris-Cl pH9.5, 0.1M NaCl, 0.05$MgCl_2$) and the detergent 0.1% Tween-20. NBT-BCIP develops into a dark blue colour in the presence of AP. Slides were left to develop at room temperature in the dark for a few hours, and sometimes overnight at 4°C, until optimal signal had appeared in the AS sections.

Slides were washed with PBS and treated for 10 min with DAPI (4',6-diamidino-2-phenylindole), a fluorescent stain that binds with DNA to show the localisation of the cell nuclei. Slides were then washed with tap water and mounted with 2 drops of glycerol mounting medium (Dako, UK) and a coverslip.

Sections were imaged using a Nanozoomer Slide Scanner (Hamamatsu, UK). Figures were compiled using NDP.view (Hamamatsu, UK) and Corel Draw.

2.3.5 Fluorescent immunohistochemistry

Fluorescent immunohistochemistry was used to label proteins in brain sections and was frequently combined with ISH for co-localisation investigations. When being combined with CISH, the steps below would follow the CISH protocol before the DAPI step, with the one exception that on the first day of the CISH 1% Triton-X100 in PBS would be used for permeating the cell membranes rather than proteinase K. To label neurons, the primary antibody HuC/D was used. Pilot experiments determined the optimal concentration of the primary and secondary antibodies.

As described in section 2.3.3 samples were fixed, cryoprotected and sectioned onto microscope slides. Slides were brought up to RT and washed with PBS. A 30 min treatment of 1% BSA and 0.1% Triton-X100 in PBS was performed to block the non-specific binding sites and permeablise the cell membranes. This was replaced with the primary antibody, HuC/D raised in mouse (1:500), in 1% BSA and 0.1% Triton-X100 in PBS, and incubated overnight at 4°C.

On the second day the slides were washed with PBS and incubated with the second antibody, anti-mouse-IgG-Alexa488, for 30 minutes in the dark at RT. This was then washed with PBS and DAPI was used to stain nuclei. The slides were finally washed with tap water and coverslips were mounted on the slides using Vectashield mounting media (Vector Labs, UK). Photos were taken using a DMLB fluorescent microscope (Leica Microsystems Ltd, Heerbrugg) and DC 300F camera (Leica Microsystems Ltd, Heerbrugg).

2.3.6 Whole mount *In Situ* Hybridisation assay

The protocol used for WISH on zebrafish larvae is based on the procedure from the Thisse lab (Thisse & Thisse 2008). Samples stored in 100% MeOH were rehydrated gradually with 5 minute incubation periods with 75% MeOH in PBT (PBS with 0.1% Tween 20), 50% MeOH in PBT, 25% MeOH in PBT and finally PBT. The samples were then treated with 10µg/ml proteinase K (Roche) to partially digest the tissue to allow for probe penetration. After washing with PBT the larvae were re-fixed in 4% PFA for 20 minutes. The samples were further washed

in PBT 5 times before a prehybridisation step to equilibriate the embryos to the hybridisation solution (hyb)(50% Formamide (Sigma, UK), 25% 20XSSC, 1% Torula RNA 50X (Sigma, UK), 0.01% Heparin (Sigma, UK), 0.1% of 20% Tween-20, 0.2% 1M citric acid, made up to volume with distilled water). The samples were incubated overnight at 65°C in hybridisation solution containing the DIG probe to allow the DIG probe to hybridise to the mRNA in the sample.

On the second day the following steps were performed at 65°C: 5 minutes in fresh hyb, 30 minutes in 50% hyb/2X SSC, 20 minutes in 2X SSC, and 5 washes in 0.2X SSC. The larvae were then cooled to RT, washed in PBS 3 times and incubated in 2% Blocking Agent in Maleic acid buffer (MAB)(Roche) at RT. This blocking step prevents the anti-DIG antibody binding to non-specific sites. The blocking solution was removed after a minimum of 3 hours and replaced with anti-DIG-AP (1:5000) in 2% blocking agent in MAB. The samples were left overnight at 4°C.

On the final day the samples were washed 4 times with PBS at RT, and then equilibrated in BM staining buffer (0.1M Tris pH9.5, 0.1% Tween 20). The embryos were then transferred to the developing substrate, BM Purple, until the colour reaction was complete. The reaction was stopped by washing with PBT. The tissue was refixed in PFA, rinsed in PBS, and finally stored in 75% glycerol in PBS at 4°C.

2.3.7 Data analysis

The CISH slides were imaged using a Nanozoomer slide scanning microscope (Hamamatsu). There is slight variation in background colour between slides and between experiments due to length of time incubating the probe. Brain regions in adult zebrafish were identified by referring to the Zebrafish Neuroanatomy Topological Atlas (Wullimann et al. 1996).

The WISH samples were imaged on an Axiovert microscope (Zeiss) and imaged using a DC 300F camera (Leica Microsystems Ltd, Heerbrugg).

2.4 Real Time quantitative PCR (qPCR)

2.4.1 Description of assay

Real time quantitative PCR (qPCR) is used to compare levels of gene expression in tissue samples under different experimental conditions. It has been used by the scientific community with increasing frequency over the past decade (VanGuilder et al. 2008). qPCR amplifies cDNA so that the original amount can be quantified. The first stage in quantifying the amount of mRNA in sample is to perform a reverse transcription reaction to convert the mRNA into cDNA. For the qPCR reaction cDNA is amplified using PCR with a fluorophore dye, in this case SYBR®Green. This dye fluoresces when intercalated with the two strands in DNA. After each PCR cycle the amount of fluorescence is measured, hence the "real-time" description. When the fluorescence is above a threshold value the cycle time (Ct) is recorded. The lower amount of cycles it takes for fluorescence to be detected, the higher amount of cDNA in the sample, and therefore higher expression of mRNA. By calculating Ct values qPCR can be used to quantify the cDNA by two methods: relative quantification and absolute quantification. Absolute quantification will determine the exact number of DNA molecules in the sample but requires samples with known quantity of DNA for calibration. Relative quantification was chosen as it is a more straightforward method (Dussault & Pouliot 2006). This compares the relative expression of the gene of interest to a reference gene (and/or to itself in another sample), which has constitutive expression in that tissue sample.

2.4.2 RNA extraction

To extract and isolate the RNA from the tissue sample the Total RNA Isolation (Trizol) reagent (Life Technologies, UK) was used. Trizol is a monophasic solution of phenol and guanidine isothiocyanate, which dissolves cell components whilst maintaining the integrity of RNA. Fresh tissue samples (e.g. dissected brains or embryos) were homogenized in an eppendorf tube with 0.5ml Trizol. The samples were then stored at -20°C until ready to proceed with the mRNA extraction step.

To separate the RNA from the DNA and other proteins the aqueous and solvent phases of the sample were separated. 100µl chloroform was added, the tube vortexed, left to stand for 2 min

at room temperature, and centrifuged at 13,200 rpm for 20 min at 4°C. This formed two distinct layers. The top colourless aqueous phase, containing the RNA, was collected and placed in a fresh tube.

To precipitate the RNA, 150µl of isopropanol was added, and the tubes stored at least overnight at -20°C, occasionally two nights was used to increase the yield of RNA collected. The tubes were then centrifuged at 13,200 rpm for 30 min at 4°C so the RNA precipitate pellet formed on the bottom of the tube. To wash this pellet the supernatant was removed and 0.5ml of 75% ethanol added, vortexed, and centrifuged at 13,200 rpm for 5 min at 4°C.

After washing, the ethanol was removed and the pellet dried at room temperature for approximately 10 minutes. It was then re-suspended in 10µl RNase-free water, quantified using a Nanodrop (UK), and stored at -20°C.

2.4.3 Reverse Transcription

To synthesise the double-stranded complementary DNA (cDNA) from the single-stranded RNA a reverse transcription reaction was run using the DNA polymerase Superscriptase II Reverse Transcription (Invitrogen, UK). 0.5µg to 2µg of RNA was added to 3µl of a master mix containing 1µl each of Random Primer (Invitrogen, UK), Oligo dT16 (Invitrogen, UK) and dNTP (Invitrogen, UK). The final volume was brought up to 10µl using RNase free water. This was heated at 65°C for 5 minutes in a Mastercycler®Pro PCR machine (Eppendorf, UK).

10µl of a second master mix was then added, containing 5µl 5X first-strand buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$] (Invitrogen, UK), 2µl 100mM dithiothreitol (DTT), 3µl RNase free water, 0.5µl RNase out (40 U/µl) (Invitrogen, UK), and 0.5µl Superscriptase II RT (200 U/µl) (Invitrogen, UK). The PCR programme was resumed with 10 min at 25°C, 60 min at 42°C, and 15 min at 70°C. The resulting cDNA was diluted with RNase free water so it would be 1:10 if the original concentration of RNA had been 2µg. Samples were stored at -20°C ready to be used for qPCR.

2.4.4 Primer design, efficiency & testing

Creating primers that accurately amplify your gene of interest is vital for the effectiveness of qPCR. Primers are single stranded oligonucleotides around 20 nucleotides in length. Two primers are designed for each gene, the forward and reverse, which produce an amplicon of 100-200 base pairs. PCR efficiency decreases with very long (>1000bp) amplicons. The GC content of both primers is optimally around 50-60% with a melting temperature of around 60°C. Primers were designed using the software Primer3, and then further analysed for additional characteristics using Oligo 6 primer analysis software, to avoid primers forming dimers or hairpins. Primers were ordered from MWG Eurofins or Sigma, put in solution to 0.1 or 1pg/ml concentration with RNase free water, and stored at -20°C.

Having efficient primers, which will reliably double the cDNA strands on each cycle, is vital to calculate the original amount of cDNA and compare different genes. To determine the efficiency of a set of primers the cDNA of a sample known to contain the gene of interest is diluted 1:10, 1:50, 1:100, 1:500, 1:1000, 1:5000, 1:10000, 1:50000, and the qPCR performed (see section 2.4.5 for further details). The standard curve is then plotted with the log of the dilution on the x axis and the mean Ct on the y axis. The slope value of this curve can be used to calculate the efficiency value (E) using the formula: E=10^(-1/slope). The percentage efficiency can be calculated: %efficiency = (E-1)*100. The general rule of a reliable, efficient primer set is an efficiency of 90-105%.

When testing new primers it is important that the amplicon is the correct sequence to confirm the specificity of the primer set to the mRNA of interest. A qPCR reaction and melting curve was run to create amplicons and indicate the presence of multiple-products, primer-dimers or possible contamination (see section 2.4.5 for further details). If a primer set was successful in creating one amplicon, this was then subcloned for size determination and sequencing.

2.4.4.1 Subcloning PCR products for sequencing

To subclone the qPCR product it was first ligated into pGEM®-TEasy by combining 5µl 2X DNA ligase buffer, 1µl pGEM®-TEasy vector (50ng), and 1µl T4 DNA ligase buffer (3U/µl) with 3µl of purified PCR product, and left overnight at 4°C.

The ligation reaction was then placed on ice to be transformed into XL10 Gold® competent cells (Invitrogen at Life Technologies Ltd). 50µl of competent cells were pipetted into a 1.5ml eppendorf tube with the ligation reaction, tapped to mix, and incubated for 30 minutes on ice. The competent cells were then heat-shocked in a water bath at 42°C for 45 seconds, followed by quenching with ice. 500µl of autoclaved LB broth 2.5% (w/v) was added to the cells and they were grown in a shaker incubator for 1 hour at 37°C. Prior to transformation, plates with LB agar (made with distilled water 3.7% (w/v) and autoclaved) and the antibiotic ampicillin (final concentration 50µg/ml, Invitrogen at Life Technologies Ltd UK), were warmed in a 37°C incubator and treated with the *lac2* substrate, IPTG/XGal (20µl 0.1M IPTG and 20µl 20µg/ml XGal, Invitrogen at Life Technologies Ltd.), for blue/white colony screening. The transformed cells were then spread onto the plates (50µl and 450µl respectively), which were inverted and incubated overnight at 37°C.

White colonies containing the vector with insert were grown in LB Amp at 37°C overnight. 1ml of this mixture was centrifuged at 13,200rpm for 2 min at RT. Plasmid preparation was then carried out using a Qiagen Mini-Plasmid Prep Kit (Qiagen Ltd.) as instructed by the manufacturer's guidelines.

The size of the product was then estimated. A restriction digestion was set-up by incubating 8 µl miniprep DNA with 1 µl 10X buffer and 1 µl restriction enzyme, for 1 hour at 37°C. The digested fragments were loaded onto a 1% agarose gel in TAE, and electrophoresed for 20 minutes, alongside a DNA ladder. Bands were visualised using a UVP Bio-Doc™ gel documentation system (Ultra-violet products Ltd.).

If the DNA was the correct length the samples from the mini-prep were sequenced by Source Bioscience Sequencing (Cambridge, UK). ClustalW2 (European Bioinformatics Institute, UK) was used align the sequence result with the expected fragment.

Target transcript & Accession No.	Primer 5' to 3'	Melting temp (°C)	GC content (%)	Efficiency (%)	Amplicon Size (bp)
Cry1a	Forward TCCAACCCTAATGGAAGCAC	57.3	50	94	109
BC124762	Reverse ACTCCTCGCTGTGTCGTTTT	57.3	50		
C-fos	Forward CAGCTCCACCACAGTGAAGA	60	55	94.6	176
NM_205569.1	Reverse GCTCCAGGTCAGTGTTAGCC	59.9	60		
Exorh	Forward GTACGCTCCGCTATCCCATA	60.1	55	100	185
NM_131212.2	Reverse ACGTGTGAAAGCCCCTACTG	60.2	55		
Per3	Forward CAGCAACGATTCCTCAGACA	57.3	50	109	116
NM_131584	Reverse GCTTGATCATGCTCCACAGA	57.3	50		
Per2	Forward TGGCTCTGGACAGAAGTGAG	59.4	55	101	120
NM_182857	Reverse GGATGTCTCGAGAATGCAAC	59.4	55		
Per1	Forward ATCCAGACCCCAATACCAC	54.5	47.4	101	248
NM_001030183	Reverse GGGAGACTCTGCTCCTTCT	58.8	57.9		
Rgr1	Forward CCTGGCTTTCTACGCCGCAG	61.1	65	87.7	155
NM_001017877.1	Reverse GGACTTGTTCTCAATAGCAGGACTCTC	58.5	48.1		
Rgr2	Forward GAGCACGTCTATCACCATCAGCT	58.7	52.2	93.3	87
NM_001024436.1	Reverse ACACCCCAGCCAATGGCAGG	63.2	65		
Rh1.1	Forward ACTTCCGTTTCGGGGAGAAC	53.8	55	96	175
NM_131084.1	Reverse GAAGGACTCGTTGTTGACAC	51.8	50		
Rh1.2	Forward GCGGTGGCTGACTTGTTTAT	60.1	50	104	165
NM_001110031.1	Reverse CTCAACAGCCAGAACAACCA	59.9	50		
rpL13a	Forward	62.7	50	93	148
NM_212784	TCTGGAGGACTGTTAGAGGTATGC	60.3	50		
	Reverse				
	AGACGGACAATCTTGAGAGCAG				
Luc	Forward ATCCATCTTGCTCCAACACC	57.3	50		123
	Reverse TTTTCCGTCATCGTCTTTCC	55.3	45		

Table 1 qPCR primer properties

2.4.5 Thermocycling and data collection

The qPCR reaction was run in blue 96-well plates (Eppendorf, UK) using a Mastercycler® EP Gradient S (Eppendorf, UK). Realplex software (Eppendorf, UK) was used to run the assay and for the initial analysis of average Ct values and melting curves.

The cDNA, primers and reaction products were put into each well, to a volume of 20µl. SYBR® Green 1 dye (Sigma, UK) was used as the fluorescent dye to specifically detect double-stranded DNA. This comes in a solution containing AmpliTaq Gold® DNA polymerase, dUTP, and a proprietary buffer. To reduce intra-assay variability a master mix of primers and SYBR® Green 1 were used. Each cDNA sample was run in triplicate to measure the intra-assay variation.

The software was programmed to run 40 cycles of 15s 95°C, 15s 58°C, and 20s 68°C (Figure 16A), at the end of each cycle the amount of fluorescence was measured. Plotting fluorescence against cycle number produces a sigmoidal amplification curve (Figure 16B). This is because PCR products are produced exponentially, in the early stages there is not enough product to be readily detectable, and in the later cycles the reaction substrates become depleted and the curve flattens. The Ct value is the cycle number when the amount of fluorescence goes over a threshold value in the exponential phase of the amplification curve.

At the end of the amplification phase the melting curve temperature was determined by running 15s 95°C, 15s 65°C, a 20 min ramp to 95°C, and 15s at 95°C (Figure 16A). As the temperature rises the double-strands of the cDNA dissociate, freeing the intercalated SYBR® Green 1 molecules and reducing the amount of fluorescence produced. The temperature when 50% of the cDNA has dissociated is termed the melting point. This point can be identified by a peak when the first derivative of the fluorescence is plotted against temperature (Figure 16C). Each peak in the melting curve represents an amplicon. If multiple peaks are produced there has been a dimerisation of primers or non-specific amplicons amplified, therefore the primers have been unspecific to one gene and those primers cannot be used.

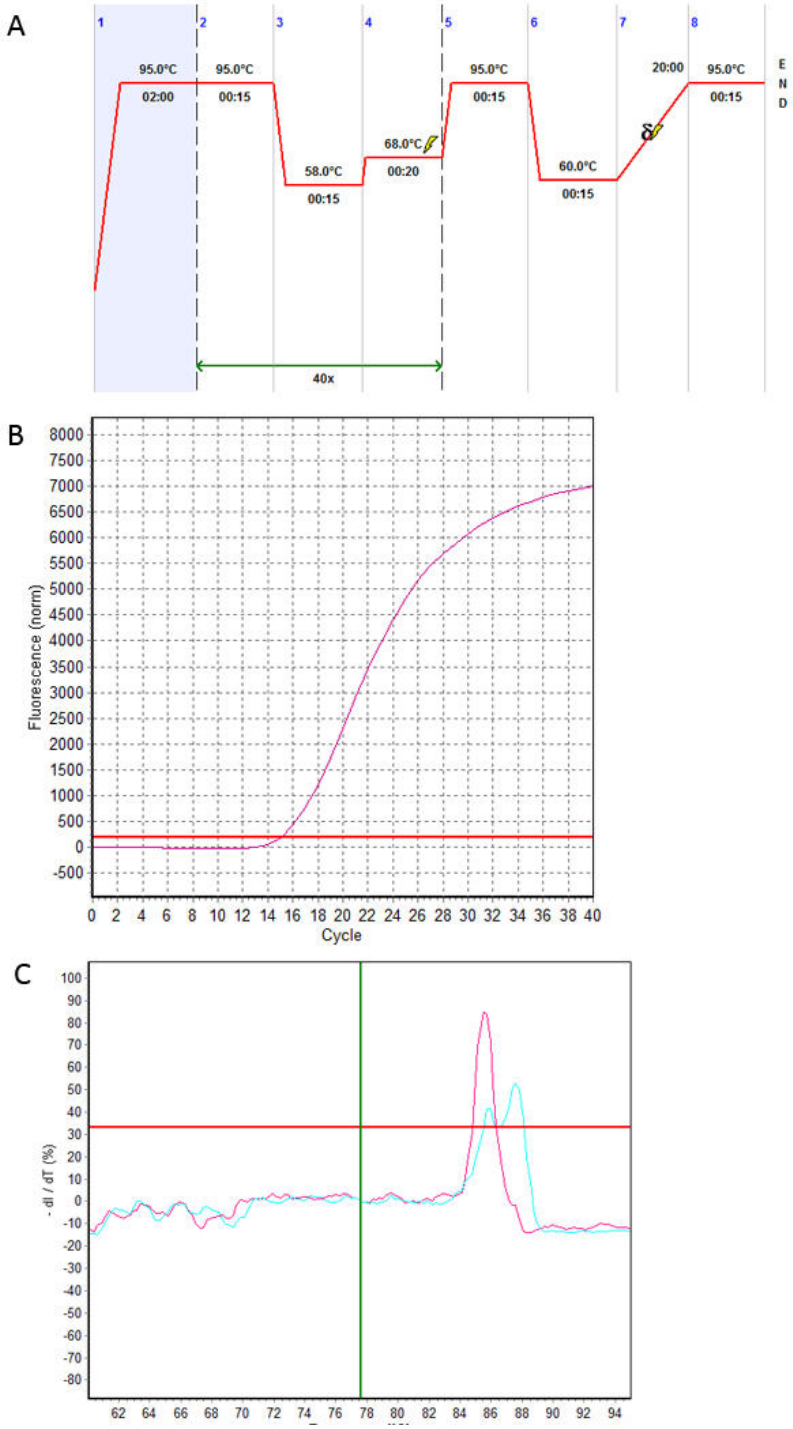


Figure 16 qPCR Settings and Analysis

A) Thermocycler set up, steps 1-4 program the PCR, and steps 5-8 the melting curve. **B)** Quantification of the Ct value, the red horizontal line indicates the threshold fluorescence value, in this example the Ct value is 15.1 **C)** Melting Curve Analysis, the pink and blue lines represent two different sets of primers; the single pink peak indicates only one product has been amplified, the double blue peak indicates that two products were amplified.

2.4.6 Normalisation of data

Using an endogenous reference control or "housekeeper gene" is vital for normalisation in the relative quantification method of analysing qPCR. Variability will arise between samples from slight differences in RNA quality and the reverse transcription reaction. By using a housekeeper gene, and normalising the gene of interest to this gene, differences in the expression of the gene of interest can be monitored accurately. Housekeeper genes are chosen due to their universal and constitutive expression in living eukaryotic cells and by not being regulated by circadian rhythms, i.e. not being rhythmically expressed or light regulated.

The ribosomal protein L13a (rpL13a) was chosen as a reference gene. rpL13a is part of the 60S subunit of a ribosome, found in the cytoplasm and used for catalysing protein synthesis. rpL13a is one of the most suitable reference genes for zebrafish tissue due to its stable gene expression (Tang et al. 2007). The housekeeper gene was run for every sample, and the sample was only used if the Ct value of the housekeeper fell within 0.3 standard deviations amongst all samples in the group.

2.4.7 Data analysis

To calculate the relative expression of the different genes in a tissue sample the $\Delta\Delta$Ct method is used. The mean Ct values from the qPCR program are exported into Excel (Microsoft) for analysis.

The mean Ct value of the housekeeping gene is subtracted from the mean Ct of the target of interest to produce the ΔCt value. The trough value in the sample set, with the largest ΔCt value, is subtracted from each of the values to produce the $\Delta\Delta$Ct (also written as ΔCt-ΔCt). The relative expression value is $2^{-\Delta\Delta Ct}$, which resolves the exponential difference between each cycle. The relative expression calculation will produce a trough value equal to 1 and all the other values will be a relative fold increase to this.

A minimum of 3 samples were made per time point and all data are presented as a mean with error bars representing the standard error of the mean (SEM).

2.5 Brain culturing

Whole brains were dissected in autoclaved PBS solution and placed into a 35mm petri dish containing L15 without phenol red media (Invitrogen at Life Technologies Ltd, UK), with 15% FCS (Biochrom AG), penicillin (100U/ml), streptomycin (100µg/ml) and gentamicin (50µg/ml) (Invitrogen at Life Technologies Ltd, UK), and sealed with parafilm. Dishes were sealed in Tupperware boxes and placed in a water bath to maintain a constant 28°C under controlled lighting conditions.

2.5.1 Validation of novel whole brain culturing technique

Culturing of zebrafish brains have been described in the literature (Tomizawa et al. 2001; Vargas et al. 2011), however in this project these methods were unsuccessful and instead a simpler, more effective technique was developed. For this technique it was important to determine whether the brain can survive in culture, maintain its morphology, and whether the clock genes were still light responsive. The technique that was developed is able to maintain the brain's structure for at least 6 days (Figure 17 and 18) and, when cultured for over 2 days, clock genes are acutely responsive to light (Figure 19).

The Tomizawa technique used a transparent polyethylene terephthalate membrane, through which the brain had contact with a conditioned CO_2 enriched media (Tomizawa et al. 2001). However in this project, brains collapsed using these membranes and this could not be avoided. Also, to maintain a consistency between buffer solutions L15 media was chosen (Invitrogen at Life Technologies Ltd, UK). This media is designed for supporting cell growth in environments without CO_2 equilibrium, and has a combination of phosphates and the amino acid L-arginine, along with L-histidine and L-cysteine, to buffer the system.

Furthermore, the Vargas aCSF recipe was tested. This has been developed for larvae and young adult zebrafish brains to be cultured for up to 6 hours for basic electrophysiological recordings (Vargas et al. 2011). However within 24 hours the brains swelled in this media and lost their structure, so was unsuitable for long-term culturing. On the contrary, the technique developed in this project is able to maintain viable brain cultures from one-year old zebrafish for at least one week.

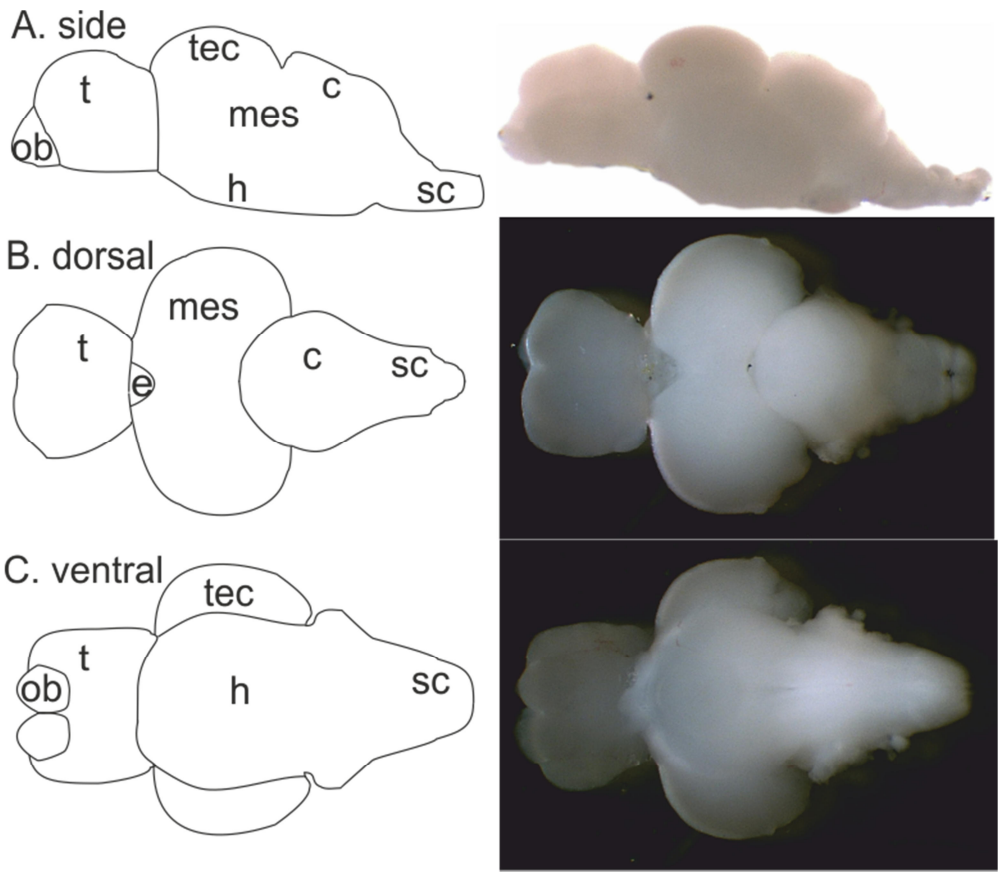


Figure 17 Cultured adult zebrafish brains maintain their structure

A whole brain culture technique was developed that maintained the brain structure for at least six days. Here is the side, dorsal, and ventral view of a brain that has been cultured for four days in L15 media.

[c, cerebellum; e, pineal; h, hypothalamus, mes, mesencephalon; ob, olfactory bulbs; sc, spinal cord; t, telencephalon; tec, optic tectum]

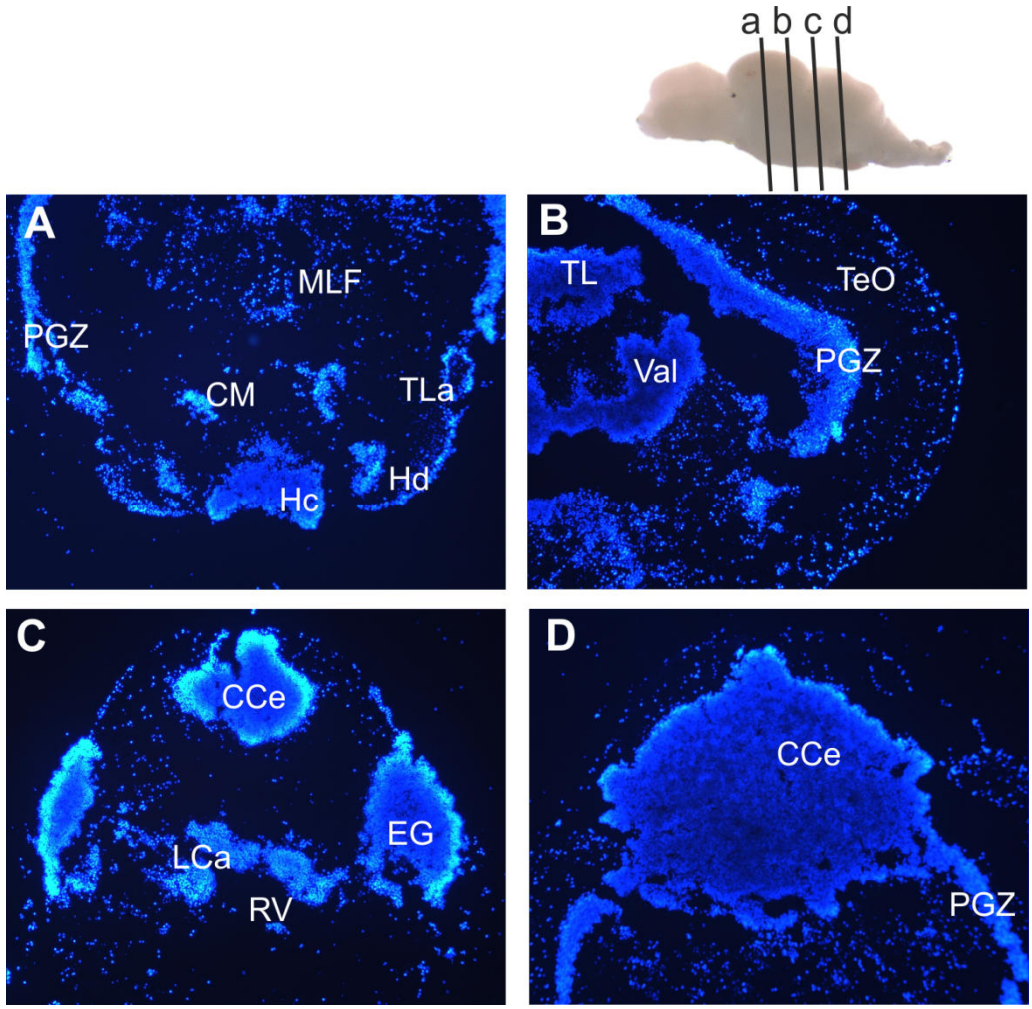


Figure 18 Cultured adult zebrafish brains maintain regional morphology

The whole brain culture technique maintained the regional morphology of the brain for at least 6 days. Here are sections of the brain, stained with DAPI to show the location of cell bodies for identification of brain regions. This brain has that has been cultured for four days in L15 media. [CCe, corpus cerebellum; CM, corpus mammilare; EG, eminentia granularis; Hc/d, caudal/dorsal zone of periventricular hypothalamus, LCa, lobus caudalis cerebelli; MLF, medial longitudinal fascicle; PGZ, periventricular grey zone of the optic tectum; RV, rhombencephalic ventricle; TeO, optic tectum; TL, torus longitudinalis; Val, valvula cerebelli]

Initially brains were collected that had been cultured overnight, however these were not consistently light responsive (data not shown). In order to determine whether the brain cultures needed to dark adapt to be light responsive, or whether the time in culture determined their light response, brain cultures were collected on day 4 after a normal LD or when dark adapted (Figure 19A). A light pulse in the late subjective night at circadian time (CT), CT22, induces increased *cry1a* mRNA expression in both dark adapted samples, and samples on a normal light dark cycle (Figure 19B). Therefore, a longer time in culture than two days was needed to monitor clock genes in the brain. This complements what is seen from *per3-luc* brain region bioluminescent traces, where in the first two days the signal is too high to monitor rhythms, as the brain is adapting to the culture conditions and recovering from surgery.

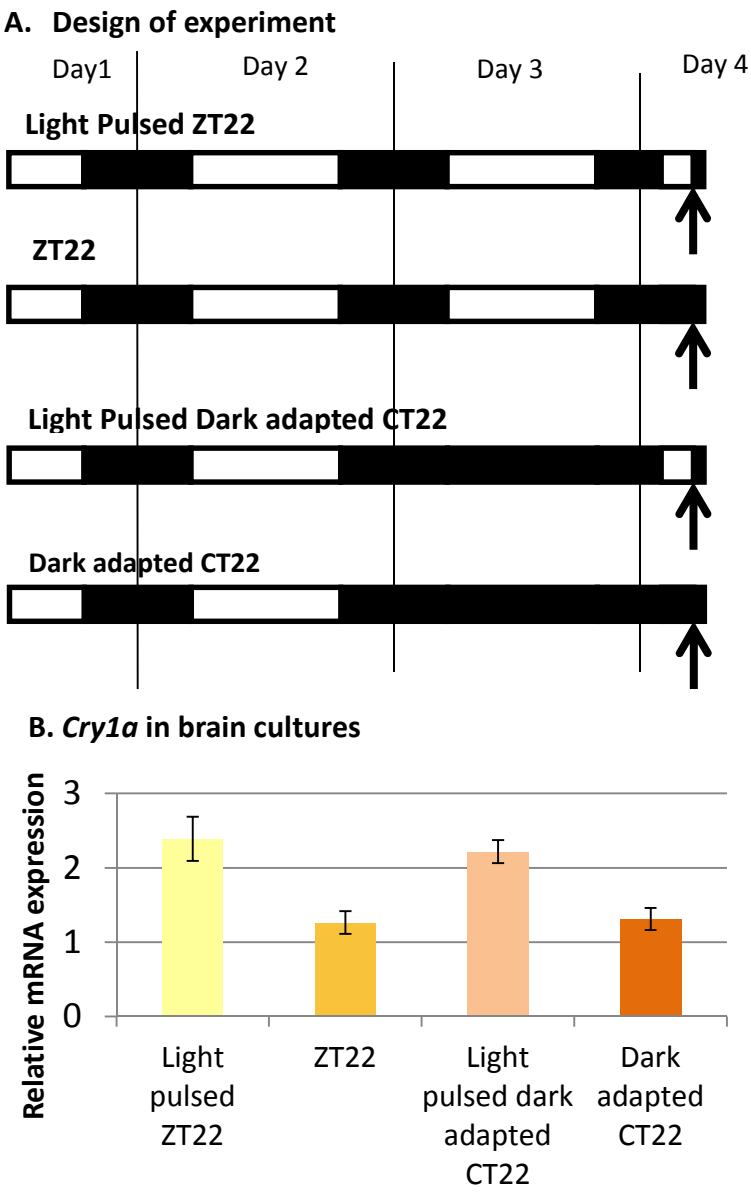


Figure 19 *Cry1a* is induced by light in brains cultured for four days

Whole brains were cultured for three days and kept on a 14:10 LD cycle (ZT) or dark adapted for one day (CT), before having a 3 hour light pulse on the fourth day. **A** Schematic outlining the LD cycles; brains were collected at the time indicated by the arrows. **B** qPCR analysis shows *cry1a* expression was induced in both samples exposed to light, and there is no significant difference between the induction in the dark adapted samples (CT) and the ZT samples ($p<0.01$, n=3, One way ANOVA with Tukey Post Test).

Chapter 3

Circadian Rhythmicity in the Zebrafish Brain

3.1 Introduction

Circadian research in zebrafish has largely focussed on the rhythmicity and light sensitivity of peripheral tissues (Whitmore, Foulkes, & Sassone-Corsi 2000). Early work in the zebrafish had demonstrated light sensitivity and rhythmicity in the pineal and more recent work has shown it to display many characteristics of a neuronal pacemaker region in the zebrafish brain (Cahill 1996; Li et al. 2012); however other neuronal pacemakers may also be present. This chapter will analyse brain nuclei throughout the zebrafish brain to determine whether there are any regions that display one of the features of a neuronal pacemaker: robust rhythmicity of a core clock gene.

Robust rhythmicity is a characteristic of all neuronal pacemakers, and can be monitored by measuring outputs of the clock, such as melatonin synthesis, or by monitoring the oscillations of molecular clock genes. Robust rhythms of *per1* were found in several regions of a cultured *per1-luc* rat brain (Abe et al. 2002). As described in the introductory chapter, one of these regions, the OB, was later shown to be a neuronal pacemaker. With the availability of the *per3-luc* zebrafish a similar initial approach, as used by Abe et al (2002), can be used to investigate rhythmicity throughout the zebrafish brain to find neuronal pacemaker candidates. *Per3* is considered a core clock gene in the zebrafish, as it is rhythmic both in LD and DD (Pando et al. 2001).

The *per3-luc* zebrafish has already been used to show that *per3* rhythms are present in the retina, heart, spleen, gill, gall bladder, pineal gland, kidney, liver, oocyte–follicle complex, and caudal fin (Kaneko, Hernandez-Borsetti, & Cahill 2006). These tissue cultures could entrain to LD cycles and free-run in DD. In all six of the tissues tested for the effects of temperature the *per3* rhythms were temperature compensated. In this study the authors mention recording bioluminescence from different parts of the brain, but did not describe any results. Therefore this chapter will begin by describing rhythms in the different subdivisions of the zebrafish brain and compare these to peripheral tissue rhythms.

Per3 has recently been examined in the nocturnal teleost species, sole (Martín-Robles et al. 2011). Using real time-PCR (RT-PCR) on different brain regions, they showed that *per3* was

expressed in all the neural and peripheral tissues examined. *Per3* was rhythmic in the optic tectum and retina, with higher amplitude oscillations in the retina. However, *per3* was not rhythmic in other brain regions, including the diencephalon which would contain the SCN. This could be because the SCN is a small structure when compared to the diencephalon and any robust rhythmicity that it contains could be masked by the desynchrony or lack of rhythmicity of neighbouring regions. Determining rhythmicity of small brain regions is a challenge when evaluating clock genes in tissues, and can be overcome by CISH on slices which reveal more detail about where the clock genes are being expressed.

Previous ISH assays on clock gene rhythms in the zebrafish have hinted at the expression of *bmal* and *clock* being restricted to certain brain areas. These included the periventricular grey zone of the optic tectum (PGZ), valvula cerebelli (Val), corpus mammilare (CM), and hypothalamus (Cermakian et al. 2000; Whitmore et al. 1998). However, the resolution of these structures was poor due to using a ^{35}S-ATP S-labelled probe for detection (Unger, Hammer, & Chenggis 1991). Also, the extent of these experiments was very limited; the entire brain was not examined for regional specific expression of clock genes. Therefore, the CISH experiments using a DIG-probe in this chapter will provide a more conclusive and more detailed analysis of rhythmic gene expression throughout the zebrafish brain.

Also, in this chapter a bioluminescent spatial imaging technique will be used to determine whether a region of the zebrafish brain can maintain robust rhythms in *per3* in constant conditions *in vitro*, in a similar way that luminescent imaging has been used to explore clock function in the mammalian SCN (Yamaguchi et al. 2003). The bioluminescent recordings from the *per3-luc* zebrafish can be directly compared to the *per3* expression analysis using CISH and qPCR on wild type zebrafish.

3.2 Aims

This chapter aims to answer:

- Do the clocks in neural and peripheral tissues exhibit the same circadian rhythmicity, in the way the tissues entrain to a LD cycle or free-run in DD?
- Are all cells throughout the zebrafish brain expressing core clock genes rhythmically, or are there localised regions of rhythmicity?
- If there are localised regions of rhythmicity, are some regions able to better maintain phase, akin to the mammalian SCN?

3.3 Methods

3.3.1 *Per3-luc* bioluminescence rhythms

Tissues from 7-month-old adult *per3-luc* were dissected and placed into a 96-well plate, and put into the Packard scintillation counter as described in Chapter 2. A section of the dorsal fin, eye (containing retina), gills, gut, kidney, and liver were placed in each well, whilst the spleen, heart, and gall bladder were placed in whole. The brain was subdivided into the rhombencephalon including spinal cord, di- and mes- encephalon, optic tectum, optic nerves (containing a piece of retina), telencephalon, and olfactory bulbs. The first day of culturing is not shown, as a large amount of bioluminescence is produced following the initial addition of the substrate, luciferin. Analysis to determine peaks and periods were done as described in Chapter 2.

3.3.2 Standard techniques

qPCR was performed as described in Chapter 2. The same calibrator was used for each of the genes studied, so direct comparisons can be made between the different conditions (for example, *in vivo vs in vitro* samples). CISH, immunohistochemistry and the whole brain culture was performed as described in Chapter 2.

3.3.3 Brain slice culture

Brains were dissected from adult *per3-luc* zebrafish and embedded into a block of low melting agarose (4%). This agarose block was affixed onto the vibratome plate using superglue, and slices 300µm thick cut in ice-cold PBS. Slices were placed into a 35mm petri dish containing L15 media with 15% FCS, P/S, gentamicin and 0.5mM luciferin, and sealed with parafilm. The dish was placed immediately in the IPD (Photek Inc) and kept at a constant 28°C. Brightfield images were taken to identify the regions and position the section under the camera. The culture was kept on a 12:12LD for two days and then kept in DD for the recording. Bioluminescent imaging began at the beginning of the second dark phase.

3.4 Results

3.4.1 Bioluminescent rhythms from isolated *per3-luc* zebrafish tissue cultures in LD cycles

The first results presented here show bioluminescent traces representing *per3* expression in numerous cultured brain regions and tissues in different lighting conditions using the *per3-luc* zebrafish. *Per3* rhythms from cultured adult *per3-luc* brain regions and organs entrain to a 24-hour period on a 12:12LD cycle with a peak between ZT4-7 (Figure 20 and 21, Table 2). When put in DD conditions all the cultures free-ran, dampening with a longer period and later phase than observed in LD ($p<0.0001$, two-tailed paired t-test, n=3-8 per tissue). Moreover, there was no difference in period or phase between the different tissues and brain regions in DD (Table 2, One way ANOVA with Tukey's multiple comparison post-test, n=3-8 per tissue), suggesting the peripheral tissues are able to entrain to the LD cycle as effectively as the brain regions. During DD the amplitude of the rhythm decreased most likely as the cells within the tissue lose synchronicity with each other, however, when returned to LD conditions the amplitude increased as the cells are resynchronised to the new LD cycle. All the tissues were able to re-entrain to the second LD cycle; therefore all the tissues contain directly light sensitive structures.

Cultures of zebrafish *per3-luc* tissues and brain regions are also able to re-entrain to new LD cycles rapidly, as shown by adapting tissues to seven 12:12LD cycles and then going directly into a 12:12 reverse light dark cycle (DL) (Figure 23 and 4). The olfactory bulb cultures had a much broader peak in the DL condition but the bioluminescent signal was still above background so the tissues were still alive (Figure 22A). When changing into the DL cycle the amplitude of the rhythm decreased in all tissues tested, suggesting that not all of the cells in the tissue were able to re-entrain to the new DL cycle.

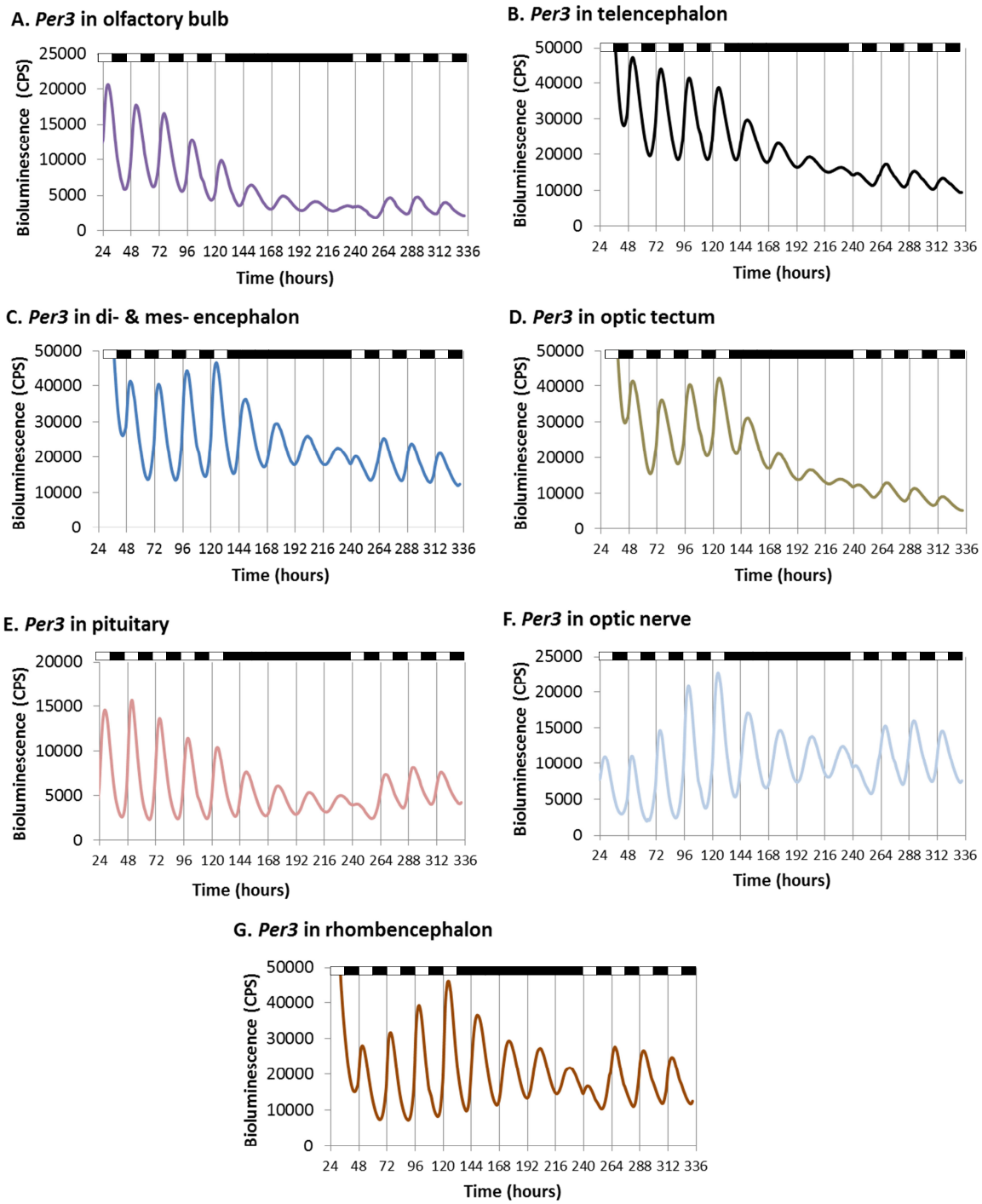


Figure 20 Bioluminescent rhythms from isolated brain cultures from *per3-luc* zebrafish

Tissues were dissected from adult *per3-luc* zebrafish and monitored for bioluminescence over 14 days. Samples were kept in 6 days of 12:12LD, 4 days of DD and 4 days back into LD. The mean bioluminescence from 3-4 tissue samples is plotted. All tissues and brain regions entrain to a 24-hour period in the LD cycle and free-run with a longer period in DD ($p<0.0001$, two-tailed paired t-test, n=3-8 per tissue). Black and white boxes indicate the lighting regime.

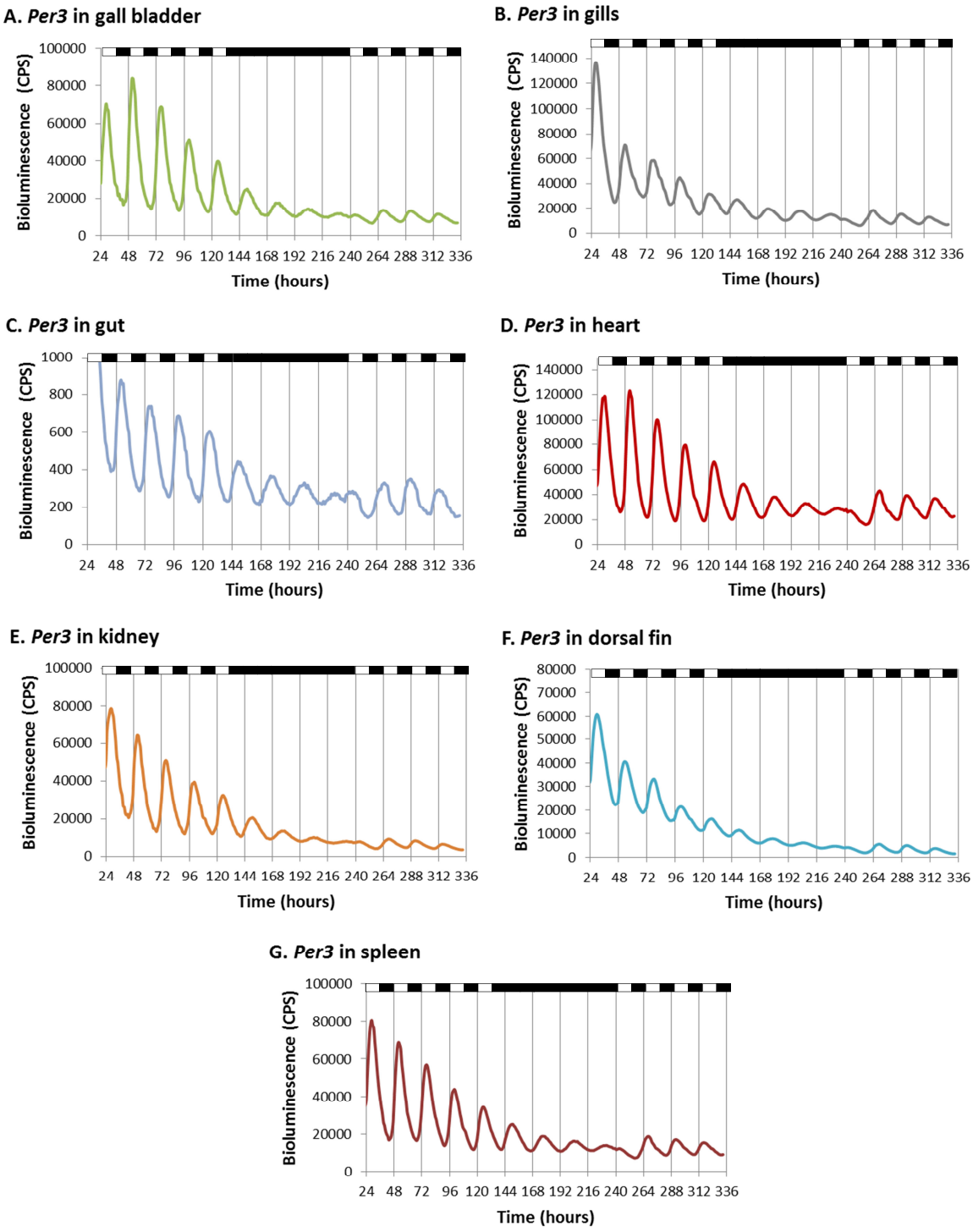


Figure 21 Bioluminescent rhythms from peripheral tissue cultures from *per3-luc* zebrafish

Tissues were dissected from adult *per3-luc* zebrafish and monitored for bioluminescence over 14 days. Samples were kept in 6 days of 12:12LD, 4 days of DD and 4 days back into LD. The mean bioluminescence from 3-4 tissue samples is plotted. All tissues and brain regions entrain to a 24-hour period in the LD cycle and free-run with a longer period in DD ($p<0.0001$, two-tailed paired t-test, n=3-4 per tissue). Black and white boxes indicate the lighting regime.

Culture	LD period (hours)	Peak (ZT)	DD free-running period (hours)	Peak (CT)
Brain region				
Olfactory Bulbs	24.3 ± 0.2	5.0 ± 0.0	27.3 ± 1.1	17.3 ± 2.6
Telencephalon	24.1 ± 0.1	5.0 ± 0.0	26.3 ± 0.4	14.0 ± 1.1
Di- & Mes- encephalon	24.3 ± 0.3	4.5 ± 0.3	26.1 ± 0.2	13.0 ± 0.6
Optic Nerve	23.5 ± 0.2	5.0 ± 0.0	26.5 ± 0.0	16.3 ± 0.6
Optic Tectum	24.0 ± 0.1	5.3 ± 0.2	25.3 ± 0.7	13.5 ± 0.4
Pituitary	24.2 ± 0.2	5.0 ± 0.0	26.7 ± 0.2	14.7 ± 0.3
Rhombencephalon	24.2 ± 0.3	4.3 ± 0.3	25.8 ± 0.2	12.7 ± 0.3
Peripheral tissues				
Dorsal Fin	24.4 ± 0.7	6.0 ± 0.4	27.3 ± 0.6	12.3 ± 3.8
Gall Bladder	24.2 ± 0.3	4.7 ± 0.3	26.5 ± 0.6	14.7 ± 0.9
Gills	24.3 ± 0.6	7.3 ± 1.6	26.8 ± 0.2	15.5 ± 1.0
Gut	24.0 ± 0.4	5.0 ± 0.0	26.1 ± 0.1	14.3 ± 0.5
Heart	24.0 ± 0.0	4.7 ± 0.3	27.0 ± 0.0	16.7 ± 0.7
Kidney	24.0 ± 0.3	5.0 ± 0.0	26.9 ± 0.2	15.8 ± 0.9
Spleen	24.9 ± 0.4	6.5 ± 0.5	27.5 ± 0.3	19.3 ± 1.1

Table 2 Periods and phases of bioluminescence rhythms in cultured tissues in 6 days of LD and 4 days of DD

Tissues were dissected from adult *per3-luc* zebrafish and monitored for bioluminescence over 14 days. Samples were kept in 6 days of 12:12LD and 4 days of DD. The mean period and phase, ± the SEM, are shown for the tissues in the last 3 days of LD or DD. All tissues entrained to a near-24-hour period in the LD conditions (One way ANOVA, n=3-8 per tissue) with the peak of the phase occurring between ZT4-7. All brain regions and peripheral tissues had lengthened periods in DD ($p<0.0001$, two-tailed paired t-test, n=3-4 per tissue) and were phase delayed ($p<0.0001$, two-tailed paired t-test, n=3-4 per tissue), and there was no significant difference between the tissues in either period or phase in DD (One-way ANOVA with Tukey's multiple comparison post-test).

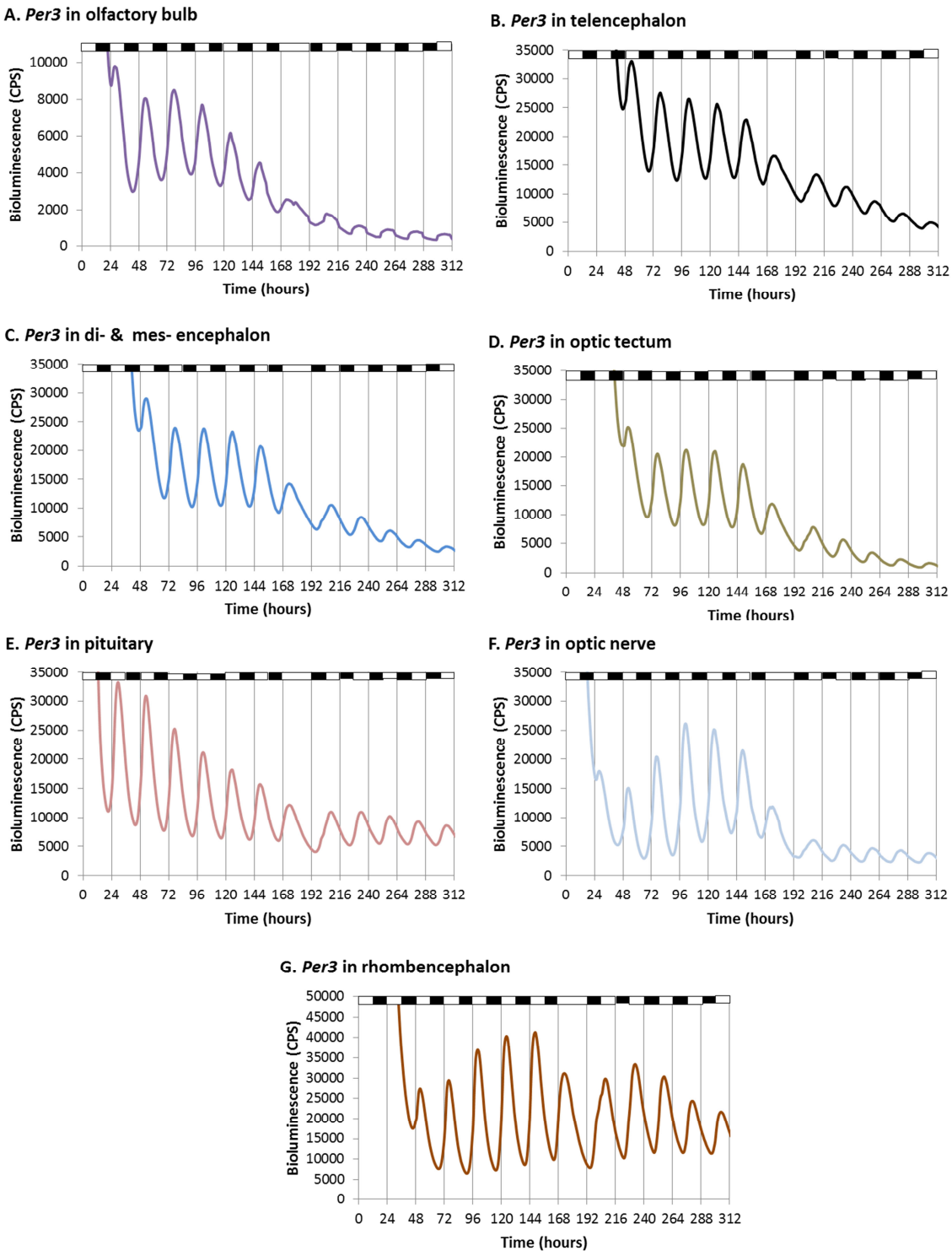


Figure 22 *Per3* rhythms in regional *per3-luc* brain cultures can re entrain to DL cycle

Brain regions were dissected from a *per3-luc* zebrafish and bioluminescence recorded for on 7 days of 12:12LD followed by five cycles of 12:12DL. The average bioluminescence are shown (n=3-8 per brain region). All regions entrained to the LD cycle with a peak around ZT4-5. All regions rapidly entrained to the DL cycle with a peak at ZT4-5, apart from the olfactory bulbs, which had a broad peak and trough and very low amplitude rhythm.

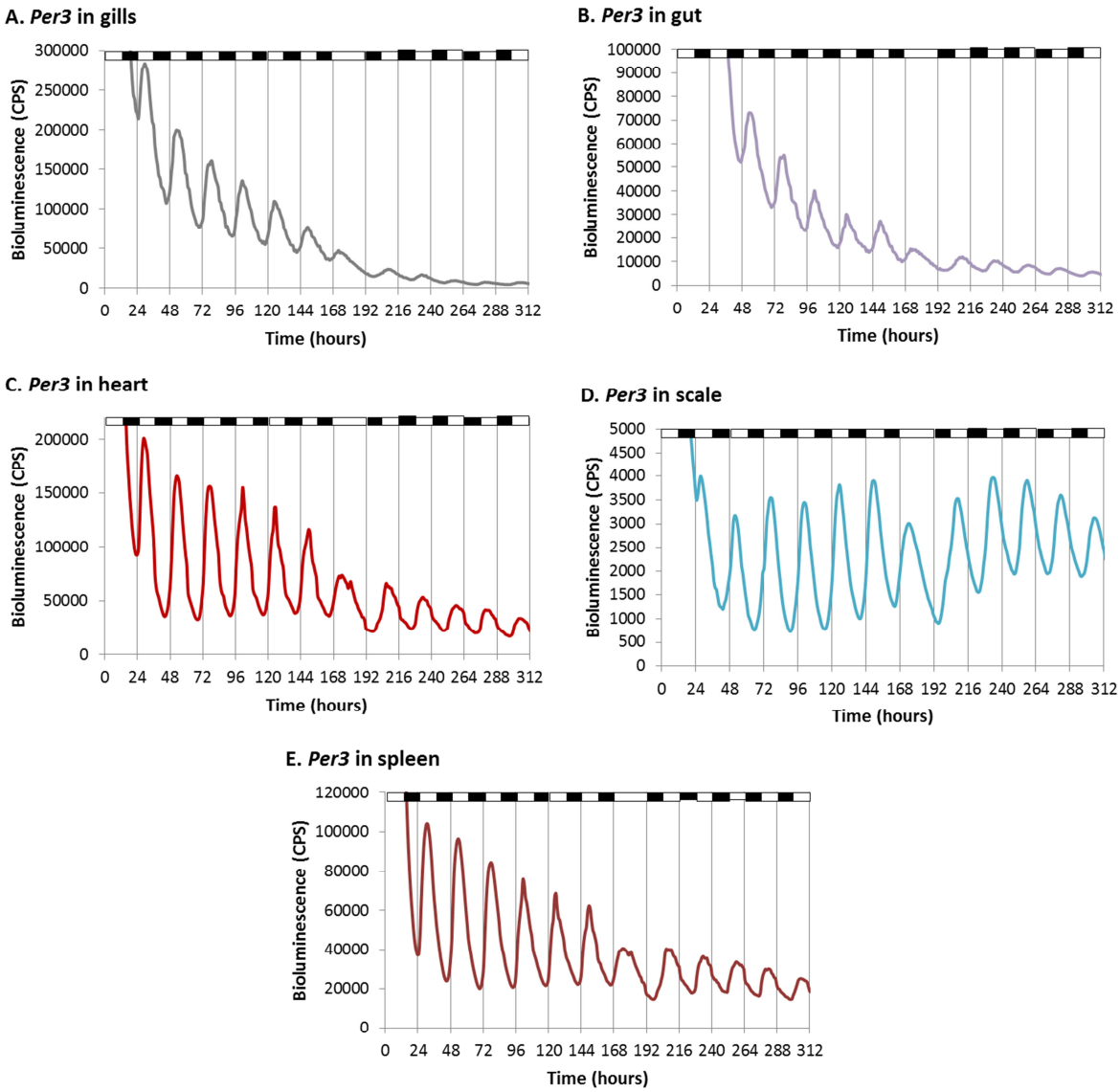


Figure 23 *Per3* rhythms in *per3-luc* peripheral tissue cultures can re-entrain to DL cycle

Tissues were dissected and cultured from a *per3-luc* zebrafish and bioluminescence recorded for on 7 days of 12:12LD followed by five cycles of 12:12DL. The average bioluminescent traces are shown (n=3-4 per tissue). All tissues entrained to the LD cycle with a peak around ZT4-6. All regions rapidly entrained to the DL cycle with a peak at ZT4-6.

3.4.2 Endogenous free-running rhythms from isolated *per3-luc* zebrafish tissue cultures in constant darkness

Zebrafish tissues may have differences in endogenous rhythm in constant conditions, which can only be detected using a longer time in DD, therefore tissues were dissected 1-2 hours before lights off and placed immediately into DD. The gut culture rapidly became desynchronised (Figure 25C). After six days in DD some of the zebrafish tissues desynchronised and the rhythm flattened out, including the gall bladder, kidney and gills (Figure 25A,B,E). However, other tissues remained rhythmic for the full 11 days monitored; these including the heart, spleen and scale, and there was no difference in the period and phase (Figure 25, One way ANOVA with Tukey's multiple comparison post-test). Similarly to the DD rhythms observed in the earlier experiment, which had first been on a LD cycle (Figure 20 and 2), these tissues displayed a robust circadian rhythm with a period longer than 24 hours.

Contrastingly to the peripheral tissues most of the brain tissues rapidly desynchronised in DD, apart from the pituitary which still had a circadian rhythm after 11 days of recording (Figure 24). This lack of robust rhythmicity found in some peripheral tissues and brain regions could be due to these tissues depending on signals generated from other neuronal pacemakers, or in part driven by humoural cues, or possibly that coupling between cellular oscillators is stronger in certain tissues/brain regions. Neuronal pacemakers like the SCN are small regions and any robust rhythmicity could be masked by larger neighbouring regions desynchronising. Therefore, it was important to next evaluate in further detail the *per3* expression and rhythmicity of the different regions of the zebrafish brain.

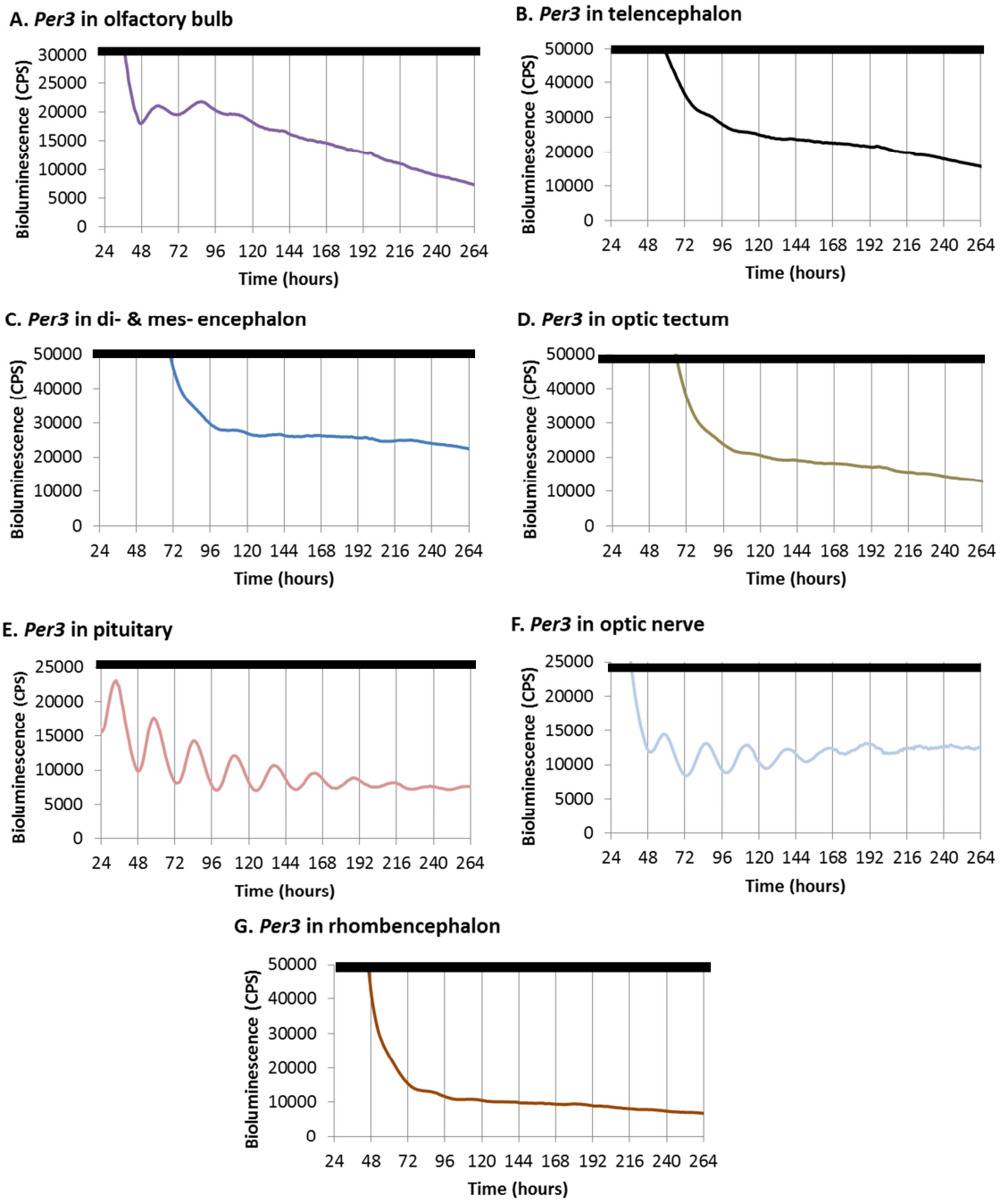


Figure 24 *Per3* rhythmicity in DD in cultured *per3-luc* brain regions

Adult *per3-luc* zebrafish were kept on 14:10 LD. Brain regions were dissected pre-lights off (approximately ZT13) and monitored for bioluminescence over 11 days in constant dark. The average bioluminescence is plotted (n=3-7 per brain region). Robust rhythmicity was observed in the pituitary. By the eleventh day a circadian rhythm could only be observed in the pituitary culture, all the others were desynchronised.

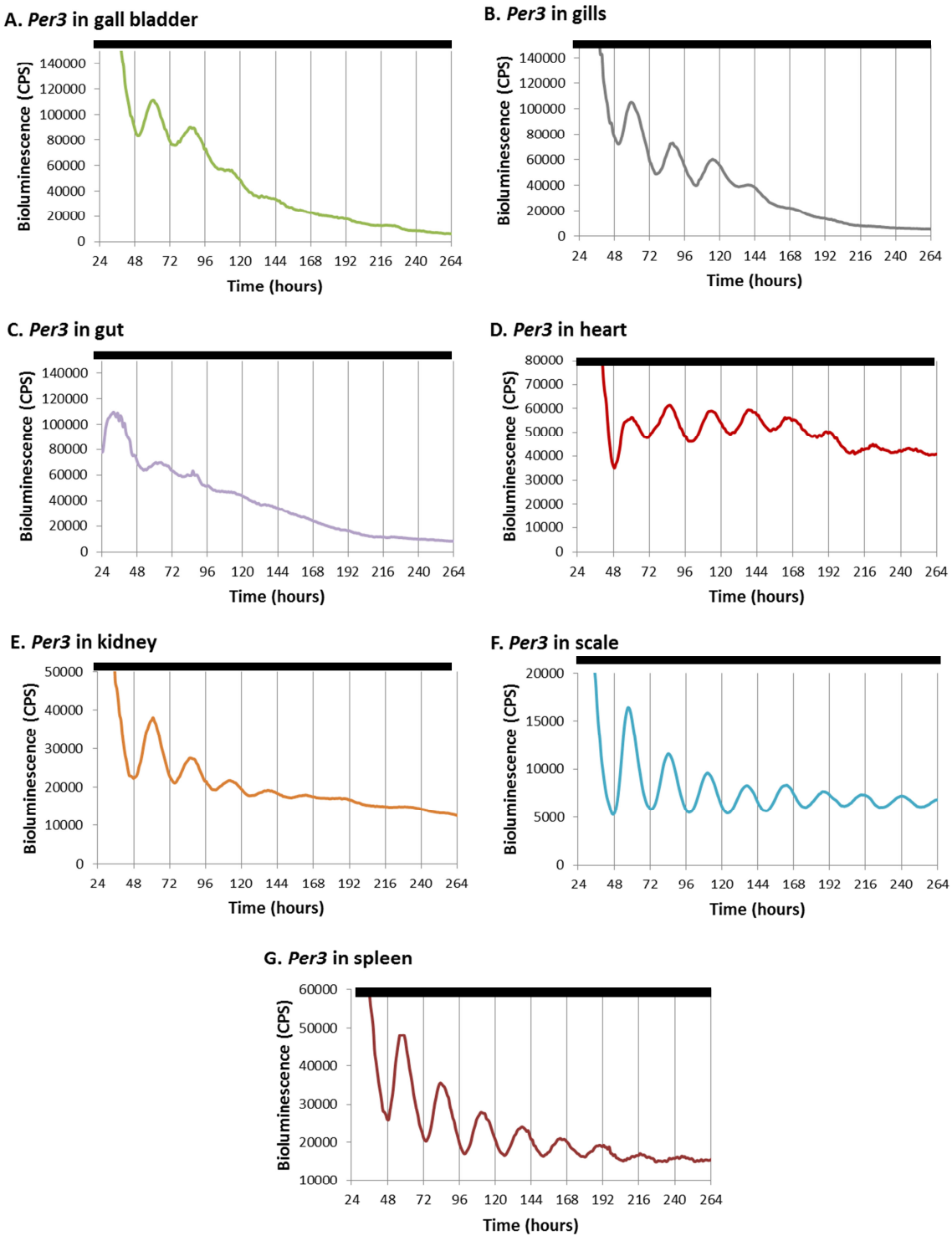


Figure 25 *Per3* rhythmicity in DD in cultured *per3-luc* organs

Adult *per3-luc* zebrafish were kept on 14:10 LD. Tissues were dissected pre-lights off (approximately ZT13) and monitored for bioluminescence over 11 days in constant dark. The average bioluminescence is plotted (n=3-4 per tissue culture). The, heart, scale and spleen all displayed robust rhythmicity throughout the recording. The gall bladder, gills, and kidney are desynchronised by six cycles, moreover, the gut rapidly became desychronised.

Culture	DD free-running period (hours)	Peak (CT)
Brain region		
Olfactory Bulbs	-	-
Telencephalon	-	-
Di- & Mes- encephalon	-	-
Optic Nerve	-	-
Optic Tectum	-	-
Pituitary	25.2 ± 0.3	14.7 ± 0.3
Rhombencephalon	-	-
Peripheral tissues		
Scale	25.9 ± 0.2	14.1 ± 1.3
Gall Bladder	-	-
Gills	-	-
Gut	-	-
Heart	25.0 ± 0.8	11.3 ± 1.4
Kidney	-	-
Spleen	28.5 ± 1.1	13.0 ± 1.3

Table 3 Period and phase of free-running rhythms from isolated *per3-luc* zebrafish tissue cultures kept in constant darkness

Tissues were dissected pre-lights off (ZT10-12) from adult *per3-luc* zebrafish and monitored for bioluminescence over eleven days in constant darkness. The mean period and phase, ± the SEM, are shown for the tissues that maintained a circadian rhythm for the full 11 days. All brain regions and peripheral tissues with a detectable rhythm had lengthened periods in DD and were phase delayed, and there was no significant difference between the rhythms in cultures in either period or phase (One way ANOVA with Tukey's multiple comparison post-test).

3.4.3 Regional *per3* expression in the zebrafish brain *in vivo*

Adult wild type zebrafish were kept on a 14:10LD cycle and brains were collected at ZT3 and ZT15. The tissues were fixed, frozen, sectioned and CISH performed to show expression of *per3* mRNA. The results from CISH agree with the bioluminescent results that *per3* was more highly expressed in the brain at ZT3 than ZT15. Interestingly, CISH revealed that *per3* was indeed expressed in specific brain nuclei throughout the brain at ZT3 (Table 4). These regions will be briefly stated here.

In the telencephalon, *per3* was expressed in the internal and external cell layers (ICL and ECL) of the OB, the dorsal zone of the dorsal telencephalic area (D), and diffusely in the lateral zone (Dl) (Figure 27). *Per3* was expressed in most regions of the ventral telencephalic area (V) (Figure 26). In the diencephalon *per3* was expressed in the SCN, and in many hypothalamic regions (Figure 28) and the preoptic nuclei. *Per3* mRNA was present in the anterior thalamic nucleus (A) but not in the central or dorsal posterior thalamic nuclei (CP or DP). There was also no *per3* expression in the pretectum.

In the mesencephalon, there was diffuse *per3* expression in the optic tectum (TeO), and strong expression throughout the PGZ and torus longitudinalis (TL) (Figure 29). The only nucleus in the tegmentum expression *per3* was the perilemniscal nucleus.

In the rhombencephalon, *per3* was expressed in the granular cellular in the lateral and medial valvula cerebelli (Val and Vam), the corpus cerebellum (CCe), eminentia granularis (EG), and lobus caudalis cerebelli (LCa) (Figure 30). The vagal lobe (LX) was the only brainstem cranial nuclei to express *per3*.

Per3 is found in the somal cytoplasm (Figure 31) and many of the brain nuclei expressing *per3*, such as the PGZ, are densely packed with cell bodies. This led to the question as to whether *per3* was expressed in all cells, but could only be detected in regions with densely packed cell bodies. However, a closer look at the optic tectum, which has also been treated with DAPI, a generic DNA stain, to show the location of nuclei/cell bodies, reveals that *per3* is not expressed in all cells (Figure 31).

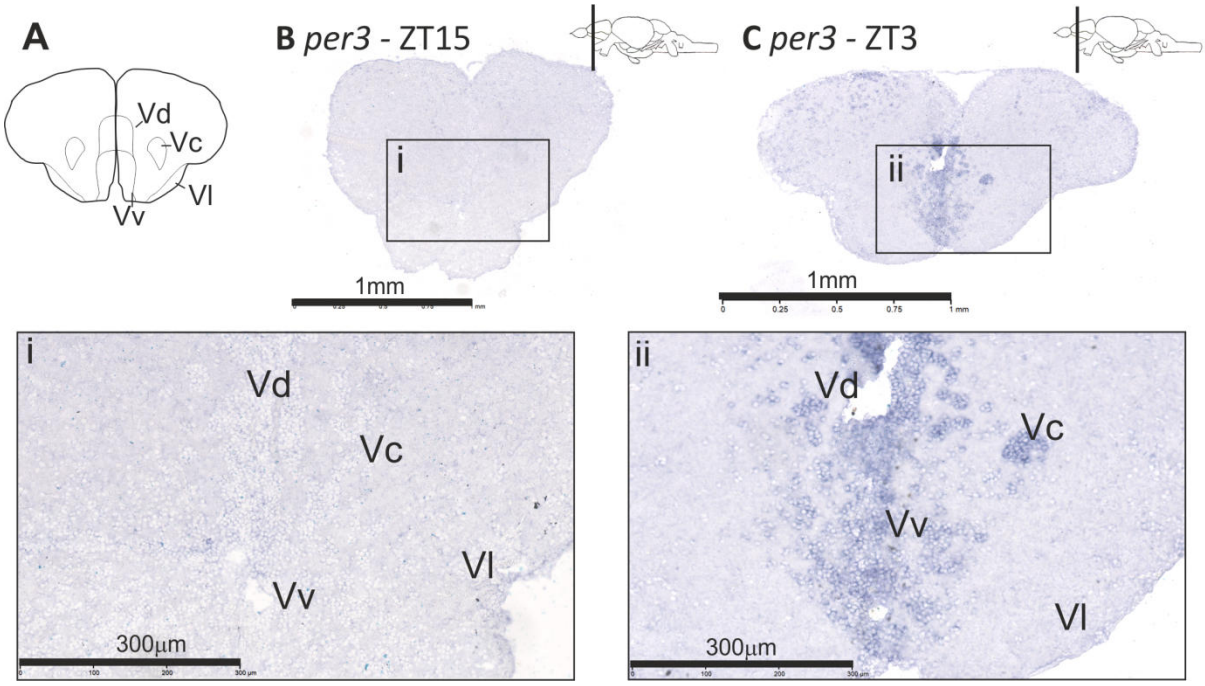


Figure 26 *Per3* expression in the anterior telencephalon

Adult zebrafish were kept on a 14:10LD cycle and brains were collected at ZT3 and ZT15. *In situ* hybridization was performed to show expression of *per3* mRNA. **A)** A schematic of the brain section containing the anterior telencephalon. **B)** At ZT15 there is no expression of *per3* in the Vc, Vd, Vl, or Vv. **C)** At ZT3 there is higher expression of *per3* in the Vc, Vd and Vv, and no expression in the Vl.

[Vc, central nuclei of V; Vd, dorsal nucleus of V; Vl, lateral nuclei of V; Vv, ventral nucleus of V.]

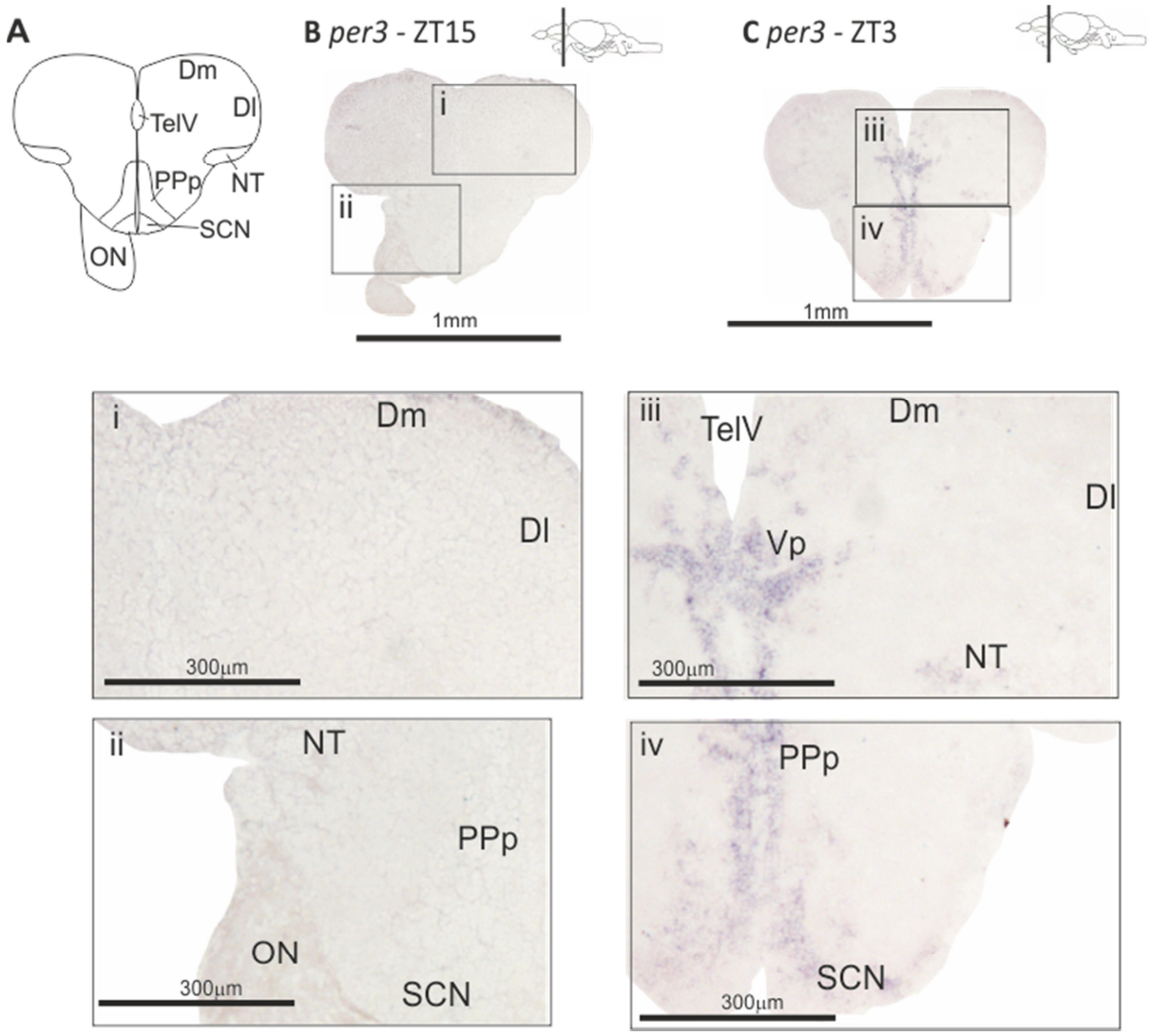


Figure 27 *Per3* mRNA expression in the telencephalon and diencephalon

Adult zebrafish were kept on a 14:10LD cycle and brains were collected at ZT3 and ZT15. *In situ* hybridization was performed to show expression of *per3* mRNA. **A)** A schematic of the brain section containing the telencephalon and diencephalon. **B)** At ZT15 there is no *per3* expression in the Dm, Dl, NT, ON, PPp or SCN. **C)** At ZT3 there is *per3* expression in the PPp, Vp and SCN. There is no expression in the Dm, and + expression in the Dl.

[Dl, lateral zone of the dorsal telencephalic area; Dm, medial zone of the dorsal telencephalic area; NT, nucleus taeniae; ON, optic nerves; PPp, parvocellular preoptic nucleus, posterior part; SCN, suprachiasmatic nucleus; TelV, telencephalic ventricle.]

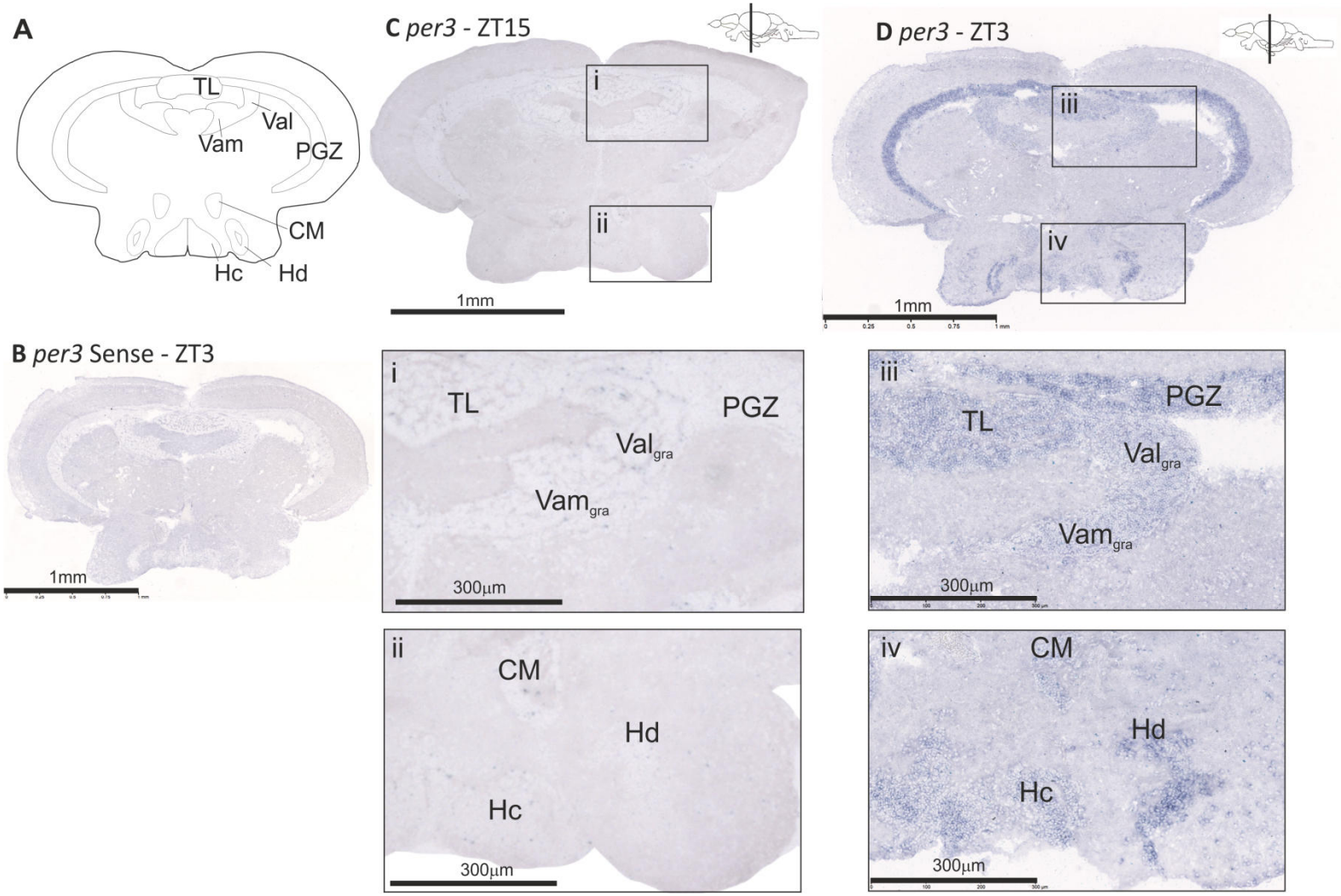


Figure 28 *Per3* expression in the mesencephalon and diencephalon

Figure 28 *Per3* expression in the mesencephalon and diencephalon

Adult zebrafish were kept on a 14:10LD cycle and brains were collected at ZT3 and ZT15. *In situ* hybridization was performed to show expression of *per3*. **A)** A schematic of the brain section containing the mesencephalon and diencephalon. **B)** The sense control shows the background signal. **C)** At ZT15 there are a small number of cells expressing *per3* in the CM, Hc, Hd, and Val_{gra}, and no *per3* detectable in the PGZ, TeO, TL, and Vam_{gra} **D)** At ZT3 there is high expression of *per3* in the CM, Hc, Hd, PGZ, TL, Val_{gra} and Vam_{gra}.

[CM, corpus mammilare; Hc, caudal zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; PGZ, periventricular grey zone of the optic tectum; TeO, optic tectum; TL, torus longitudinalis; $Val_{gra,}$ granular layer of the lateral division of valvula cerebelli; $Vam_{gra,}$ granular layer of the medial division of valvula cerebelli]

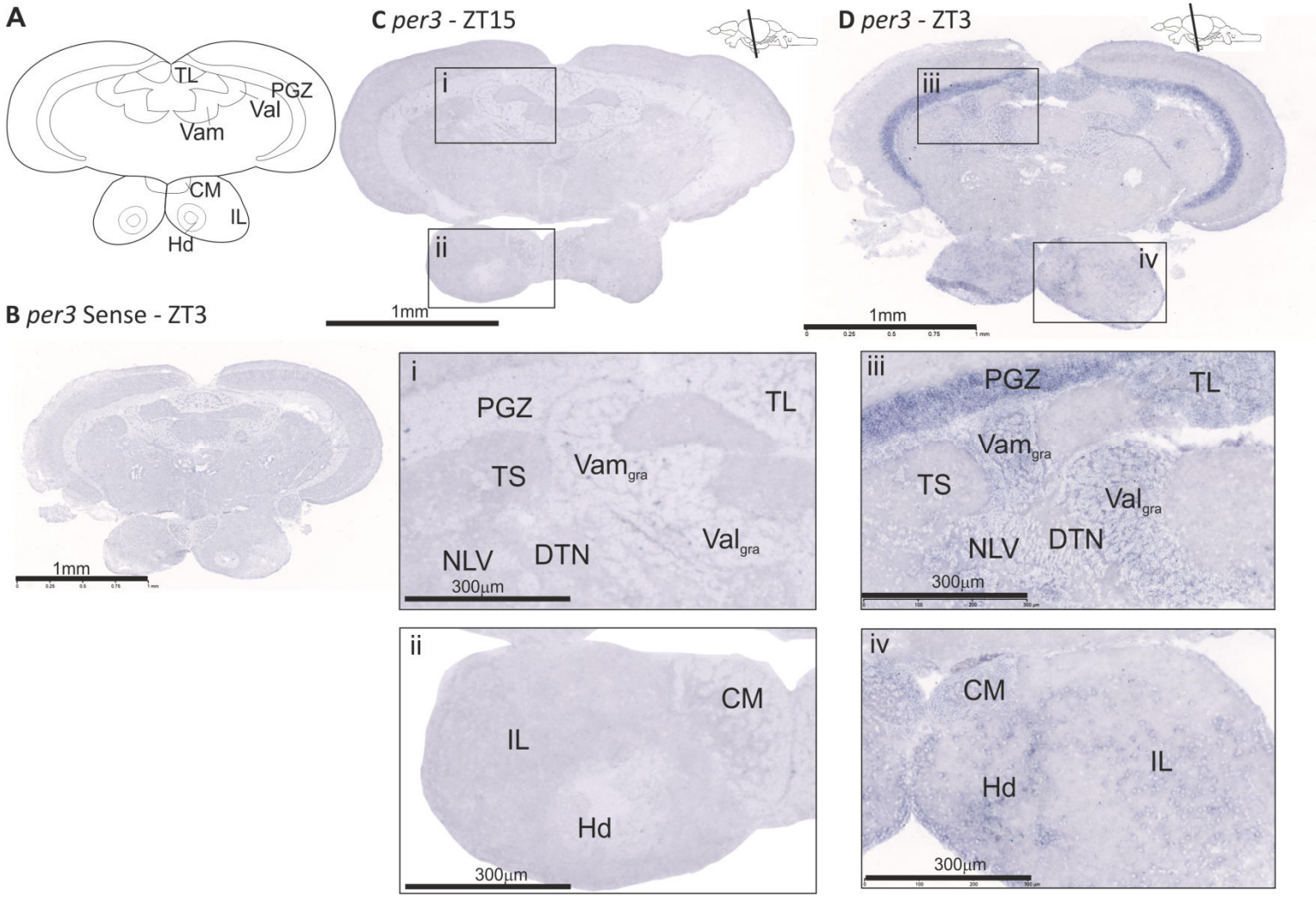


Figure 29 *Per3* expression in the posterior mesencephalon and diencephalon

Figure 29 *Per3* expression in the posterior mesencephalon and diencephalon

Adult zebrafish were kept on a 14:10LD cycle and brains were collected at ZT3 and ZT15. *In situ* hybridization was performed to show expression of *per3*. **A)** A schematic of the brain section containing the posterior mesencephalon and diencephalon. **B)** The sense control shows the background signal **C)** At ZT15 there is no expression of *per3* in CM, DTN, Hd, IL, PGZ, TL, TS, Val_{gra} and Vam_{gra}. **D)** At ZT3 there is raised expression of *per3* in the CM, DTN, Hd, IL, NLV, PGZ, TL, Val_{gra} and Vam_{gra}. There was expression in the ventricular lining cells in the TS.
[CM, corpus mammilare; DTN, dorsal tegmental nucleus; Hd, dorsal zone of periventricular hypothalamus; IL, inferior lobe; NLV, nucleus lateralis valvulae; PGZ, periventricular grey zone of the optic tectum; TeO, optic tectum; TS, torus semicircularis; $Val_{gra,}$ granular layer of the lateral division of valvula cerebelli; $Vam_{gra,}$ granular layer of the medial division of valvula cerebelli]

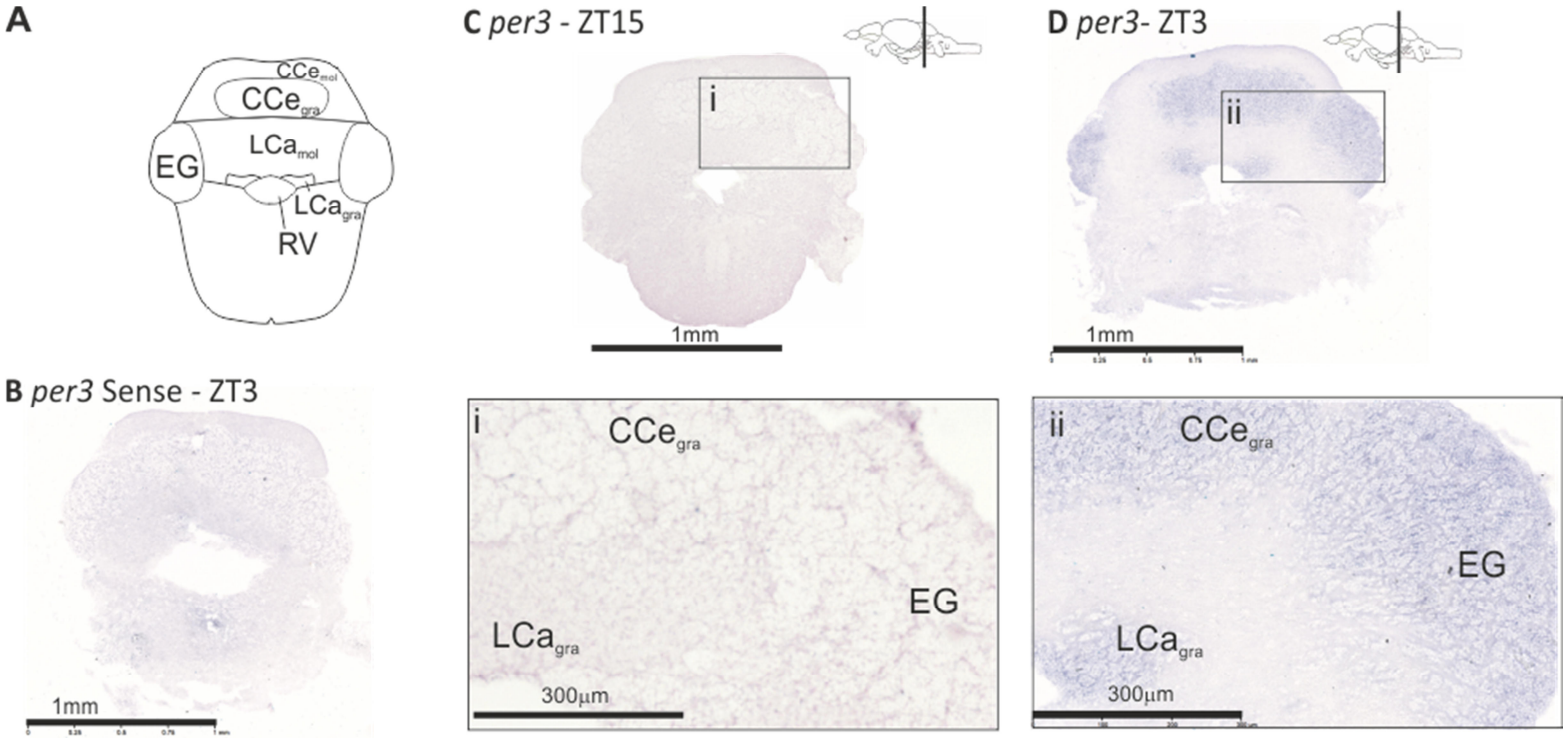


Figure 30 *Per3* expression in the rhombencephalon

Adult zebrafish were kept on a 14:10LD cycle and brains were collected at ZT3 and ZT15. CISH was performed to show expression of *per3*. **A)** A schematic of the brain section containing the rhombencephalon. **B)** The sense control shows the background signal **C)** The dark control shows no expression of *per3* in CCe_{gra}, EG, and LCa_{gra} **D)** In the light pulsed sample there is raised expression of *per3* in the CCe_{gra}, EG, and LCa_{gra} [CCe_{gra}, granular layer of the corpus cerebelli; EG, eminentia granularis; and LCa_{gra}: granular layer of the lobus caudalis cerebelli; RV, rhombencephalic ventricle]

Zebrafish brain nuclei	Per3 (ZT15)	Per3 (ZT3)
Telencephalon		
OB, Olfactory Bulbs		
ICL, internal cellular layer	-	++
ECL, external cellular layer of olfactory	-	++
GL, glomerular layer	-	-
D, Dorsal telencephalic area		
Dc, central zone of D	-	-
Dd, dorsal zone of D	-	++
Dl, lateral zone of D	-	+
Dm, medial zone of D	-	-
Dp, posterior zone of D	-	-
V, Ventral telencephalic area		
Cv, commissural nucleus of V	-	-
Vd, dorsal nucleus of V	-	++
Vv, ventral nucleus of V	-	++
Vp, postcommissural nucleus of V	-	++
Vs, supracommissural nucleus of V	-	++
Vc, central nuclei of V	-	++
Vl, lateral nuclei of V	-	-
EN, entopeduncular nucleus	-	++
NT, nucleus taeniae	-	++
Diencephalon		
Area preoptica		
CO, optic chiasm	-	-
OT, optic tract	-	-
PPa, parvocellular preoptic nucleus, anterior part	-	++
PPd, dorsal part of the pretectal diencephalic cluster	-	++
PPp, parvocellular preoptic nucleus, posterior part	-	++
SCN, suprachiasmatic nucleus	-	++
Epithalamus		
Ha, ventral and dorsal habenula	-	++
Thalamus		
VM, ventromedial thalamic nucleus	-	++
VL, ventrolateral thalamic nucleus	-	++
A, anterior thalamic nucleus	-	++
DP, dorsal posterior thalamic nucleus	-	-
CP, central posterior thalamic nucleus	-	-
ZL, zona limitans	-	++
Pretectum		
PS, superficial pretectal nuclei	-	-
CPN, central pretectal nucleus	-	-
APN, accessory pretectal nucleus	-	-
PO, posterior pretectal nucleus	-	-

Zebrafish brain nuclei	Per3 (ZT15)	Per3 (ZT3)
DAO/VAO, dorsal/ventral accessory optic nuclei	-	-
PPv, periventricular pretectal nucleus, ventral part	-	-
Posterior tuberculum		
Tpp, periventricular nucleus of the posterior tuberculum	-	-
TLa, torus lateralis	-	++
CM, corpus mamillare	+	++
PTN, posterior tuberal nucleus	-	++
PVO, paraventricular organ	-	++
PGl/a, lateral and anterior preglomerular nuclei	-	++
PGm, medial preglomerular nucleus	-	++
Hypothalamus		
ATN, anterior tuberal nucleus	-	++
CIL, central nucleus of the inferior lobe	-	++
DIL, diffuse nucleus of the inferior lobe	-	+
Hc, caudal zone of periventricular hypothalamus	+	++
Hd, dorsal zone of periventricular hypothalamus	+	++
Hv, ventral zone of periventricular hypothalamus	-	++
LH, lateral hypothalamic nucleus	-	++
Mesencephalon		
Superior and inferior colliculi		
TeO, optic tectum	-	+
PGZ, periventricular grey zone	-	++
TL, torus longitudinalis	-	++
TS, torus semicircularis	-	+
LLF, lateral longitudinal fascicle	-	-
MNV, mesencephalic nucleus of trigeminal nucleus	-	-
Nmlf, nucleus of the medial longitudinal fascicle	-	-
PCN, paracommissural nucleus	-	-
Tegmentum		
DTN, dorsal tegmental nucleus	-	-
EW, Edinger-Westphal nucleus	-	-
NLV, nucleus lateralis valvulae	-	++
NIII, Oculomotor nucleus	-	-
NLL, nucleus of lateral lemniscus	-	-
PL, perilemniscal nucleus	-	++
SR, superior raphe nucleus;	-	-
SRF, superior reticular formation	-	-
NIn, nucleus interpeduncularis	-	-
NI, nucleus isthmus	-	-
LC, Locus coeruleus	-	-
SGN, secondary gustatory nucleus	-	-

Zebrafish brain nuclei	Per3 (ZT15)	Per3 (ZT3)
Rhombencephalon		
Cerebellum		
$Val_{gra,}$ granular layer of lateral division of valvula cerebelli	+	++
$Val_{mol,}$ molecular layer of lateral division of valvula cerebelli	-	-
$Vam_{gra,}$ granular layer of medial division of valvula cerebelli	-	++
$Vam_{mol,}$ molecular layer of medial division of valvula cerebelli	-	-
$CCe_{gra,}$ granular layer of corpus cerebellum	-	++
$CCe_{mol,}$ molecular layer of corpus cerebellum	-	-
EG, eminentia granularis	-	++
$LCa_{gra,}$ granular layer of lobus caudalis cerebelli	-	++
$LCa_{mol,}$ molecular layer of lobus caudalis cerebelli	-	-
Medulla oblongata		
brainstem cranial nuclei (except LX)	-	-
LX, vagal lobe	-	++
Gc, griseum centrale	-	-

Table 4 *Per3* is expressed in region-specific brain nuclei of the zebrafish

This table describes the mRNA expression of *per3* in various brain nuclei of wild type zebrafish as shown using chromogenic *in situ* hybridization. Zebrafish were kept on a 14:10LD cycle and brains dissected at ZT3 (the peak of *per3* expression) and ZT15 (the trough). (-, no detectable signal; +, signal found in some cells in the region; ++, signal found in many cells in the region)

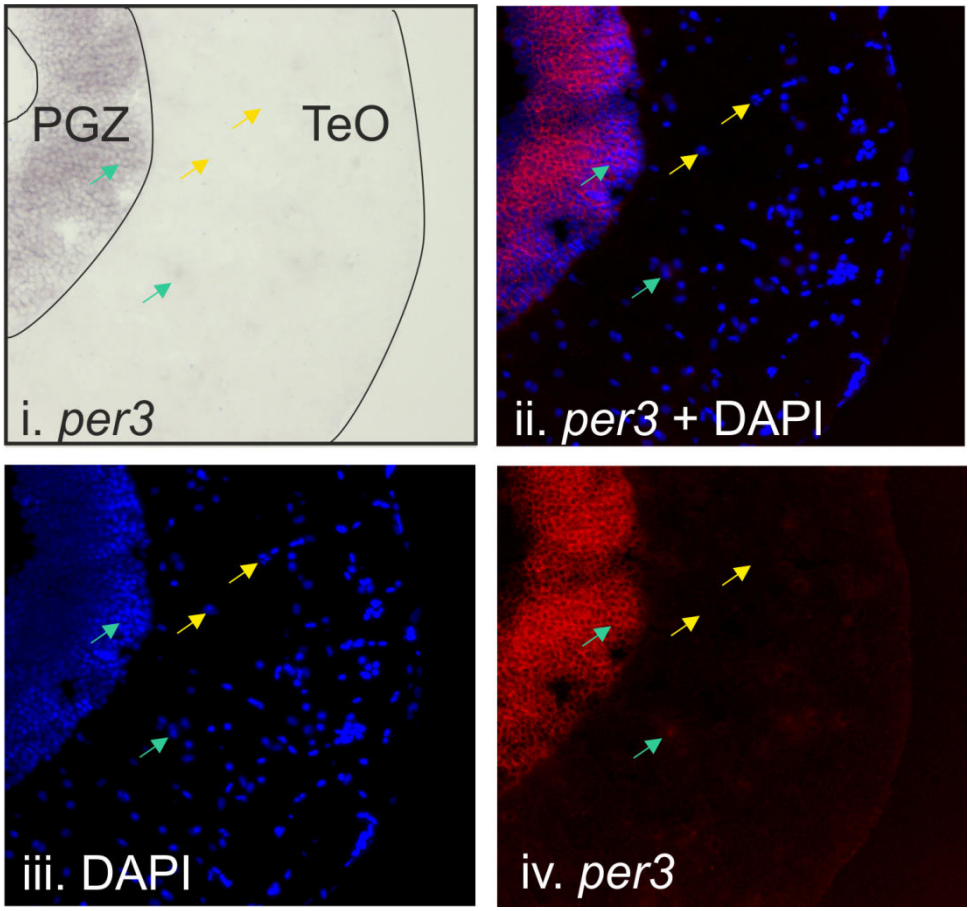


Figure 31 *Per3* is located in the somal cytoplasm

Adult zebrafish were kept on a 14:10LD cycle and brains were collected at ZT3. CISH was performed to show expression of *per3*, DAPI was added to the section to show the location of the cell bodies. *Per3* is highly expressed in cells in the PGZ; however it cannot be detected using CISH in most cells within the other layers of the TeO (20X magnification). **i)** CISH reveals *per3* is expressed in the PGZ and in some cells in the other layers of the TeO (green arrows). In these cells *per3* is located in the somal cytoplasm. **ii)** *Per3* and DAPI merge show *per3* is not detectable in all the cells (yellow arrows). **iii)** DAPI shows the location of cell bodies. **iv)** False coloured CISH *per3* expression.

[TeO, optic tectum; PGZ, periventricular grey zone of the optic tectum]

3.4.4 Temporal *per3* expression in the zebrafish brain *in vitro*

The *per3-luc* rhythms show that the brain can be directly light responsive and rhythmic in culture conditions. The CISH results show that this *per3* expression can be found in discrete regions in the brain. One aim of this project was to monitor the rhythmicity of these regions in culture conditions, ultimately, to determine whether there is an SCN-like region that retains strong rhythmicity within its network of cells. Therefore, a novel brain culturing technique was developed, and as part of the validation for this technique, the endogenous expression of *per3* throughout the whole brain, was compared between *in vivo* and *in vitro* conditions.

Expression levels of *per3* mRNA were quantified *in vivo* using qPCR on whole brain samples collected from wild type zebrafish kept in two days of 14:10LD followed by two days in DD (Figure 32). In LD the peak of *per3* expression is at ZT3, with a trough at ZT15, with an approximate 15-fold difference ($p<0.0001$, One way ANOVA, n=7-10). In DD, this phasing remains the same ($p<0.0001$, One way ANOVA, n=3-5), although the amplitude between peak and trough is halved to 7-fold.

Per3 mRNA was then quantified from whole brain cultures over 3-5 days of culture. The phase of the per3 rhythms is the same *in vitro* as *in vivo,* with a peak at ZT3 and trough at ZT15 ($p<0.001$, One way ANOVA, n=3-4) (Figure 33). This was observed for at least the 5 days measured in this experiment. Unlike in the *in vivo* situation, in the whole brain cultures in DD, *per3* rhythmicity rapidly dampens so that a peak and trough cannot be observed within 2 days ($p<0.0001$, One way ANOVA, n=3-4, with Dunnett's multiple comparison post-hoc test) (Figure 33B). Furthermore, the amount of *per3* mRNA at the peak of expression is not as high *in vitro* as *in vivo.*

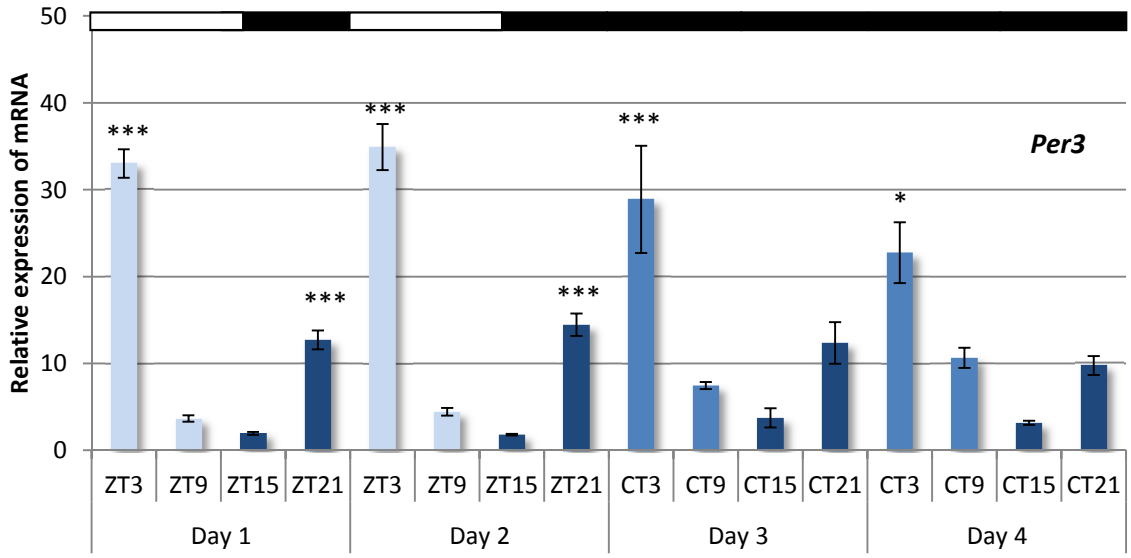


Figure 32 *Per3* is a highly rhythmic circadian clock gene in whole brains *in vivo*

Zebrafish were kept on 2 days of 14:10 LD followed by 2 days of DD, and brains dissected at the times indicated. RNA was extracted and qPCR was performed to evaluate the relative expression of *per3* mRNA. In LD there was a peak at ZT3 and trough at ZT15 ($p<0.0001$, One way ANOVA, n=7-10). In DD there was also a peak at CT3 and trough at CT15 ($p<0.0001$, One way ANOVA, n=3-5). The stars represent the statistical significance from the post-hoc Dunnett's multiple comparison test, which used the calibrator day 1 ZT15 for LD conditions, and day 4 CT15 for DD conditions. The above white black bars represent the lighting conditions, with the different shades of blue representing light, dark or subjective dark phases.

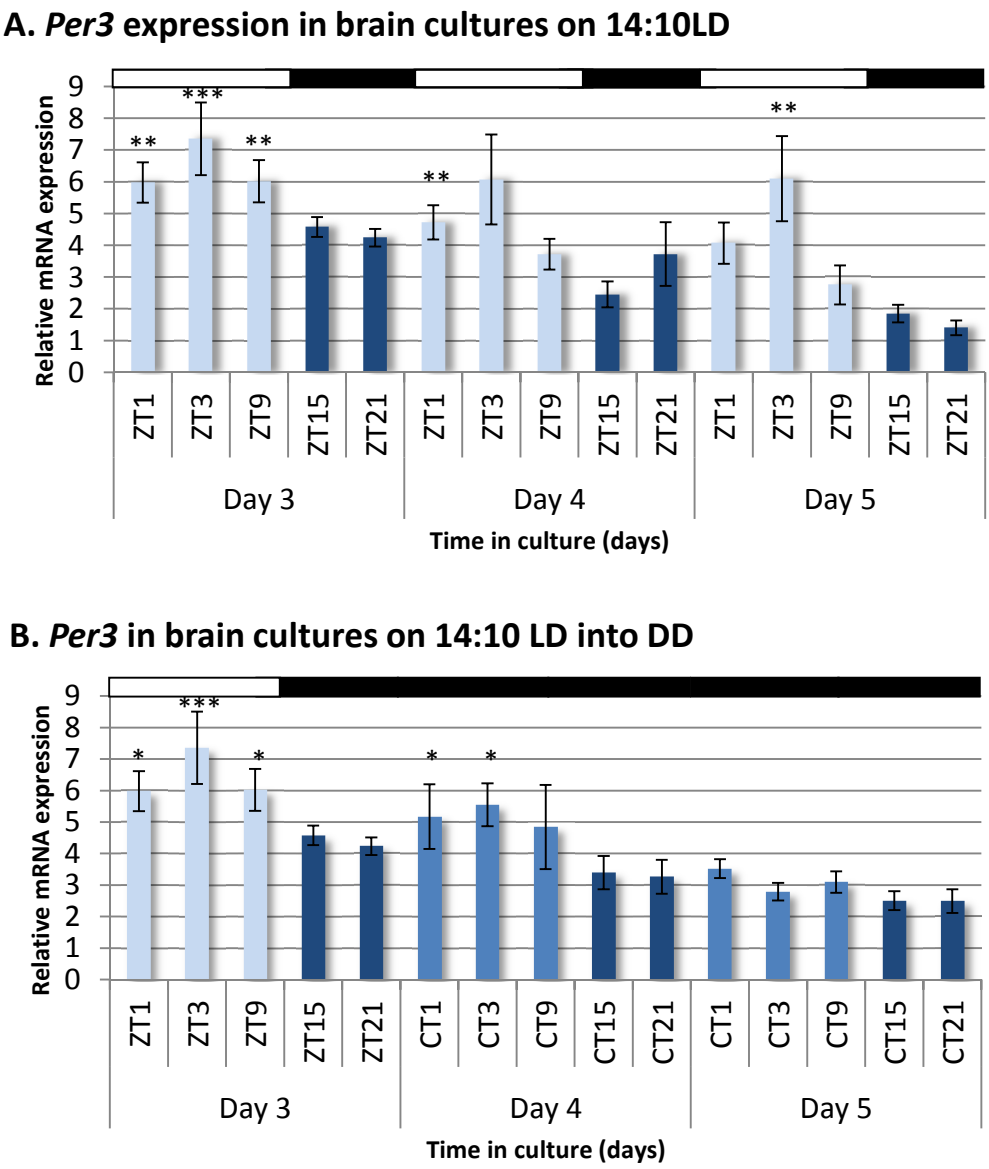


Figure 33 Expression of *per3* in cultured brains under different light conditions

Whole brains were dissected, cultured and kept on a 14:10 LD cycle for 3 days. Samples were collected at the times indicated, mRNA extracted and qPCR performed to determine the expression of *per3* mRNA. **A.** Samples were kept in 14:10 LD. Peak at ZT3, trough at ZT15 ($p<0.001$, One-way ANOVA, n=3-4. The results from the Dunnett's multiple comparison post-hoc test are on the graph, showing the statistical significance between timepoints compared to the trough, ZT21, on day 5) **B.** During day 4 and 5 the brain cultures were put in darkness. There was a peak at CT3 on the first day of darkness but the rhythmicity did not persist into the second day ($p<0.0001$, One way ANOVA, n=3-4. The results from the Dunnett's multiple comparison post-hoc test are on the graph, showing the statistical significance between timepoints compared to the trough, CT21, on day 5). The above white black bars represent the lighting conditions, with the different shades of blue representing light, dark or subjective dark phases.

3.4.5 Regional *per3* expression in the zebrafish brain *in vitro*

The next step was to determine whether the same localised expression of *per3* remained in the whole brain cultures *in vitro*. CISH was performed on sections of a whole brain culture kept on a 14:10LD cycle for 3 days and collected at ZT3. The results showed that the cultures also showed regional expression of *per3* as in the *in vivo* state (Figure 34). Some of these regions included the TL, Val_{gra}, $Val_{mol,}$ PGZ, hypothalamus, which match with the regions expressing *per3* mRNA *in vivo*.

Finally, the *per3* rhythms in a cultured *per3-luc* brain slice were recorded spatially using luminescent imaging in the IPD system. The results of this dynamic high-resolution spatial imaging revealed localised regions of rhythmicity. In one recording the PGZ was observed retaining a more robust rhythm than the Hv (Figure 35). Therefore, this technique is also able to show differences in rhythmicity between different brain nuclei in the zebrafish brain.

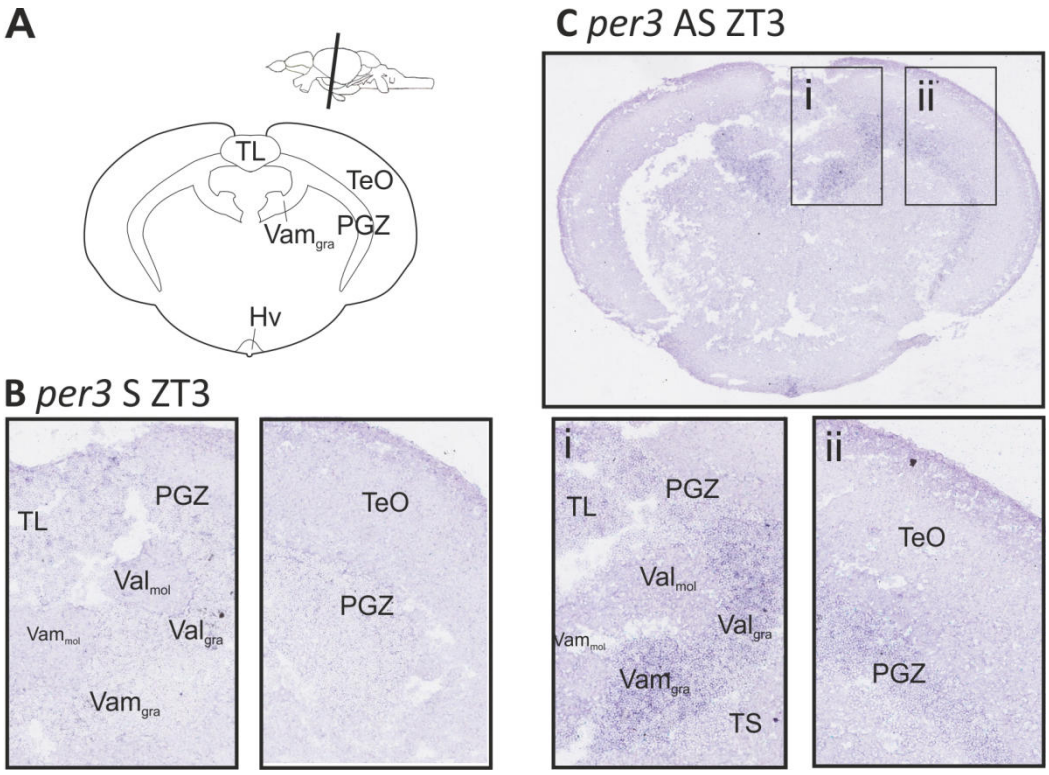


Figure 34 *Per3* expression in regions of a cultured zebrafish brain

Zebrafish brains were cultured for 3 days on a 14:10LD and then collected on day 4 at ZT3, fixed, frozen and sectioned. CISH was performed for the detection of *per3* mRNA. Per3 is found in specific brain nuclei, matching what is seen in the in vivo situation. **A** Schematic of the brain regions shown. **B)** The *per3* sense probe shows the background staining. **C)** *Per3* mRNA is detected in specific nuclei: TL, Val_{gra}, Val_{mol} and PGZ. It is not expressed in the TeO and TS. Inserts are at 20X magnification.

[PGZ: periventricular grey zone of the optic tectum; TeO: optic tectum; TS: torus semicircularis; Val_{gra}: granular layer of the lateral division of valvula cerebelli; Vam_{gra}: granular layer of the medial division of valvula cerebelli]

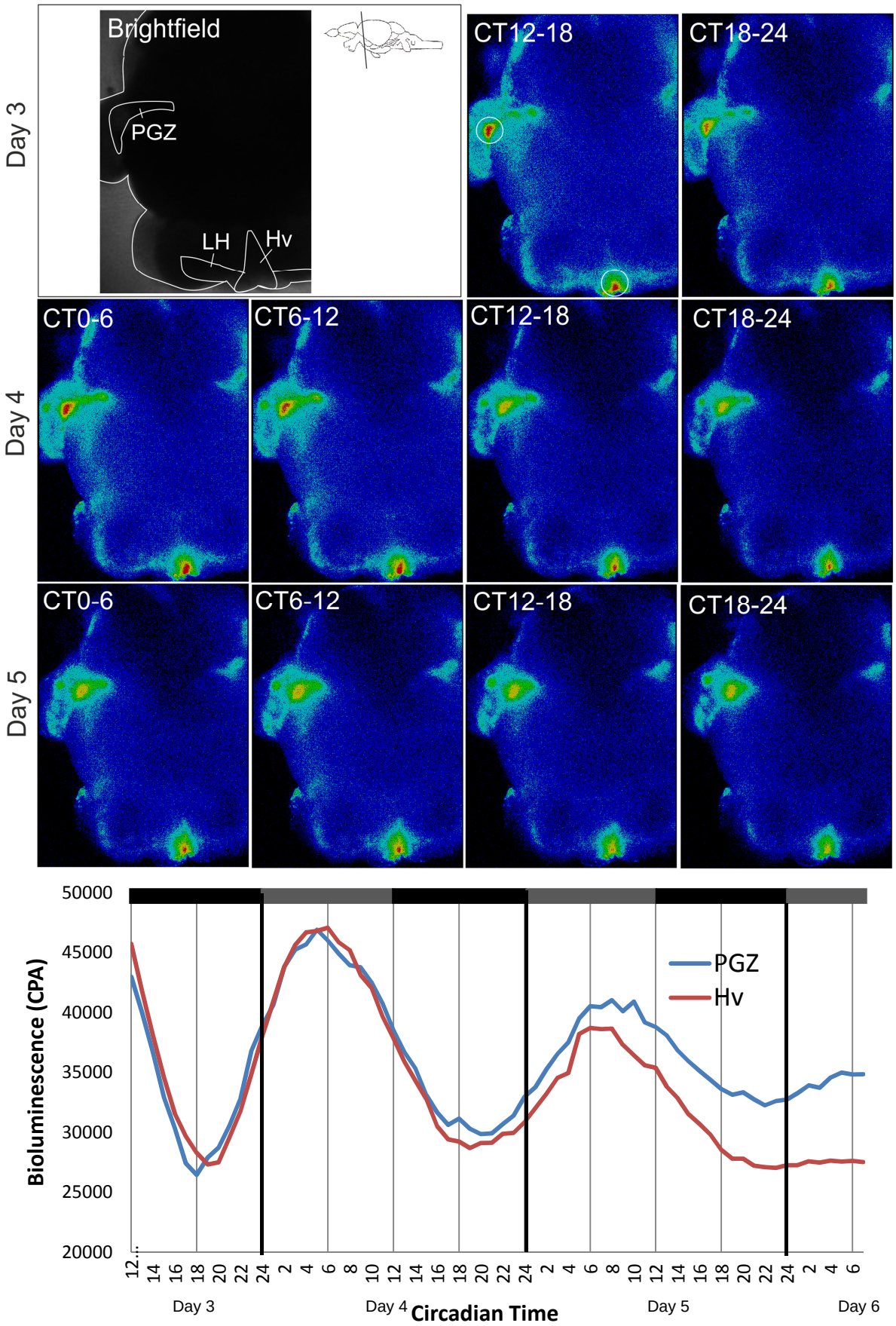


Figure 35 Regional *per3* rhythmicity in a *per3-luc* brain culture

Figure 17 Regional *per3* rhythmicity in a *per3-luc* brain culture

A cross section of a *per3-luc* brain was cultured at a constant 28°C on an IPD. The slice was kept on a 14:10LD cycle for three days. A brightfield image was taken before the beginning of the dark phase of day 3, when bioluminescence then began to be monitored as the tissue was put into DD conditions. Circadian rhythms of *per3* expression could be observed in two regions: the PGZ and Hv. The rhythmicity of these regions peaked at CT5 on day 4 and CT7 on day 5 as the clocks in these regions free-ran. Differences could be seen between these two regions on day 6 when *per3* expression began to rise in the PGZ but not in the Hv, which was likely becoming desynchronised.

[Hv, ventral zone of the periventricular hypothalamus; PGZ, periventricular grey zone of the optic tectum]

3.5 Discussion

This chapter has evaluated the circadian rhythmicity in the zebrafish brain by looking at the dynamic and spatial expression of the clock gene *per3*. This is the first presentation of high resolution *per3-luc* rhythms in the zebrafish brain, shown both dynamically and spatially. It is also the first report of the regional specific expression of *per3* in the zebrafish brain. Together this data provides indication that neuronal pacemakers may be found throughout the zebrafish brain.

3.5.1 *Per3* rhythms in central *vs* peripheral tissues

Presented here are the first demonstrations of dynamic recordings of circadian rhythms from *per3-luc* brain parts. These results show that all brain regions contained directly light sensitive structures that enabled the cultures to entrain to LD cycles. Furthermore, the results from these bioluminescent traces confirm previously reported findings that *per3-luc* peripheral tissues also entrain directly to LD cycles in culture (Kaneko et al. 2006). All these findings build on early work showing that clock genes are expressed in the brain and in tissues throughout the zebrafish body and these tissues can entrain directly to a LD cycle (Whitmore et al. 1998).

There are many similarities between the light entrainment properties of peripheral tissues and brain regions. When switching from a 12:12LD to a 12:12DL cycle all the tissues and brain regions show a rapid entrainment to the new cycle, except the olfactory bulbs, which re-entrained but with a broad peak in the light phase. On a LD cycle the rhythms of central and peripheral tissues are indistinguishable, with a peak between ZT3-5. Moreover, there is no significant difference in the free-running properties of peripheral tissues and brain regions in DD after entrainment with six LD cycles.

Contrastingly, differences in the free-running properties between peripheral tissues have been reported (Kaneko et al. 2006). In those experiments, the cultures were exposed to one cycle of LD before entering six days of DD, whereas in the experiments reported here the tissues were exposed to six LD cycles prior to entering DD for four days. To explore whether the number of LD cycles exposed to the culture prior to DD affects the robustness of the endogenous free-running rhythm, the next experiments used tissues dissected at ZT13 and cultured them directly

into DD without exposure to a LD cycle. Indeed, in this case a distinct difference could be found between the tissue cultures in DD. Peripheral tissues such as the heart, spleen and scale maintain a robust rhythm of *per3* in DD, whereas many brain regions and the gut have very weak circadian rhythms. Therefore the number of entraining cycles applied to the cultures is important for maintaining the endogenous rhythmicity subsequently in DD. This rapid dampening of neural tissue immediately entering DD conditions was rather unexpected, especially compared to the more robust oscillations seen in the pituitary gland and optic nerves. The brain appears to oscillate robustly *in vivo*, as measured by qPCR, so this near instant loss of rhythmicity could reflect very weak coupling between cellular clocks in these brain regions, such that they rapidly move out of phase. Presumably, if this was true, then oscillator coupling would be stronger in the pituitary gland. It is also possible that the brain is more sensitive to dissection than other tissues, and that surgery disrupts rhythmicity in these areas more easily than say, for example, in the pituitary gland or heart.

Greg Cahill, who created the *per3-luc* transgenic zebrafish, showed robust endogenous rhythms from the heart, spleen and gall bladder after six days in DD (Kaneko et al. 2006). These results are replicated in the findings reported here, although continuing recording for eleven days meant the rhythm from the gall bladder desynchronised. The robust rhythmicity and direct light sensitivity of some of the peripheral tissues suggest that the cells within the tissues contain strongly coupled autonomous clocks. This has been the view of peripheral tissues in the zebrafish community (Vatine et al. 2011).

However, the many tissues that lacked a robust rhythm for a long period in culture may demonstrate a potential need in the zebrafish for signals from neuronal pacemakers to drive or at least coordinate rhythms in the periphery. Neuronal pacemakers may be present in the zebrafish brain, driving rhythms in these tissues, but in the culture used in this assay, the signal from these putative neuronal pacemakers may be cut-off by severed neuronal tracts, and therefore rapid desynchronisation could occur. Of course, there is also the possibility of humoural factors providing synchrony to these peripheral clocks, on top of the direct light sensitivity seen in culture. In fact, brains cultured without the pineal in LD display a robust circadian rhythmic expression of *per3*, which suggests there are other neuronal pacemakers in

the zebrafish. Therefore, it was next necessary to evaluate the expression of *per3* spatially to determine whether there were any localised areas of rhythmicity that could represent putative neuronal pacemaker or central clock structures.

3.5.2 *Per3* expression in specific brain nuclei

CISH analysis revealed that *per3* is expressed in particular cells in discrete brain nuclei. The results in this chapter provide a comprehensive list of discrete brain regions that express *per3*. This regional expression of *per3* is rhythmic, with expression being higher at ZT3 and lower at ZT15 in all brain regions. Of course, to determine if there were slight phase differences between neuronal nuclei these experiments would need to be performed at much higher temporal resolution, or dynamic imaging approaches need to be used. Nonetheless, there is a low basal level of *per3* detectable by CISH and qPCR at ZT15, and this agrees with findings from the *per3-luc* traces and qPCR data, where the amount of bioluminescent never reaches zero.

Earlier work hinted that the clock genes *bmal* and *clock* were localised in certain brain regions, however due to the technique used there was insufficient fine resolution to do a detailed analysis (Cermakian et al. 2000; Whitmore et al. 1998). The finding that *per3* is regionally expressed, and in the same areas reported in this literature, validates these early findings.

The spatial expression of *per1* has been mapped throughout the hamster brain using ISH with radioactive isotopes at ZT4, a time when high *per1* expression is found in the SCN (Yamamoto, Shigeyoshi, & Ishida 2001). Similar to the findings presented here, *per1* was localised in specific regions. There were high levels of expression in the SCN, granular cells of the ICL, tenia tecta, olfactory tubercle, piriform cortex, and hippocampal gyrus dentatus. Moderate levels of *per1* were found in the anterior paraventricular thalamic nucleus, caudate-putamen, inferior collicus, pontine nuclei, inferior olive, nucleus of the solitary tract, and granular cell layers of the cerebellum.

The rat has also been shown to have spatial expression of the rhythmic mammalian clock genes *per1* and *per2* (Matsui, Takekida, & Okamura 2005). The localisation of *per1* and *per2* is similar to *per1* expression in the hamster, although differences may be explained by the limited number of timepoints studied in the hamster. For example, when further examining the temporal

expression, some regions showed reversed expression profile to that of the SCN. There is high *per1* signal at CT4 and low at CT16 in the OB, granular layer of the hippocampal gyrus dentatus, and SCN. In contrast, moderate *per1* signal was found in other brain regions with an opposite phase, these regions included the tuberculum olfactorium, piriform cortex, supraoptic nucleus, hypothalamic ventromedial nucleus, arcuate nucleus, stratum pyramidale of the Ammon's horn, and the granular layer of the cerebellar cortex. *Per2* signal was also high in the SCN at CT4 and low at CT16. However, all the other regions had an inverse expression, low at CT4 and high at CT16. On the other hand, zebrafish *per3* was high at ZT3 and low at ZT15 in all regions, with no region expressing an inverse temporal rhythm.

Why do these particular zebrafish brain regions have high levels of *per3* gene expression, rather than expression throughout the brain? One suggestion is that these regions may have a high expression level because they are receiving light input from the retina, as with the mammalian SCN. Indeed, *per3* is expressed in many regions that are reported to receive retinofugal inputs in zebrafish, including the ventral thalamic nuclei, PGZ, and Vd (Bally-Cuif & Vernier 2010). However, not all regions that receive input from the retina express *per3*, for example, pretectal regions such as the central pretectal nuclei (CPN). Furthermore, there are many regions that express *per3* that have not been reported to be receive retinofugal inputs in teleosts, such as the hypothalamus, TL, preglomerular nuclei, and valvula cerebelli (Northcutt & Wullimann 1987). The role of the retina in effecting circadian rhythms will be further explored using eyeless larvae in chapter 6, but it appears that retinofugal inputs are not necessary for *per3* expression.

Perhaps the localised expression of *per3* is due to particular neurotransmitter cell locations. Dopamine is the core neurotransmitter in the mammalian SCN for regulating circadian rhythms and responding to photic inputs (Novak & Nunez 1998). Therefore it is plausible that the regions expressing clock genes may share the share neurotransmitter cell types. Tyrosine hydroxylase, a key enzyme for the synthesis of dopamine, was used to label dopamine cells throughout the zebrafish (Rink & Wullimann 2001). Dopamine cells can be found in regions that express *per3*, including the SCN, Hc, Hv, PPa and Vd. However, dopamine cells are not located in many other regions expressing *per3*, including the PG, Hd and PGZ. These regions instead express many

of the other neurotransmitter cell types, for example the PGZ consists of serotonergic (Norton, Folchert, & Bally-Cuif 2008), GABAergic (Yokogawa et al. 2007) and adrenergic cells (Ruuskanen et al. 2005). Therefore the regional expression of *per3* is unlikely due to it being produced by a particular neurotransmitter cell type.

The zebrafish expresses hypocretin receptors (hcrtr), which respond to the wake promoting neuropeptide hypocretin (hcrt), that are located in specific regions of the brain and are thought to be important for the regulation of sleep (Yokogawa et al. 2007). There appears to be remarkable overlap with regions that express *hcrtr* and *per3*, including the ventral thalamic nuclei, PGZ, hypothalamus, and cerebellum. There are a few notable differences including the expression of *hcrtr* in the griseum centrale, which does not express *per3*. However, the relationship between the expression of clock genes and the sleep-regulatory regions of the zebrafish brain is worth further exploration. It is feasible that a strong expression of clock genes in regions regulating sleep would be advantageous and harmonious to the regulation of circadian rhythms.

It is possible that some of these regions expressing high levels of clock genes may represent neuronal pacemaker candidates. To further determine whether these regions have a robust endogenous rhythmicity, which is a necessary characteristic of a neuronal pacemaker, the next experiments explored the expression of *per3* in the zebrafish brain *in vitro*.

3.5.3 *Per3* rhythmicity *in vitro*

A whole brain culturing approach was developed to examine the expression of clock genes *in vitro*. Endogenous *per3* is rhythmic in whole brain cultures on a LD with the same phase as seen *in vivo* and in *per3-luc* cultured brain parts. It is also expressed in the same localised regions. However, the amplitude of the *per3* rhythm was lower *in vitro* than *in vivo* and the rhythm rapidly dampened in DD as seen in cultured *per3-luc* brain parts, unlike the *in vivo* results. There could be a number of causes for this difference in *per3* expression *in vitro* and *in vivo*. During the culturing process, damage to neuronal tracts or cell death could result in neuronal pacemakers generally being less robust, and no longer able to communicate to other regions. Or the neuronal pacemakers can only communicate to a small number of other regions and the robustness of this rhythmicity is masked by the large number of cells in the brain which

are not being driven by these pacemakers. Furthermore, the culturing condition could alter the oscillator networks by changing coupling strength among cells and regions.

It could also be the case that the brain may be reliant on other structures such as the retina or the pineal to drive rhythms within it, and without them the whole brain becomes arrhythmic. However, this is unlikely as CISH on cultured brain tissue reveals *per3* remains localised to specific regions in the brain, without an intact retina or pineal. Also, the *per3-luc* cultures show entrainment and rhythmicity *in vitro*, which also argues against the need to eye and pineal.

High levels of clock genes do not always correlate with rhythmicity in brain regions (Abe et al. 2002). Therefore the last experiments described in this chapter explored whether these regions remain rhythmic *in vitro* by imaging the spatial bioluminescence from *per3-luc* brain slices.

3.5.4 Spatial imaging of *per3-luc* rhythms in a brain slice

The results from *per3-luc* brain slices are the first demonstration of spatial high-resolution circadian rhythms in a zebrafish tissue culture. The results confirm that *per3* is expressed in localised regions in culture, and go further to show that this regional expression remains rhythmic *in vitro*. Though these are only pilot studies at this time, these data demonstrate the utility of this approach in zebrafish, much as it has been employed in mouse. The initial data suggest that some regions are able to maintain a more robust circadian rhythm than others *in vitro* in constant conditions. One region, the PGZ remained rhythmic after 3 days in DD, a time when *per3* expression was shown using qPCR to be arrhythmic globally in whole brain cultures.

Do these robustly rhythmic regions represent regulatory areas? The regions with robust rhythmic expression of *per3* may represent neuronal pacemaker structures with the zebrafish brain. However, it will need to be determined whether these regions are able to drive rhythmicity in other regions, or whether they control the physiology of that brain nucleus locally.

These results provide insight into the temporal regulation of circadian rhythms in the zebrafish brain. It appears that not all peripheral tissues have autonomous clocks and there is likely a role for several brain regions to be neuronal pacemakers. It will next be important to determine whether these rhythmic regions are directly light sensitive, and this will be explored in the next chapter.

3.6 Conclusions

- Molecular oscillators in both neural and peripheral tissues can entrain to LD cycles *in vitro*, confirming previous observations.
- Long-term DD recordings show there are differences between the endogenous circadian rhythms in tissues. Peripheral tissues like the heart and spleen are better able to maintain rhythmicity in constant conditions, although others like the gut rapidly desynchronise.
- There are localised regions of rhythmicity in the adult zebrafish brain, and these represent potential neuronal pacemaker structures.
- The rhythmic clock gene, *per3,* does not appear to be expressed in all cells in the zebrafish brain.
- Some regions are able to maintain a strong circadian rhythm in DD in culture, for example the periventricular grey zone of the optic tectum (PGZ).

Chapter 4

Light sensitivity of the zebrafish brain

4.1 Introduction

In chapter 3 it was shown that the molecular clock in the zebrafish brain can be directly entrained by light in culture, and there were particular regions of the adult zebrafish brain that highly express the rhythmic clock gene, *per3*. This chapter will further explore the expression of clock genes by examining the quantitative and spatial expression of key light induced clock genes, *cry1a* and *per2,* in the zebrafish brain.

As mentioned in the introduction, the *cry* and *per* genes play the role of negative repressors in the molecular core clock loop by disrupting the formation of the CLOCK:BMAL heterodimer. Amongst this core clock loop in the zebrafish, the *cry1a* and *per2* transcription factors are unique in containing D-box elements in their promoter, which allow light to directly drive their expression (Vatine et al. 2009). The expression of *cry1a* and *per2* is thus indicative of a cell being light sensitive. The pathways through which light regulation occurs has not been fully elucidated, but amongst the candidates thought to be involved is the D-box enhancer element binding factor, *thyroid embryonic factor 1* (*tef1*) (Vatine et al. 2009). Although there are many transcription factors that are regulated by light, and there may be a number of other transcription factors that interact with D-boxes to regulate *cry1a* and *per2* (Weger et al. 2011).

The key role that *cry1a* plays in light entrainment of the zebrafish clock has been examined using a c*ry1a-luc* cell line (Tamai, Young, & Whitmore 2007). This study shows that light can directly induce the expression of *cry1a* to 'reset' the clock. Furthermore, overexpression of *cry1a* causes the repression of *per1* rhythmicity in the same way as constant light (LL), which demonstrates how *cry1a* can act as a potent repressor of the clock.

Unlike *cry1a* and the other *per* genes in the zebrafish, *per2* is not rhythmically expressed in constant darkness, but is only upregulated in the presence of light (Cahill 2002; Pando et al. 2001). Nevertheless, functional *per2* is necessary for optimal clock function and generating circadian rhythms; without *per2*, high amplitude rhythms are not achievable on a LD cycle. *Per2* can be induced by light as early as day 1 postfertilisation (Tamai et al. 2004), and is necessary for entrainment and rhythmic melatonin production from the pineal in embryos (Ziv & Gothilf 2006).

There are a few differences in the way the CRY1A and PER2 proteins interact with the CLOCK:BMAL dimer. CRY1A binds to key regions on CLOCK and BMAL necessary for the formation of a CLOCK:BMAL dimer, thereby competing with CLOCK and BMAL to prevent the dimer forming and thus blocking the transcription of the molecular repressor components of the loop. CRY1A also blocks the transactivation domain of BMAL (Tamai et al. 2007). In contrast, PER2 is unable to bind to either CLOCK or BMAL individually, but only does so when CLOCK and BMAL form a heterodimer (Hirayama 2003). A detailed study using cultured cell lines and immunofluorescence have shown that zebrafish CRY1A and PER2 also interact with CLOCK and BMAL in different locations in the cell (Hirayama 2003). CRY1A primarily interacts in the nucleus, whereas PER2 acts to induce the heterodimer CLOCK:BMAL into the cytoplasm. However, co-immunoprecipitation assays further revealed that CRY1A and PER2 can co-operate and both bind to the CLOCK:BMAL complex at the same time to create a ternary structure, which localizes in the nucleus and the cytoplasm.

A few studies have investigated the spatial expression of these light sensitive clock genes in the zebrafish. In the zebrafish larvae *per2* is expressed throughout the larval body and cranial regions (Ziv et al. 2005) and in particular high levels of expression can be seen in the pineal, pituitary, OB at 28 hpf using WISH (Ziv & Gothilf 2006). *Cry1a* has been shown using RNase protection and Northern Blot assays to be rhythmic throughout the adult zebrafish body with high expression at ZT3 in the brain and eyes (Kobayashi et al. 2000). Both *cry1a* and *per2* are found in cell lines derived from embryos (Cermakian et al. 2002; Pando et al. 2001) and in adult skeletal muscle (Amaral & Johnston 2012).

However, there has not yet been a detailed analysis of *cry1a* and *per2* expression in the adult zebrafish brain. In addition, it is not known whether all regions of the brain are directly light responsive.

4.2 Aims

The experiments in this chapter aim to answer:

- Is the whole adult zebrafish brain equally light-sensitive, or are there localised regions of enhanced light sensitivity? Do these areas overlap with the *per3* regions of expression described in the previous chapter?
- Are the key light induced clock genes, *cry1a* and *per2,* upregulated in brain cultures in response to light pulses?

4.3 Methods

A 3-hour light pulse starting at CT19 was chosen for the detection of *cry1a* and *per2*. 3 hours has shown to be a sufficient length of time for detection of upregulated of *per2* (Cermakian et al. 2002) and *cry1a* (Tamai et al. 2007) by light. This is also a time during the dark phase, so light will cause a phase shift in rhythms by the upregulation of *per2* and *cry1a*. The light intensity was approximately 800µW/cm^2.

In chapter 3, bioluminescent recordings showed that the heart was able to retain strong amplitude circadian rhythms in culture for up to 14 days. Therefore, where appropriate, the levels of expression of *cry1a* and *per2* in the brain were compared to the heart as a representative peripheral tissue.

Whole brains were cultured as described in Chapter 2; whole hearts were cultured in the same way. Sample collection, CISH, WISH and qPCR were also performed as described in Chapter 2.

4.4 Results

4.4.1 *Cry1a* and *per2* light induction *in vivo*.

Firstly, the acute light induction of key light induced clock components, *cry1a* and *per2,* were quantified *in vivo* in the brain, heart, and different parts of the brain. A 3-hour light pulse, between CT19 and CT22, was given to zebrafish in their normal environment, before tissues were dissected and mRNA extracted and quantified using qPCR. *Cry1a* and *per2* were induced by light *in vivo* in all samples in the whole brain, heart and all different brain parts (Figure 36).

The light pulsed zebrafish had significantly higher levels than the dark controls in both the heart and brain for *cry1a* ($p=0.0014$, Two way ANOVA, n=3) and *per2* ($p=0.0017$, Two way ANOVA, n=3). Furthermore, the brain also had a higher induction than the heart of *cry1a* ($p=0.0025$, Two way ANOVA, n=3), and *per2* ($p=0.0015$, Two way ANOVA, n=3).

All brain parts showed an increase in *cry1a* in the light pulsed samples ($p<0.0001$, Two way ANOVA, n=3), and *per2* ($p=0.0015$, Two way ANOVA, n=3). Additionally, there was no significant difference between the different brain regions in either *cry1a* or *per2* expression (Two way ANOVA, n=3).

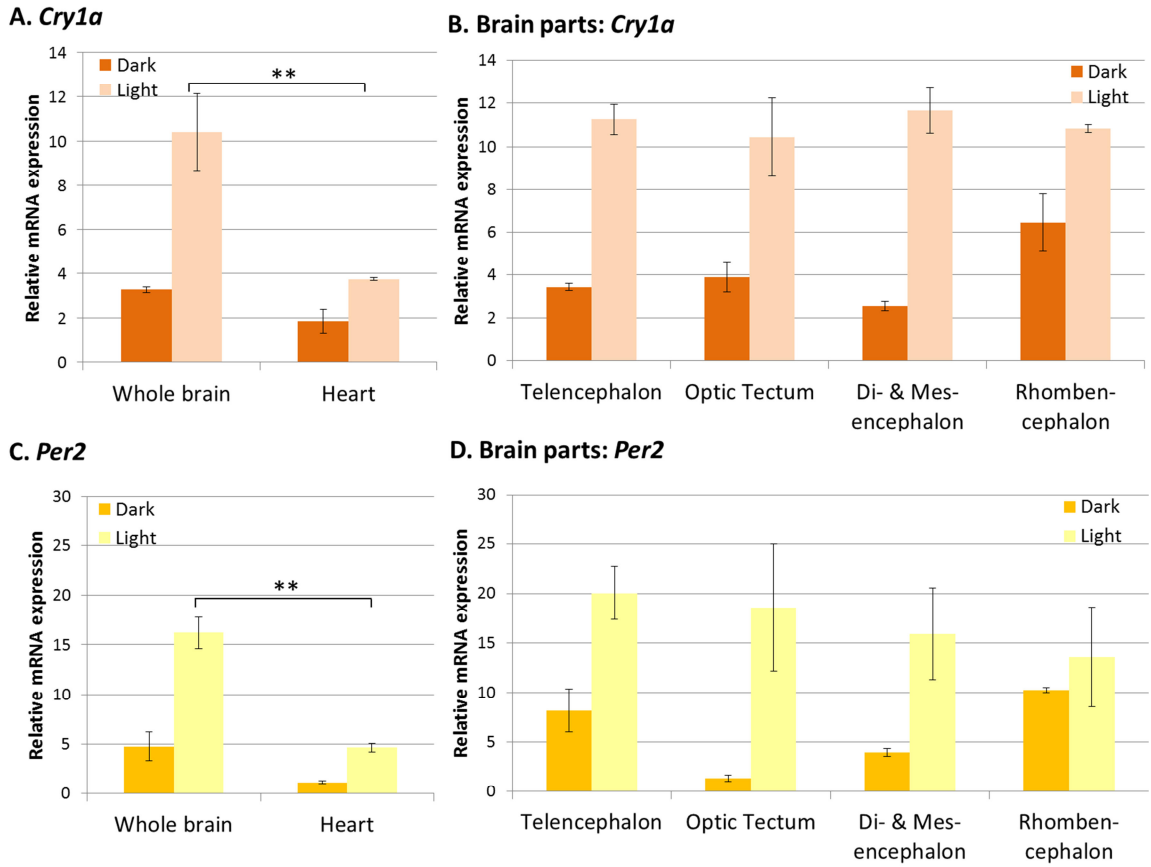


Figure 36 *Cry1a* and *Per2* are light-induced in the brain and heart of zebrafish

Adult zebrafish were kept in the dark for 3 days and then either exposed to light for 3 hours or remained in the dark. The zebrafish were killed at CT22 and their brain and hearts dissected, RNA extracted and qPCR performed. All samples showed the light responsive genes, *cry1a* and *per2*, were increased in light pulsed tissues compared to dark control. **A)** The light pulsed zebrafish had significantly higher c*ry1a* levels in both the heart and brain (p=0.0014, Two way ANOVA, n=3), however the brain had a higher induction (p=0.0025, Two way ANOVA, n=3). **B)** All brain parts showed an increase in *cry1a* in the light pulsed samples (p<0.0001, Two way ANOVA, n=3), however there was no significant difference between the different brain regions (Two way ANOVA, n=3) **C)** The light pulsed zebrafish had significantly higher *per2* levels in both the heart and brain (p=0.0017, Two way ANOVA, n=3), however the brain had a higher induction (p=0.0015, Two way ANOVA, n=3). **D)** All brain parts showed an increase in *per2* in the light pulsed samples (p=0.0015, Two way ANOVA, n=3), however there was no significant difference between the different brain regions (Two way ANOVA, n=3).

4.4.2 *Cry1a* and *per2* light induction in region-specific brain nuclei

The whole zebrafish brain was then examined to determine whether there were specific regions of the brain expressing the light-inducible clock genes, *cry1a* and *per2,* by using CISH. As in the previous experiment, zebrafish were exposed to light *in vivo* for 3 hours and their brains dissected at CT22. CISH was performed to show the localised expression of *cry1a* and *per2*, comparing samples that had been light pulsed (LP) to CT22 dark controls. Both *cry1a* and *per2* were found highly expressed in discrete brain nuclei (Figures 36-48, Table 5). There was a considerable overlap between regions that expressed both genes, however not all brain regions expressed one or both.

In the telencephalon, *per2* expression was increased in the LP in the internal cell layers (ICL) and whereas *cry1a* expression was increased in the external cell layers (ECL) of the OB (Figure 37 and Figure 43). Both *cry1a* and *per2* were expressed in cells throughout the dorsal telencephalic area (D), except the dorsal zone (Dd) and *cry1a* was not found in the central zone (Dc) (Figure 38 and Figure 44). In the ventral telencephalic area *cry1a* and *per2* were found in the same regions except the nucleus taeniae (NT); however neither were expressed in the lateral nuclei (Vl) or entopeduncular nucleus (EN) (Figure 39 and Figure 45).

In the diencephalon *cry1a and per2* were both expressed in the preoptic nuclei, epithalamus and thalamus. None of the nuclei in the pretectum expressed either *cry1a* or *per2*. All nuclei in the posterior tuberculum expressed *cry1a* except the torus lateralis (TLa) (Figure 41), whereas *per2* was also not expressed in the periventricular nucleus of the posterior tuberculum (Tpp) or the corpus mammilare (CM) (Figure 47). In the hypothalamus, *cry1a* and *per2* were found in the caudal, dorsal, and ventral zones of the periventricular hypothalamus (Hc, Hd, Hv) and the lateral hypothalamic nucleus (Figure 41). Additionally, *per2* was also expressed in the inferior lobe (Figure 47).

In the mesencephalon, there was scattered expression of *per2* in the optic tectum (TeO); however, there was strong expression of both *cry1a* and *per2* throughout the PGZ and torus longitudinalis (TL) (Figure 40 and Figure 46). The only nucleus in the tegmentum expressing *cry1a* and *per2* was the perilemniscal nucleus (PL) (Figure 41 and Figure 47).

In the rhombencephalon, *cry1a* and *per2* was expressed in the granular cellular layers in the lateral valvula cerebelli (Val), medial valvula cerebelli (Vam), the corpus cerebellum (CCe), eminentia granularis (EG), and lobus caudalis cerebelli (LCa) (Figure 42 and Figure 48). The vagal lobe (LX) was the only brainstem cranial nuclei to express *cry1a* and *per2*.

Co-staining the sections with DAPI to show the location of cell bodies revealed that the light responsive genes are detectable in the somal cytoplasm but could not be detected in all cells using CISH (Figure 49). This was particularly clear in brain nuclei such as the LLF and the optic nerve fibres (Figure 49iii, iv).

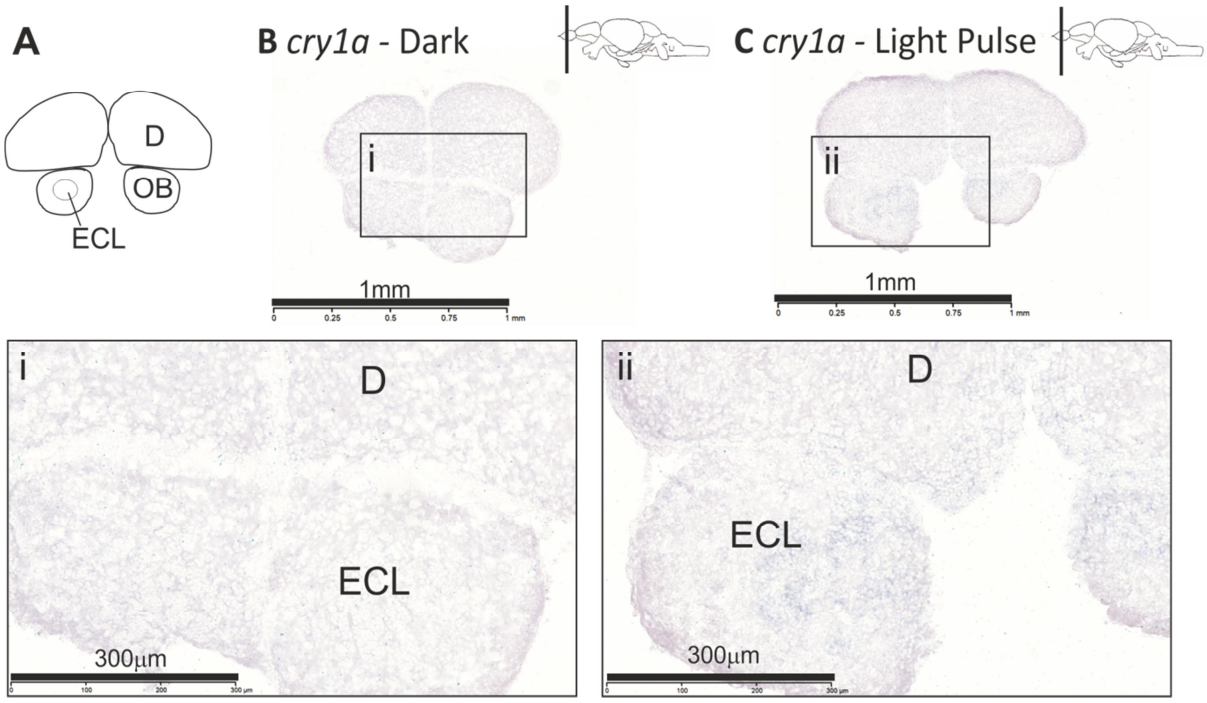


Figure 37 *Cry1a* expression in olfactory bulbs of a light pulsed zebrafish

A zebrafish was light pulsed for 3 hours and brain removed at CT22. CISH was performed to show expression of *cry1a*. **A)** A schematic of the brain section. **B)** The dark control shows no *cry1a* expression in the D or ECL. **C)** In the light pulsed sample there is higher expression of *cry1a* in the ECL, and diffuse expression in the D.

[D, dorsal telencephalic area; ECL, external cellular layer of olfactory bulb]

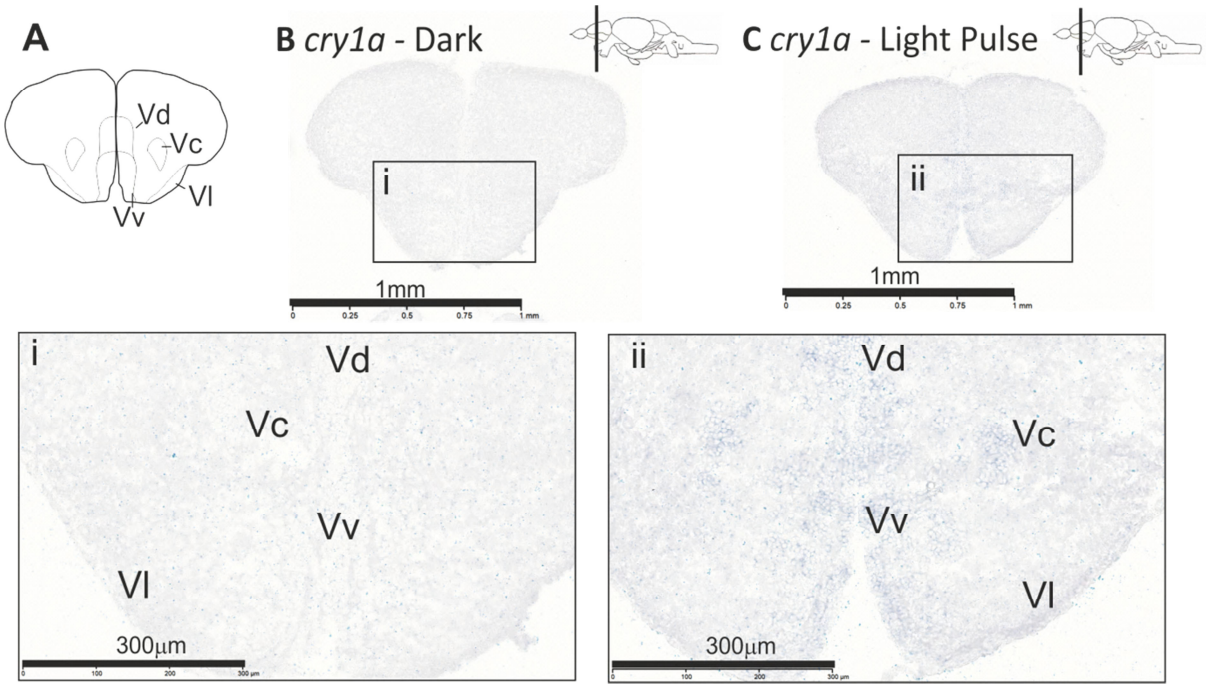


Figure 38 *Cry1a* expression in the anterior telencephalon of a light pulsed zebrafish

A zebrafish was light pulsed for 3 hours and brain removed at CT22. CISH was performed to show expression of *cry1a*. **A)** A schematic of the brain section. **B)** The dark control shows no expression of *per2* in the Vc, Vd, Vl, or Vv. **C)** In the light pulsed sample there is higher expression of *cry1a* in the Vc, Vd and Vv, and no expression in the Vl.

[Vc, central nuclei of V; Vd, dorsal nucleus of V; Vl, lateral nuclei of V; Vv, ventral nucleus of V.]

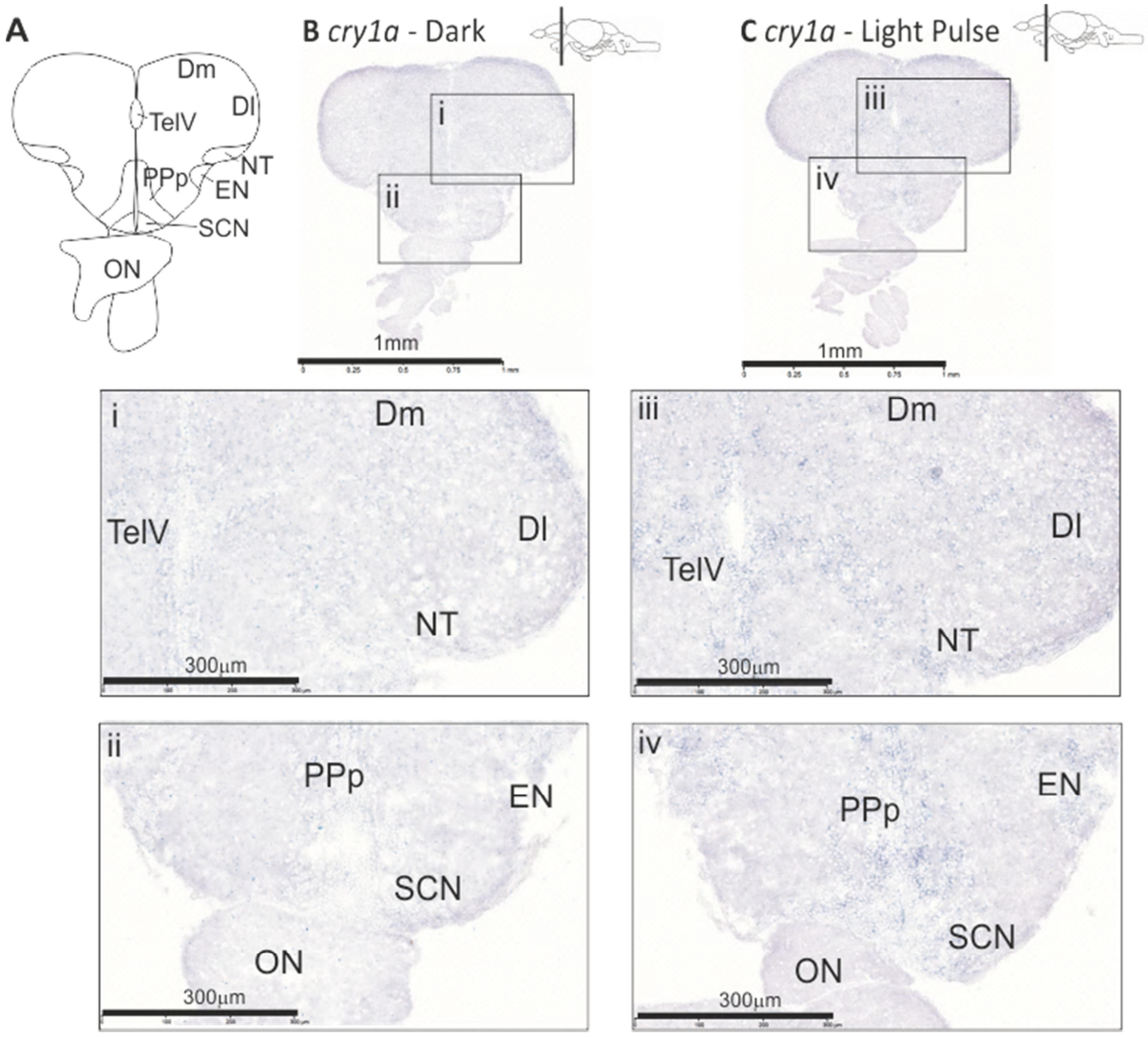


Figure 39 *Cry1a* expression in the diencephalon and telencephalon of a light pulsed zebrafish

A zebrafish was light pulsed for 3 hours and brain removed at CT22. CISH was performed to show expression of *cry1a*. **A)** A schematic of the brain section. **B)** The dark control shows low *cry1a* expression in scattered cells in the Dl, Dm and PPp however none in the EN, SCN or ON. **C)** In the light pulsed sample there is higher, yet still scattered, expression of *cry1a* in the Dm and Dl. There is expression in the EN, NT, PPp, and SCN. There remains no expression in the ON.

[Dl, lateral zone of the dorsal telencephalic area; Dm, medial zone of the dorsal telencephalic area; NT, nucleus taeniae; ON, optic nerves; PPp, parvocellular preoptic nucleus, posterior part; SCN, suprachiasmatic nucleus; TelV, telencephalic ventricle]

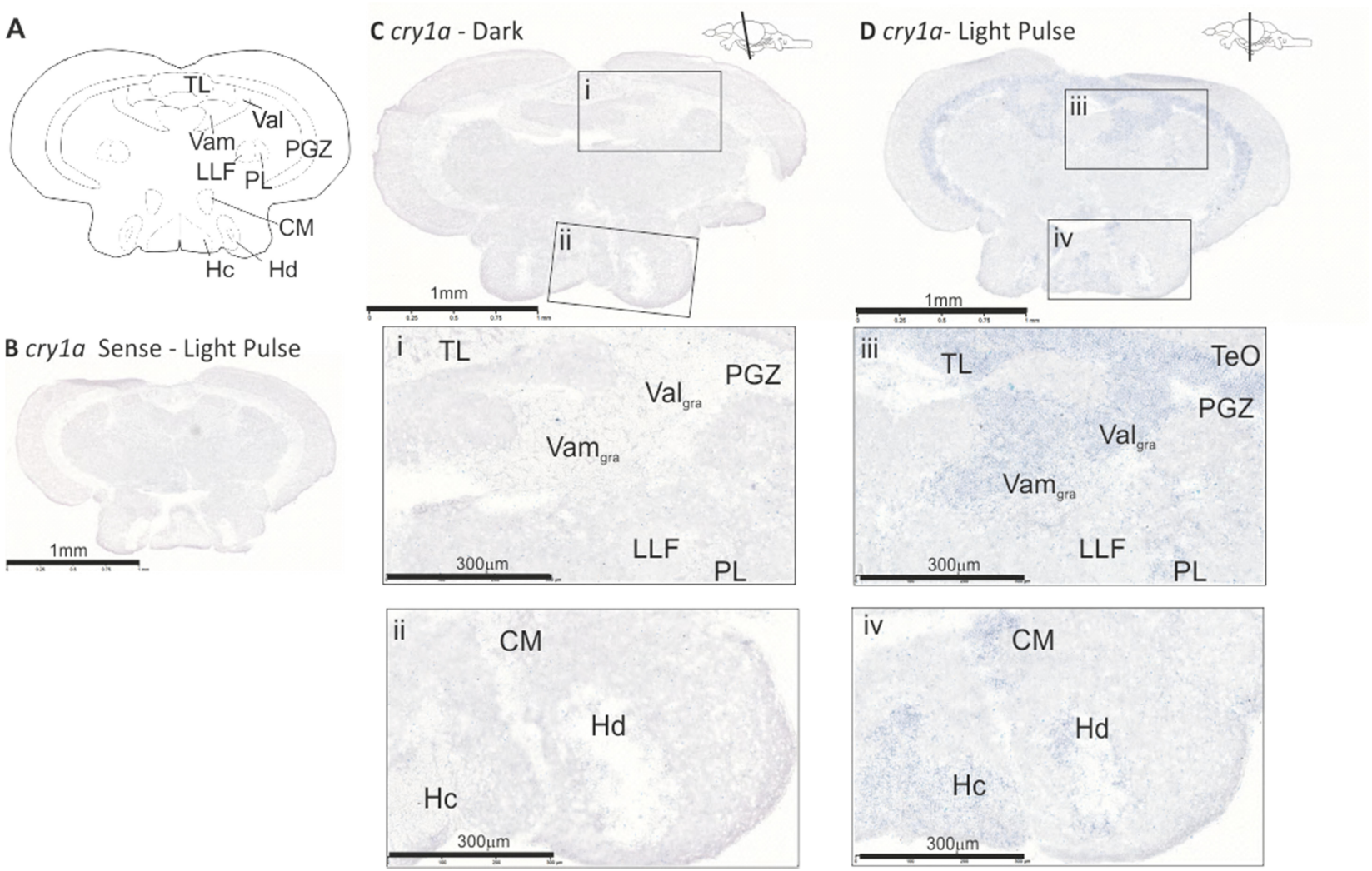


Figure 40 *Cry1a* expression in the diencephalon and mesencephalon of a light pulsed zebrafish

Figure 40 *Cry1a* expression in the diencephalon and mesencephalon of a light pulsed zebrafish

A zebrafish was light pulsed for 3 hours and brain removed at CT22. CISH was performed to show expression of *cry1a*. **A)** A schematic of the brain section containing the mesencephalon. **B)** The sense control shows the background signal **C)** The dark control shows no expression of *cry1a* in the CM, Hc, Hd, LLF, PGZ, PL, TeO, TL, Val_{gra} and Vam_{gra} **D)** In the light pulsed sample there is high expression of *cry1a* in the CM, Hc, Hd, PL PGZ, TL, Val_{gra} and Vam_{gra}. There is no expression in the LLF and TeO.

[CM: corpus mamillare; Hc: caudal zone of periventricular hypothalamus; Hd: dorsal zone of periventricular hypothalamus; LLF: lateral longitudinal fascicle; PL: perilemniscal nucleus; PGZ: periventricular grey zone of the optic tectum; TeO: optic tectum; TL: torus longitudinalis; Val_{gra}: granular layer of the lateral division of valvula cerebelli; Vam_{gra} granular layer of the medial division of valvular cerebelli]

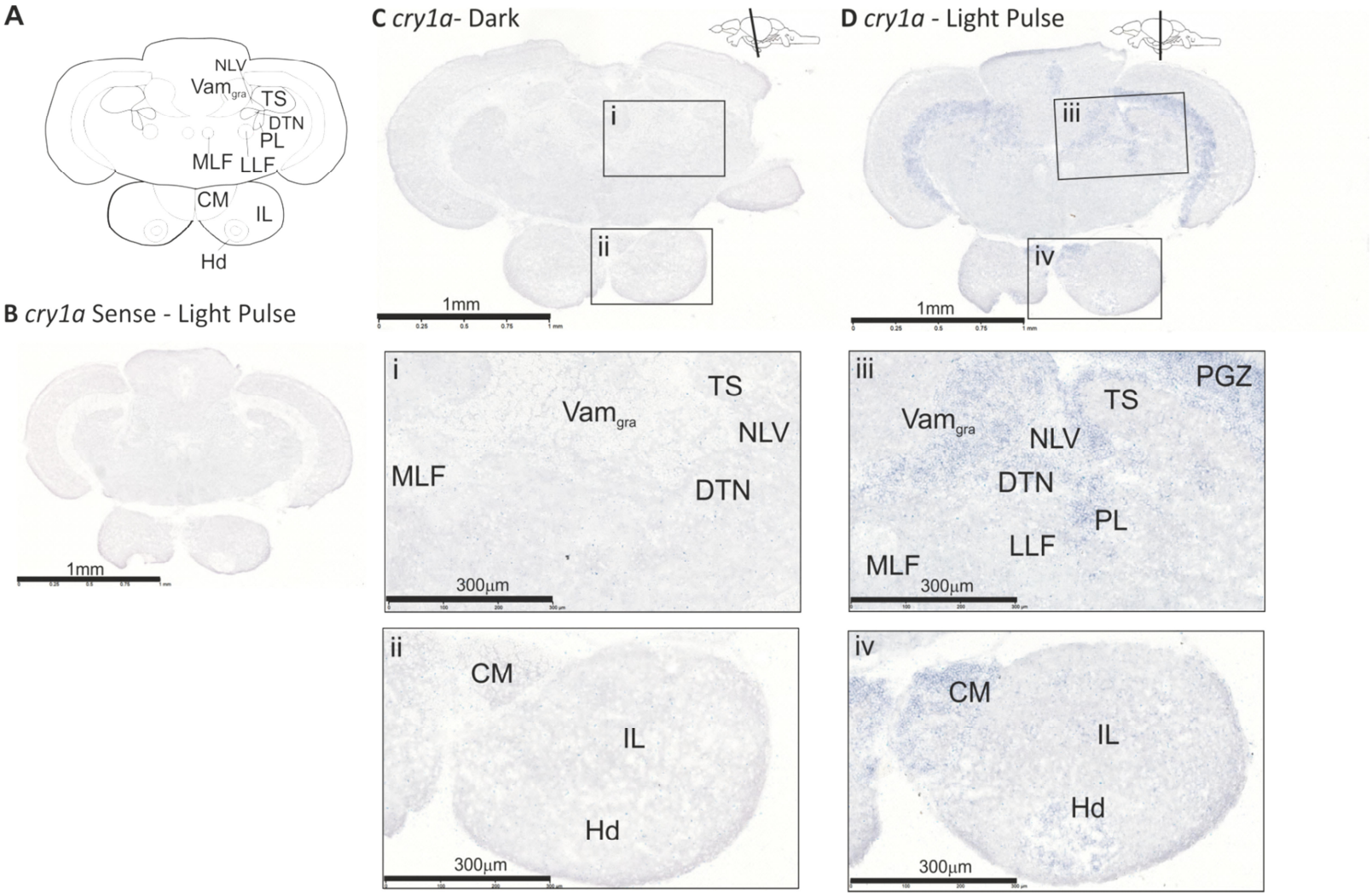


Figure 41 *Cry1a* expression in the diencephalon and posterior mesencephalon of a light pulsed zebrafish

Figure 41 *Cry1a* expression in the diencephalon and posterior mesencephalon of a light pulsed zebrafish

A zebrafish was light pulsed for 3 hours and brain removed at CT22. CISH was performed to show expression of *cry1a*. **A)** A schematic of the brain section containing the posterior mesencephalon. **B)** The sense control shows the background signal **C)** The dark control shows no expression of *cry1a* in CM, DTN, Hd, IL, LLF, MLF, NLV, PL, TS Vam_{gra}. **D)** In the light pulsed sample there is raised expression of *cry1a* in the CM, DTN, Hd, LLF, MLF, NLV, PL, and Vam_{gra}. There was expression in the ventricular lining cells in the TS, and no expression in the IL.

[CM, corpus mammilare; DTN, dorsal tegmental nucleus; Hd, dorsal zone of periventricular hypothalamus; IL, inferior lobe; LLF, lateral longitudinal fascicle; MLF, medial longitudinal fascicle; NLV, nucleus lateralis valvulae; PL, perilemniscal nucleus; TS, torus semicircularis; Vam_{gra}, granular layer of the medial division of valvula cerebelli]

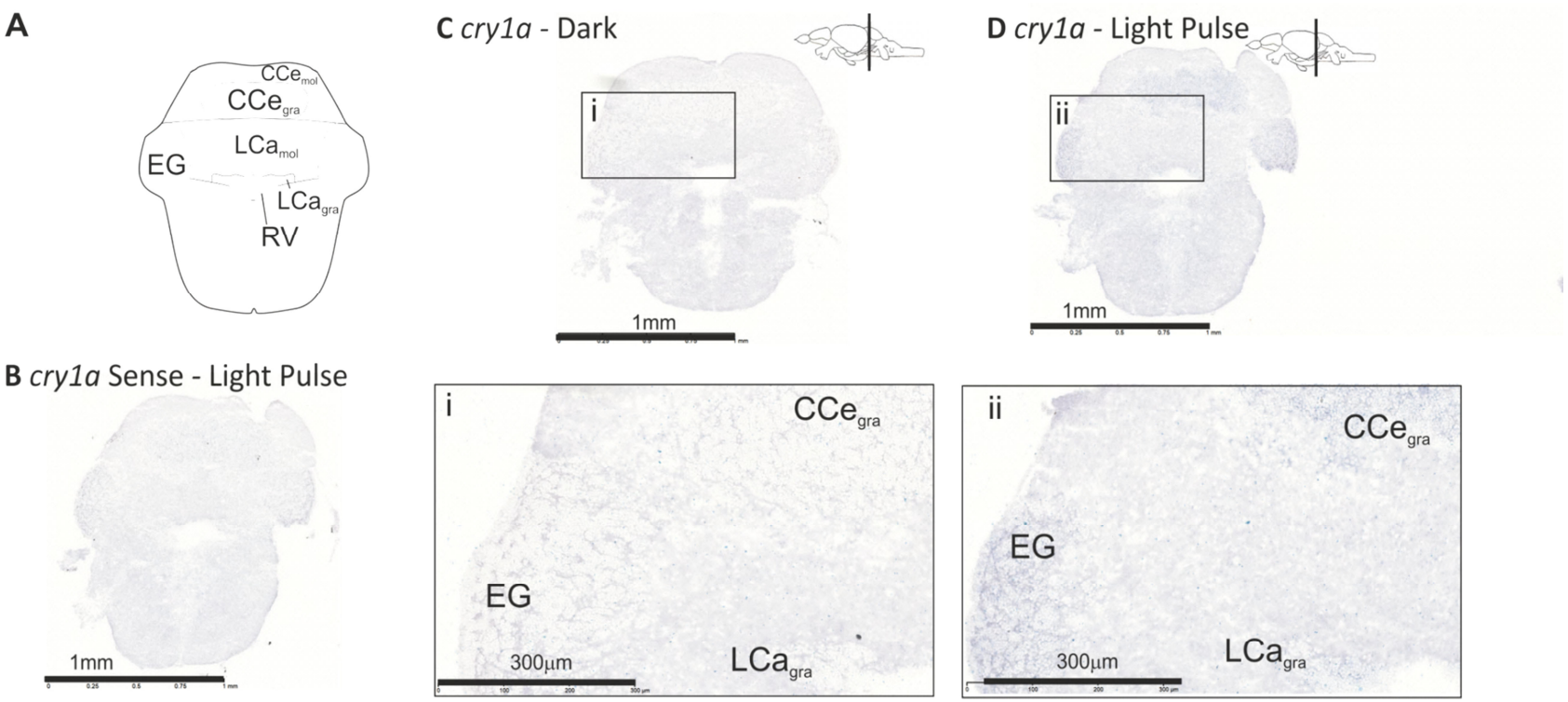


Figure 42 *Cry1a* expression in the rhombencephalon of a light pulsed zebrafish

A zebrafish was light pulsed for 3 hours and brain removed at CT22. CISH was performed to show expression of *cry1a* mRNA. **A)** A schematic of the brain section containing the hindbrain. **B)** The sense control shows the background signal **C)** The dark control shows no expression of *cry1a* in CCe_{gra}, EG, and $LCa_{gra.}$ **D)** In the light pulsed sample there is raised expression of *cry1a* in the CCe_{gra}, EG, and LCa_{gra}. [CCe_{gra}, granular layer of the corpus cerebelli; EG, eminentia granularis; and LCa_{gra}, granular layer of the lobus caudalis cerebelli; RV, rhombencephalic ventricle]

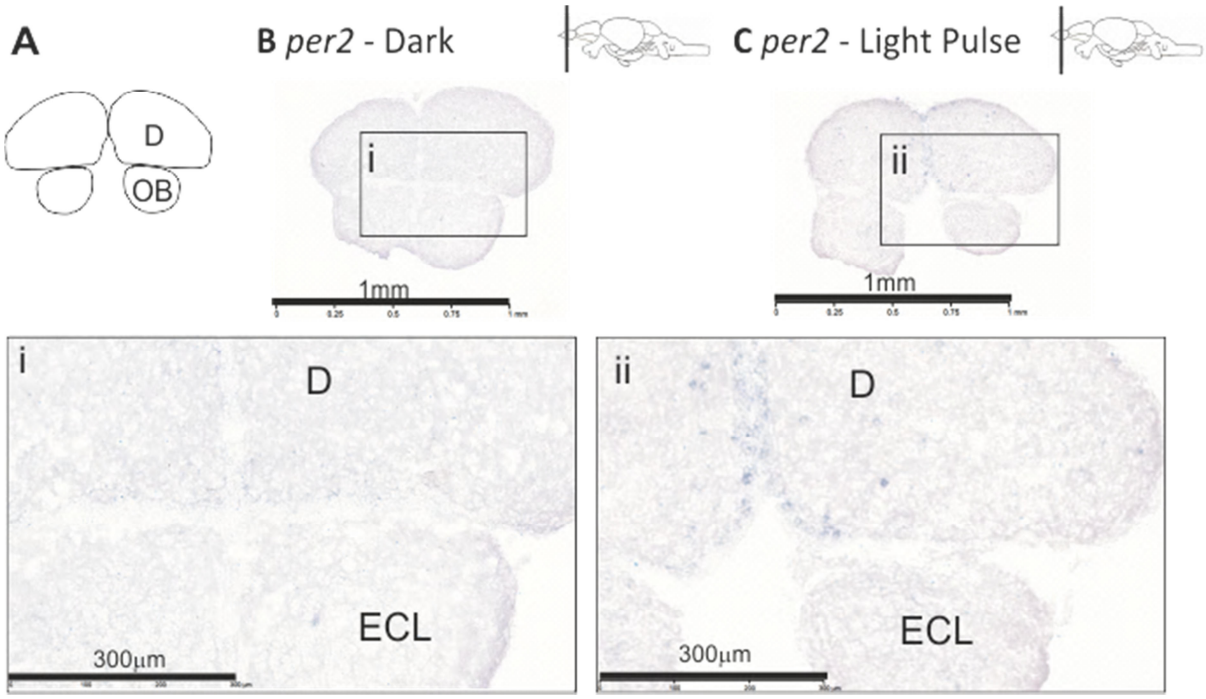


Figure 43 *Per2* expression in olfactory bulbs of a light pulsed zebrafish

A zebrafish was light pulsed for 3 hours and brain removed at CT22. *In situ* hybridization was performed to show expression of *per2*. **A)** A schematic of the brain section. **B)** The dark control shows minimal expression of *per2* in the D and ECL. **C)** In the light pulsed sample there is higher expression of *per2* in the D but none in the ECL.

[D, dorsal telencephalic area; ECL, external cellular layer of olfactory bulb; OB, olfactory bulbs]

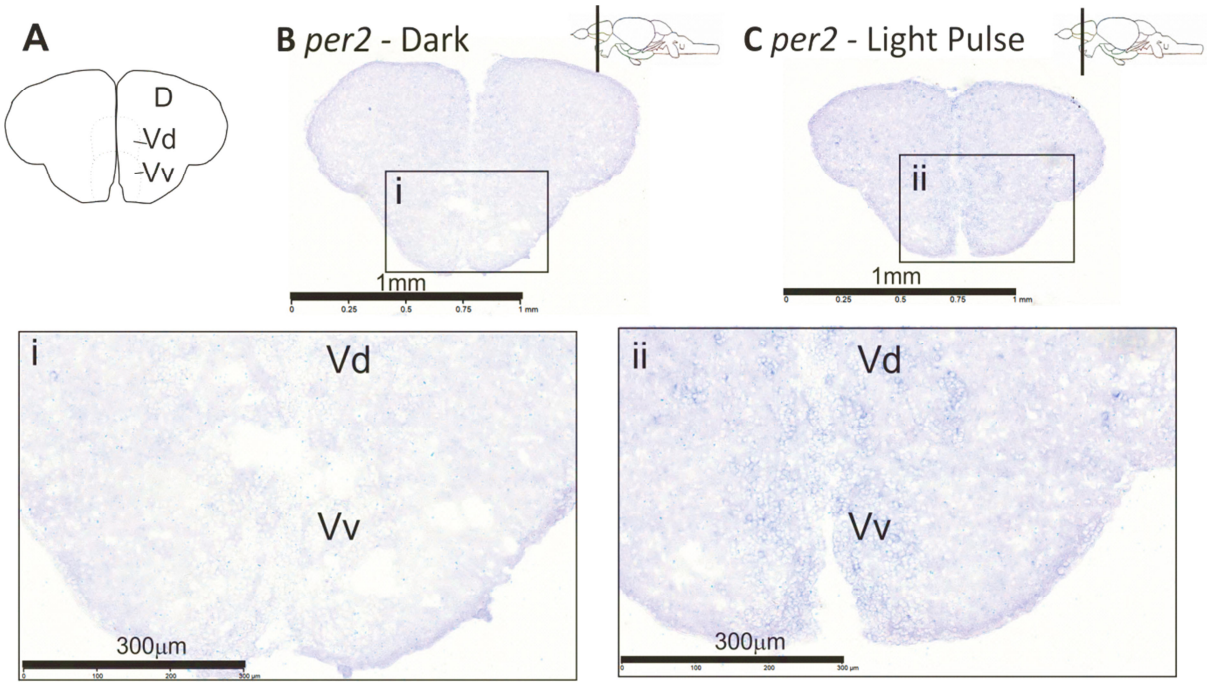


Figure 44 Per2 expression in the anterior telencephalon of a light pulsed zebrafish

A zebrafish was light pulsed for 3 hours and brain removed at CT22. *In situ* hybridization was performed to show expression of *per2*. **A)** A schematic of the brain section. **B)** The dark control shows no expression of *per2* in the Vd or Vv. **C)** In the light pulsed sample there is higher expression of *per2* in the Vd and Vv.

[D, dorsal telencephalic area; Vd, dorsal nucleus of V; Vv, ventral nucleus of V.]

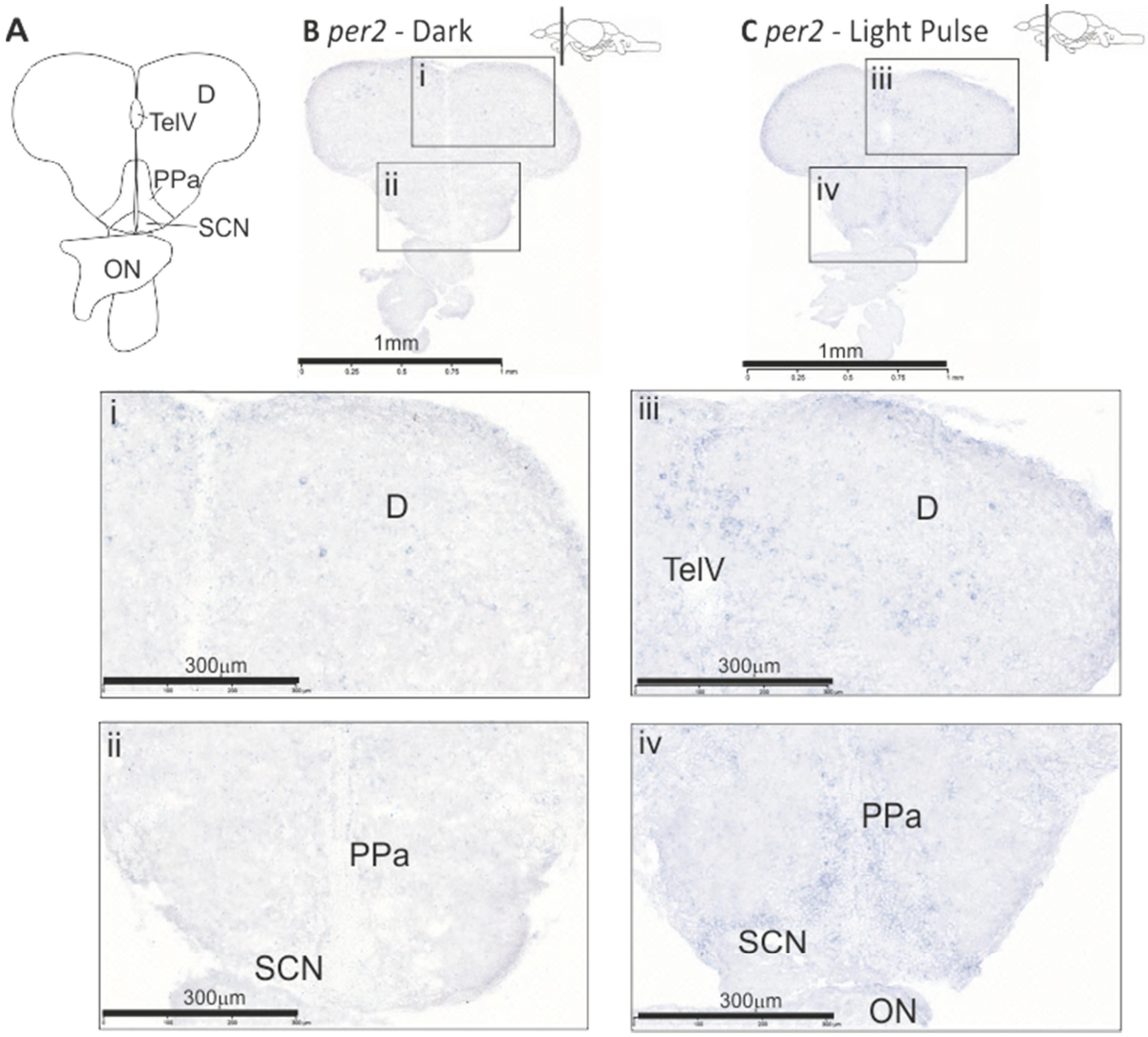


Figure 45 *Per2* expression in the diencephalon and telencephalon of a light pulsed zebrafish

A zebrafish was light pulsed for 3 hours and brain removed at CT22. *In situ* hybridization was performed to show expression of *per2*. **A)** A schematic of the brain section. **B)** The dark control shows scattered cells expressing per2 in the D, however none in the PPa or SCN. **C)** In the light pulsed sample there is higher expression of *per2* in the D, PPa, and SCN, although there is no expression in the ON.

[D,: dorsal telencephalic area; ON, optic nerves; PPa, parvocellular preoptic nucleus, anterior part; SCN, suprachiasmatic nucleus; TelV, telencephalic ventricle.]

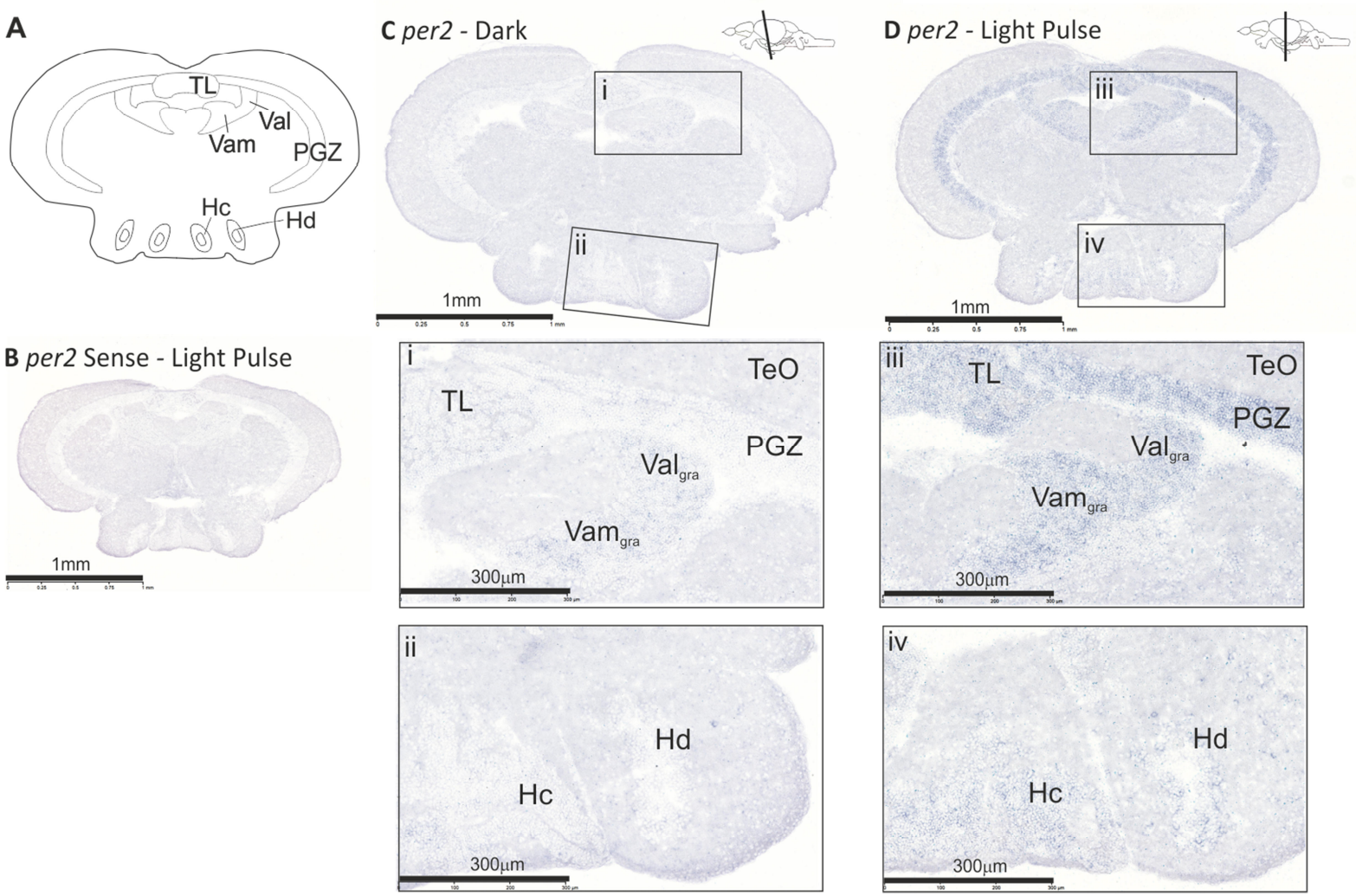


Figure 46 *Per2* expression in the diencephalon and mesencephalon of a light pulsed zebrafish

Figure 46 *Per2* expression in the diencephalon and mesencephalon of a light pulsed zebrafish

A zebrafish was light pulsed for 3 hours and brain removed at CT22. *In situ* hybridization was performed to show expression of *per2*. **A)** A schematic of the brain section containing the mesencephalon. **B)** The sense control shows the background signal **C)** The dark control shows no expression of *per2* in the Hc, Hd, TL, TeO, PGZ, and minimal expression in the granular layer of the Val and Vam **D)** In the light pulsed sample there is raised expression of *per2* in the Hc, Hd, PGZ, TeO, TL, Val_{gra} and Vam_{gra}.

[Hc, caudal zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; PGZ, periventricular grey zone of the optic tectum; TeO, optic tectum; TL, torus longitudinalis; $Val_{gra,}$ granular layer of the lateral division of valvula cerebelli; $Vam_{gra,}$ granular layer of the medial division of valvula cerebelli]

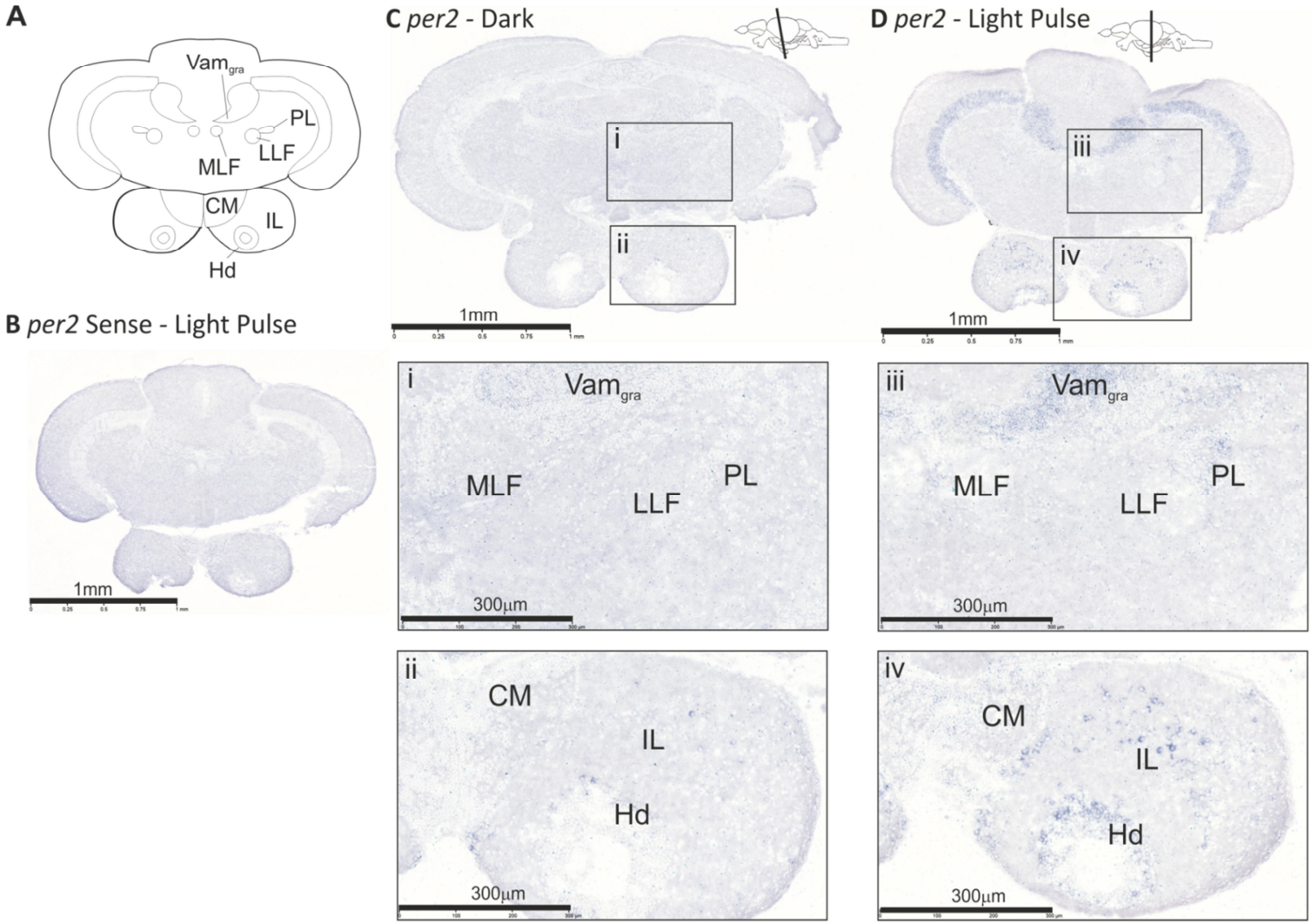


Figure 47 *Per2* expression in the posterior diencephalon and mesencephalon of a light pulsed zebrafish

Figure 47 *Per2* expression in the posterior diencephalon and mesencephalon of a light pulsed zebrafish

A zebrafish was light pulsed for 3 hours and brain removed at CT22. *In situ* hybridization was performed to show expression of *per2*. **A)** A schematic of the brain section containing the mesencephalon. **B)** The sense control shows the background signal **C)** The dark control shows no expression of *per2* in CM, LLF, MLF, PL, and minimal expression in the IL, Hd, and granular layer of the Vam **D)** In the light pulsed sample there is raised expression of *per2* in the Hd, IL, LLF, MLF, PL, and Vam_{gra}. There was minimal expression in the CM.

[Hd, dorsal zone of periventricular hypothalamus; IL, inferior lobe; LLF, lateral longitudinal fascicle; MLF, medial longitudinal fascicle; PL, perilemniscal nucleus; $Vam_{gra,}$ granular layer of the medial division of valvula cerebelli]

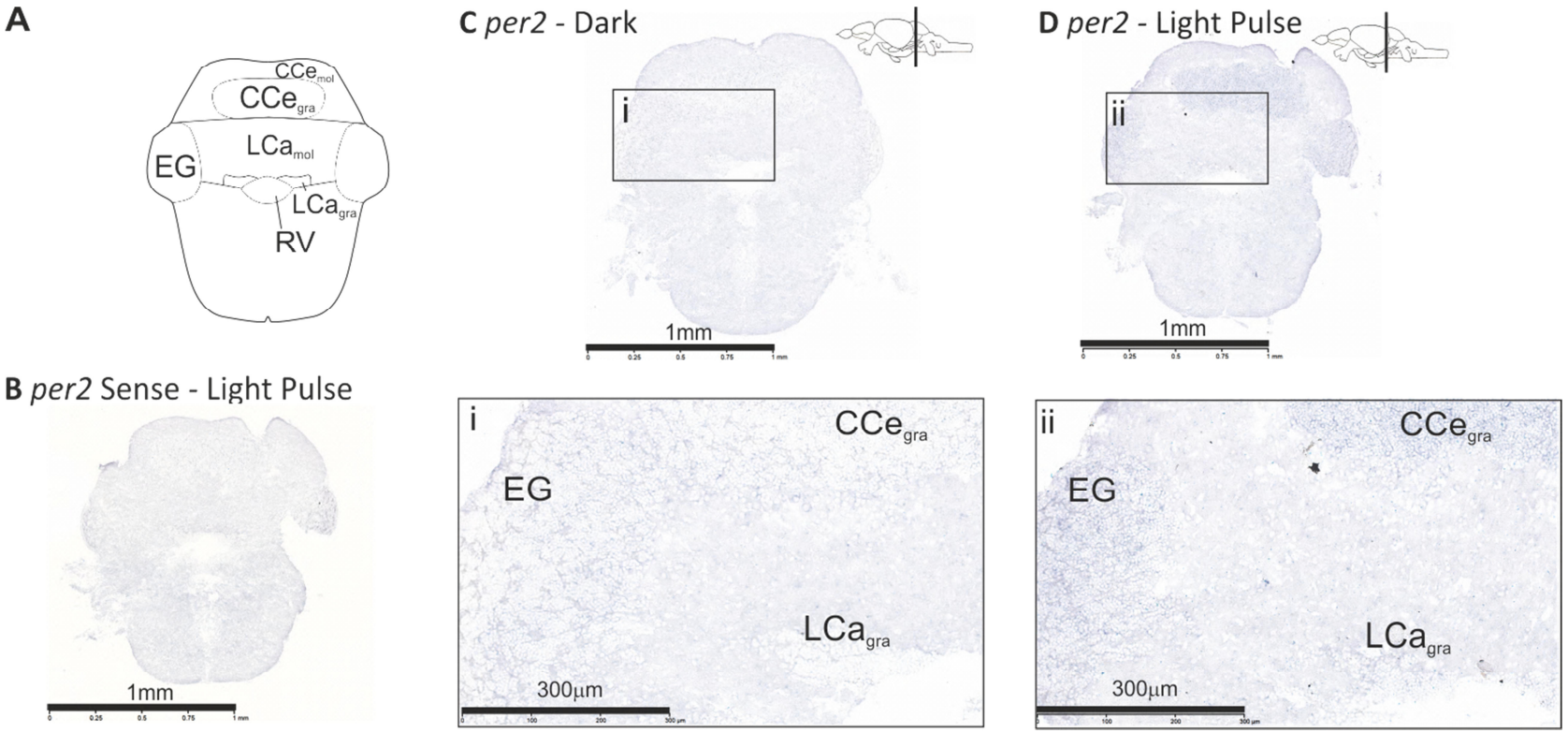


Figure 48 *Per2* expression in the rhombencephalon of a light pulsed zebrafish

A zebrafish was light pulsed for 3 hours and brain removed at CT22. *In situ* hybridization was performed to show expression of *per2*. **A)** A schematic of the brain section containing the hindbrain. **B)** The sense control shows the background signal **C)** The dark control shows no expression of *per2* in CCe_{gra}, EG, and LCa_{gra} **D)** In the light pulsed sample there is raised expression of *per2* in the CCe_{gra}, EG, and LCa_{gra} [$CCe_{gra,}$ granular layer of the corpus cerebelli; EG, eminentia granularis; and $LCa_{gra,}$ granular layer of the lobus caudalis cerebelli; RV, rhombencephalic ventricle]

Zebrafish brain nuclei	*Cry1a* CT22 (D)	*Cry1a* (LP)	*Per2* CT22 (D)	*Per2* (LP)
Telencephalon				
OB, Olfactory Bulbs				
ICL, internal cellular layer	-	-	-	++
ECL, external cellular layer	-	++	+	+
GL, glomerular layer	-	-	-	-
D, Dorsal telencephalic area				
Dc, central zone of D	-	-	-	+
Dd, dorsal zone of D	-	-	-	-
Dl, lateral zone of D	+	+	+	++
Dm, medial zone of D	+	+	+	++
Dp, posterior zone of D	-	++	-	+
V, Ventral telencephalic area				
Cv, commissural nucleus of V	-	-	-	-
Vd, dorsal nucleus of V	-	++	-	++
Vv, ventral nucleus of V	-	++	-	++
Vp, postcommissural nucleus of V	-	++	-	++
Vs, supracommissural nucleus of V	-	++	-	++
Vc, central nuclei of V	-	++	-	++
Vl, lateral nuclei of V	-	-	-	-
EN, entopeduncular nucleus	-	+	-	-
NT, nucleus taeniae	-	++	-	-
Diencephalon				
Area preoptica				
CO, optic chiasm	-	-	-	-
OT, optic tract	-	-	-	-
PPa, parvocellular preoptic nucleus, anterior part	-	++	-	++
PPd, dorsal part of the pretectal diencephalic cluster	-	++	-	++
PPp, parvocellular preoptic nucleus, posterior part	+	++	-	++
SCN, suprachiasmatic nucleus	-	++	-	++
Epithalamus				
Ha, ventral and dorsal habenula	-	++	-	++
Thalamus				
VM, ventromedial thalamic nucleus	-	++	-	++
VL, ventrolateral thalamic nucleus	-	++	-	++
A, anterior thalamic nucleus	-	++	-	++
DP, dorsal posterior thalamic nucleus	-	++	-	++
CP, central posterior thalamic nucleus	-	++	-	++
ZL, zona limitans	-	++	-	++
Pretectum				
PS, superficial pretectal nuclei	-	-	-	-
CPN, central pretectal nucleus	-	-	-	-
APN, accessory pretectal nucleus	-	-	-	-
PO, posterior pretectal nucleus	-	-	-	-
DAO/VAO, dorsal/ventral accessory optic nuclei	-	-	-	-

Zebrafish brain nuclei	*Cry1a* CT22 (D)	*Cry1a* (LP)	*Per2* CT22 (D)	*Per2* (LP)
PP, periventricular pretectal nucleus	-	+	-	+
Posterior tuberculum				
Tpp, periventricular nucleus of the posterior tuberculum	-	++	-	-
TLa, torus lateralis	-	-	-	-
CM, corpus mamillare	-	++	-	-
PTN, posterior tuberal nucleus	-	++	-	++
PVO, paraventricular organ	-	++	-	++
PGl/a, preglomerular lateral/anterior nucleus	-	++	-	++
PGm, preglomerular medial nucleus	-	++	-	++
Hypothalamus				
ATN, anterior tuberal nucleus	-	-	-	-
Hc, caudal zone of periventricular hypothalamus	-	++	-	++
Hd, dorsal zone of periventricular hypothalamus	-	++	+	++
Hv, ventral zone of periventricular hypothalamus	-	++	-	++
LH, lateral hypothalamic nucleus	-	++	-	++
IL, inferior lobe	-	-	+	++
Mesencephalon				
Superior and inferior colliculi				
TeO, optic tectum	-	-	-	+
PGZ, periventricular grey zone	-	++	-	++
TL, torus longitudinalis	-	++	-	++
TS, torus semicircularis	-	-	-	-
LLF, lateral longitudinal fascicle	-	-	-	-
MNV, mesencephalic nucleus of trigeminal nucleus	-	-	-	-
Nmlf, nucleus of the medial longitudinal fascicle	-	-	-	-
PCN, paracommissural nucleus	-	-	-	-
Tegmentum				
DTN, dorsal tegmental nucleus	-	-	-	-
EW, Edinger-Westphal nucleus	-	-	-	-
NLV, nucleus lateralis valvulae	-	-	-	-
NIII, Oculomotor nucleus	-	-	-	-
NLL, nucleus of lateral lemniscus	-	-	-	-
PL, perilemniscal nucleus	-	++	-	++
SR, superior raphe nucleus;	-	-	-	-
SRF, superior reticular formation	-	-	-	-
NIn, nucleus interpeduncularis	-	-	-	-
NI; nucleus isthmus	-	-	-	-
LC, Locus coeruleus	-	-	-	-
SGN, secondary gustatory nucleus	-	-	-	-

Zebrafish brain nuclei	*Cry1a* CT22 (D)	*Cry1a* (LP)	*Per2* CT22 (D)	*Per2* (LP)
Rhombencephalon				
Cerebellum				
$Val_{gra,}$ granular layer of lateral division of valvula cerebelli	-	++	+	++
$Val_{mol,}$ molecular layer of lateral division of valvula cerebelli	-	-	-	-
$Vam_{gra,}$ granular layer of medial division of valvula cerebelli	-	++	+	++
$Vam_{mol,}$ molecular layer of medial division of valvula cerebelli	-	-	-	-
$CCe_{gra,}$; granular layer of corpus cerebellum	-	++	-	++
$CCe_{mol,}$ molecular layer of corpus cerebellum	-	-	-	-
EG, eminentia granularis	-	++	-	++
LCa_{gra}, granular layer of lobus caudalis cerebelli	-	++	-	++
$LCa_{mol,}$ molecular layer of lobus caudalis cerebelli	-	-	-	-
Medulla oblongata				
brainstem cranial nuclei (except LX)	-	-	-	-
LX, vagal lobe	-	++	-	++
Gc; griseum centrale	-	-	-	-

Table 5 *Cry1a* and *Per2* is expressed in region-specific brain nuclei in light pulsed zebrafish

This table describes the mRNA expression profile of *cry1a* and *per2* in various brain nuclei of light pulsed (LP) or CT22 dark (D) control wild type zebrafish as shown using chromogenic *in situ* hybridization. In the LP samples zebrafish were given light for 3 hours and brain removed at CT22. [-, no detectable signal; +, signal found in some cells in the region; ++, signal found in many cells in the region]

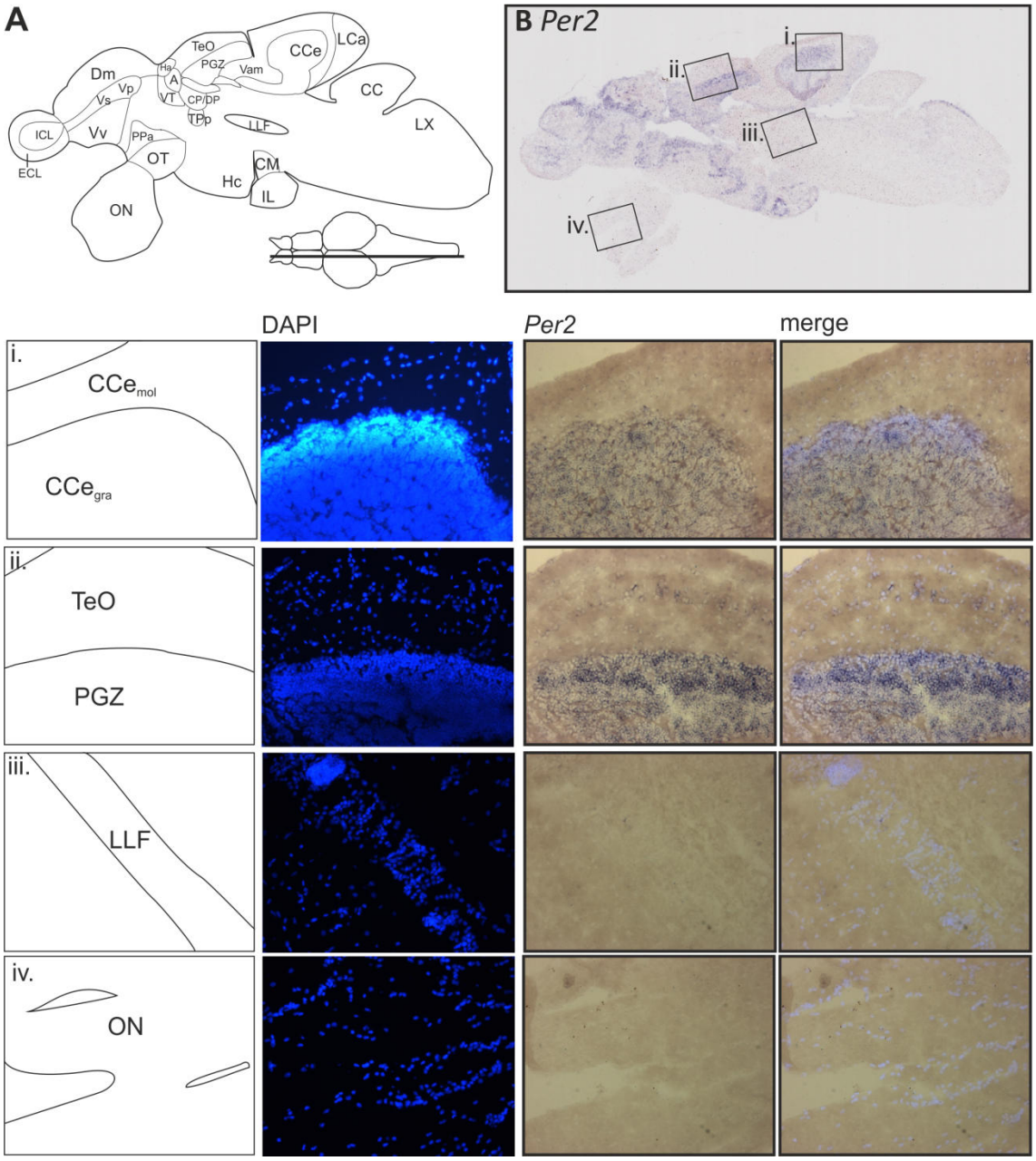


Figure 49 *Per2* is not detectable in all cells

An adult zebrafish was light pulsed for 3 hours and brain removed at CT22. CISH was performed to show expression of *per2*, and DAPI was added to show the location of the cell bodies. **i)** and **ii)** show *per2* is located in the somal cytoplasm in cells in the CCegra and PGZ. **iii)** and **iv)** show *per2* cannot be detected in cells in the LLF or ON. Inserts are shown at 20X magnification. [A, anterior thalamus; ATN, anterior tuberal nucleus; CC, crista cerebellis; CCe, corpus cerebellum; CM, corpus mammilare; CP/DP, central and dorsal posterior thalamic nucleus; Dm, medial zone of the dorsal telencephalon; ECL, external cellular layer of olfactory bulb; Ha, habenula; ICL, internal cellular layer of olfactory bulb; IL, inferior lobe; LCa, lobus caudalis cerebelli; LX, vagal lobe; ON, optic nerve; OT, optic tract; PPa, anterior parvocellular preoptic nucleus; PGZ, periventricular grey zone; TeO, optic tectum; TPp, periventricular nucleus of the posterior tuberculum; Vs/p, supracommissural and postcommissural nucleus of the ventral telencephalon, VT, ventral tegmentum; Vv, ventral nucleus of the ventral telencephalon]

4.4.3 *Cry1a* and *per2* light induction in cultured adult brains

In this project a novel brain culturing technique has been developed for monitoring the whole brain over several days, for the detection of pacemaker candidates. To determine how the brain is responding to light in these culture conditions we quantified the expression of *cry1a* and *per2* in whole brain cultures. Cultured hearts were also investigated as a peripheral tissue control.

Wild type adult zebrafish brain parts, whole brains and hearts were cultured in L15-media for four days. Samples were exposed to a 3 hour light pulse or kept in the dark. Samples were collected at CT22, RNA extracted, and qPCR performed. The expression of light responsive genes *cry1a* and *per2* were increased in all cultures exposed to light. (Figure 50)

Cry1a and *per2* is induced by light in both the brain and heart ($p<0.0001$, Two way ANOVA, n=3-5), with the heart having a higher induction of *cry1a* than the brain ($p<0.01$, Two way ANOVA, n=3-5), although there is no difference in *per2* between the two tissues (Two way ANOVA, n=3-5).

The light pulsed brain part cultures had significantly higher *cry1a* levels than the dark controls ($p<0.0001$, Two way ANOVA, n=3) and *per2* levels ($p=0.0013$, Two way ANOVA, n=3). Additionally, there was variation between the expression in the different brain regions in *cry1a* ($p<0.001$, Two way ANOVA, n=3) and *per2* ($p=0.0016$, Two way ANOVA, n=3), with the telencephalon producing a higher *cry1a* and *per2* induction ($p<0.05$, Bonferroni multiple comparisons post-test).

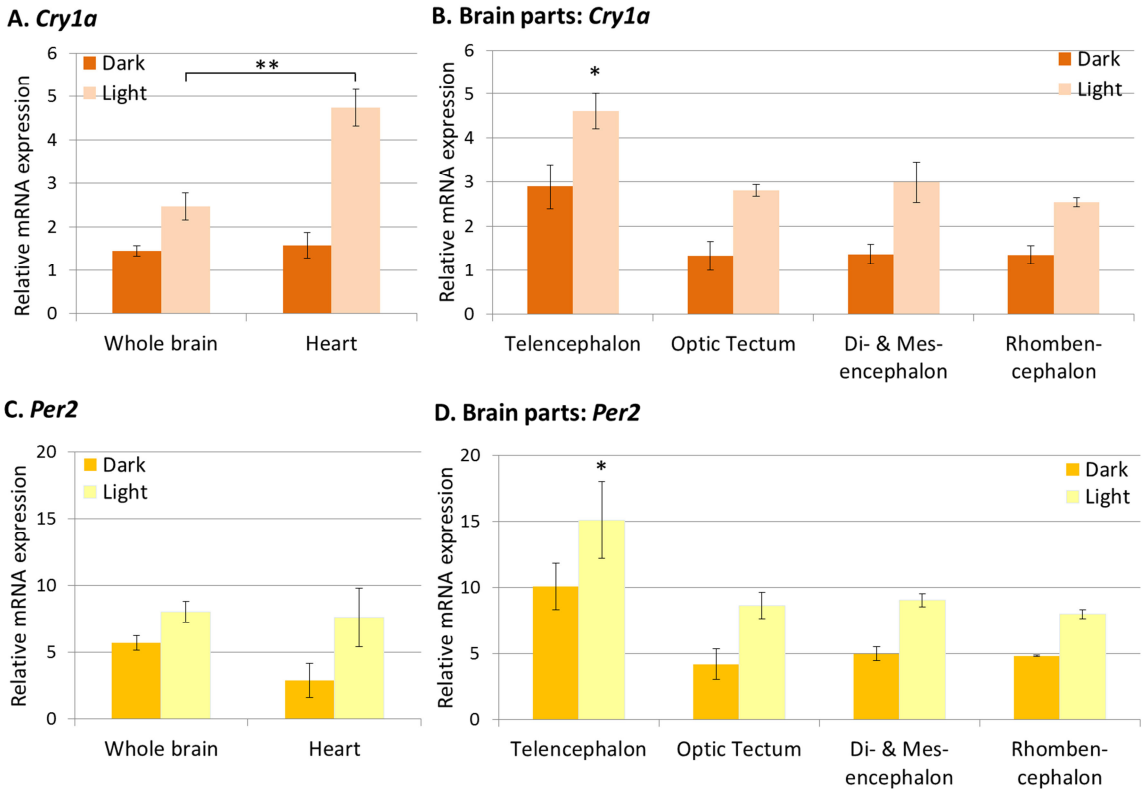


Figure 50 Expression of light responsive genes in cultured adult tissues

Wild type adult zebrafish brain parts, whole brains and hearts were cultured in L15-media. Samples were exposed to a 3 hour light pulse or kept in the dark. Samples were collected at CT22, RNA extracted, and qPCR performed. The expression of light responsive genes *cry1a* and *per2* were increased in all cultures exposed to light. **A)** *Cry1a* is induced by light in both the brain and heart ($p<0.0001$, Two way ANOVA, n=3-5), with the heart having a higher induction than the brain ($p<0.01$, Two way ANOVA, n=3-5). **B)** The light pulsed brain part cultures had significantly higher *cry1a* levels than the dark controls ($p<0.0001$, Two way ANOVA, n=3), however there was variation between the expression in the different brain regions ($p<0.001$, Two way ANOVA, n=3), with the telencephalon producing a higher *cry1a* induction ($p<0.05$, Bonferroni multiple comparisons post-test) **C)** *Per2* is induced by light in both the brain and heart ($p<0.0001$, Two way ANOVA, n=3-5), with no difference in induction between the two tissues (Two way ANOVA, n=3-5). **D)** The light pulsed brain part cultures had significantly higher *per2* levels ($p=0.0013$, Two way ANOVA, n=3), additionally there was variation between the expression in the different brain regions ($p=0.0016$, Two way ANOVA, n=3), with the telencephalon producing a higher induction of *per2* expression ($p<0.05$, Bonferroni multiple comparisons post-test).

4.5 Discussion

This chapter has evaluated the quantitative and spatial expression of light responsive clock genes, *cry1a* and *per2,* in the zebrafish brain. *Cry1a* and *per2* are the only light responsive core clock genes, but not the only light responsive genes, identified so far in the zebrafish (Vatine et al. 2011) and were therefore chosen to examine the light sensitivity of the circadian system in the zebrafish brain. It was necessary to investigate the expression of both genes as CRY1A and PER2 interact differently as well as cooperatively as light-induced transcriptional repressors in the entrainment pathway of the molecular circadian clock (Hirayama 2003; Tamai et al. 2007). The most interesting findings of this study show for the first time that *cry1a* and *per2* are expressed in regional specific areas in the zebrafish brain.

Cry1a and *per2* were upregulated by light in all zebrafish tissues and samples used in these experiments, which supports many previous reports for their importance as strongly light-induced transcription factors (Vallone et al. 2005). The results here show that the expression of *cry1a* and *per2* can be observed in both the brain and heart in response to light, with low basal levels during the night. This agrees with many other studies detailing the properties of *cry1a* and *per2* in embryonic cell lines (Cermakian et al. 2002; Pando et al. 2001; Tamai et al. 2007) and adult tissues (Amaral & Johnston 2012). In culture, the zebrafish brain retained the capability to express *cry1a* and *per2* in response to light. This corroborates previous findings that the zebrafish has light responsive structures throughout its body, and that this light sensitivity is not restricted to the classical photosensitive areas, the retina and pineal (Whitmore, Foulkes, & Sassone-Corsi 2000).

Similar to the findings on *per3* expression in brain nuclei, described in chapter 3, both *cry1a* and *per2* were found highly expressed in particular brain nuclei (see appendix for comparative table). This was an encouraging result as it suggests these regions may be important in the regulation of circadian rhythms. Many regions express both rhythmic and light responsive genes, including the SCN region, other nuclei in the preoptic area, hypothalamus, posterior tuberculum, the PGZ, and granular cell regions of the cerebellum. In addition, *cry1a* and *per2* are also expressed in the same regions that *bmal* and *Clock* have been reported in: the PGZ, valvula cerebelli and hypothalamus (Cermakian et al. 2000; Whitmore et al. 1998). Neuronal

pacemakers are able to entrain to the environment as well as produce a near-24 hour rhythm to drive rhythms in other areas. In *Drosophila*, which also has directly light sensitive tissues, rhythms of *per* can be detected in a few central brain regions, the optic lobes, the eyes, and head sensory tissues (ocelli, antennae, maxillary palps, and at the tip of the proboscis) (Rachidi et al. 1997). Most of these neuronal regions are also pacemaker structures, such as the lateral neurons, or project to pacemaker structures (Hall 1995). The light responsive and rhythmic zebrafish regions may thus represent candidate neuronal pacemakers.

Nevertheless, there were exceptions to this co-localised expression in the zebrafish. The light responsive clock genes were expressed in the Tpp, DP and CP, but these regions did not express *per3*. This suggests that these areas can detect light, but might not possess a functional circadian clock. Furthermore, the ATN, Dd, EN and TLa expressed *per3* but not *cry1a* and *per2*. Raising the rather fascinating question of how these particular areas might entrain directly to light, that is of course assuming that they do. It must be noted that the regions that did not appear to express *cry1a* and *per2* may be due to the cells expressing them at a low level that could not be detected using the CISH method. A more sensitive method, such as qPCR, may detect mRNA if these regions can be accurately dissected. However, it is also plausible that not all cells within the zebrafish brain are directly light sensitive, and they may rely on communication from other light responsive cells to entrain to exogenous LD cycles. Indeed, there are several regions of the brain that *cry1a*, *per2* or *per3* cannot be detected, including many nuclei in the pretectum, tegmentum and brainstem. It may be that these regions require signals from neuronal pacemakers for entrainment. Although, multiple replications of clock genes in the zebrafish means there is not yet sufficient evidence to say these regions do not express clock genes. Nonetheless, the expression of only certain clock genes in some regions hints at the fine tuning of circadian regulation in the zebrafish. Further examinations of the expression of other clock genes will provide a more complete understanding of the circadian regulation in the brain; however, the data so far allows for a network of multiple neuronal pacemakers and light responsive regions may exist.

The question still remains as to why some regions express clock genes like *cry1a* and *per2* more highly than others, and the same possible explanations can be put forward as with the

expression of *per3*. Is it due to direct retinal input or a specific neurotransmitter cell type? As with *per3*, it is unlikely solely due to retinal inputs as several of the light responsive regions have not been reported to have primary retinofugal terminal projections in teleosts, including the hypothalamus, TL, preglomerular nuclei, and valvula cerebelli (Northcutt & Wullimann 1987). Neither is it likely to be one neurotransmitter cell type involved as the light responsive regions contain a diverse range of neurotransmitter cell types. Is it due to the location of a particular cell type, neurons or glia? This will be explored in the next chapter.

Another suggestion for the regional expression of *cry1a* and *per2* might be the location of the regions from the ventricles, which are packed with glia and allows regions closer contact with nutrients and to signal hormonally. Indeed, many of the proliferation zones in the zebrafish that show production of new cells are close to ventricles (Zupanc, Hinsch, & Gage 2005). Similarly many regions expressing *cry1a* and *per2* are close to ventricles and many of the regions that do not appear to express *cry1a* and *per2* are further away from ventricles. The ventricular lining epithelial glia have been shown to express *per2* in mice (Guilding et al. 2009). However not all light responsive zebrafish nuclei are neighbouring ventricles, such as the perilemniscal nucleus (PL), which is within the tegmentum. Therefore the closeness of nuclei to ventricles does not provide a complete explanation for the light sensitivity of the region.

The light sensitive regions are found throughout the zebrafish brain, including many regions of the hypothalamus. This is consistent with findings of deep brain photoreceptors in other species (Foster & Soni 1998). In the zebrafish environmental light should be able to penetrate through the skull and reach all regions of the brain. In fact, even teleosts with thick tissue layers covering a considerably bony skull, such as the eel, have been shown to have measurable amounts of light penetrate to at least the ventral surface of the diencephalon (Hartwig & Veen 1979).

One unifying property of these light responsive regions could be the expression of particular photoreceptors. Although, the inherent light sensitivity of given areas might vary with differences in their photopigment expression, both in terms of type and levels. There have been many candidate photoreceptors put forward for the zebrafish, including *cry4*, opsins, and flavin-containing oxidases (Vatine et al. 2011). It is likely that a number and variety of these

photoreceptors act in parallel in the zebrafish brain, and the varying levels of *cry1a* and *per2* expression may be due to the different spatial expression of these photoreceptors. Thus the expression of photosensitive pigments in the zebrafish brain will be explored in chapter seven.

In vivo the zebrafish brain was more light responsive than the heart, expressing a higher proportion of *cry1a* and *per2* compared to basal levels and this could be due to the brain expressing a different variety or number of photoreceptors. Contrastingly, *in vitro* the heart had a higher light induction of *cry1a* than the brain. There could be a number of reasons for this including cell death in the culturing conditions, reducing the ratio of the number of photoreceptive to non-photoreceptive cells in the brain. It could also be possible that some regions of the brain rely on retinal inputs for the expression of *cry1a* and *per2*. The best way to address this without the complications of artificial culturing conditions is to use eyeless zebrafish *in vivo.* There are mutants available for studying the effects of eyes in larvae, and this will be used later in this project to address how retinal inputs effect clock gene and photoreceptor expression in the larval brain.

Overall, these results provide the first glimpse into the complexities of the regional expression of light sensitive clock genes in the adult zebrafish brain. Whether these properties exist exclusively in neurons will be addressed in the next chapter.

4.6 Conclusions

- *Cry1a* and *per2*, light responsive clock genes, are expressed in specific adult brain regions in response to light.
- Regions expressing *cry1a* and *per2* include the thalamus, SCN, hypothalamus, PGZ, granular regions in the cerebellum, and the vagal lobe.
- Many regions expressing *cry1a* and *per2* also express the clock gene, *per3*, and these may represent directly light responsive neuronal pacemakers.
- Cultured brains, hearts, and brain parts also exhibit an induction of *cry1a* and *per2* in response to light, suggesting the light-sensing structures are within these tissues, as has been previously demonstrated.

Chapter 5

Circadian rhythmicity and light sensitivity of adult zebrafish neurons

5.1 Introduction

Chapters 3 and 4 showed that the cellular clocks in the zebrafish brain can be directly re-entrained by light, and there are particular regions of the adult zebrafish brain that highly express the clock genes, *cry1a*, *per2*, and *per3*. This chapter will determine whether zebrafish neurons express clock genes and will also explore how light entrains the molecular clock by examining a molecular marker of neuronal firing, *c-fos*.

Clock genes are rhythmically expressed in mammalian SCN neurons and this correlates with a robust circadian rhythm in action potential firing. The mammalian SCN expresses all the mammalian clock genes, with a high expression of *per* in the early morning (Yamaguchi et al. 2003). Likewise the rhythmic neurons in the SCN are electrically active during the day with a slow and steady pace, and silent during the night (Colwell 2011; Herzog & Schwartz 2002). This is contrary to the expression patterns observed in most other non-pacemaker rhythmic brain regions, which in general have a high expression of *per* in the night (Matsui, Takekida, & Okamura 2005) , and have a peak firing activity in the night (Inouye & Kawamura 1979). The zebrafish has not yet been examined to determine whether any regions exhibit an endogenous neuronal activity rhythm that could be indicative of a neuronal pacemaker region.

An alternative method to electrophysiology to examine the neuronal firing pattern of brain regions, though less direct and extensive, is to measure the expression of *c-fos*. *C-fos* is an immediate early gene that is often expressed when neurons fire action potentials, and therefore is an indirect marker of recent neuronal activity. It can be induced in a cell as a response to photic stimulation and can be used to study the light entrainment of central neuronal pacemakers (Caputto & Guido 2000). Furthermore, *c-fos* can be used as a molecular marker for the mammalian SCN. Cultured explants from *c-fos-luc* mice show that the SCN expresses robust rhythms of *c-fos* reflecting the endogenous rhythmic firing pattern of the SCN (Geusz et al. 1997).

Detailed investigations into *c-fos* expression have revealed several aspects as to how the SCN functions. The mammalian SCN is made up of a heterogeneous population of cells that exhibit *c-fos* in different ways. The ventrolateral SCN region, which is referred to as the “core” part of the SCN, is the retinorecipient part of the SCN, and is the region that initially responds to light

input with an induction of *c-fos* (Karatsoreos et al. 2004). The core SCN relays the light information to the rest of the SCN, and is critical for phase-setting and synchronising the SCN (Yamaguchi et al. 2003) and maintaining rhythmicity in behaviour and physiology (Kriegsfeld, LeSauter, & Silver 2004). The dorsomedial SCN region, or "shell", does not respond to light pulses, but does have a robust endogenous circadian rhythm of *c-fos,* that is high at night and low during the day (Sumová et al. 1998).

Induction of *c-fos* expression in the core SCN usually correlates with when a light pulse is given at a time to phase shift the circadian clock. For example, it is acutely expressed in the mammalian SCN when a light pulse is given during the dark when it will produce a phase delay or phase advance in the behavioural activity rhythm (Kornhauser, Mayo, & Takahashi 1996). This photic *c-fos* induction is rapid: a 30-min exposure to light in the dark phase is sufficient to produce a high expression of *c-fos* in the rat core SCN (Colwell 2011), fitting with the definition of *c-fos* as an immediate early gene.

C-fos deficient mice take far longer to entrain to LD cycles than wild type (Honrado et al. 1996), and intracerebral application of antisense *c-fos* oligonucleotides blocks light-induced phase shifts in rat wheel-running rhythms (Wollnik et al. 1995). This suggests *c-fos* plays an important role in the normal entrainment of the mammalian circadian system to light, and is therefore an important gene to investigate the light mediated signalling mechanism to the circadian clock.

C-*fos* CISH has been shown to be a suitable method for evaluating stimulus-induced neuronal activity in most, if not all, neurons of the adult zebrafish brain (Lau et al. 2011). *C-fos* has a very low basal expression in the zebrafish brain (Lau et al. 2011) and is therefore likely to be an appropriate gene to study light entrainment in the zebrafish. Furthermore, C-FOS protein has been shown to be upregulated by a 30-min light pulse in zebrafish cell lines, with levels peaking after 2 hours and returning to baseline after 4 hours (Hirayama et al. 2005). Though at this time, there is no functional data proving that *c-fos* is essential for phase shifting the zebrafish circadian clock.

As *c-fos* has been used as a marker for the mammalian SCN, an examination of *c-fos* throughout the zebrafish brain may provide clues identifying neuronal pacemakers in the zebrafish. Furthermore, the zebrafish brain is directly light responsive, and therefore

understanding the expression of *c-fos* may provide further understanding into how light entrains circadian rhythms in the zebrafish brain.

5.2 Aims

The results of this chapter aim to answer:

- Do all zebrafish neurons express clock genes? Is this widespread in all neurons?
- What is the temporal and spatial expression profile of *c-fos* in the zebrafish brain on a LD cycle?
- Is there an endogenous rhythm of *c-fos* in the zebrafish brain, akin to that seen in the mammalian dorsomedial SCN region?
- Can a light pulse given at a time capable of phase shifting the clock, induce *c-fos* in zebrafish? If so, does this occur in specific brain regions?

5.3 Methods

RNA extraction, brain culturing, fluorescent immunohistochemistry, CISH, and qPCR were performed as described in chapter two. A 30min light pulse at CT22 was chosen as it is a time when light is able to most highly induce *c-fos* in hamster (Guido et al. 1999) and generates a phase delay in zebrafish light-responsive cell lines.

5.4 Results

5.4.1 *Per3* is detected in some, but not all, neurons

To explore whether *per3* is expressed by neurons, fluorescent immunohistochemistry using the neuronal marker HuC/D was used alongside CISH for *per3* mRNA detection in sections of zebrafish brain dissected at ZT3 from zebrafish kept on a 14:10 LD. DAPI was also added for the detection of all cell nuclei. *Per3* mRNA was expressed in neurons in some regions, including the PGZ (Figure 51). However, *per3* was not detectable in all neurons, as shown in Figure 52, where there are many neurons in brain nuclei in the mesencephalon that did not co-localise with *per3* expression. Therefore, *per3* is detected in some, but not all, neurons.

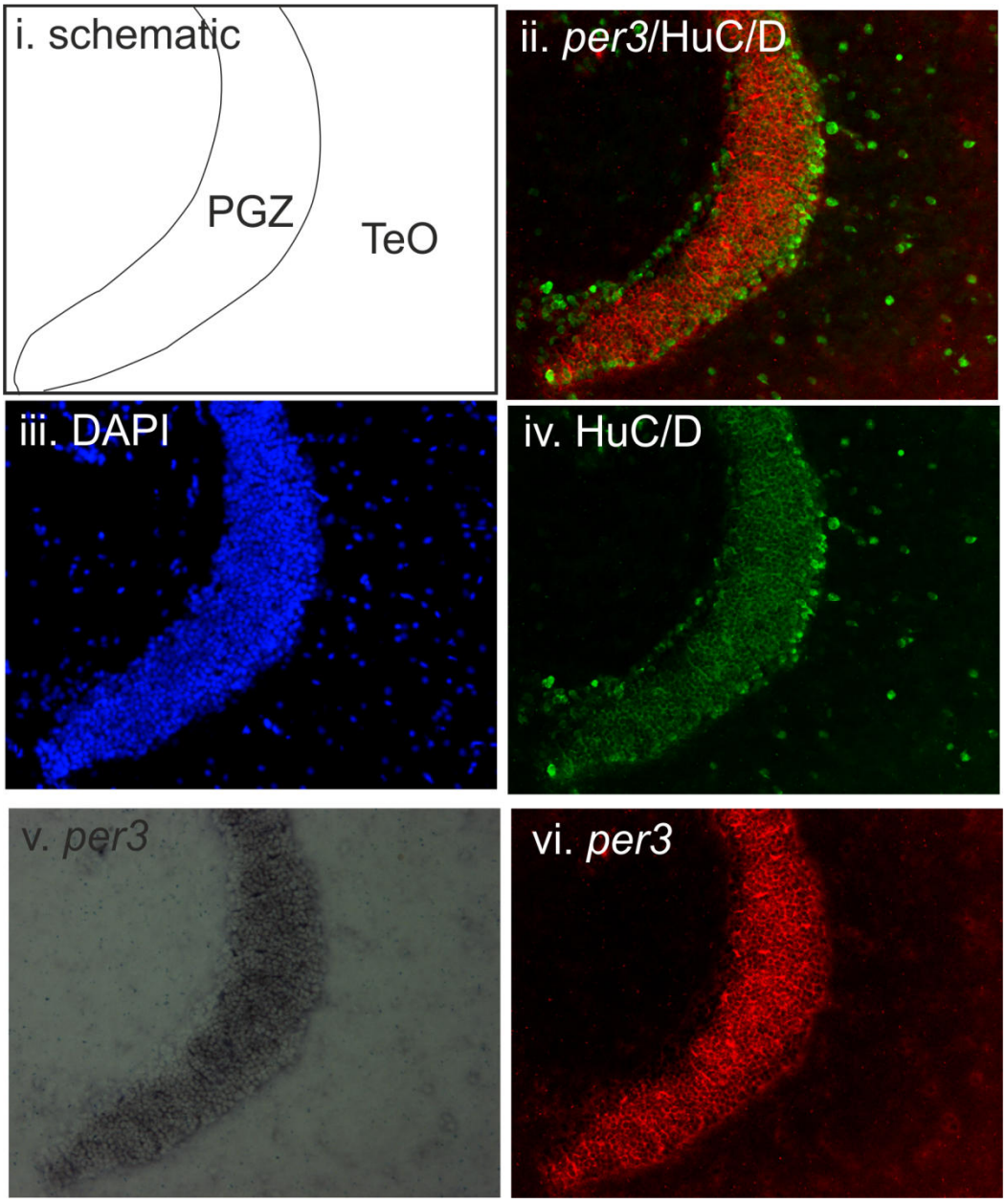


Figure 51 *Per3* mRNA is co-localised with HuC/D in some neuronal regions

Brains were dissected at ZT3, fixed, frozen and sectioned. CISH was performed for the localization of *per3* mRNA and fluorescent immunohistochemistry to show the location of HuC/D as a marker for neurons. DAPI was added to show the location of the cells. **i)** This is a schematic to show the brain nuclei in the mesencephalon examined. **ii)** *Per3* is expressed in neuronal cells in the PGZ. **iii)** Cell nuclei are shown by DAPI. **iv)** HuC/D staining shows neuronal cell bodies in the different brain nuclei. **v)** *Per3* mRNA is located in the PGZ. **vi)** *Per3* signal is false coloured for co-localisation with HuC/D. Sections are shown at 20X magnification. [TeO, optic tectum; PGZ, periventricular grey zone of the optic tectum]

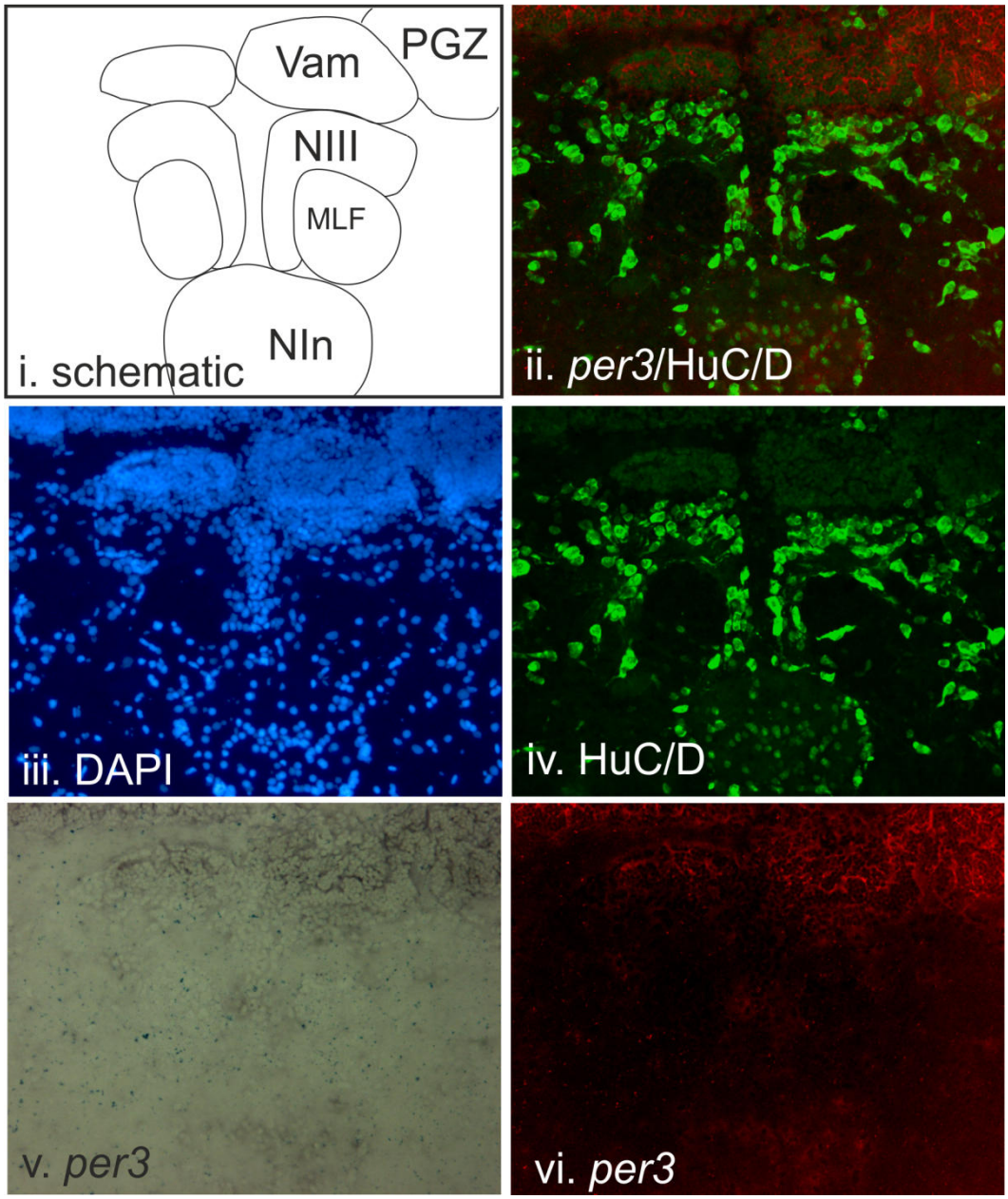


Figure 52 *Per3* mRNA is not co-localised with HuC/D in all neuronal regions

Brains were dissected at ZT3, fixed, frozen and sectioned. CISH was performed for the localization of *per3* mRNA and fluorescent immunohistochemistry to show the location of HuC/D as a marker for neurons. DAPI was added to show the location of all the cells. **i)** This is a schematic to show the brain nuclei in the mesencephalon examined. **ii)** *Per3* is expressed in neuronal regions in the Vam and PGZ, but not in the tegmental nuclei NIII or NIn. **iii)** Cell nuclei are shown by DAPI. **iv)** HuC/D marks neurons in the different brain nuclei. **v)** *Per3* mRNA is located in the Vam and PGZ but not in the NIII, MLF or Nin. **vi)** *Per3* signal is false coloured for co-localisation with HuC/D. Sections are shown at 20X magnification.

[MLF, medial longitudinal fascicle; NIII, oculomotor nucleus; NIn, nucleus interpeduncularis; PGZ, periventricular grey zone of the optic tectum; Vam, medial division of valvula cerebelli]

5.4.2 *C-fos* expression *in vivo*

To determine whether light directly affects neuronal activity in the brain the marker for neuronal excitability, *c-fos* expression, was quantified in zebrafish brains *in vivo* on a 14:10 LD into DD (Figure 53). *C-fos* is acutely upregulated when there is a change in lighting conditions, the hour after lights on (ZT1) or lights off (ZT15) ($p<0.0001$, One way ANOVA with Dunnett's multiple comparison post-test, n=4-9). During DD, the expression of *c-fos* throughout the brain exhibits an endogenous, free-running circadian rhythm with a peak at CT21 ($p<0.001$, One way ANOVA with Dunnett's multiple comparison post-test, n=4-9), which rapidly dampens and cannot be detected on the second day in DD.

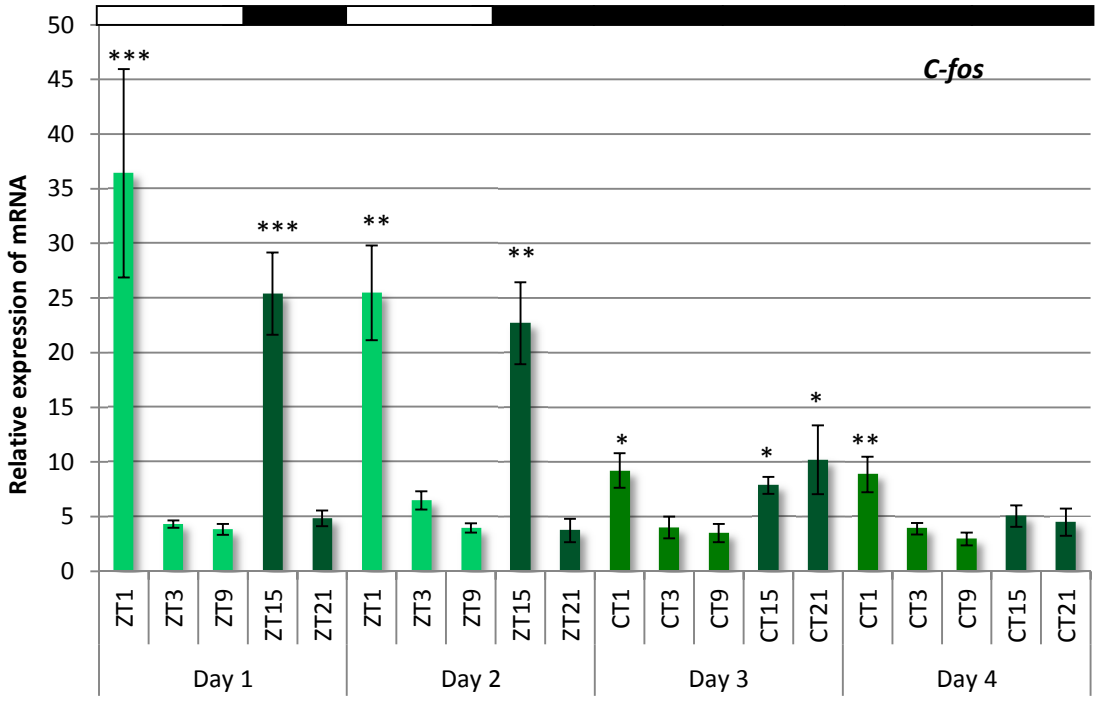


Figure 53 *C-fos* mRNA expression in the brain of zebrafish

Zebrafish were kept on a 14:10 LD schedule and transferred into DD for two days. Brains were dissected at the times indicated, and *c-fos* mRNA quantified using qPCR. **In LD** there was a peak at ZT1 and ZT15 on day 1 and 2, ($p<0.0001$, One way ANOVA, n=4-9. The stars in the graph refer to the results of the Dunnett's multiple comparison post test, which compared samples to Day 1 ZT9). **In DD** there was a peak at CT21 on day 3 and trough at CT9 on day 3 and day 4 ($p<0.001$, One way ANOVA, n=4-9. The stars in the graph refer to the Dunnett's multiple comparison post test, which compared samples to CT9 on day 4). The above white and black bars indicate the lighting schedule, and the shades of green reflect the light, dark and subjective dark phases.

5.4.3 Neuronal activity increases at the beginning of the dark phase

To explore further the induction of *c-fos* in the first hour of the dark phase, regions of the brain were analysed for *c-fos* expression. Brains were dissected from adult zebrafish on a 14:10LD after one hour into the dark phase, ZT15, and at time during the light when *c-fos* expression was low, ZT3. Samples were fixed, frozen, sectioned and CISH was performed for the detection of *c-fos*.

C-fos was upregulated in specific regions at ZT15 compared to ZT3. The description of all the regions is detailed in Table 6.

In the telencephalon at ZT3 there was diffuse expression of *c-fos* in the Vd and Vv. However at ZT15 more cells expressed *c-fos* in the Vd and VV, and furthermore, *c-fos* was also expressed in the ICL, Dl, Dm, Vp, Vs, Vc, EN and NT (Figure 54).

In the diencephalon at ZT3 *c-fos* was detectable in cells in the VM, PG, Hc, and Hv. At ZT15 all these regions had more cells expressing *c-fos*, and in addition, *c-fos* was expressed in the preoptic nuclei, habenula, all thalamic nuclei, most regions of the posterior tuberculum (excluding the torus lateralis), and in the hypothalamic ATN, DIL, Hd and Hv (Figure 55).

In the mesencephalon at ZT3 a few cells in the PGZ and TS express *c-fos*. At ZT15 *c-fos* expression increased in the PGZ and TS and is also found expressed in the TL, DTN, NLV, PL, NI, and SGN (Figure 55 and 56).

In the rhombencephalon *c-fos* is not detectable at ZT3, however at ZT15 *c-fos* is expressed in the granular cells of the Val, Vam, CCe, EG and LCa (Figure 57).

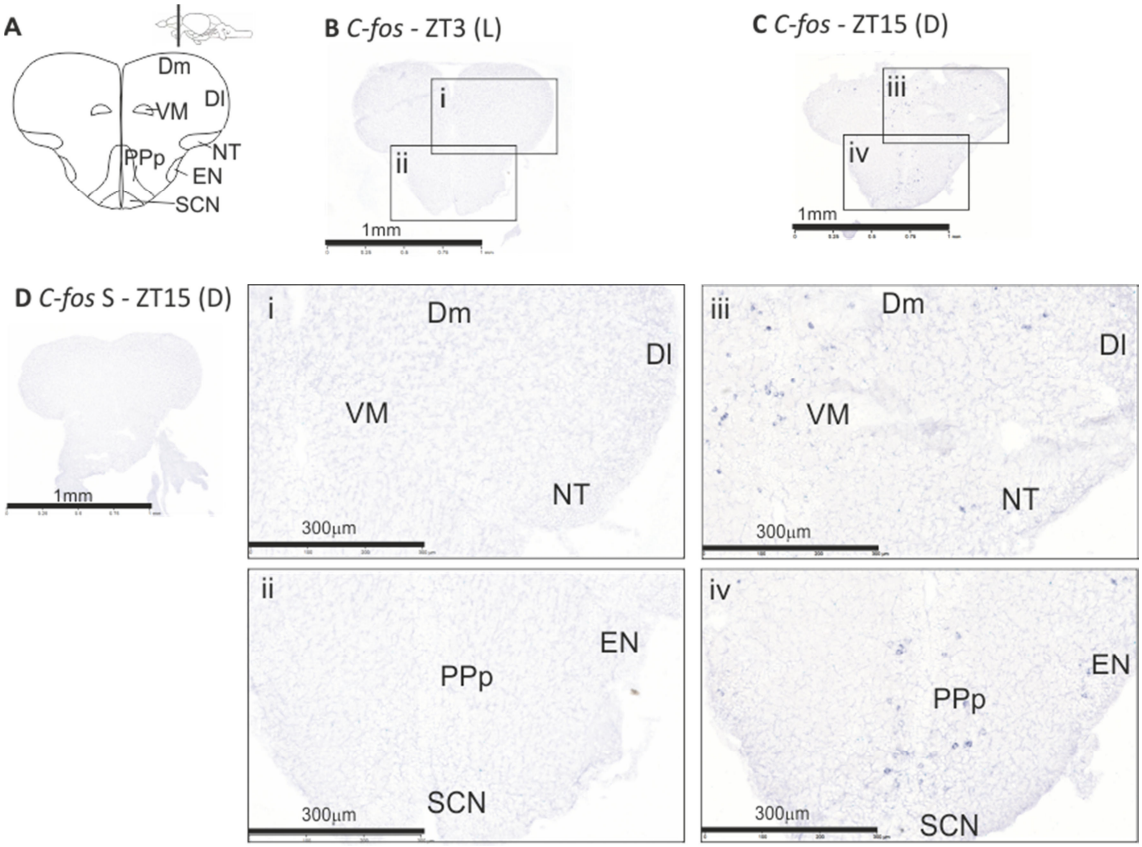


Figure 54 *C-fos* expression in the di- and tel- encephalon at ZT3 and ZT 15

Brains were dissected from adult zebrafish on a 14:10LD at ZT3 and ZT15. Samples were fixed, frozen, sectioned, and CISH performed to determine levels of *c-fos* mRNA as an indicator of neuronal activity. **A)** A schematic of the brain section containing the di- and tel- encephalon. **B)** During the light phase (L) at ZT3 *c-fos* is not detectable in the dorsal telencephalic area or the EN, NT, PPp, VM or SCN **C)** One hour into the dark phase (D) at ZT15 *c-fos* is expressed in cells within the Dm, Dl, EN, NT, PPp, VM or SCN **D)** The sense control shows the background signal

[Dl, lateral zone of dorsal telencephalon; Dm, medial zone of dorsal telencephalon; EN, entopeduncular nucleus; NT, nucleus taeniae; SCN, suprachiasmatic nucleus; VM, ventromedial thalamic nucleus; PPp, posterior periventricular pretectal nucleus;]

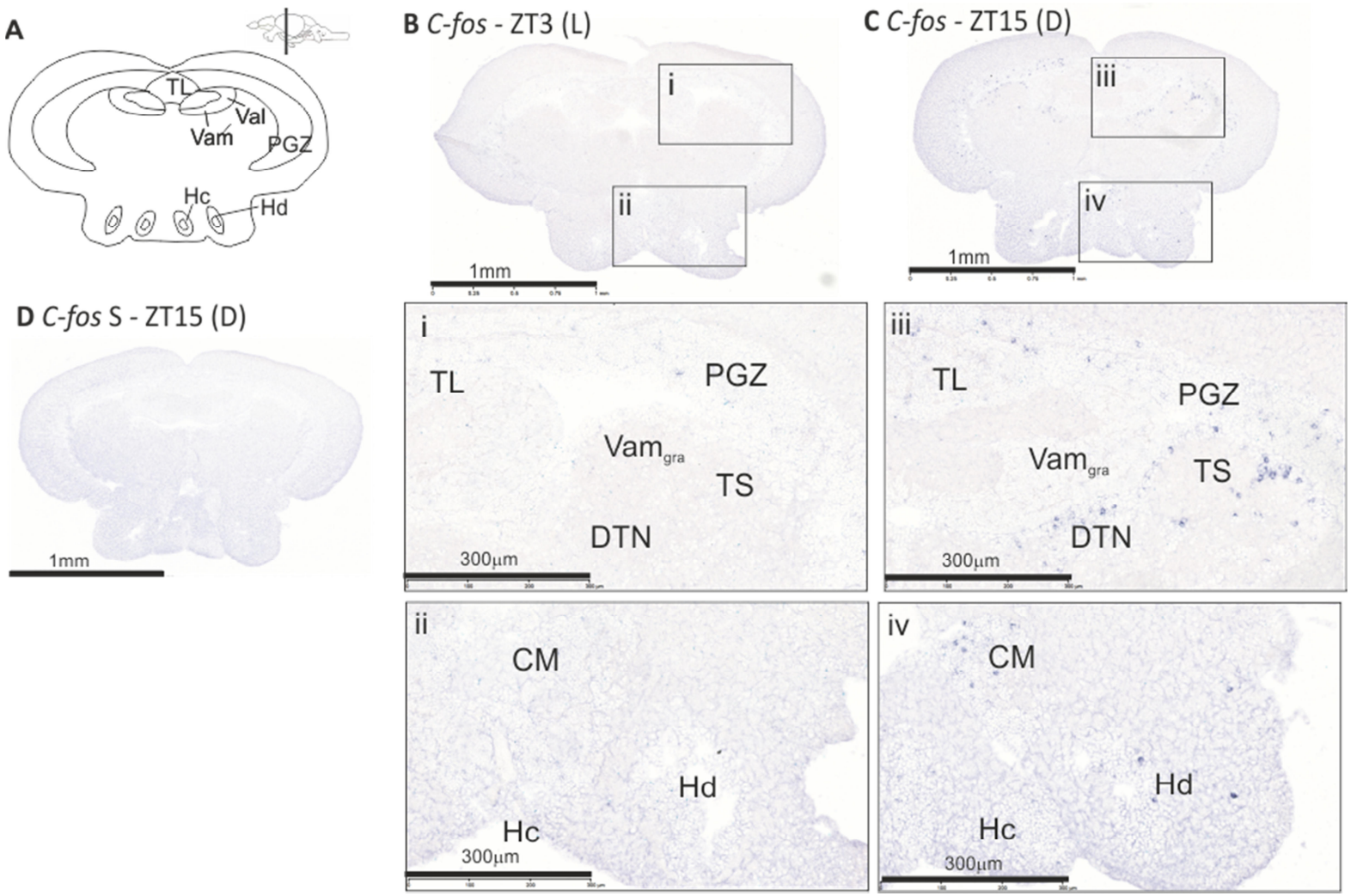


Figure 55 *C-fos* expression in the di- and mes- encephalon at ZT3 and ZT15

Figure 55 *C-fos* expression in the di- and mes- encephalon at ZT3 and ZT15

Brains were dissected from adult zebrafish on a 14:10LD at ZT3 and ZT15. Samples were fixed, sectioned, and CISH performed to determine levels of *c-fos* mRNA as an indicator of neuronal activity. **A)** A schematic of the brain section containing the **B)** During the light phase (L) at ZT3 a few cells within the PGZ express *c-fos*, however no *c-fos* can be detected in the CM, DTN, Hc, Hd, TL, or Vam_{gra}. **C)** One hour into the dark phase (D) at ZT15 *c-fos* is expressed in the CM, DTN, Hd, TL, TS, and Vam_{gra}, however it is not detectable in the Hc. **D)** The sense control shows the background signal.

[DTN, dorsal tegmental nucleus, Hc: caudal zone of periventricular hypothalamus; Hd: dorsal zone of periventricular hypothalamus; PGZ: periventricular grey zone of the optic tectum; TeO: optic tectum; TL: torus longitudinalis; TS: torus semicircularis; Val_{gra}: granular layer of the lateral division of valvula cerebelli; Vam_{gra} granular layer of the medial division of valvula cerebelli]

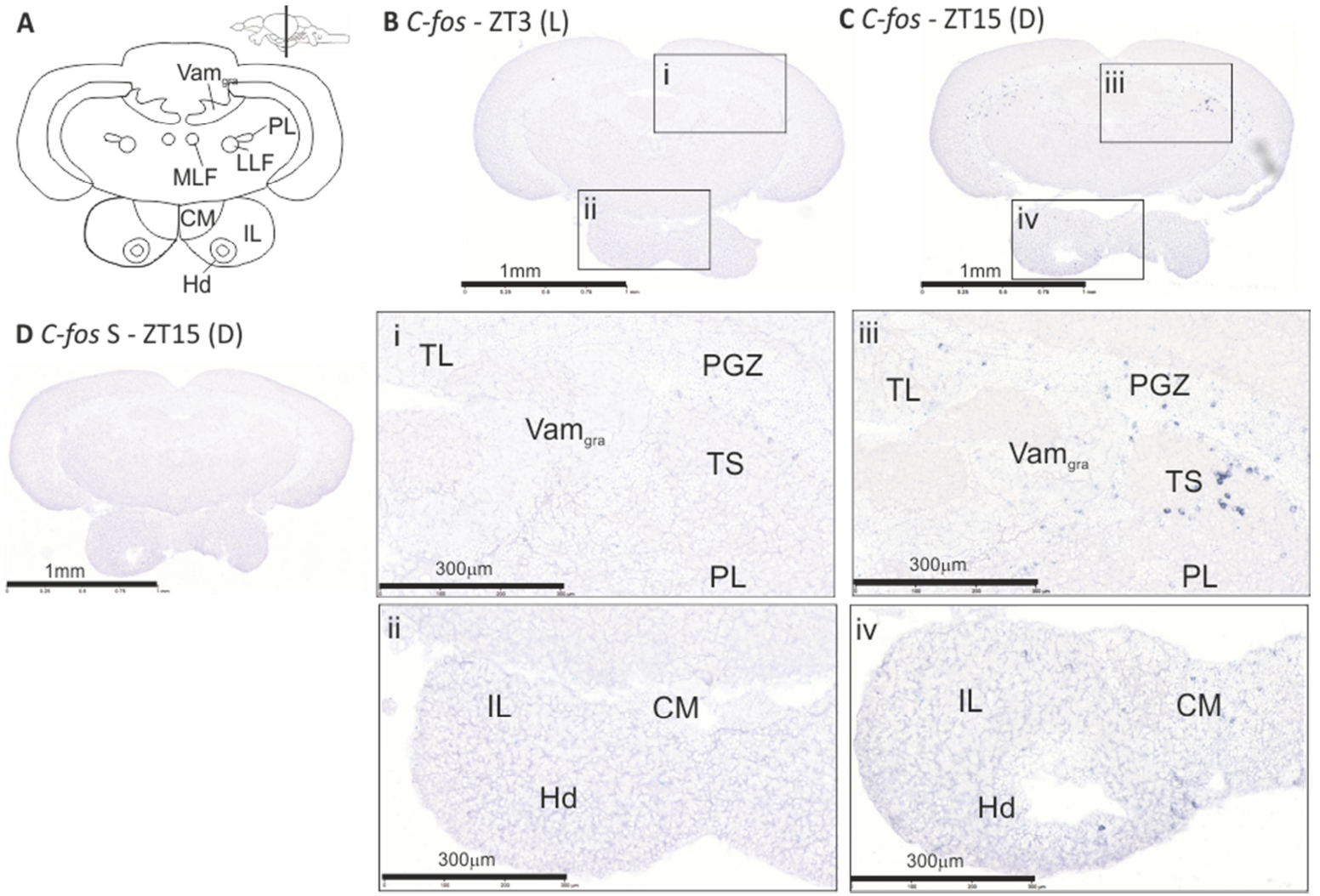


Figure 56 *C-fos* expression in the hypothalamus and mesencephalon at ZT3 and ZT15

Figure 56 *C-fos* expression in the hypothalamus and mesencephalon at ZT3 and ZT15

Brains were dissected from adult zebrafish on a 14:10LD at ZT3 and ZT15. Samples were fixed, sectioned, and CISH performed to determine levels of *C-fos* mRNA as an indicator of neuronal activity. **A)** A schematic of the brain section containing the hypothalamus and mesencephalon. **B)** In the light phase (L) at ZT3 *c-fos* is detectable at low levels in a few cells of the TS, but is not found in the CM, Hd, IL, PL, TL or Vam_{gra}. **C)** One hour into the dark phase (D) at ZT15 *c-fos* is expressed in the CM, Hd, IL, PL, PGZ, TL, TS, and Vam_{gra} **D)** The sense control shows the background signal.

[CM: corpus mammilare; DTN: dorsal tegmental nucleus; Hd: dorsal zone of periventricular hypothalamus; IL: inferior lobe; LLF: lateral longitudinal fascicle; MLF: medial longitudinal fascicle; PL: perilemniscal nucleus; Vam_{gra} granular layer of the medial division of valvula cerebelli]

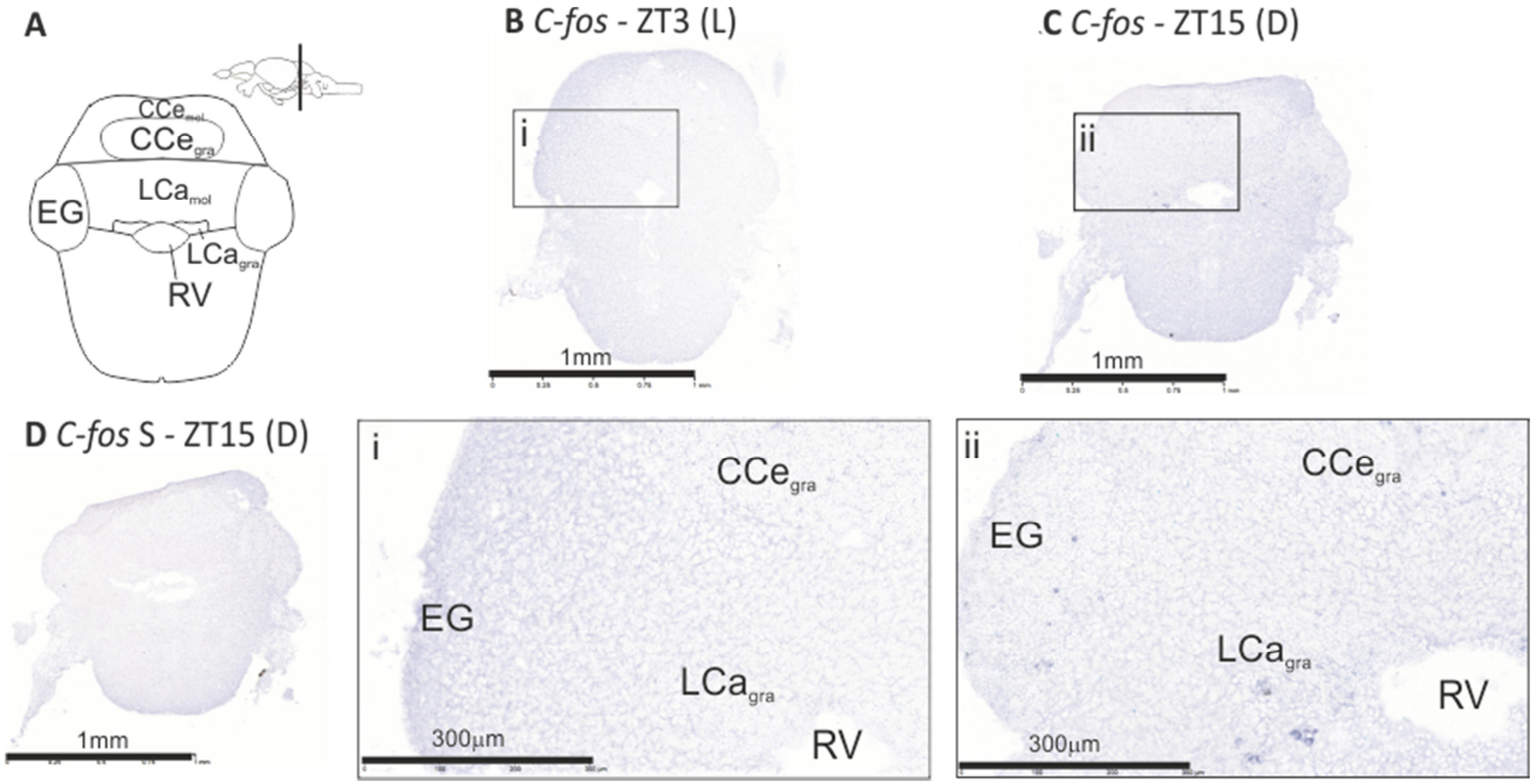


Figure 57 *C-fos* expression in the rhombencephalon at ZT3 and ZT15

Brains were dissected from adult zebrafish on a 14:10LD at ZT3 and ZT15. Samples were fixed, sectioned, and CISH performed to determine levels of *C-fos* mRNA as an indicator of neuronal activity. **A)** A schematic of the brain section. **B)** In the light phase (L) at ZT3 there is no expression of *c-fos* in CCe_{gra}, EG, and LCa_{gra} **D)** One hour into the dark phase (D) at ZT 15 there is expression of *c-fos* in the CCe_{gra}, EG, and $LCa_{gra.}$ **D)** The sense control shows the background signal [CCe_{gra}: granular layer of the corpus cerebelli; EG: eminentia granularis; and LCa_{gra}: granular layer of the lobus caudalis cerebelli; RV: rhombencephalic ventricle]

5.4.4 Neuronal activity is increased by light

To investigate the effect of light on *c-fos* further a short 30-min light pulse was given at CT22, a time of day that would phase shift the clock, and the quantitative and spatial expression of *c-fos* examined.

C-fos was induced by light in the zebrafish brain *in vivo* (Figure 58). *C-fos* expression was five-fold higher in the brains of the light pulsed zebrafish compared to dark controls ($p<0.0001$, unpaired two-tailed t-test, n=7-8).

A spatial examination using CISH revealed that light directly increases neuronal activity in specific regions of the brain (Figures 59-64, Table 6). Interestingly, there was induced *c-fos* expression in the SCN region of the zebrafish brain (Figure 61). Other regions which displayed upregulated *c-fos* expression were found in the telencephalon, thalamus, and hypothalamus. There was no detectable spatial expression of *c-fos* in the rhombencephalon (Figure 64).

There was a diffuse basal expression of *c-fos* throughout many regions of the brain of the fish that had not been light pulsed, showing these regions are constitutively active during the night.

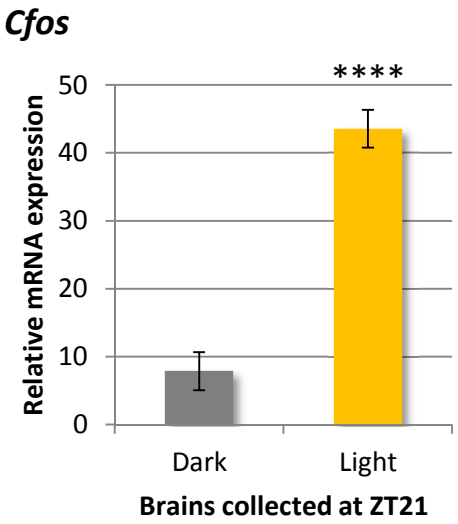


Figure 58 *C-fos* expression shows increased neuronal activity in the brain of light pulsed zebrafish

Adult zebrafish on a 14:10LD were given a 30 minute light pulse or kept in the dark at ZT21. Brains were dissected, RNA extracted, and qPCR performed to determine levels of *c-fos* mRNA as an indicator of neuronal activity. *C-fos* expression was five-fold higher in the brains of the light pulsed zebrafish ($p<0.0001$, unpaired two-tailed t-test, n=7-8).

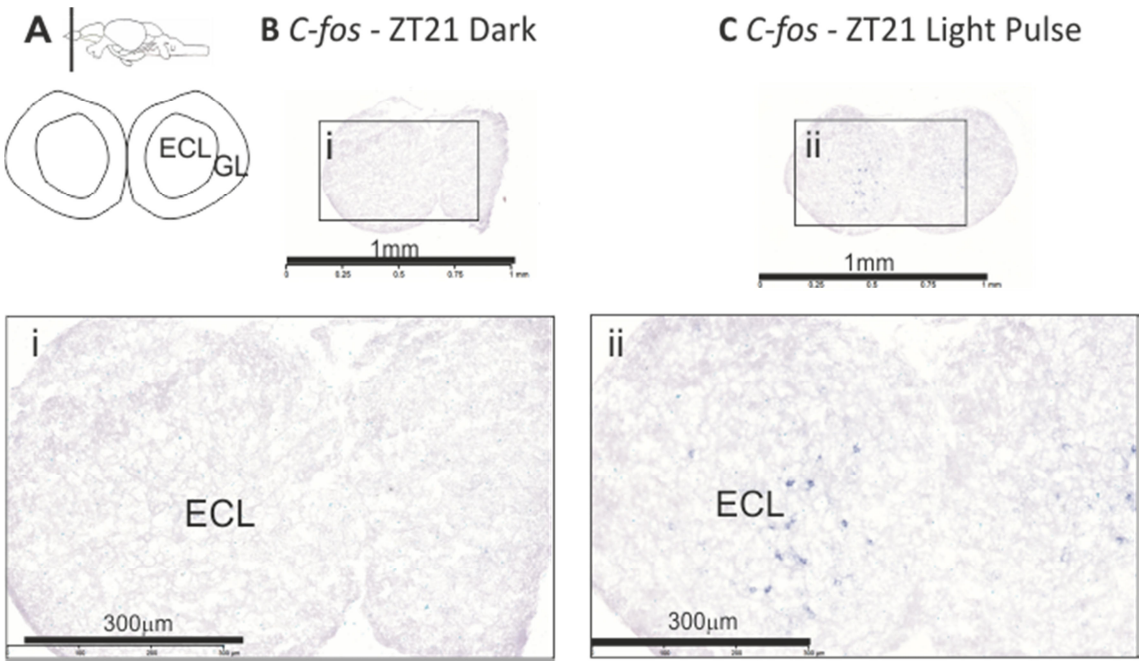


Figure 59 *C-fos* expression in the olfactory bulbs in light pulsed zebrafish

Adult zebrafish on a 14:10LD at ZT21 were given a 30 minute light pulse or kept in the dark. Brains were dissected, fixed, sectioned, and CISH performed to determine levels of *c-fos* mRNA as an indicator of neuronal activity. **A)** A schematic showing the olfactory bulbs. **B)** There is no expression *C-fos* in cells in the olfactory bulbs at ZT21. **C)** *C-fos* expression is induced by light in the ECL.

[ECL, external cellular layer of olfactory bulb including mitral and ruffed cells; GL, glomerular layer]

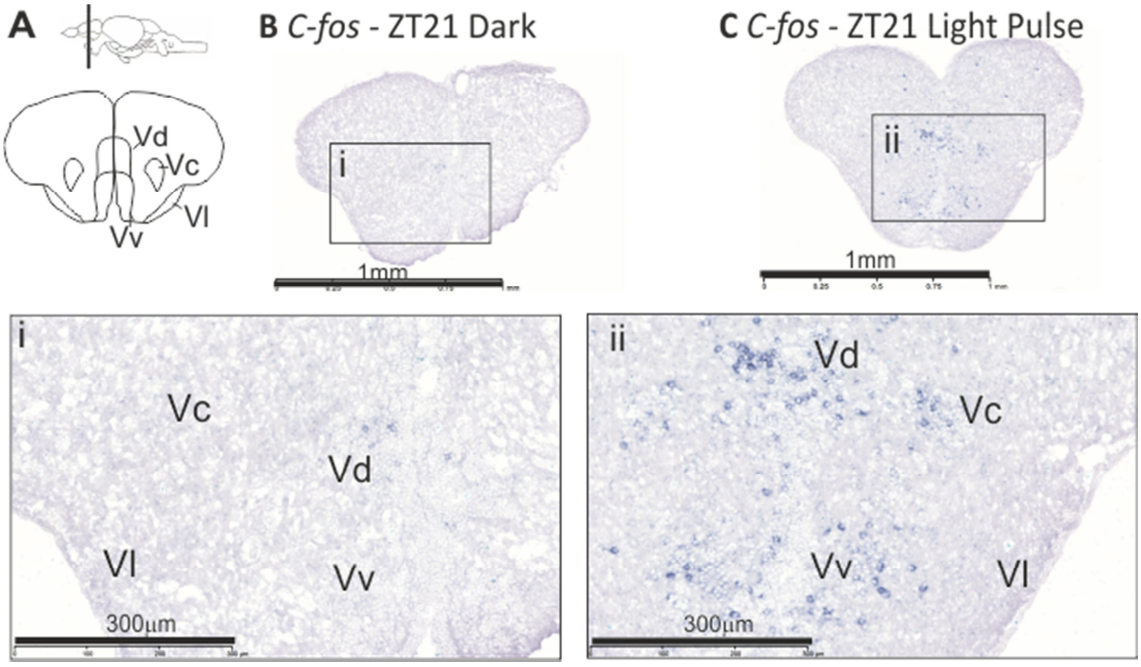


Figure 60 *C-fos* expression in the telencephalon of a light pulsed zebrafish

Adult zebrafish on a 14:10LD at ZT21 were given a 30 minute light pulse or kept in the dark. Brains were dissected, fixed, frozen, sectioned, and CISH performed to determine levels of *c-fos* mRNA as an indicator of neuronal activity. **A)** A schematic showing the ventral telencephalon. **B)** There is minimal expression *c-fos* in cells in the Vd at ZT21. **C)** *C-fos* expression is induced by light in the Vc, Vd, and Vv. There remains no expression in the Vl. [Vc/d/l/v, central/dorsal/lateral/ventral nucleus of the ventral telencephalic area]

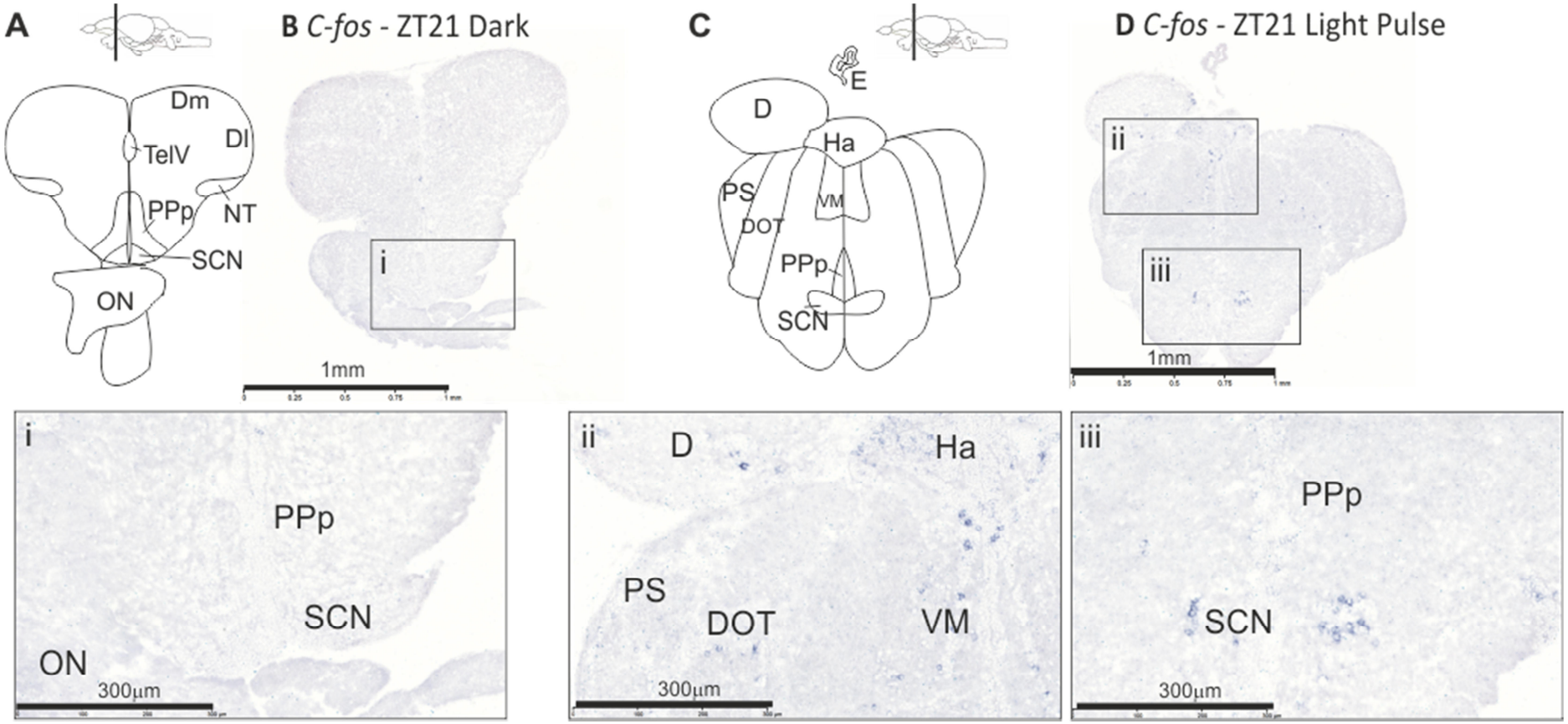


Figure 61 *C-fos* expression in the diencephalon of a light pulsed zebrafish

Figure 61 *C-fos* expression in the diencephalon of a light pulsed zebrafish

Adult zebrafish on a 14:10LD at ZT21 were given a 30 minute light pulse or kept in the dark. Brains were dissected, fixed, frozen, sectioned, and CISH performed to determine levels of *c-fos* mRNA as an indicator of neuronal activity. **A)** A schematic showing the posterior telencephalon in the dark sample. **B)** There is no expression *c-fos* in cells in the SCN, ON and PPp at ZT21. **C)** A schematic showing the diencephalon including the OT in the light pulsed sample. **D)** *C-fos* expression is induced by light in D, Ha PS, DOT, VM, and SCN. There remains no expression in the PPp.

[D, dorsal telencephalon; DOT, dorsal optic tract; E, pineal; Ha, habenula; OT, optic tract; ON, optic nerve; SCN, suprachiasmatic nucleus; VM, ventromedial thalamic nucleus; PPp, posterior periventricular pretectal nucleus; PS, superficial pretectal nuclei]

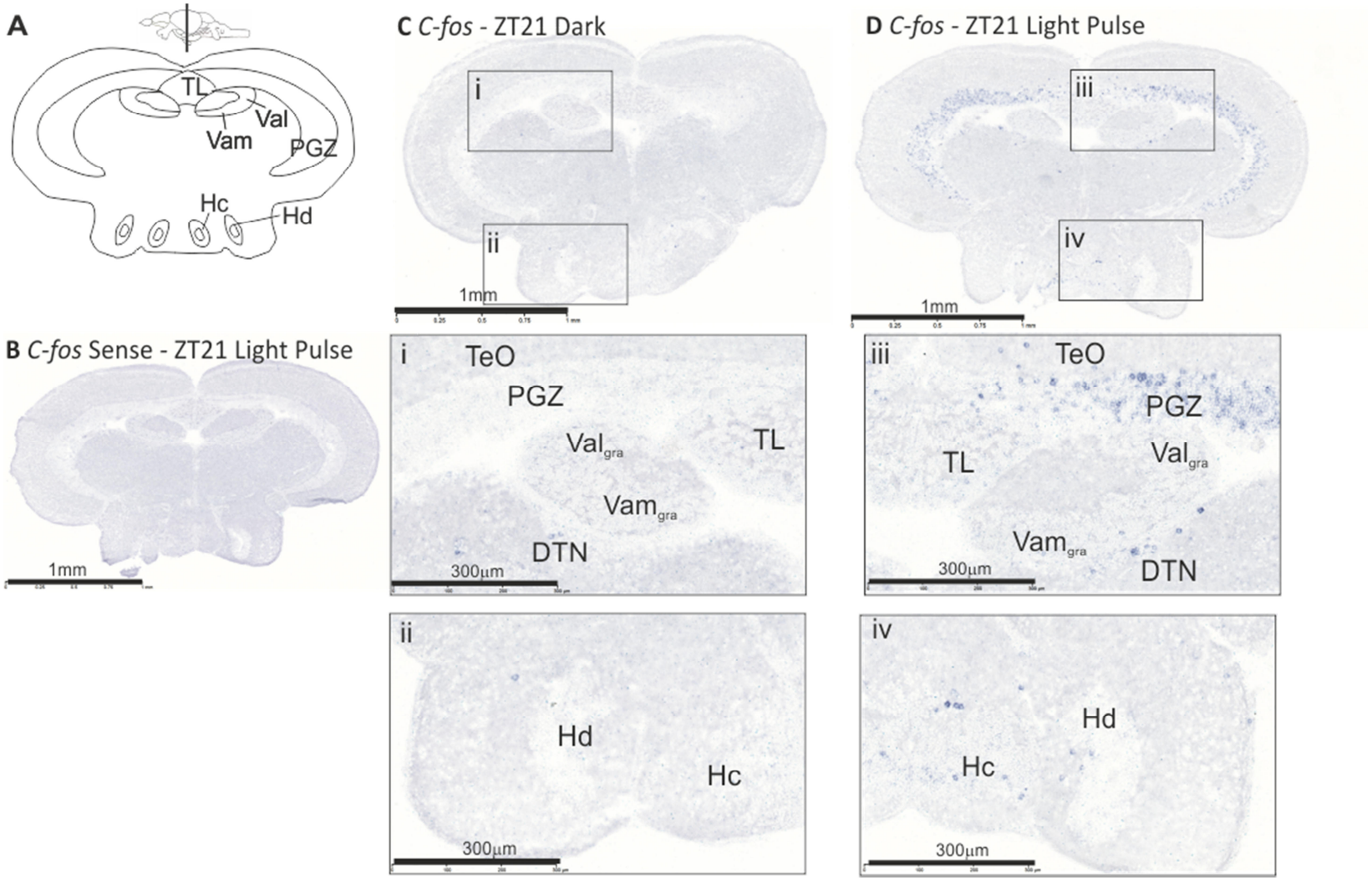


Figure 62 *C-fos* expression in the mesencephalon and diencephalon of light pulsed zebrafish

Figure 62 *C-fos* expression in the mesencephalon and diencephalon of light pulsed zebrafish

Adult zebrafish on a 14:10LD at ZT21 were given a 30 minute light pulse or kept in the dark. Brains were dissected, fixed, frozen, sectioned, and CISH performed to determine levels of *c-fos* mRNA as an indicator of neuronal activity. **A)** A schematic showing the posterior mesencephalon. **B)** The sense control shows the background staining. **C)** There is minimal expression *c-fos* in cells in the DTN and Hd at ZT21. **D)** *C-fos* expression is induced by light in the DTN, PGZ, TeO, Hc and Hd. There remains no expression in the TL or valvula cerebelli.
[DTN, dorsal tegmental nucleus, Hc, caudal zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; PGZ, periventricular grey zone of the optic tectum; TeO: optic tectum; TL, torus longitudinalis; Val_{gra}, granular layer of the lateral division of valvula cerebelli; Vam_{gra} granular layer of the medial division of valvula cerebelli]

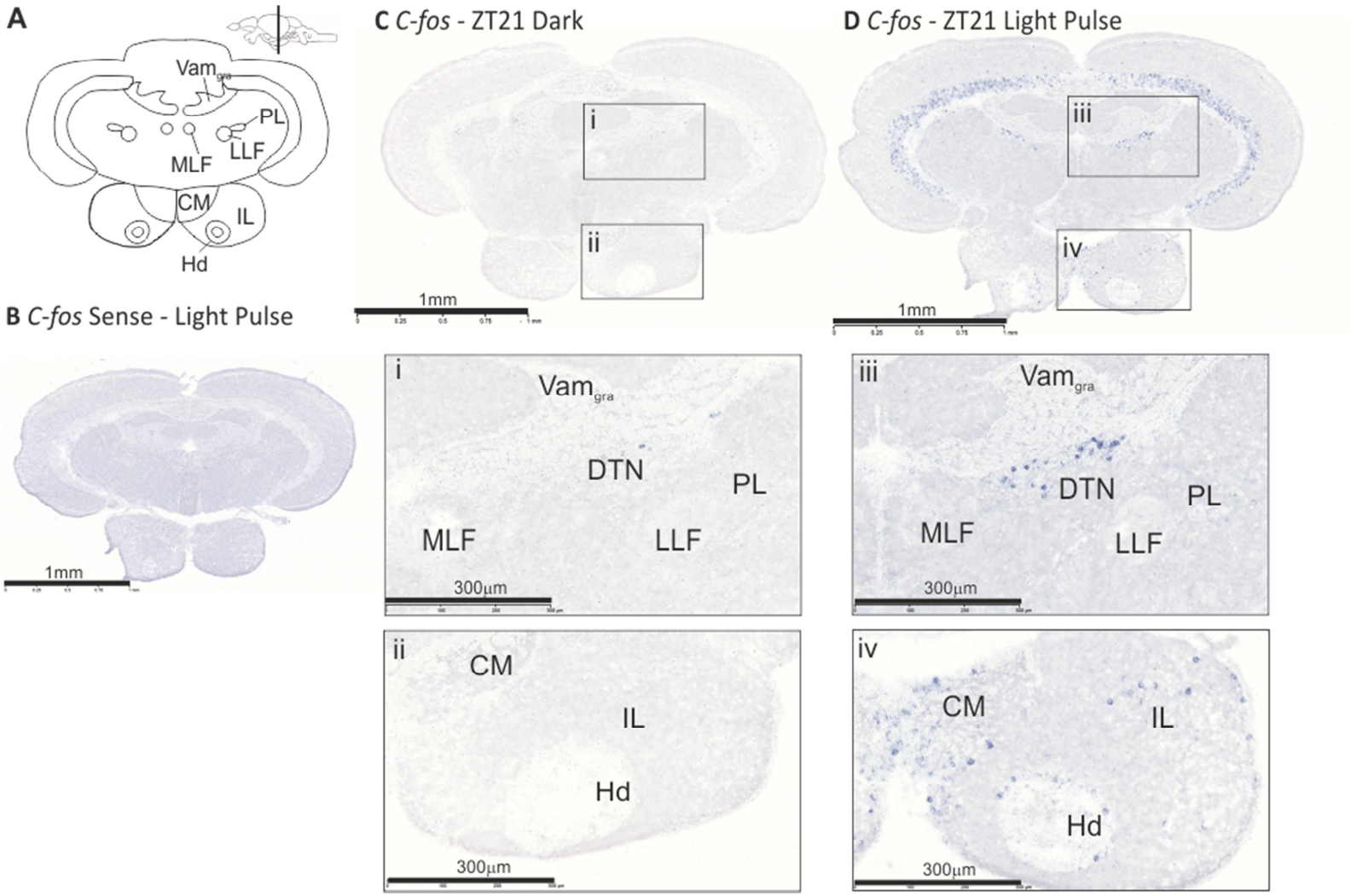


Figure 63 *C-fos* expression in the posterior mesencephalon and diencephalon of a light pulsed zebrafish

Figure 63 *C-fos* expression in the posterior mesencephalon and diencephalon of a light pulsed zebrafish

Adult zebrafish on a 14:10LD at ZT21 were given a 30 minute light pulse or kept in the dark. Brains were dissected, fixed, frozen, sectioned, and CISH performed to determine levels of *c-fos* mRNA as an indicator of neuronal activity. A) A schematic showing the posterior mesencephalon. B) The sense control shows the background staining. C) There is minimal expression *c-fos* in cells in the DTN at ZT21. D) In the light pulsed zebrafish several brain nuclei increase their expression of *c-fos*, DTN, CM, Hd, IL. There is no increase in expression in the LLF, MLF, PL, or Vam.

[CM, corpus mammilare; DTN, dorsal tegmental nucleus; Hd, dorsal zone of periventricular hypothalamus; IL, inferior lobe; LLF, lateral longitudinal fascicle; MLF, medial longitudinal fascicle; PL, perilemniscal nucleus; $Vam_{gra,}$ granular layer of the medial division of valvula cerebelli]

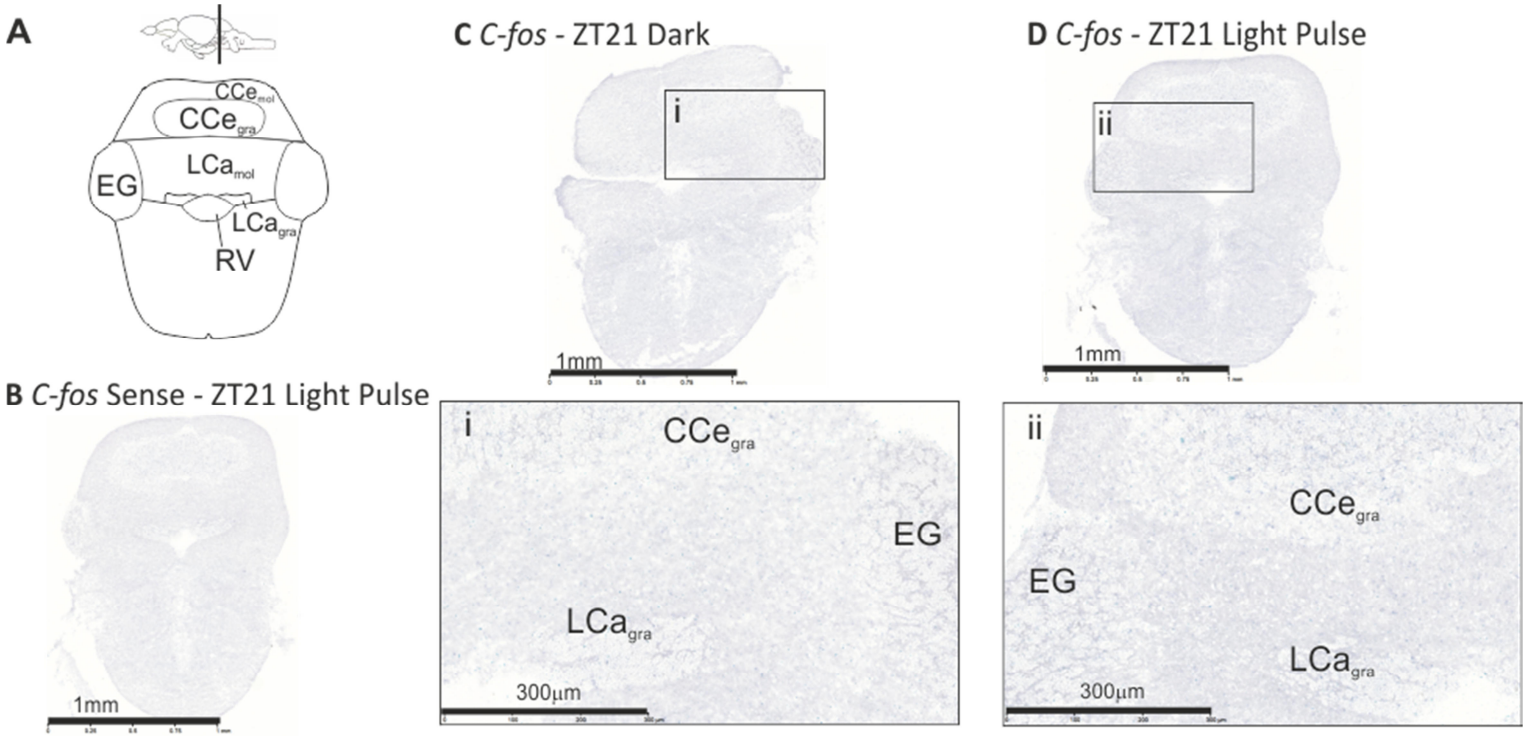


Figure 64 *C-fos* expression in the rhombencephalon of a light pulsed zebrafish

Adult zebrafish on a 14:10LD at ZT21 were given a 30 minute light pulse or kept in the dark. Brains were dissected, fixed, frozen, sectioned, and CISH performed to determine levels of *C-fos* mRNA as an indicator of neuronal activity. **A)** A schematic of the brain section containing the rhombencephalon. **B)** The sense control shows the background signal **C)** The dark control shows no expression of *c-fos* in CCe_{gra}, EG, and LCa_{gra} **D)** In the light pulsed sample there is also no expression of *c-fos* in the CCe_{gra}, EG, and LCa_{gra} [CCe_{gra}, granular layer of the corpus cerebelli; EG, eminentia granularis; and LCa_{gra}, granular layer of the lobus caudalis cerebelli; RV, rhombencephalic ventricle]

Zebrafish brain nuclei	ZT3 (L)	ZT15 (D)	ZT21 (D)	ZT21 (LP)
Telencephalon				
OB, Olfactory Bulbs				
ICL, internal cellular layer	-	++	-	-
ECL, external cellular layer	-	-	-	+
GL, glomerular layer	-	-	-	-
D, Dorsal telencephalic area				
Dc, central zone of D	-	-	-	+
Dd, dorsal zone of D	-	-	-	+
Dl, lateral zone of D	-	++	-	+
Dm, medial zone of D	-	++	+	+
Dp, posterior zone of D	-	-	-	-
V, Ventral telencephalic area				
Cv, commissural nucleus of V	-	-	-	-
Vd, dorsal nucleus of V	+	++	+	++
Vv, ventral nucleus of V	-	++	-	++
Vp, postcommissural nucleus of V	+	++	-	++
Vs, supracommissural nucleus of V	-	++	-	++
Vc, central nuclei of V	-	++	-	++
Vl, lateral nuclei of V	-	-	-	-
EN, entopeduncular nucleus	-	+	-	+
NT, nucleus taeniae	-	+	-	-
Diencephalon				
Area preoptica				
CO, optic chiasm	-	-	-	-
OT, optic tract	-	-	-	+
PPa, parvocellular preoptic nucleus, anterior part	-	++	-	+
PPd, dorsal part of the pretectal diencephalic cluster	-	++	-	-
PPp, parvocellular preoptic nucleus, posterior part	-	++	-	-
SCN, suprachiasmatic nucleus	-	+	-	+
Epithalamus				
Ha, ventral and dorsal habenula	-	++	-	+
Thalamus				
VM, ventromedial thalamic nucleus	+	++	-	++
VL, ventrolateral thalamic nucleus	-	++	-	++
A, anterior thalamic nucleus	-	++	-	++
DP, dorsal posterior thalamic nucleus	-	++	-	++
CP, central posterior thalamic nucleus	-	++	-	++
ZL, zona limitans	-	+	-	-

Zebrafish brain nuclei	ZT3 (L)	ZT15 (D)	ZT21 (D)	ZT21 (LP)
Pretectum				
PS, superficial pretectal nuclei	-	-	-	+
CPN, central pretectal nucleus	-	-	-	+
APN, accessory pretectal nucleus	-	-	-	-
PO, posterior pretectal nucleus	-	-	-	-
DAO/VAO, dorsal/ventral accessory optic nuclei	-	-	-	-
PP, periventricular pretectal nucleus	-	-	-	+
Posterior tuberculum				
Tpp, periventricular nucleus of the posterior tuberculum	-	++	-	+
TLa, torus lateralis	-	-	-	-
CM, corpus mammilare	-	++	-	+
PTN, posterior tuberal nucleus	-	++	-	++
PVO, paraventricular organ	-	++	-	-
PGl/a, lateral and anterior preglomerular nuclei	-	++	-	-
PGm; medial preglomerular nucleus	+	++	-	+
Hypothalamus				
ATN, anterior tuberal nucleus	-	++	-	-
CIL, central nucleus of the inferior lobe	-	-	-	-
DIL, diffuse nucleus of the inferior lobe	+	++	+	++
Hc, caudal zone of periventricular hypothalamus	-	-	-	++
Hd, dorsal zone of periventricular hypothalamus	-	+		+
Hv, ventral zone of periventricular hypothalamus	+	++	+	++
LH, lateral hypothalamic nucleus	-	-	-	-
Mesencephalon				
Superior and inferior colliculi				
TeO, optic tectum	-	-	-	+
PGZ, periventricular grey zone	+	++	+	++
TL, torus longitudinalis	-	++	-	-
TS, torus semicircularis	+	++	-	-
LLF, lateral longitudinal fascicle	-	-	-	-
MNV, mesencephalic nucleus of trigeminal nucleus	-	-	-	-
Nmlf, nucleus of the medial longitudinal fascicle	-	-	-	-
PCN, paracommissural nucleus	-	-	-	-
Tegmentum				
DTN, dorsal tegmental nucleus	-	++	-	++
EW, Edinger-Westphal nucleus	-	-	-	-
NLV, nucleus lateralis valvulae	-	+	-	+
NIII, Oculomotor nucleus	-	-	-	-
NLL, nucleus of lateral lemniscus	-	-	-	-

Zebrafish brain nuclei	ZT3 (L)	ZT15 (D)	ZT21 (D)	ZT21 (LP)
PL, perilemniscal nucleus	-	++	-	-
SR, superior raphe nucleus;	-	-	-	-
SRF, superior reticular formation	-	-	-	-
NIn, nucleus interpeduncularis	-	-	-	-
NI, nucleus isthmus	-	+	-	-
LC, Locus coeruleus	-	-	-	-
SGN, secondary gustatory nucleus	-	++	-	-
Rhombencephalon				
Cerebellum				
$Val_{gra,}$ granular layer of lateral division of valvula cerebelli	-	++	-	-
$Val_{mol,}$ molecular layer of lateral division of valvula cerebelli	-	-	-	-
$Vam_{gra,}$ granular layer of medial division of valvula cerebelli	-	++	-	-
$Vam_{mol,}$ molecular layer of medial division of valvula cerebelli	-	-	-	-
CCe_{gra}, granular layer of corpus cerebellum	-	++	-	-
CCe_{mol}, molecular layer of corpus cerebellum	-	-	-	-
EG, eminentia granularis	-	++	-	-
$LCa_{gra,}$ granular layer of lobus caudalis cerebelli	-	++	-	-
$LCa_{mol,}$ molecular layer of lobus caudalis cerebelli	-	-	-	-
Medulla oblongata				
brainstem cranial nuclei	-	-	-	-
Gc, griseum centrale	-	-	-	-

Table 6 *C-fos* expression in zebrafish brain nuclei

Brains were dissected from adult zebrafish on a 14:10LD, either at ZT3 in the light phase (L), ZT15 and ZT21 in the dark phase (D), or after a 30 minute light pulse (LP) at ZT21. Brain samples were fixed, sectioned, and CISH performed to determine levels of *c-fos* mRNA as an indicator of neuronal activity. This table details the *c-fos* expression in various nuclei throughout the zebrafish brain. *C-fos* was induced an hour after lights off at ZT15, and after a light pulse in the late dark phase, ZT21. [-, no detectable signal; +, signal found in some cells in the region; ++, signal found in many cells in the region]

5.5 Discussion

5.5.1 Circadian gene expression in neurons

Close examination of brain nuclei that express *per3* shows that this expression is co-localised with neuronal cell bodies. This is in accordance with studies of mammalian and *Drosophila* neurons, which have observed *per* expression in neurons, and fits with the role of *per3* as a transcriptional repressor (Hall 1995; Yamaguchi et al. 2003). However, *per3* was not detected in all zebrafish neurons. This is consistent with findings throughout this project that some neural areas do not appear to have a high expression of clock genes. These areas may still receive circadian regulation from other pacemaker regions, but based on the expression analysis of one key core clock gene, don't appear to possess a molecular circadian clock.

These data must be interpreted carefully because the co-localisation was done using CISH rather than fluorescent ISH. The fluorescent ISH technique was attempted using DIG-labelled probes and an anti-DIG-POD secondary antibody. Regrettably the secondary antibody lacked specificity in the zebrafish brain sections, with high background staining using the sense probes, and was therefore deemed unreliable and CISH used instead. Combining CISH and IF does not allow the co-localisation to be viewed on the same plane on a confocal microscope, and confirm with full confidence that the mRNA is in the same cell as the protein. However, the localisation of *per3* mRNA in regions densely packed with neuronal cell bodies provides reasonable confidence that in some regions *per3* is expressed in neurons and in other neuronal regions cannot be detected.

The next step would be to determine whether clock genes were expressed in other cell types in the zebrafish brain. Non-neuronal cells have been shown to express clock genes in other species. *Per* has been detected in *Drosophila* glia (Jackson 2011), and mice glia express *per2* (Guilding et al. 2009). In fact, the mice glial cells expressing *per2* are hypothalamic epithelial glia that line the central brain cavities and are part of the ventricle walls. As many of the regions expressing clock genes in zebrafish are found in regions close to the ventricles, the epithelial glia may also be implicated in regulating circadian rhythms. In future investigations it might be possible to co-localise zebrafish clock proteins with glial makers such as glial fibrillary acidic

protein (GFAP). However, one of the general problems of zebrafish as a model system, over mouse, is the relative lack of good, specific antibodies.

5.5.2 Neural response to changing light conditions

An important finding of this present work is that there are at least four types of induction of *c-fos* in the zebrafish brain in response to different lighting conditions. Firstly, there is striking induction of *c-fos* an hour after lights-on. Secondly, there is another strong induction of *c-fos* an hour after lights-off. Thirdly, there is a low amplitude endogenous rhythm with higher *c-fos* expression in the subjective night. Finally, a light pulse during the late subjective night will induce *c-fos* expression. These four types of *c-fos* light induction are also seen in various mammalian neural (Onodera et al. 1999; Rusak et al. 1990; Schwartz et al. 1994; Sumová et al. 1998) and retinal tissues (Nir & Agarwal 1993), however they have not all yet been observed in the same cell type. This wide range of responses might be due to the possibility that the zebrafish brain contains several types of photosensitive cells.

Closer analyses of the regional specific expression of *c-fos* in the zebrafish brain reveal that different regions have a *c-fos* induction at lights-off compared to regions that are induced by a light pulse. For the regions that had no detectable levels of *c-fos*, this could suggest that these areas either have a very rapid, transient response to light, and were no longer firing at the end of the 30 minute light pulse, or they do not respond to changes in the lighting conditions. There was a difference between brain regions that showed an induction of *c-fos* at lights-off and when exposed to a light pulse at ZT21. Most notably, the TL, PL, ATN, and granular cell layers in the rhombencephalon exhibit *c-fos* induction at lights off but not after a light pulse in the subjective night, and all also show clock gene expression (appendix).

Probably the tissue with the most similar *c-fos* induction to the zebrafish brain is the mammalian retina, which contains numerous photoreceptor cell types. On a 12:12LD the mouse retina has been shown to have an increase in *c-fos* expression in the first hour both after lights-on, and in the first half of the dark phase (Nir & Agarwal 1993). Further experiments in rat show that different retinal cell types are responsible for the light induced short lived and the prolonged dark-activated *c-fos* expression. In the ganglion layer and the inner nuclear layer (INL), which consists of rod bipolar, cone bipolar, horizontal and amacrine cells, a 30-min exposure to light

induces *c-fos* expression, but no response is seen when entering dark. On the other hand, the outer nuclear layer (ONL), which contains the cell bodies of the rod and cone granular cells, produces no increase in *c-fos* after acute light exposure however there is an increase in *c-fos* expression at the beginning of the dark period (Yoshida, Kawamura, & Imaki 1993). Similarly, investigation into the avian chick retina shows that a light pulse in the night induces *c-fos* in the ganglion cells but decreases *c-fos* expression in the photoreceptor cells (Caputto & Guido 2000). In the present results the zebrafish brain shows a different spatial expression of *c-fos* induction at the beginning of the dark phase, ZT15, than in a LP at ZT21, which agrees with the earlier hypothesis that the zebrafish brain contains several types of photosensitive cells that express *c-fos* in different conditions. Although, there may be yet more regions involved in the light response at ZT0, and future studies are therefore recommended to further elucidate the different regions involved in light entrainment.

The abrupt transition of lights on and off is, of course, an unnatural stimulus, as in the natural environment a twilight signal is normally observed at dawn and dusk. An induction of *c-fos* after lights on and off has been reported in the SCN of gerbils, but only when a twilight signal is given. Gerbils on a LD cycle with abrupt transitions between lights on and off only show a dawn *c-fos* induction (Cooper et al. 1998), which, as will be detailed later, is common for the mammalian SCN. The altered induction of *c-fos* using the twilight conditions is accompanied by a changing pattern of locomotor activity. It is likely the twilight signal is being interpreted differently by photosensitive cells and this causes a second *c-fos* induction at dusk. To further understand the mechanism of light to regulate the circadian clock it would be interesting to study the effects of twilight on locomotor activity, clock gene expression and *c-fos* expression in zebrafish.

Lights causes an acute induction of *c-fos* in the mammalian core SCN both at lights-on on a normal LD cycle (Schwartz et al. 1994) and when a light pulse is given during the dark phase (Rusak et al. 1990; Schwartz, Aronin, & Sassone-Corsi 2005). However, no induction of *c-fos* is observed immediately prior to lights-off in the rat core SCN on a 12:12LD (Schwartz et al. 1994), on a 16:8LD, 8:16LD or even when using natural light twilight signals (Ják, Sumová, &

Illnerová 2000). Therefore the regions in the zebrafish brain that are responding to a lights-on stimulus are behaving more like the mammalian SCN.

It should be noted that there is a discrepancy in the effects of lights-off in the hamster core SCN, which has an induction of *c-fos* at the beginning of the dark phase of a long day 16:8LD (Yan & Silver 2008), despite no induction occurring on a 14:10LD (Guido et al. 1999) or 8:16LD (Yan & Silver 2008). This suggests that *c-fos* can be induced in the hamster core SCN in response to lights-off but the length of the light period is critical. It has previously been noted that there are several differences in the circadian rhythmicity of spontaneous neuronal firing the hamster SCN compared to rat and mouse, of which *c-fos* induction at lights-off is also different. Therefore, there may be have been a divergence in the central clock mechanism in these rodents (Burgoon, Lindberg, & Gillette 2004).

A recent study has examined *c-fos* expression in the adult zebrafish brain (Lau et al. 2011). The experiments were designed to examine the brain regions controlling zebrafish behaviour to avoid brightly lit areas in the tank. The authors used CISH to show regions that had an induced *c-fos* expression when exposed to a 30 min light pulse, presumably during the day but this was not stated. These regions show remarkable similarity to the results presented here with a light pulse at ZT21. The exception to this was *c-fos* induction in the ATN, LH and cerebellum, which was not observed in the ZT21 light pulse. However, both the ATN and cerebellum show *c-fos* induction at the beginning of the dark phase at ZT15. Therefore, *c-fos* expression appears to be a reliable and consistent reporter of light induction in zebrafish brain regions.

More detailed analysis of the mammalian retinal ganglion layer shows a further different response to light onset in the different ganglion cell types. In the retinal ganglion cells that project to the SCN, light at dawn cause a sustained induction of C-FOS until lights off at dusk. Whereas in non-RHT projecting retinal ganglion cells, light induces a transient induction of C-FOS, which decays within three hours (Hannibal et al. 2001). In the present study, total *c-fos* levels in the zebrafish brain returned to basal levels within three hours of the lights on at ZT3, demonstrating that the strong *c-fos* induction is mostly caused by cells that have a transient induction. However, the CISH analysis reveals that some brain regions continued to express *c-fos* at ZT3. Whether these cells are responding to the light signal has not been fully addressed

in this experiment, as equivalent dark controls and longer term light samples were not examined. A more detailed investigation to monitor cells for transient or long-term responses to light in zebrafish brain would be worthwhile to characterise the light sensitivity of the brain.

5.5.3 Rhythmic *c-fos* expression in the zebrafish brain

The present study shows that a rhythm of *c-fos* persists in the zebrafish brain for at least one day into constant darkness, with a peak at CT21. The rat retinal ONL, which does not show an induction with a light pulse, has a circadian expression of *c-fos* in DD, with a peak in the dark phase (Yoshida et al. 1993). As in the zebrafish, the *c-fos* peak in the rat retinal ONL in DD was not as high as seen on a LD cycle. These findings are in accordance with the temporal expression of the C-FOS protein in the rat retina, which increases sixty-fold 5 hours after the onset of darkness kept on a 14:10 LD (Humphries & Carter 2004). When put into DD the rhythm of C-FOS persists, peaking in the late dark phase, but with lower amplitude. This shows that the expression of *c-fos* in the zebrafish brain and mammalian ONL can be strongly influenced by the environmental LD cycle and this can mask the endogenous rhythm.

SCN explants from *c-fos-luc* mice SCN have near-24 hour rhythms in *c-fos* expression for at least five days in culture (Geusz et al. 1997). Contrastingly, rhythmicity was not observed in the anterior hypothalamus, suggesting the SCN *c-fos* rhythm is part of its neuronal pacemaker characteristics. Further work pinpointed this rhythmicity to the dorsomedial part of the caudal SCN, the shell, in rats (Sumová et al. 1998). *C-fos* has an endogenous rhythm which is high during the early subjective day and low in the early subjective night. Whereas the retinorecipient core SCN region, and specifically the gastrin-releasing peptide cells within, has a circadian-gated rhythm in sensitivity to light pulses that induce *c-fos* expression, which is restricted to the night and early morning when light can phase shift locomotor rhythms (Karatsoreos et al. 2004). The difference of the phase of *c-fos* rhythm between rat and zebrafish could be explained by the rat being nocturnal, and the zebrafish diurnal. This provides further evidence that neuronal pacemakers exist in the zebrafish brain that have a rhythmic induction of *c-fos*.

The different expression of *c-fos* in different cell populations in the SCN suggests that these cells function differently. This corresponds with the expression of clock genes, which show in the gastrin-releasing peptide-containing cells, *per* and *c-fos* mRNA are both upregulated in

response to a light pulse but do not have a detectable endogenous rhythms in the core SCN (Karatsoreos et al. 2004).

Further work performing CISH in the zebrafish brain will be useful to determine in which regions this endogenous rhythmicity occurs. This may provide further clues to determine the circadian regulatory structures in the zebrafish brain.

5.5.4 Light input pathways and *c-fos* expression

How do these regions with upregulated *c-fos* compare to areas shown to have retinofugal projections? As with the expression of *cry1a*, *per2*, and *per3* there are regions that show *c-fos* induction that are reported to receive retinofugal inputs in zebrafish, including the ventral thalamic nuclei, PGZ, and Vd (Bally-Cuif & Vernier 2010). However, there are many regions that show *c-fos* induction that have not been reported to be receive direct retinofugal inputs in teleosts, such as the hypothalamus, TL, preglomerular nuclei, and valvula cerebelli (Northcutt & Wullimann 1987).

What is the influence of retinal inputs on the light induction of *c-fos* in the zebrafish brain? As the zebrafish brain is directly light responsive it would be interesting to distinguish whether the regional induction of *c-fos* is due to local light sensitivity or due to retinofugal inputs. However, this question cannot be addressed using brain cultures that lack retinal inputs, because *c-fos* is strongly expressed in apoptosis pathways, which will likely mask any effect of the light.

This work has laid the foundations that neuronal excitability can be induced by light in certain brain regions in the zebrafish. As *c-fos* can also be a marker for other stimulus-induced neuronal activity, such as stress, this research will need to be further validated with electrophysiological examination. The next step would be to perform *in vitro* electrophysiological recordings to directly monitor these firing patterns. Multimicroelectrode plate arrays (MMEP) have been widely used to investigate the circadian effects of SCN firing (Herzog et al. 1997). Brain cultures can be grown on the plate, and multi-unit activity can be measured from a number of neurons. A direct response of changing firing rate to a light stimulus will represent a novel and highly interesting phenomenon. Furthermore, a *c-fos-luc* zebrafish transgenic would

be a useful tool to explore the acute effects of changing light conditions and the regional expression of *c-fos* in further detail.

This chapter has shown that neurons can express clock genes are implicated in the light entrainment of the circadian clock. Most importantly, this data shows strong evidence for multiple types of photosensitive cells in the zebrafish brain, which respond to different light in different conditions. Chapter 6 will explore whether different types of photosensitive cells can be described in the zebrafish brain through the expression of different photopigments. However, first an investigation into the larvae brain will examine circadian rhythmicity and light sensitivity during development and will further explore the role of retinal inputs.

5.6 Conclusions

- *Per3* is expressed by some, but not all, neurons.
- *C-fos*, a marker of neuronal activity, is upregulated by the transition from light to dark and dark to light on a normal LD cycle.
- *C-fos* is rhythmic in the brain during DD, with a peak around CT21.
- *C-fos* is upregulated in specific adult brain regions by a short light pulse in the late dark phase. These regions include the ventral telencephalic area, PGZ, SCN, hypothalamus, and thalamic nuclei.
- Different areas show high *c-fos* levels in the hour after lights off, including the posterior tuberculum, and granular cells of the rhombencephalon. However, some of the same regions are induced including the ventral telencephalic area, PGZ, SCN, hypothalamus, and thalamic nuclei.
- The different photic responses of *c-fos* are indicative of multiple photoreceptor types throughout the brain.

Chapter 6

Circadian rhythmicity and light sensitivity of the zebrafish larval brain

6.1 Introduction

Chapter 3 has shown that *per3* is highly expressed in specific brain nuclei in the adult zebrafish brain. This chapter will extend this investigation further and explore whether *per3* is also expressed in the same manner in the developing larvae. As part of this the *per3-luc* zebrafish will be evaluated for its effectiveness for monitoring high-resolution clock rhythms in larval fish. Chapter 4 describes a higher induction of light responsive genes in zebrafish *in vivo* compared to *in vitro*, which may be due to the lack of retinal or pineal inputs in culture. In this chapter, by using mutant larvae lacking eyes and pineal, experiments will address whether retinal or pineal inputs are important for entrainment of the zebrafish, to help understand whether adult brain cultures may respond differently to light due to lacking this inputs. Furthermore, the expression of *c-fos* will be investigated to determine whether neuronal activity increases in response to changes light condition in larvae as it does in the adult.

6.1.1 Circadian function in zebrafish larvae

The circadian clock is functional very early in zebrafish development. When the zebrafish embryo is still in the blastula phase, many of the clock genes are expressed and maternally inherited (Dekens & Whitmore 2008; Ziv & Gothilf 2006). These include *clock1*, *bmal1*, *per1b*, and *per2*. *Cry1a,* however, remains absent until the embryo is exposed to light (Dekens & Whitmore 2008). Whereas, *per1b* transcription starts on the first day of development, even in embryos raised in constant darkness (Dekens & Whitmore 2008). Nevertheless, as in adult tissues, light is required for the synchronisation of individual cellular *per1b* rhythms in larvae.

There is evidence to suggest that the light sensitivity of the eyes and pineal are not essential for circadian rhythmicity. Indeed, zebrafish embryos in the gastrulation stage where the eyes and pineal have not yet developed, show an acute light response in the induction of *per2* expression (Dekens & Whitmore 2008; Tamai et al. 2004). The developing zebrafish offers a useful model for studying acute light responses, and the availability of eyeless, *mbl*, and pineal-less, *flh*, mutant larvae provides a valuable tool.

Some components of the molecular clock are regulated differently in the embryo to the adult. *Clock1* and *bmal1* are rhythmic in the adult zebrafish, but constant during development in

embryos despite being raised on a LD cycle until at least 5 dpf (Dekens & Whitmore 2008). Therefore, there may also be other differences in the regulation of circadian rhythms and light sensitivity in larvae.

Numerous circadian output rhythms have been demonstrated in zebrafish larvae. Locomotor rhythms in zebrafish larvae are rhythmic (Cahill, Hurd, & Batchelor 1998). Synaptic plasticity is also rhythmic with larval brain hypocretin (hcrt) neurons having a circadian rhythm in the number of synapses formed by their axons (Appelbaum et al. 2010). *C-fos* will be investigated to determine whether the zebrafish brain responds to changes in lighting conditions as it does in the adult, where high expression is observed one hour after a light change (chapter 5).

The *per3-luc* zebrafish was created by Cahill and colleagues for the high-resolution monitoring of the zebrafish molecular clock, however initial results in larvae show bioluminescent rhythms begin several days after *per3* is first expressed (Kaneko & Cahill 2005). Therefore, a temporal examination of the molecular expression of both *per3* and *luc* will be carried out in this chapter to confirm that the *luc* is accurately reporting *per3* expression. Furthermore, the spatial expression of *per3* will be monitored in the *per3-luc* larvae using both CISH and spatial bioluminescent imaging.

6.1.2 Spatial expression of core circadian genes in larvae

WISH analysis has been used to examine the expression of clock genes in zebrafish larvae. Experiments in early embryos show that *per1b* and *clock1* are expressed ubiquitously at 2 dpf (Dekens & Whitmore 2008), with no discrete regional *per1b* expression in the brain or retina. Another study compared the spatial and temporal expression of *per1a* and *per1b* in older 5 dpf larvae and reported high levels in the brain and retina (Wang 2008). This study showed *per1a* is discretely expressed in the telencephalon, retina and diencephalon, both in the dark and light phase, but with a marked increase in the light phase. On the other hand, *per1b* was not detectable in the dark phase, but was constitutively expressed throughout the entire brain and retina in the light phase, as seen in the earlier stages. Another clock gene, *per2,* is highly expressed in the pituitary, OB and pineal in early embryos from 2 dpf (Delaunay et al. 2003; Ziv & Gothilf 2006).

Early work has shown *per3* can be detected from the 1 dpf, with WISH assays showing high expression in the retina and brain (Delaunay et al. 2000). However, in light of the new findings of regional expression of *per3* expression in the adult zebrafish brain (chapter 3), *per3* expression in larvae will be re-examined in this chapter using a WISH, and also examined using qPCR and CISH to produce a detailed spatial and temporal understanding of the expression pattern of *per3* in larvae.

6.2 Aims

The next series of experiments aim to answer:

- Is *per3* expressed throughout the larvae brain, like *per1b*, or in specific regions akin to either larval *per1a* or adult *per3*?
- Do the classical photosensitive areas, such as the eyes and pineal, have a role in entraining circadian rhythms during zebrafish development?
- What are the spatial and temporal characteristics of *per3* expression in *per3-luc* larvae *in vivo*?
- Do larvae show an increase of *c-fos* when exposed to different light-dark conditions, as seen in the adult zebrafish? If so, is this expression effected by a lack of eyes, i.e. does visual light detection produce a different neuronal response than non-visual light detection?

6.3 Methods

For RNA extraction 10-15 larvae were pooled for each sample. Identification of the regions in the zebrafish larvae brain was performed using the Atlas of Early Zebrafish Brain Development (Mueller & Wullimann 2005) and FishNet (Bryson-Richardson et al. 2007). CISH sections sections were imaged on a Leica SCN400 slidescanner.

6.4 Results

6.4.1 *Per3* expression in wild type larvae

The expression of *per3* was monitored in 4 dpf wild type larvae raised on a 12:12 LD cycle. WISH analysis confirmed *per3* is strongly expressed in the entire brain and eyes of the larvae at ZT3 (Figure 65A-D). At ZT15, the trough of *per3* expression in adult tissues, *per3* can no longer be observed in the head, but it is detectable in the gut. Further analysis using qPCR on samples separating the head of the larvae and the rest of the body confirmed that *per3* is expressed throughout the body, but as was also shown by WISH, the expression was greatest in the head at ZT3. Also, *per3* was detectable at ZT15 in the head but at levels approximately 150 fold lower than ZT3 (Figure 65E, p=0.0019, One way ANOVA with Dunnett's multiple comparison post-test, n=3).

On a 12:12LD cycle *per3* expression levels were rhythmic in samples of entire wild type larvae, with a peak at ZT3 and trough at ZT15 (Figure 65F, p<0.0001, One way ANOVA, n=3-4). When raised in LL the rhythm of *per3* was abolished and there was no significant difference between the different timepoints (One way ANOVA, n=3-4), moreover the expression of *per3* was kept at mid-level between the levels observed at the peak and trough on a LD cycle (Figure 65F).

For a more detailed analysis of the expression of *per3* in the larval brain, 4 dpf wild type larvae raised on a 12:12LD were dissected at ZT3 fixed, frozen and sectioned and CISH performed. As shown in the WISH experiments, *per3* was highly expressed in the eye and throughout the zebrafish brain, however this assay also showed that *per3* was expressed in all neural regions with cell bodies. *Per3* was detected in the cytoplasm of the cell soma, and the regions that lacked staining are areas populated with neuropil or plexiform layers, i.e. areas containing unmyelinated axons, dendrites and glia and no cell bodies (Figure 66).

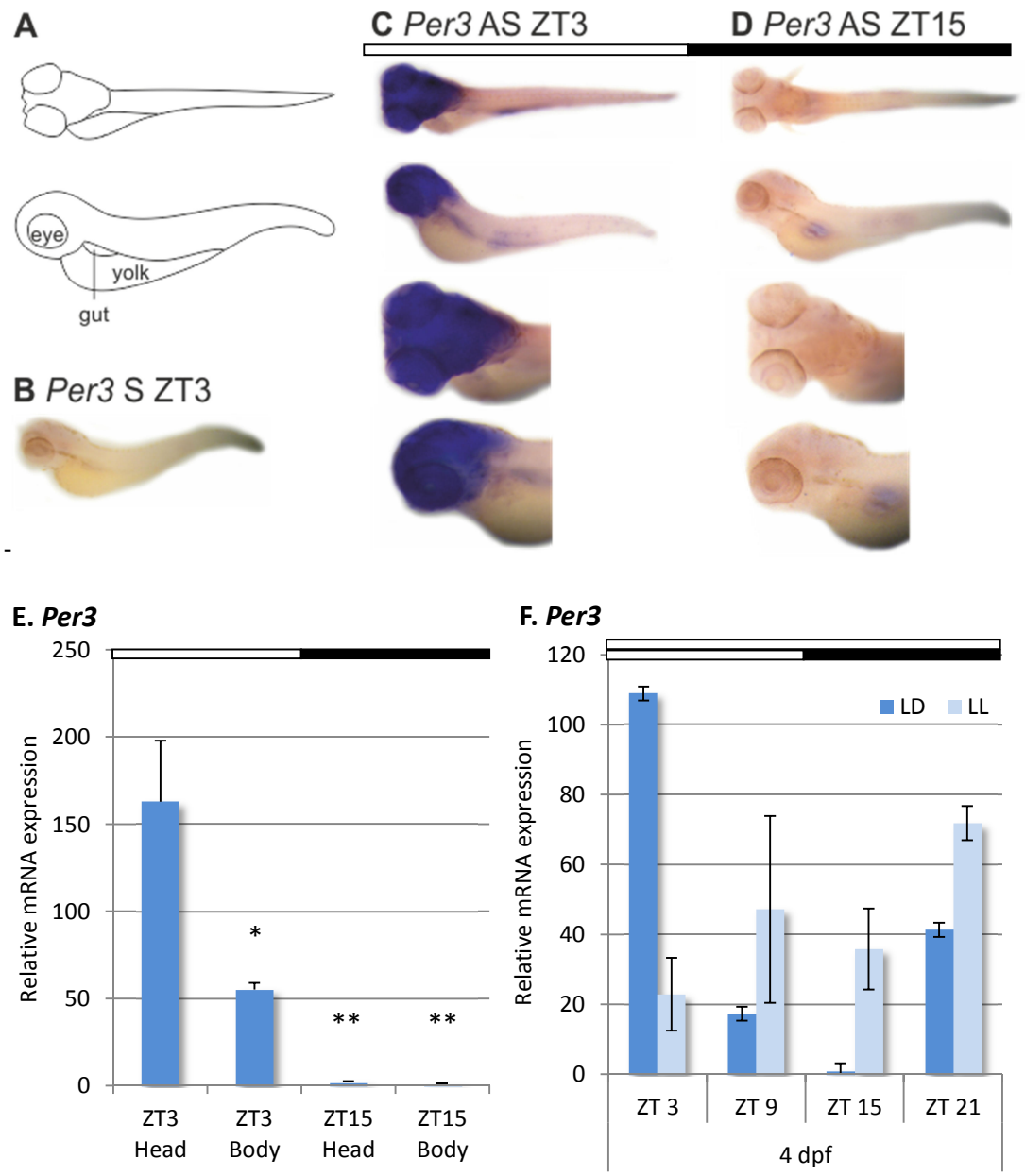


Figure 65 *Per3* is highly expressed in the zebrafish larvae head

Zebrafish larvae were raised in 12:12 LD conditions at a constant 28°C, and collected on 4 dpf. **A)** Anatomical schematic of the zebrafish larvae. **B)** Zebrafish larvae were fixed and WISH was performed to detect *per3* mRNA. The sense DIG probe shows the background signal. **C)** *Per3* is highly expressed in the head of larvae zebrafish at ZT3. **D)** *Per3* expression is undetectable in the head at ZT15, however it is found in the gut. **E)** qPCR confirms *per3* is still detectable in the rest of the body at ZT3, but at lower levels than in the head, and that the expression of *per3* is higher at ZT3 than ZT15 in both the head and body ($p=0.0019$, One way ANOVA with Dunnett's multiple comparison post-test, n=3. The stars represent the statistically significance of the post test using the ZT3 head for comparison). **F)** Zebrafish show a strong 24-hour rhythm in *per3* expression during LD (One way ANOVA, $p< 0.0001$, n=3-4), and this rhythm is abolished when raised in LL (One way ANOVA, ns, n=3-4). The levels of *per3* mRNA remain around the LD mid-level during LL.

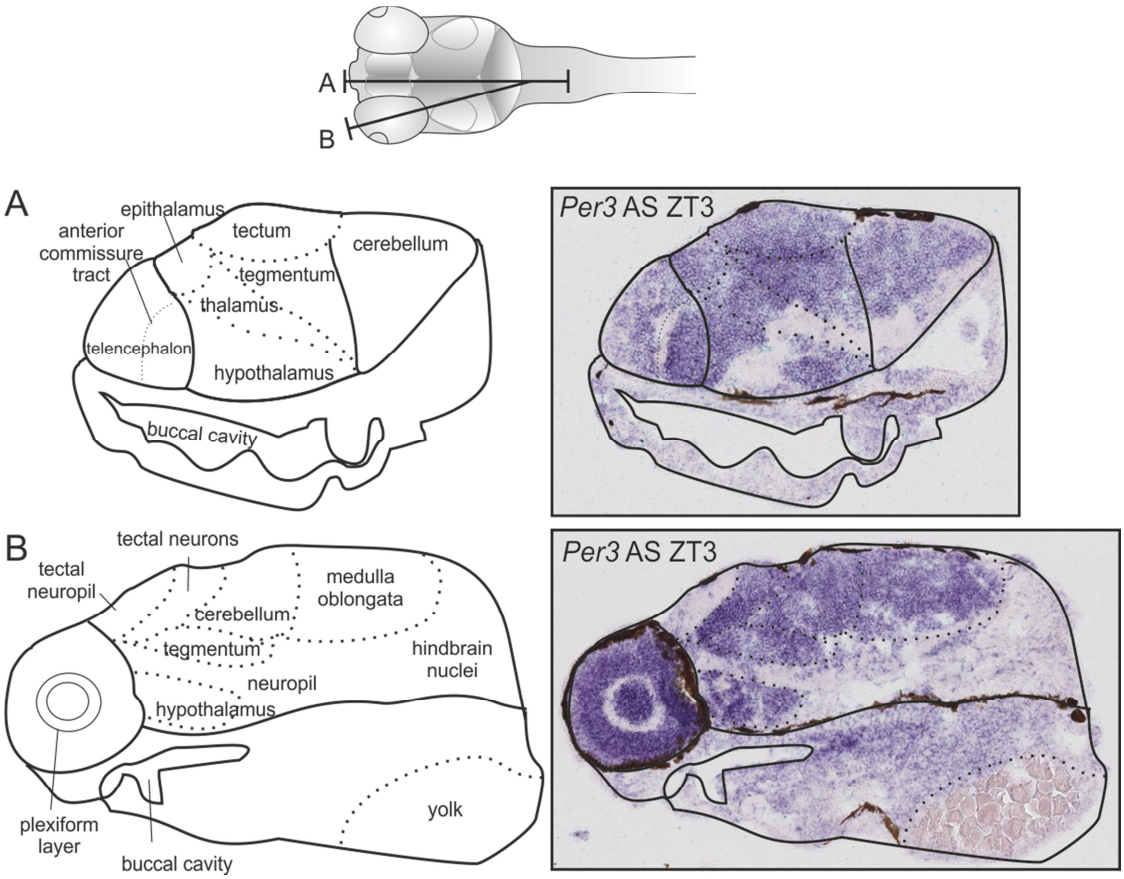


Figure 66 *Per3* is expressed in all regions of the zebrafish larval brain

Wild type zebrafish larvae were raised in 12:12 LD conditions at a constant 28°C, and collected at ZT3 on 4 dpf. Larvae were fixed, frozen and sectioned and CISH performed for *per3* detection. Schematics show the approximate location of different brain regions in the **A)** medial and **B)** lateral sagittal planes. *Per3* mRNA is highly expressed in the eye and throughout the zebrafish brain in all regions. *Per3* is detectable in the cytoplasm of the cell soma, and the regions that appear to lack staining are areas of neuropil.

6.4.2 *Per3* expression in *mbl* larvae

To determine whether the eyes, which are a classical photosensitive area, have a role in entraining circadian rhythms during zebrafish development, the eyeless mutant *mbl* larval was investigated. qPCR analysis revealed that the temporal expression of *per3* in 4-6dpf larvae was the same in wild type and *mbl* larvae, with a reoccurring peak at ZT3 and trough at ZT15 (Figure 67A and B, Two way ANOVA, $p<0.001$, $n=3$).

CISH analysis on 4 dpf *mbl* larvae revealed *per3* expression at ZT3 in all the same regions of the brain as seen in wild type larvae (Figure 67C). Also like wild type larvae, the detection of *per3* mRNA in *mbl* larvae was restricted to the somal cytoplasm.

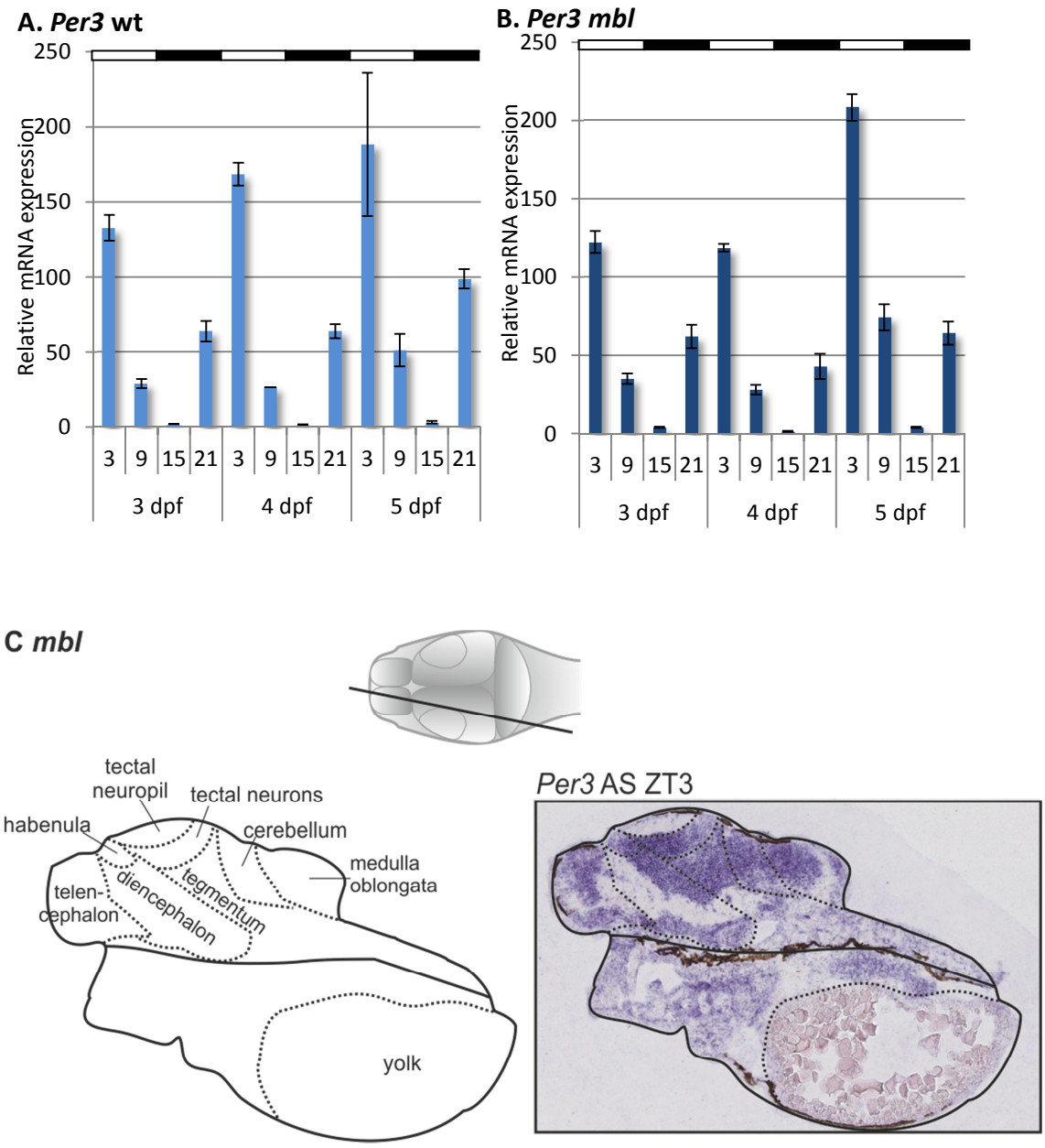


Figure 67 *Per3* expression is the same in wt and *mbl* larvae

Zebrafish wild type (wt) and masterblind (*mbl*) larvae were collected at ZT3, 9, 15, 21, on 3-5dpf. RNA was extracted. Expression of the core clock gene *period3* was examined by qPCR. **A & B** Both wt and *mbl* zebrafish larvae displayed rhythmic *per3* expression with the same peak at ZT3 and trough at ZT15 but there was no significant difference between wt and *mbl* (Two way ANOVA, $p<0.001$, $n=3$). **C** *Mbl* larvae were raised in 12:12 LD conditions at a constant 28°C, and collected at ZT3 on 4 dpf. Larvae were fixed, frozen and sectioned and CISH for *per3* detection. A schematic shows the approximate location of the different brain regions. As in the wt larvae, *per3* was highly expressed throughout all regions of the zebrafish brain. *Per3* is expressed in the cytoplasm of the cell soma, and the regions that lack staining are areas of neuropil.

6.4.3 *Per3* expression in *per3-luc* larvae

Per3-luc zebrafish larvae were raised in 12:12 LD conditions at a constant 28°C, and collected at ZT3, 9, 15 and 21 on 4 dpf for quantification of *per3* and *luc* mRNA. *Per3* and *luc* is expressed in a rhythmic fashion with a peak at ZT3 and trough at ZT15 in *per3-luc* larvae (Figure 68, $p=0.0014$ and $p<0.0001$ respectively, One way ANOVA with Tukey's multiple comparison post-test, n=3). This is the same temporal expression profile of *per3* as observed in adult tissues (Chapter 3). This demonstrates that in larvae the expression of *luc* is accurately reporting the expression of *per3*.

Further analysis to determine the regional expression of *per3* using CISH, reveals that as with wild type larvae *per3* is also expressed throughout the cell bodies in all the regions of the brain and eye (Figure 69).

Spatial expression of bioluminescence from *per3-luc* larvae should provide further detail of the rhythmicity of different brain regions. *Per3-luc* were embedded at 3dpf in low melt agarose with 0.5 mM luciferin in egg water and recorded for spatial bioluminescence for two days. Total bioluminescence shows *per3* is rhythmic in DD with a peak at CT6 (n=6), this is the same rhythm as seen in the qPCR experiments (Figure 70). However, dorsal and sagittal spatial views of larvae show that the bioluminescence signal is restricted to the olfactory pits. This does not correspond with the results from the CISH, which do not show extraordinarily high expression of *per3* in the anterior telencephalic regions. This will be considered later in the discussion section of this chapter.

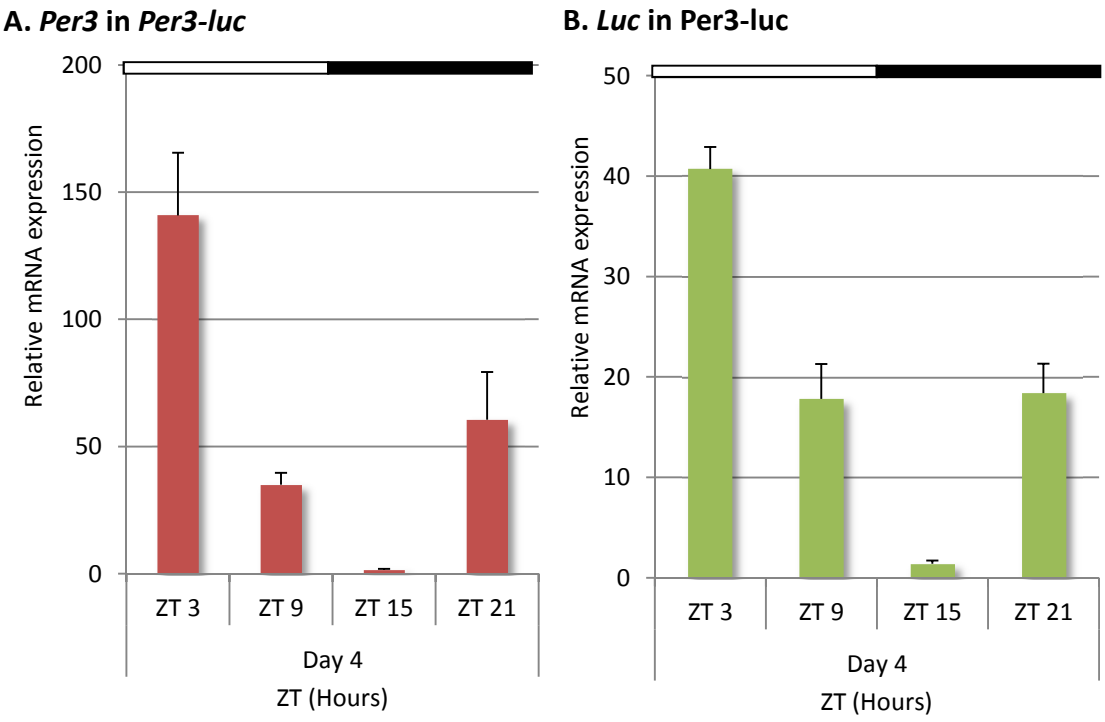


Figure 68 *Luc* expression accurately reports *per3* expression in *per3-luc* larvae

Per3-luc zebrafish larvae were raised in 12:12 LD conditions at a constant 28°C, and collected at ZT3, 9, 15 and 21 on 4 dpf. **A)** *Per3* is expressed in a rhythmic fashion with a peak at ZT3 and trough at ZT15 in *per3-luc* larvae ($p=0.0014$, One way ANOVA with Tukey's multiple comparison post-test, n=3) **B)** *luc* is also rhythmic in *per3-luc* larvae with the same temporal profile as *per3*, a peak at ZT3 and trough at ZT15 ($p<0.0001$, One way ANOVA with Tukey's multiple comparison post-test, n=3).

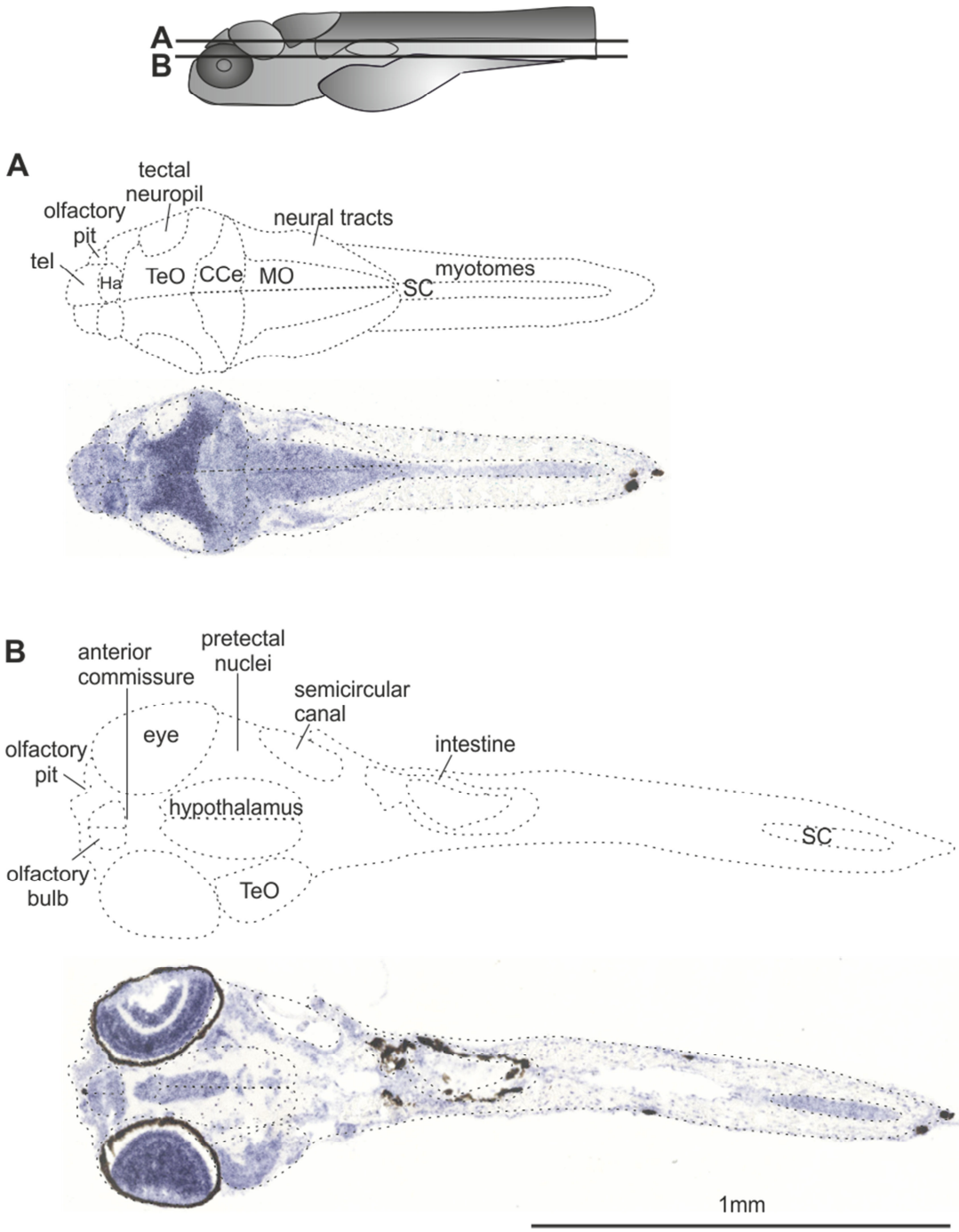


Figure 69 *Per3* is spatially expressed in the same regions in *per3-luc* larvae as wild type

Per3-luc zebrafish larvae were raised in 12:12 LD conditions at a constant 28°C, and collected at ZT3 on 4 dpf. Larvae were fixed, frozen and sectioned and CISH performed for *per3* detection. **A)** Dorsal and **B)** ventral sections of *per3-luc* larvae show that, as seen in wild type larvae, *per3* is highly expressed in the eye and throughout the zebrafish brain in all regions. Schematics show the approximate location of the different brain regions. *Per3* is expressed in the cytoplasm of the cell soma, and the regions that appear to lack staining are areas of neuropil.

[CCe, corpus cerebellum; Ha, habenula; MO, medulla oblongata; SC, spinal cord; tel, telencephalon; TeO, optic tectum]

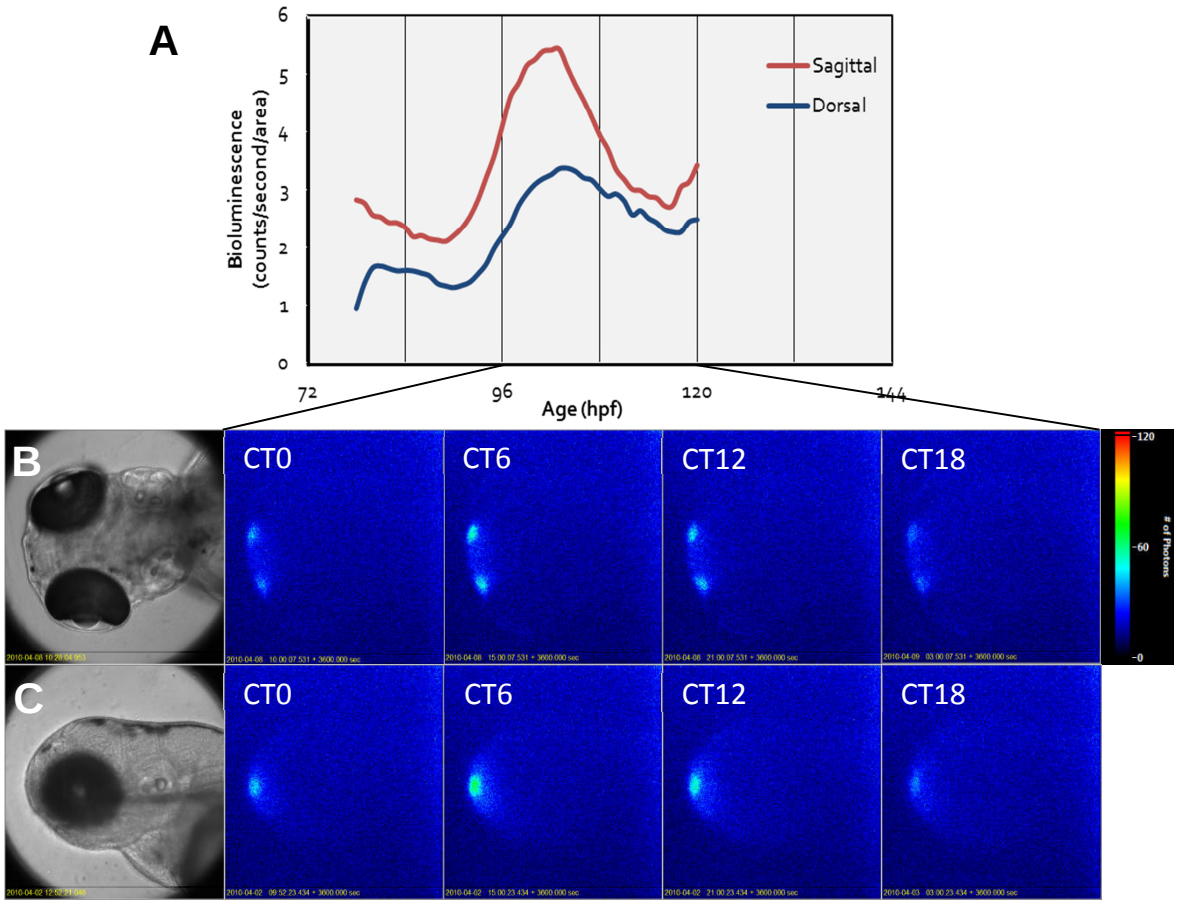


Figure 70 Spatial bioluminescence from *per3-luc* larvae

Per3-luc zebrafish larvae were raised in 12:12 LD conditions at a constant 28°C, at 3 dpf they were embedded in low melt agarose with 0.5 mM luciferin in egg water and recorded for bioluminescence using an IPD. The colour scale shows the number of photons in each one hour bin. **A)** Total bioluminescence shows *per3* is rhythmic in DD with a peak at CT6 (n=6). **B)** Dorsal and **C)** sagittal spatial views of larvae showed that the bioluminescence signal is restricted to the olfactory pits.

6.4.4 Bioluminescent recording from *per3-luc* larvae

Per3-luc mbl, *per3-luc flh*, and *per3-luc mbl flh* were bred alongside *per3-luc* siblings. All larvae were kept on a 12:12LD cycle and temporal bioluminescence was monitored every hour between 72 and 120 hours. All larvae expressed rhythmic *per3* with an overall 24-hour period with no significant difference between period lengths (Figure 71A, One way ANOVA with Tukey multiple comparison post-test, *per3-luc* n=52, *per3-luc mbl* n=42, *Per3-luc flh* n=42, *per3-luc mbl flh* n=15). There was no significant difference between the phases of rhythmicity in the different larvae (Figure 71B, One way ANOVA with Tukey's multiple comparison post-test).

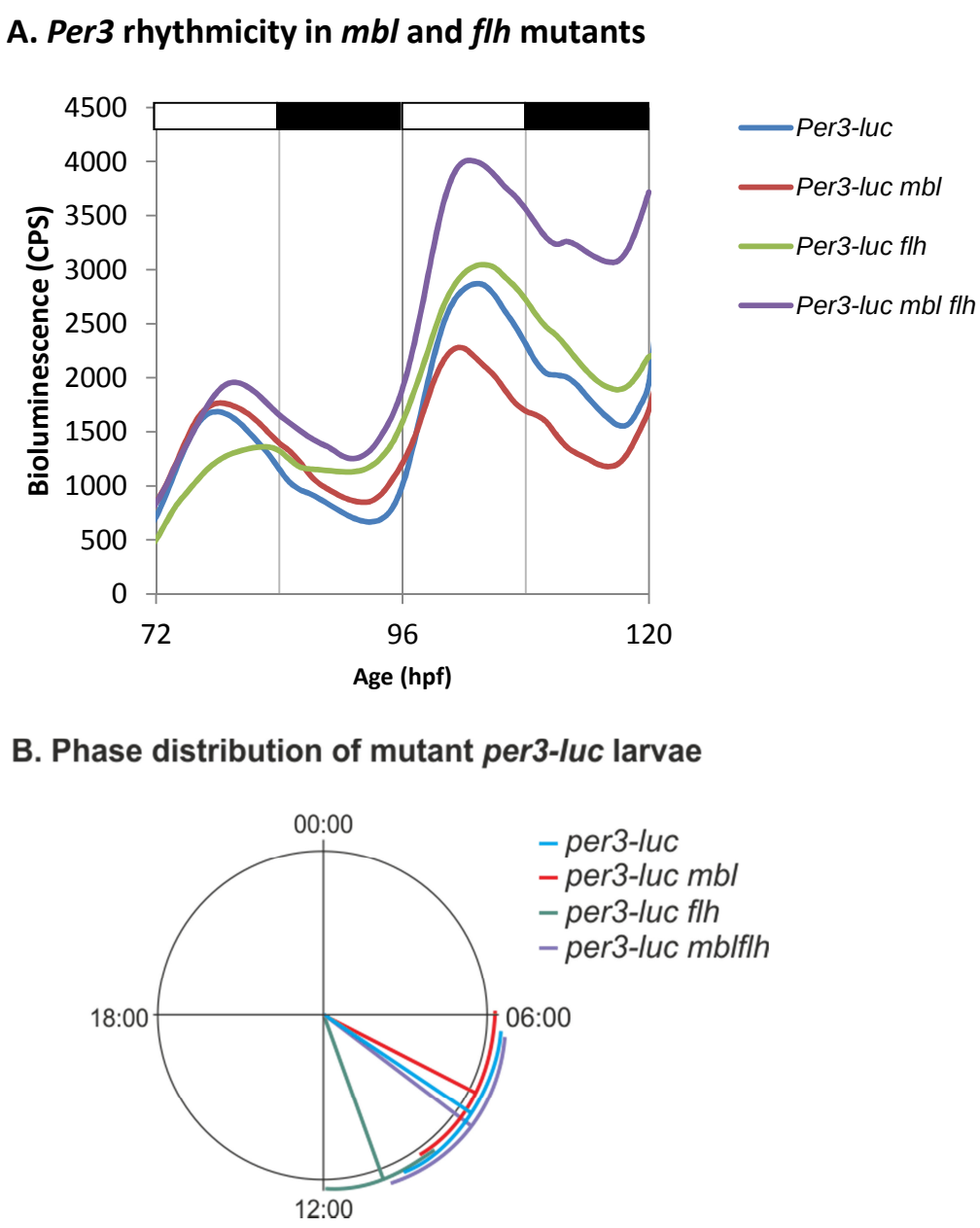


Figure 71 *Per3* expression in *mbl* and *flh* mutants

Per3-luc larvae were kept on a 12:12LD cycle and bioluminescence was monitored every hour between 72 and 120 hours. **A)** All larvae expressed rhythmic *per3* with an overall 24-hour period and no significant difference between them (One way ANOVA with Tukey multiple comparison post-test, *per3-luc* (n=52), *per3-luc mbl* (n=42), *Per3-luc flh* (n=42), *per3-luc mbl flh* (n=15)) **B)** The mean phase with confidence intervals for the different mutants were plotted on a circular phase distribution graph. There was no significant difference between the phase of rhythmicity in the different larvae (ns, One way ANOVA with Tukey's multiple comparison post-test).

6.4.5 *C-fos* expression in larvae

Wild type and *mbl* larvae were investigated to determine whether the expression of *c-fos* is induced by a change in lighting condition as it is in the adult, and whether eyes influence this expression. Larvae on a 12:12LD cycle were collected at the end of 3 dpf into 4 dpf and RNA extracted to determine the level of *c-fos*.

There was no significant response to the change in lighting conditions in *c-fos* expression in wt larvae as there is in in the adult (Figure 72A, One way ANOVA, n=3-6).

There was also no response in the change in lighting condition in *mbl* larvae, although a high expression at ZT21 was observed (Figure 72B, $p=0.01$, One way ANOVA with Tukey's multiple comparisons test, n=3-6). Furthermore, *c-fos* expression was higher in the *mbl* larvae compared to the wt ($p<0.05$, Two way ANOVA, n=3-6).

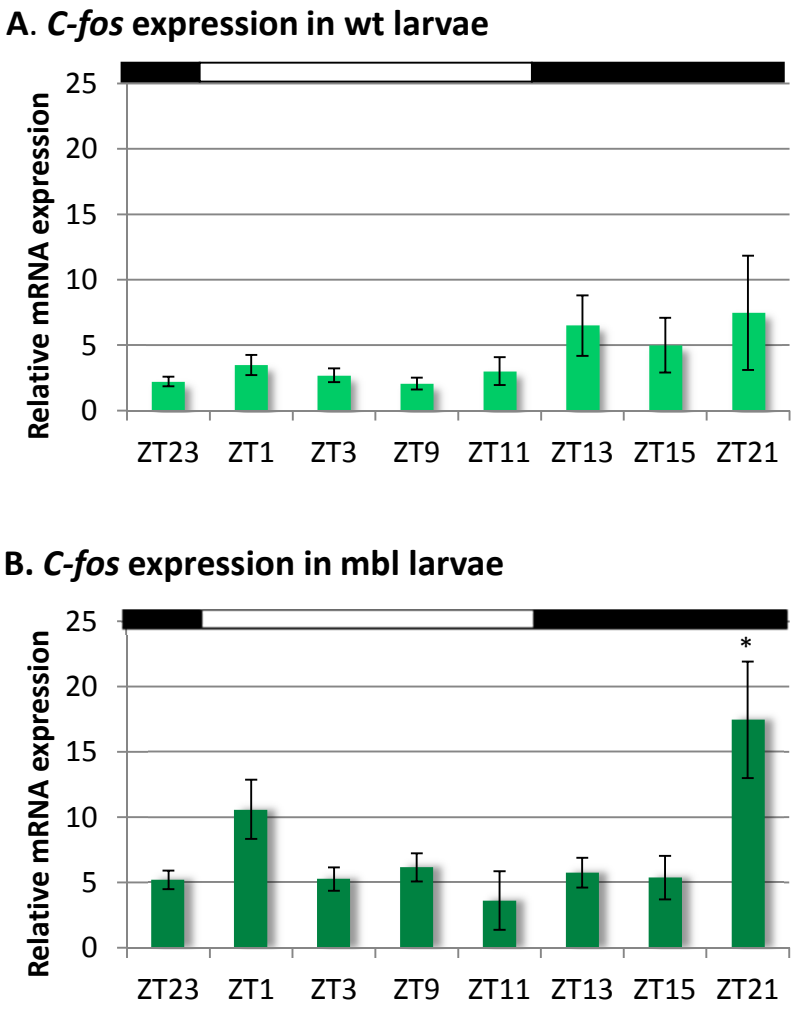


Figure 72 Expression of *c-fos* mRNA in wt and *mbl* larvae

Larvae on a 12:12LD cycle were collected at the end of 3 dpf and into 4 dpf and RNA extracted to determine the levels of *c-fos*. **A)** There was no response to the change in lighting conditions in *c-fos* expression in wt larvae (One way ANOVA with Tukey's multiple comparisons test, n=3-6). **B)** *Mbl* larvae had a raised c-fos expression at ZT21 ($p=0.01$, One way ANOVA with Tukey's multiple comparisons test, n=3-6). *C-fos* expression was higher in the *mbl* larvae during the light phase compared to the wt ($p<0.05$, Two way ANOVA, n=3-6). Error bars represent the SEM. The LD condition is indicated by the white and black bars.

6.5 Discussion

Earlier chapters in this thesis have described the discovery of a novel spatial expression of clock genes in the adult zebrafish brain. This chapter has detailed the expression of *per3* in the larval brain and further evaluated the use of bioluminescent imaging for *per3-luc* larvae. Larvae were also examined for the expression *c-fos*, and for the effects of having no eyes or pineal.

6.5.1 *Per3* expression in larval brain

Shown here is the first report of *per3* expression in the larval brain examined using CISH to reveal the distinct regions of the brain. These results show that unlike the adult brain, *per3* expression is not restricted to certain regions in the developing larval brain and instead is found in all regions of the brain containing cell bodies. However, the CISH results also reveal that *per3* is detectable in the same cellular location in adults and larvae, in the somal cytoplasm rather than neuropil areas. When comparing *per3* expression between larvae and adult it is important to note that some brain structures that highly express *per3* in the adult are not yet distinct at the larval stage observed. Furthermore, changes in spatial expression of clock genes are observed in other species during development. Notably, the spatial expression of *per* and *tim* changes in the Drosophila CNS through embryonic, larval and adult stages and, moreover, there are changes in the number of glia-expressing clock genes (Ruiz et al. 2010). Additionally, in the rat retina the spatial distribution of spontaneous and light-induced *c-fos* and *per1* gene expression changes during early postnatal development (Matejů, Sumová, & Bendová 2010). Further analysis of *per3* expression in later developmental stages would be useful in determining if the differentiation of neuronal regions coincides with any changes in circadian rhythmicity or light sensitivity.

The qPCR results also show that *per3* is rhythmically expressed throughout the larvae body and head. The rhythm of this expression appears the same as observed in adult tissues, with a peak at ZT3 and trough at ZT15. This rhythm is abolished in constant light conditions, as is seen in other clock genes such as *cry1a* and *per1*, which becomes arrhythmic in constant light in zebrafish cell lines (Tamai, Young, & Whitmore 2007; Vallone & Gondi 2004). Many output rhythms in the zebrafish are also abolished in LL, including sleeping (Yokogawa et al. 2007) and

changes in rod and cone light sensitivity (Li & Dowling 1998). Therefore, the arrhythmicity of *per3* in LL shows that the molecular clock has been repressed, and that, importantly, expression levels of *per3* are accurately reflecting the properties of the core molecular clock in the larval zebrafish.

Altogether these results indicate that the spatial and temporal expression of *per3* is more like *per1b* than *per1a* in the 4 dpf larval head (Wang 2008). In other tissues the strong temporal expression of *per3* may not always have a high amplitude rhythm. *Per3* was unmistakably detectable using CISH in the gut at both ZT3 and ZT15, which suggests that further investigation of the isolated larval gut and other peripheral organs may reveal a lower amplitude *per3* rhythm as seen in the head. Therefore, the expression of *per3* in the central tissues may be quite different to peripheral tissues in larvae and requires further investigation.

The CISH larvae *per3* results confirm findings from WISH reported here and by other groups that *per3* is constitutively expressed in the whole brain and retina (Delaunay et al. 2000). Why is there such a strong expression of *per3* in the developing larvae head? One possibility is *per3* may be playing a role in the timing of neural development. There are examples in other species of circadian genes playing roles in development. In *Xenopus*, *clock* activates the expression of *otx-2*, which is vital for the specification and development of the head (Morgan 2002). Furthermore, in *Xenopus*, *clock* also activates *pax-6*, a key gene for eye development (Morgan 2004). In the *Caenorhabditis elegans* worm, the homolog of *period*, *lin-42,* is a developmental timing gene that is affected by the homologs of the *Drosophila* circadian gene *timeless* and *doubletime*, *tim-1* and *kin-20* (Banerjee et al. 2005). Therefore, circadian genes may also play vital roles in the spatial and temporal regulation of neural patterning during development. This raises the possibility that circadian rhythms and early development may be intimately linked. Further studies would be needed to distinguish the roles of zebrafish clock genes in development, and the strong expression of *per3* in the larvae head makes it an interesting candidate for this further research.

6.5.2 Larval *per3-luc* zebrafish

The *per3-luc* zebrafish was created by Greg Cahill for the high resolution monitoring of molecular clock rhythms in adult and larvae (Kaneko & Cahill 2005). However, the first

investigations of *per3-luc* larvae could not detect rhythms in *per3-luc* larvae until 4 dpf, despite molecular analysis showing expression starting at 40 hpf. The authors hypothesised that this could be a fault with the transgene, however further investigations carried out in this chapter reveal that it is more likely that the shortcoming is with the uptake of the substrate for the bioluminescence, luciferin, into the embryo.

The results presented here suggest that there is no fault with *per3* expression or the report of this expression using *luc*. Molecular analyses reveal that the expression of *per3* and *luc* were both rhythmic in *per3-luc* larvae. Furthermore, CISH analysis reveals *per3* is expressed throughout the larval brain in somal cytoplasm in *per3-luc* zebrafish as in wild type. However, bioluminescent signal was not detected throughout the brain and eyes using spatial imaging, and instead the signal was localised to the olfactory pits. In some *per3-luc* larvae signal was detected throughout the entire head and body (data not presented), but these larvae were unhealthy and died soon after, probably due to damaged skin that allowed the penetration of harmful bacteria or toxins. Consequently, it is likely that luciferin was not penetrating the larval vitelline membrane, and only being taken up in the olfactory pits, which are ordinarily used by larvae to sense the chemical nature of the surrounding fluid. Therefore, the bioluminescent signal from *per3-luc* larvae may not be representative of *per3* expression throughout the larvae when using this method.

To overcome the lack of penetration of luciferin throughout the zebrafish larvae, trials could be done injecting luciferin into the heart. However, this may not provide a sufficient or continuous supply of luciferin and another delivery system may need to be devised.

In conclusion, it is likely that the bioluminescent signals from the adult *per3-luc* tissues are accurately reporting the expression of *per3*, but recordings from *per3-luc* larvae must be interpreted with caution at this time.

6.5.3 Circadian rhythmicity in larvae lacking eyes and pineal

Results from *per3luc* larvae lacking either eyes or pineal or both, showed that a normal *per3* rhythm can be seen in these mutant larvae. Although care must be applied in the interpretation of these experiments as it is likely that the luciferin has not penetrated the whole larvae and

therefore the bioluminescent signal is not representative of the entire larvae. However, it can be reasoned that the eyes and pineal are not essential for all rhythmicity within the developing zebrafish. This is in accordance with previous studies that have shown that larvae lacking eyes and pineal can still respond to an abrupt loss of light illumination (Fernandes et al. 2012). Additionally, peripheral tissues are directly light sensitive and can remain rhythmic in culture (Whitmore, Foulkes, & Sassone-Corsi 2000). Therefore, although a role for the eyes and pineal in circadian regulation cannot be ruled out, these structures are not essential for the functioning of circadian clocks in larvae zebrafish.

CISH and qPCR using the eyeless mutant, *mbl*, revealed that the spatial and temporal expression of *per3* is unchanged in larvae that do not have eyes compared to wild type larvae with eyes. These results concur with previously reports that circadian rhythm outputs are unaffected by a lack of eyes, for example the visual motor response in dark photokinesis is unaltered in larvae with surgically ablated eyes (Fernandes et al. 2012).

A previous investigation using other retinal defected zebrafish mutants has attempted to decipher the role of ocular photoreception in regulating circadian rhythms. The functionally blind zebrafish mutants, *lakritz*, which lack the retinal ganglion cell layer, have unaffected cell cycle rhythms in peripheral tissues (Dickmeis et al. 2007). However, the mutant *chokh/rx3*, which has an impaired eye development, did show attenuated circadian S-phase rhythm, despite core circadian gene expression of *clock* and *per1b* being unaltered (Dickmeis et al. 2007). The authors suggest this result may be due to another aspect of the mutation in the *chokh/rx3,* that of the glucocorticoid signalling pathway, rather than the effect of ocular light impairment. This is something to be wary of using all genetic mutants, and therefore highlights the importance of studying multiple genetic mutants before drawing firm conclusions.

The investigation into the role of the eyes and pineal in this present chapter, alongside previous findings, suggest that the eyes are unlikely to be playing a prominent role in light detection for functioning of the molecular circadian clock in larvae. Further investigation into the pineal will be necessary to understand its role in regulating circadian rhythms in larvae, although it appears that rhythmicity in some regions can persist without a pineal.

6.5.4 Larval *c-fos* expression

The increases in *c-fos* expression observed in adult brains when lighting conditions change were not observed in larvae. There may be a few reasons for this difference in light sensitivity between larvae and adult *c-fos* expression. Firstly, brain regions that respond to changes in light conditions may have not fully differentiated and developed in the larvae. Another possibility is that functional neural links between the eyes and specific regions of the brain may not yet have been established at this early developmental stage. Future experiments should examine slightly older larvae, around 7-8 dpf, when individuals are capable of active feeding.

Secondly, a change in *c-fos* may only be observed sooner after the change in larval zebrafish, and by an hour later when the sampling took place, the expression had returned to baseline. Support for this comes from experiments monitoring locomotor activity in larvae. When placed in the dark larvae have been reported to increase locomotor activity, this phenomena is termed visual motor response. This has recently been shown to be triggered by a group of neurons in the preoptic area of the hypothalamus (Fernandes et al. 2012). This elevated rate of locomotor activity is very short, returning to baseline within 10 minutes, and *c-fos* expression reporting this activity may not be detectable an hour after lights off. Future experiments will need to address this possibility.

Like *per3*, the temporal expression of *c-fos* was also unaltered in *mbl* larvae, however expression of *c-fos* was higher at each time point investigated. One explanation for the overall raised level of *c-fos* in *mbl* larvae could be due to the eyes suppressing *c-fos* expression and this masks higher levels of *c-fos* in other regions of the larvae. Or perhaps brain activity increases to compensate for the loss of light sensitivity from the eyes. However, *c-fos* can be induced by other stimuli including hypoxia (Walker, Lee, & Nitsos 2000) and apoptosis (Preston et al. 1996). Therefore, many other factors may also affect the *c-fos* expression in this mutant, for example increased cell death in this lethal mutation, and therefore more experiments, perhaps using other mutants, would be needed to examine the effect of ocular photoreception on *c-fos* expression.

Overall, this chapter has expanded the understanding of the circadian rhythmicity and light sensitivity of the larvae zebrafish brain. The next and final data chapter of this project will further explore the zebrafish larvae brain, alongside the adult brain, to evaluate the expression of putative photopigments which might account for this light sensitivity.

6.6 Conclusions

- *Per3* is prominently expressed in the head and eyes of larval zebrafish at the peak of its expression.
- *Per3* is expressed in all larval brain regions, unlike adult *per3* or larval *per1a*.
- The spatial and temporal expression of *per3* is unaltered in mutant larvae lacking eyes.
- Temporal *per3* rhythms are detectable in mutant larvae lacking both eyes and pineal.
- *Per3-luc* embryos and larvae are unsuitable for bioluminescent monitoring due to poor luciferin penetration. However, *per3* expression is normal in these larvae.
- The increase in *c-fos* expression observed in adult brains when lighting conditions change is not observed in larvae.

Chapter 7

Opsin expression in the adult zebrafish brain and larvae

7.1 Introduction

Chapter 3 has shown that cultures of zebrafish brain and organ tissues are directly light sensitive, and chapter 6 demonstrated that zebrafish larvae lacking eyes did not have altered clock gene expression. These results indicate that the zebrafish brain contains structures for light detection, which have yet to be identified. Chapters 4 and 5 describe a difference in light sensitivity in different regions of the zebrafish brain; one potential mechanism for this could the different expression of photosensitive molecules expressed in particular regions. Opsins are a particular group of light responsive proteins known to play a role in phototransduction. This chapter details an investigation into opsins in the adult and larval zebrafish brain.

7.1.1 Extra-ocular photoreceptors

In mammals non-visual photoreceptors are exclusively active in the retina. However, in other vertebrates non-visual photoreceptors have been found in other brain regions, such as the pineal and deep brain areas (Foster & Soni 1998). As mentioned in the introduction chapter, these extra-ocular photosensitive regions are thought to be critical for the photoentrainment of circadian biology.

Furthermore, extra-ocular photoreceptors are also important for photoperiodism, i.e. the detection of seasonal changes in photoperiod. In a teleost ayu sweetfish, *Plecoglossus altivelis*, gonadal development is stimulated in short days and this persists in eyeless and pinealectomised sweetfish (Masuda, Iigo, & Aida 2005).

In addition, extra-ocular photoreceptors are responsible for changes in skin darkness when light conditions are changed. For example, in zebrafish larvae, pigment dispersion, which describes when pigments spread into the cytoplasm to cause a darkening of the skin, persists in isolated tails when larvae are exposed to the dark (Shiraki, Kojima, & Fukada 2010).

The location and characterisation of many extra-ocular photoreceptors responsible for these different roles remains unknown. This chapter will focus on characterising and localising a few of the photoreceptors in the zebrafish opsin superfamily.

7.1.2 Teleost opsin expression

Opsins are a superfamily of light-sensitive membrane-bound heptahelical receptors that are important in both visual and non-visual photodetection in mammals and other vertebrate and invertebrate species (Foster & Bellingham 2004). They are distinguished by an ability to bind a vitamin A chromophore, retinal, which attaches to an amino acid residue in the seventh α-helix (Foster & Bellingham 2002). In the zebrafish genome sequences for 43 different gene opsins have been identified (Wayne Davis, personal communication). It is not yet known how many of these are expressed in different teleost tissues, and which ones may have a role in phototransduction (Peirson, Halford, & Foster 2009). This section will briefly describe these different opsins and what is currently known about them in zebrafish.

There have been several visual opsins identified in zebrafish, which are grouped as cone-opsins (*opn1*) or rod-opsins (*opn2*). The rod opsin is named rhodopsin (*rh1.1*), and a second rhodopsin-like opsin has been identified, *rh1.2* (Morrow, Lazic, & Chang 2011). The cone opsins are also subdivided by their amino acid sequence and spectral characteristics into two long-wave-sensitive (*lws1* and *lws2*), four middle wavelength sensitive or rhodopsin-like (*rh2.1*, *rh2.2*, *rh2.3*, *rh2.4*), and two short-wave-sensitive (*sws1*, which is sensitive to UV and *sws2*, which is sensitive to blue light) (Chinen et al. 2003). The peak absorption spectra of these visual opsins vary from the UV part of the spectrum (355nm) to the red end (558nm) (Chinen et al. 2003).

There have been many non-visual opsins identified in zebrafish. *Opn3* was originally termed encephalopsin as it shows strong expression in the mouse brain (Blackshaw & Snyder 1999), however it was later renamed panopsin as it was also expressed in the retina (Halford et al. 2001). *Opn3* is found in both the brain and eyes of zebrafish larvae (Shiraki et al. 2010).

Melanopsin (*opn4*) is probably the most studied non-visual opsin in mammals, as it is found in the retinal ganglion cells that directly project to the SCN (Gooley et al. 2001; Hannibal et al. 2002). There are five melanopsin isoforms in the zebrafish all expressed in the retina; they are classified on the basis of their greater similarity with either the mammalian (*Opn4m*) or non-mammalian (*Opn4x*) melanopsins (Davies et al. 2011). Recent data shows that *opn4m1*

expressing cells in the hypothalamus are responsible for detecting changes of light that allow for the visual motor response in larval zebrafish (Fernandes et al. 2012).

The vertebrate ancient (*va*) opsin was first localised in salmon (Soni & Foster 1997). It was so named because, at its discovery, the phylogenic tree suggested that *va* opsin was diverged from a common ancestor at the beginning of vertebrate evolution. However, the further discovery of non-visual opsins put many as even older (Davies, Hankins, & Foster 2010). There are three *va* homologs in zebrafish, one with a short isoform (*vas*), and two with long isoforms *va1* (*valopa*) and *va2* (*valopb*) (Kojima, Mano, & Fukada 2000). Both are dominantly expressed in the eye and brain and have been found differentially expressed in developing larvae (Kojima & Torii 2008).

Several opsins have been discovered through investigating the photoreceptive properties of the pineal in different species. Parapinopsin was first identified in the pineal of the cat fish (Blackshaw & Snyder 1997). There are two homologs found in the zebrafish genome, and the second has been found in zebrafish larval pineal (Shiraki et al. 2010). Another pineal opsin is extraretinal rhodopsin-like, or exorhodopsin (*exorh*), which was first located in the zebrafish pineal (Mano, Kojima, & Fukada 1999). *Exorh* is a duplication of *rh1.1*, which is found in the retina, and the two opsins share a 74% amino acid (Mano et al. 1999).

Studies on another photoreceptive structure, the parietal eye of a lizard, have identified a green-sensitive opsin, named parietopsin (Su et al. 2006). This opsin is expressed in the larval zebrafish pineal (Shiraki et al. 2010).

Whilst most opsins are G protein-coupled receptors, not all opsins activate G-proteins. Ironically retinal G protein-coupled receptor (*rgr*) opsin, which was named before its function was fully elucidated, is one opsin that does not activate G proteins (Wenzel et al. 2005). Rather, *rgr* uses photic energy to convert its bound retinal chromophore from its all-*trans*-retinal state into the 11-*cis*-retinal configuration, which is then released and may be used to regenerate other photoreceptors' light sensitivity (Hao & Fong 1999). There have been two *rgr* opsins found in the zebrafish genome, *rgr1* (Davies et al. 2011) and *rgr2* (Leung, Ma, & Dowling 2007), however the tissue expression patterns have not yet been resolved (Shiraki et al. 2010).

Several opsins genes have been identified in the genome (Davies et al. 2011) but have not yet been detected in zebrafish tissues (Shiraki et al. 2010). Including, neuropsin (*opn5*), *opn9* and peropsin (*rrh*), also known as retinal pigment epithelium-derived rhodopsin homolog.

A recently discovered opsin group, named *novo,* have been identified in the zebrafish genome but have not yet been analysed for their expression patterns (Wayne Davis, personal communication).

Teleost Multiple Tissue (*tmt*) opsin has been suggested as one candidate for the photic regulation of peripheral clocks in zebrafish (Moutsaki et al. 2003). As its name implies, it has been found in many tissues in the zebrafish: the telencephalon, optic tectum, hindbrain, eyes, heart and kidney, as well as very early stage blastula embryos and embryonic cell lines (Moutsaki et al. 2003). This expression pattern fits with that expected of a circadian photopigment, however further studies are required before claiming that *tmt* is the key photopigment in the zebrafish circadian timing system. Recent work in the Somalian cavefish has suggested that TMT, as well as melanopsin, can function as photopigments capable of entraining the teleost circadian clock (Cavallari et al. 2011).

7.1.3 Pineal: the classic photosensitive teleost brain region

The pineal has long been known to be important in the photo detection in the adult teleost brain. J.Z. Young published the first detailed report on the function of photoreceptors in the pineal complex in fishes using the lamprey as a teleost model (Young 1935). Young showed a pronounced daily rhythm of skin colour change, which free ran in DD, and was suppressed in LL. Pinealectomy interrupted this rhythm and caused constant maximum darkening. This was among the first experiments to show the importance of the pineal in the maintenance of daily rhythms in vertebrates.

Exorh has been shown to be a predominant photoreceptive molecule in the zebrafish pineal (Mano et al. 1999). However, it has not yet been determined whether *exorh* is expressed in other regions of the zebrafish brain, and this will be addressed in this chapter. Furthermore preliminary investigations will also explore whether the adult pineal expresses other opsins that are highly expressed in the adult brain.

Exorh has been shown to be rhythmically expressed in the pineal of zebrafish embryos, with a peak at ZT18 (Pierce et al. 2008). NanoString (a new method used for measuring gene expression levels) results from day 1 and 2 old embryos also confirm this raised expression during the night, with a ZT15 peak (David Whitmore, personal communication). In this chapter it will be determined whether other opsins are rhythmic in the developing larvae, and if so, whether these opsins remain rhythmic in the adult zebrafish brain. It is also of interested whether opsin rhythmicity in the larvae brain can persist when there are no retinal inputs. To address this, the eyeless zebrafish mutant, *mbl,* will be used.

7.1.4 Declaration

This work was carried out as part of a collaboration with Mark Hankin's group in Oxford. The NanoString experiment was performed by David Whitmore, but all other results shown here were gained solely by the author.

7.2 Aims

- Which opsins are highly expressed in the adult zebrafish brain and where are they located?
- Are these highly expressed opsins rhythmic in the adult zebrafish brain?
- Are these highly expressed opsins also present in the larvae brain? Are they rhythmic in zebrafish larvae?
- Can the expression profile of these opsins alter in zebrafish larvae with no eyes?
- Can constant light alter the expression of these opsins in zebrafish larvae?

7.3 Methods

7.3.1 NanoString

The NanoString nCounter gene expression system is used to quantify a large number of mRNA transcripts expressed in each sample, rather than having to do numerous, individual qPCR experiments (Geiss et al. 2008). The NanoString experiments were performed by David Whitmore and collaborators. Briefly, samples were collected and mRNA extracted. The brain extraction did not distinguish the inclusion of the pineal or optic nerves, which may contain part of the retina. 100ng of total RNA was used in the NanoString. In the hybridisation step two probes (each approximately 50bp) are hybridised for each mRNA; one probe is a reporter with a "barcode" and carries the signal and the second "capture" probe is used to immobilise the complex for data collection. All the probes for all the mRNA sequences are hybridised at the same time. Following hybridisation, the excess probes are washed off and the remaining complexes are bound and immobilised to an nCounter cartridge by the capture probe. The reporter probes contain a coloured sequence and can be detected in a digital analyser, which allows for each target molecule to be counted.

7.3.2 Sample collection

Brains were dissected carefully for the CISH technique to ensure the pineal and optic nerves remained attached. However, for the qPCR experiments the brain dissection process excluded the pineal or optic nerves.

7.3.3 qPCR

To determine the relative mRNA expression a dCt/dCt calibrator is used from the sample set. In the adult brain experiments the same calibrator is used for all genes, to allow for comparison of the different genes with the samples. In the larvae experiments a calibrator is chosen from one sample for each gene, so comparisons of gene expression can be made between the *mbl* and wt samples or between the LD and LL conditions, but not quantitatively between the different genes. *Per1b* and *per3* are both rhythmic zebrafish clock genes used to show the circadian clock was functional in these tissues. In the adult *per1b* was used as the rhythmic control as this

was being used throughout the adult opsin project. However, in the larvae *per3* was used so comparison can be made between these results and chapter 6 of this project.

7.3.4 Chromogenic *in situ* hybridisation

Experiments were carried out using the protocol outlined in Chapter 2. Plasmids containing sequences for the opsin genes were kindly provided by Wayne Davis.

7.4 Results

7.4.1 Relative and temporal expression of opsins in the adult zebrafish brain

Zebrafish brains were assessed for their expression of opsins using the NanoString technique. Brain samples were dissected from adult wild type zebrafish at ZT3. NanoString results reveal *exorh* and *rgr1* to be the most abundantly expressed of the opsins in the adult zebrafish brain (Figure 73). There was large inter-sample variation in the expression of some opsins; this may be explained by the brain extraction technique used, which did not distinguish whether the pineal or optic nerves were attached or not.

It was next explored whether these highly expressed opsins, *exorh* and *rgr1*, were also rhythmic in the adult zebrafish brain. In addition, the opsins *rh1.1*, *rh1.2*, and *rgr2* were investigated. RNA was extracted from zebrafish brains collected every six hours over two days on a 14:10LD cycle and qPCR was performed. These brain samples did not include the pineal or optic nerves. None of the opsins tested, *exorh*, *rh1.1*, *rh1.2*, *rgr1*, or *rgr2*, displayed a 24-hour rhythm in the adult brain (Figure 74B-F), despite the samples having a functional circadian clock, as shown by the rhythmic expression of the core clock gene *per1b* (Figure 74A). Overall the qPCR results showed moderately high levels of *rgr1* expressed in the brain, and comparably low levels of *exorh* and *rgr2*, and extremely low levels of *rh1.1* and *rh1.2*.

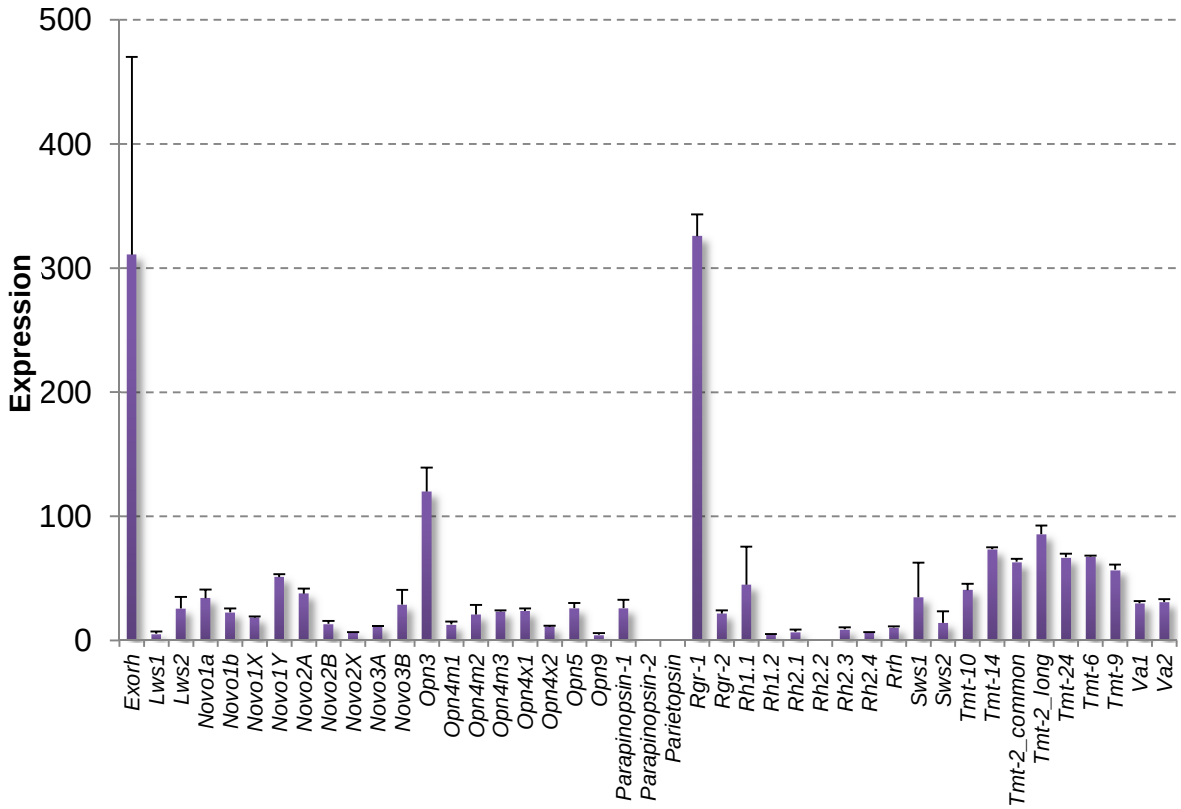


Figure 73 Zebrafish opsin mRNA expression in the adult zebrafish brain

One-year old adult zebrafish were kept on a 14:10 LD. Brains were dissected at ZT3 and RNA extracted. Nanostring was performed with probes for the 43 different opsins already identified in the zebrafish genome. *Exorh* and *rgr1* were the most abundant genes expressed, followed by *opn3*, and the *tmt*s. n=3, error bars show SEM.

NB. No discretion was made when dissecting the brain whether to include pineal or optic nerves, which may have skewed expression of some of the genes such as *exorh* and led to large error bars between samples.

[Lws, long-wave-sensitive; Novo, Novel opsins; Opn, opsin; Opn4, melanopsin, Rgr, retinal G-protein coupled receptor; Rh, rhodopsin; Rrh, retinal pigment epithelium-derived rhodopsin homolog; Sws, short-wave-sensitive; Tmt, teleost multiple tissue; Va, vertebrate ancient]

Data courtesy of David Whitmore

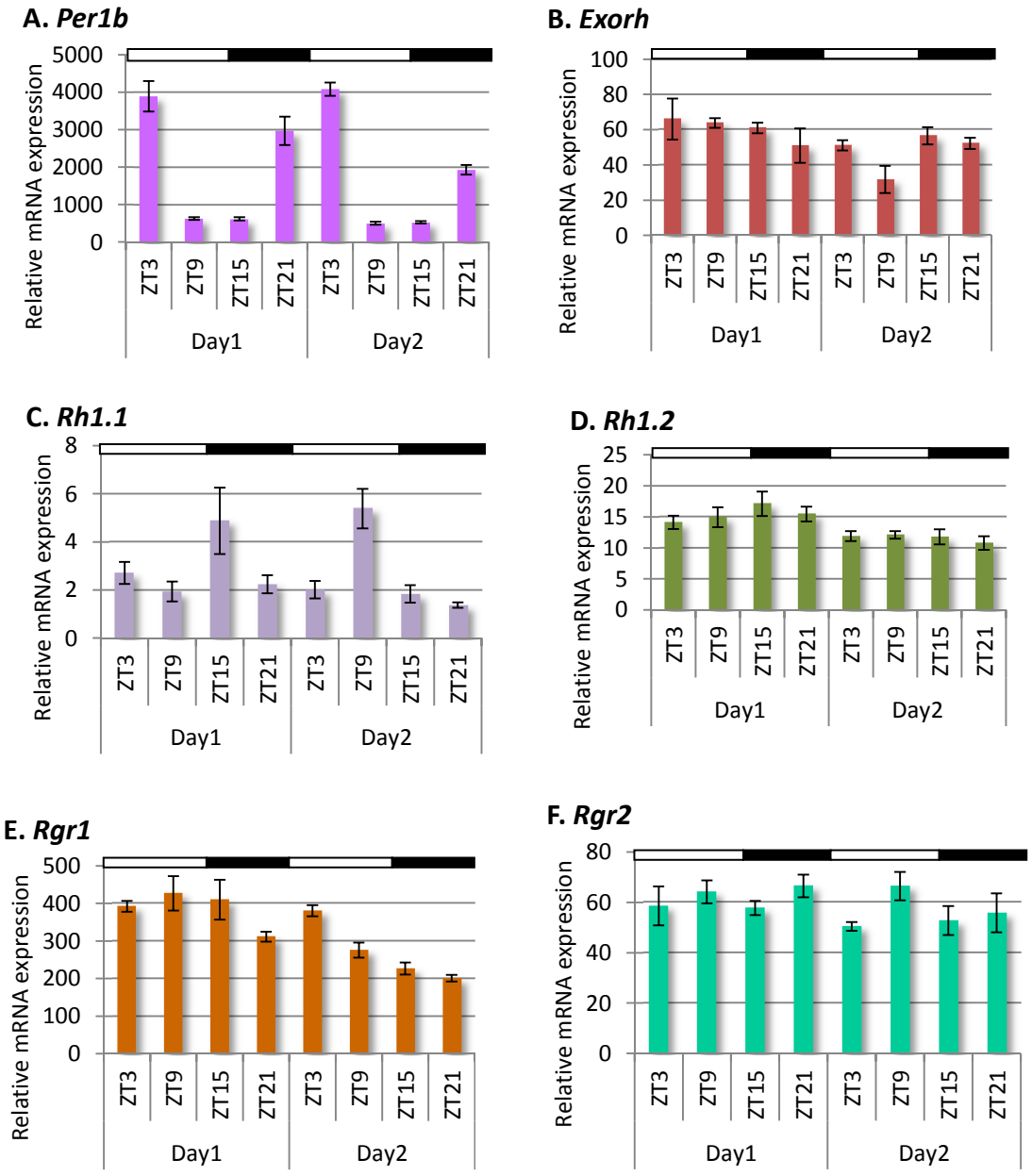


Figure 74 Expression levels of opsins throughout the adult zebrafish brain

A The adult zebrafish brain is rhythmic, as shown by the *period1* expression (One way ANOVA, $p<0.001$). **B-F T**here is no 24-hour rhythm in any of the opsin genes tested (One way ANOVA, ns), and there is low levels of *exorh* and *rgr2*, extremely low levels of *rh1.1* and *rh1.2*.

Each time point had 4 samples, each representing RNA from 1 adult brain, excluding pineal, optic nerve, and pituitary. All samples are relative to the calibrator, a ZT9 sample in day 1 *RH1.1*. Error bars represent the SEM. The 14:10LD regime is indicated by the white and black bars. [Rgr, retinal G protein-coupled receptor; Rh, rhodopsin.]

7.4.2 Spatial expression of opsins in the adult zebrafish brain

A spatial examination of opsin expression in regions of the adult zebrafish brain was performed using CISH on brains dissected at ZT3 (Table 7). *Exorh* mRNA was only found in the pineal (Figure 75B), if it was expressed in other brain regions it was at a low and diffuse level that could not be detected by the CISH technique. The inconsistency of this result with the abundant expression found using the NanoString technique could be due to inclusion of the pineal in the samples provided for the NanoString technique, it is likely that the large amount of *exorh* in these results is due to pineal contamination. *Rgr1* mRNA was found in the pineal (Figure 75D), dorsal telencephalon, thalamus (Figure 77), and many other brain nuclei (Figure 76). *Rh1.1* and *rgr2* mRNA were not detectable in any specific regions, they could be constitutively expressed everywhere and/or at a low level that was not detectable using CISH.

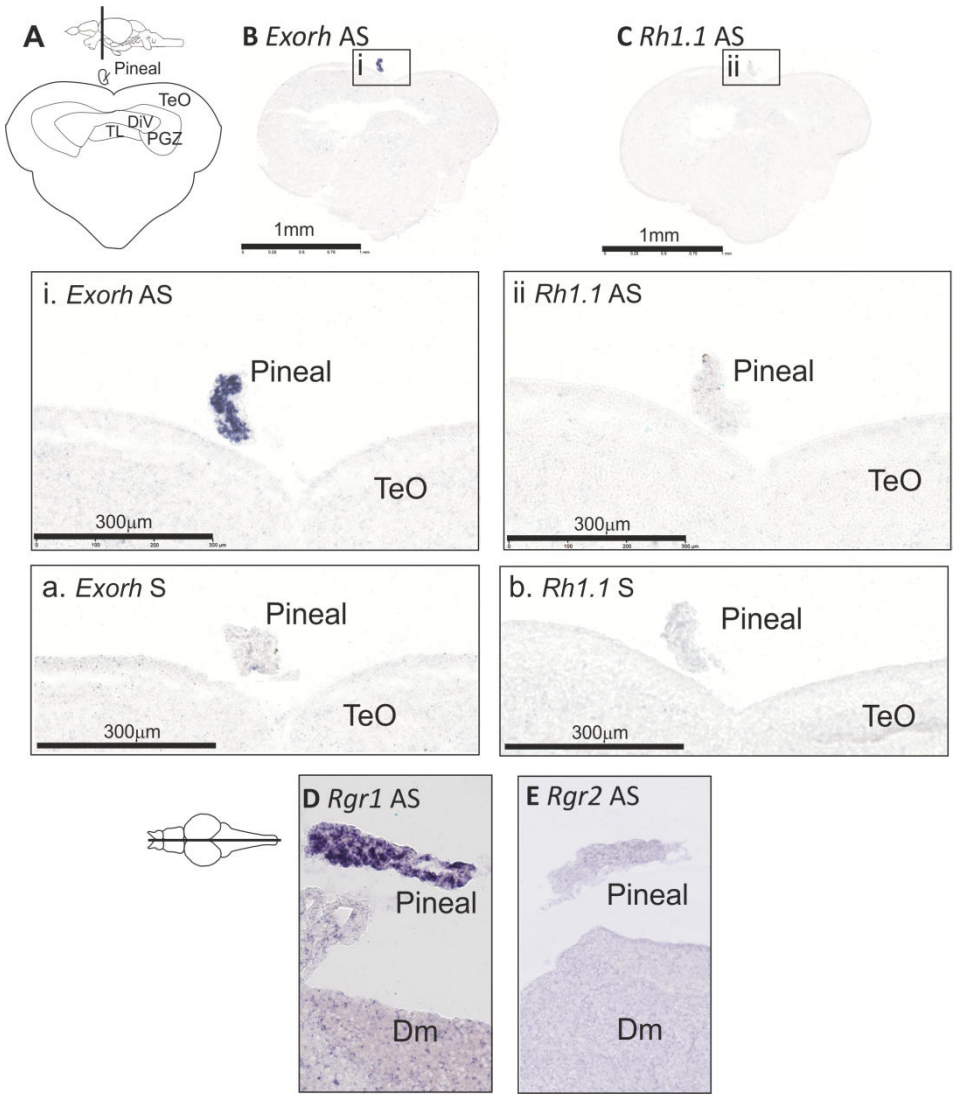


Figure 75 Opsin mRNA expression in the pineal

Brains were dissected at ZT3, fixed, frozen and chromogenic *in situ* hybridisation performed for detection of opsin mRNA. **A)** A schematic for the brain regions in B and C. The samples for D and E were cut in the sagittal plan. **B)** *Exorh* mRNA is strongly expressed in the pineal. The background staining using the sense probe is shown in a. **C)** *Rh1.1* mRNA is not present in the pineal. The background staining is shown in b. **D)** *Rgr1* and **E)** *Rgr2* is not expressed in the pineal. Scale bars are shown for B and C. D and E are shown at 20X magnification.

The *rgr* sections were imaged on a Leica SCN400 slidescanner.

[DiV, diencephalic ventricle; Dm, medial zone of the dorsal telencephalon; Exorh, exorhodopsin; Rgr, retinal G-protein coupled receptor; Rh, rhodopsin; S, sense DIG-probe control; PGZ, periventricular gray zone; TeO, optic tectum; TL, torus longitudinalis]

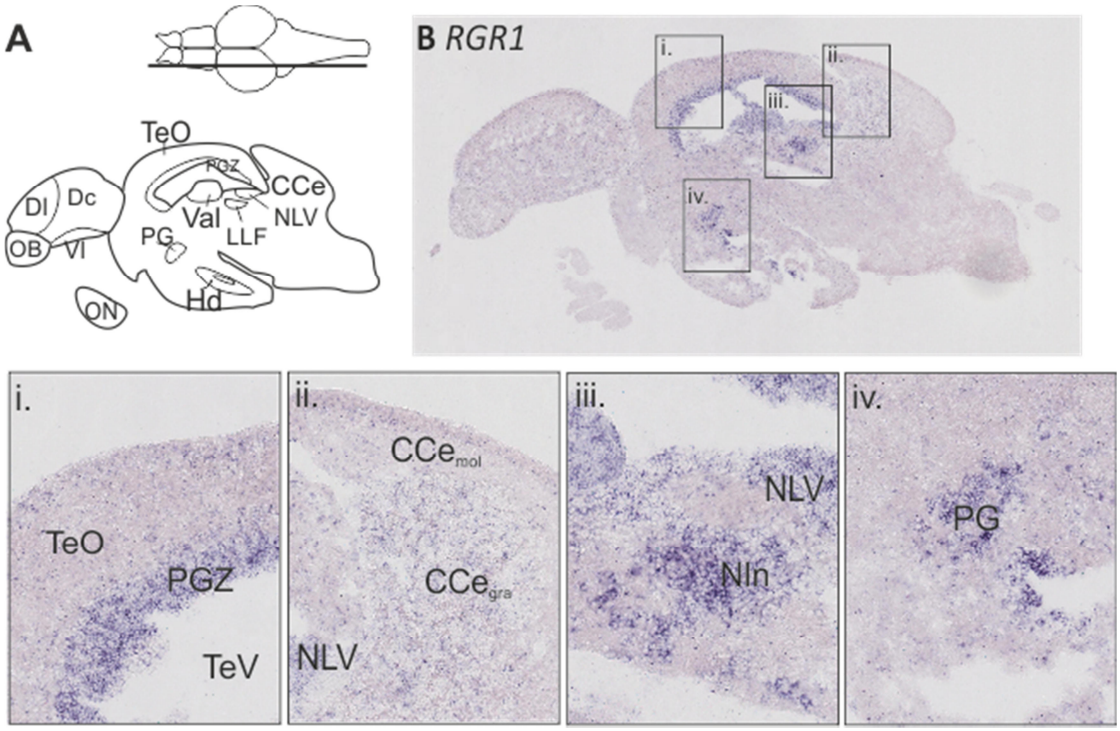


Figure 76 *Rgr1* expression in the zebrafish

Brains were dissected at ZT3, fixed, frozen and chromogenic *in situ* performed for detection of *Rgr1* mRNA. Inserts i-iv are 20X magnification. **A)** The brain was cut in the sagittal plane. The schematic shows the main brain nuclei in the section. **B)** *Rgr1* mRNA is expressed in discrete brain nuclei. **i)** There was diffuse staining in the TeO and strong staining in the PGZ. **ii)** There was diffuse expression in the CCe_{gra}. **iii)** There was strong staining for RGR1 in the LLF and NLV. **iv)** There was strong expression in the PG.

These sections were imaged on a Leica SCN400 slidescanner.

[Dc, cental zone of the dorsal telencephalon; Dl, lateral zone of the dorsal telencephalon; CCe_{gra}, granular layer of the corpus cerbelli; Hd, dorsal zone of periventricular hypothalamus; NIn, nucleus interpenduncularis; NLV, nucleus lateralis valvulae; OB, olfactory bulb; PG, preglomerular nucleus; PGZ, periventricular gray zone; TeO, optic tectum]

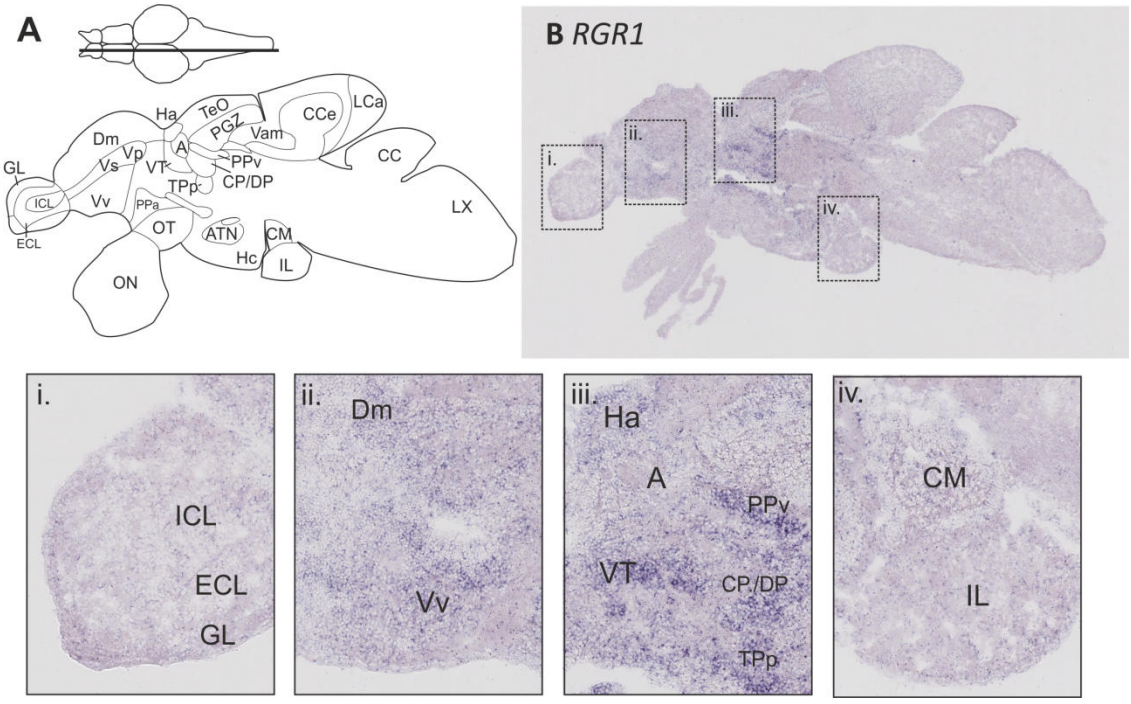


Figure 77 *Rgr1* is expressed in thalamic nuclei

Brains were dissected at ZT3, fixed, frozen and CISH performed for detection of *rgr1* mRNA. Inserts i-iv are 20X magnification. **B)** *Rgr1* mRNA is expressed in discrete brain nuclei. **i)** There no staining in the olfactory bulbs in either the ICL, ECL or GL. **ii)** There was diffuse expression in the Dm and Vv. **iii)** There was strong staining for *rgr1* in thalamic nuclei the VT, CP, DP, TPp, and in the PPv. There was low expression in the Ha and diffuse expression in the A. **iv)** There was no expression in the CM or IL.

These sections were imaged on a Leica SCN400 slidescanner.

[A, anterior thalamus; ATN, anterior tuberal nucleus; CC, crista cerebellis; CCe, corpus cerebellum; CM, corpus mammilare; CP/DP, central and dorsal posterior thalamic nucleus; Dm, medial zone of the dorsal telencephalon; ECL, external cellular layer of olfactory bulb; Ha, habenula; ICL, internal cellular layer of olfactory bulb; IL, inferior lobe; LCa, lobus caudalis cerebelli; LX, vagal lobe; ON, optic nerve; OT, optic tract; PPv, ventral periventricular pretectal nucleus; PGZ, periventricular grey zone; TeO, optic tectum; TPp, periventricular nucleus of the posterior tuberculum; Vs/p, supracommissural and postcommissural nucleus of the ventral telencephalon, VT, ventral tegmentum; Vv, ventral nucleus of the ventral telencephalon]

Zebrafish brain nuclei	*Exorh*	*Rh1.1*	*Rgr1*	*Rgr2*
Telencephalon				
OB, Olfactory Bulbs				
ICL, internal cellular layer	-	-	-	-
ECL, external cellular layer	-	-	-	-
GL, glomerular layer	-	-	-	-
D, Dorsal telencephalic area				
Dc, central zone of D	-	-	+	-
Dd, dorsal zone of D	-	-	+	-
Dl, lateral zone of D	-	-	+	-
Dm, medial zone of D	-	-	++	-
Dp, posterior zone of D	-	-	+	-
V, Ventral telencephalic area				
Cv, commissural nucleus of V	-	-	-	-
Vd, dorsal nucleus of V	-	-	-	-
Vv, ventral nucleus of V	-	-	++	-
Vp, postcommissural nucleus of V	-	-	+	-
Vs, supracommissural nucleus of V	-	-	-	-
Vc, central nuclei of V	-	-	-	-
Vl, lateral nuclei of V	-	-	-	-
EN, entopeduncular nucleus	-	-	-	-
NT, nucleus taeniae	-	-	++	-
Diencephalon				
Area preoptica				
CO, optic chiasm	-	-	-	-
OT, optic tract	-	-	-	-
PPa, parvocellular preoptic nucleus, anterior part	-	-	++	-
PPp, parvocellular preoptic nucleus, posterior part	-	-	++	-
SCN, suprachiasmatic nucleus	-	-	++	-
Epithalamus				
Ha, ventral and dorsal habenula	-	-	++	-
Pineal	++	-	++	-
Thalamus				
VM, ventromedial thalamic nucleus	-	-	++	-
VL, ventrolateral thalamic nucleus	-	-	++	-
A, anterior thalamic nucleus	-	-	+	-
DP, dorsal posterior thalamic nucleus	-	-	++	-
CP, central posterior thalamic nucleus	-	-	++	-
ZL, zona limitans	-	-	++	-
Pretectum				
PS, superficial pretectal nuclei	-	-	-	-
CPN, central pretectal nucleus	-	-	-	-
APN, accessory pretectal nucleus	-	-	-	-

Zebrafish brain nuclei	*Exorh*	*Rh1.1*	*Rgr1*	*Rgr2*
PO, posterior pretectal nucleus	-	-	-	-
DAO/VAO, dorsal/ventral accessory optic nuclei	-	-	-	-
PPv, periventricular pretectal nucleus, ventral part	-	-	++	-
Posterior tuberculum				
Tpp, periventricular nucleus of the posterior tuberculum	-	-	++	-
TLa, torus lateralis	-	-	-	-
CM, corpus mammilare	-	-	-	-
PTN, posterior tuberal nucleus	-	-	++	-
PVO, paraventricular organ	-	-	++	-
PGl/a, lateral and anterior preglomerular nuclei	-	-	++	-
PGm, medial preglomerular nucleus	-	-	++	-
Hypothalamus				
ATN, anterior tuberal nucleus	-	-	+	-
Hc, caudal zone of periventricular hypothalamus	-	-	-	-
Hd, dorsal zone of periventricular hypothalamus	-	-	++	-
Hv, ventral zone of periventricular hypothalamus	-	-	++	-
LH, lateral hypothalamic nucleus	-	-	-	-
IL, inferior lobe	-	-	-	-
Mesencephalon				
Superior and inferior colliculi				
TeO, optic tectum	-	-	+	-
PGZ, periventricular grey zone	-	-	++	-
TL, torus longitudinalis	-	-	-	-
TS, torus semicircularis	-	-	-	-
LLF, lateral longitudinal fascicle	-	-	-	-
MNV, mesencephalic nucleus of trigeminal nucleus	-	-	-	-
Nmlf, nucleus of the medial longitudinal fascicle	-	-	-	-
PCN, paracommissural nucleus	-	-	-	-
Tegmentum				
DTN, dorsal tegmental nucleus	-	-	-	-
EW, Edinger-Westphal nucleus	-	-	-	-
NLV, nucleus lateralis valvulae	-	-	++	-
NIII, Oculomotor nucleus	-	-	-	-
NLL, nucleus of lateral lemniscus	-	-	++	-
PL, perilemniscal nucleus	-	-	-	-
SR, superior raphe nucleus;	-	-	-	-
SRF, superior reticular formation	-	-	-	-
NIn, nucleus interpeduncularis	-	-	++	-
NI, nucleus isthmus	-	-	-	-
LC, Locus coeruleus	-	-	-	-
SGN, secondary gustatory nucleus	-	-	-	-

Zebrafish brain nuclei	*Exorh*	*Rh1.1*	*Rgr1*	*Rgr2*
Rhombencephalon				
Cerebellum				
Val_{gra}, granular layer of lateral division of valvula cerebelli	-	-	++	-
Val_{mol}, molecular layer of lateral division of valvula cerebelli	-	-	-	-
$Vam_{gra,}$ granular layer of medial division of valvula cerebelli	-	-	++	-
$Vam_{mol,}$ molecular layer of medial division of valvula cerebelli	-	-	-	-
CCe_{gra}, granular layer of corpus cerebellum	-	-	+	-
CCe_{mol}, molecular layer of corpus cerebellum	-	-	+	-
EG, eminentia granularis	-	-	+	-
LCa, lobus caudalis cerebelli	-	-	-	-
Medulla oblongata				
brainstem cranial nuclei (except LX)	-	-	+	-
Gc, griseum centrale	-	-	+	-

Table 7 Opsin mRNA expression in zebrafish brain nuclei

This table describes the mRNA expression of *exorh*, *rh1.1 rgr1*, and *rgr2*, in various brain nuclei of adult wild type zebrafish as shown using chromogenic *in situ* hybridization. Zebrafish were kept on a 14:10LD cycle and brains dissected at ZT3. [-, no detectable signal; +, signal found in some cells in the region; ++, signal found in many cells in the region]

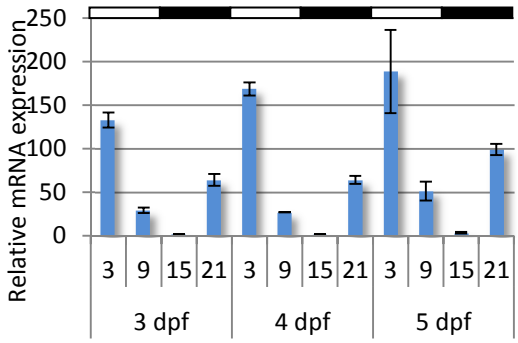
7.4.3 Opsin expression during larval development

Larvae zebrafish, wt and *mbl* on a 12:12LD, were collected every six hours between 3-5 dpf. RNA was extracted and the expression of the rhythmic clock gene *per3* and opsin genes were examined by qPCR. All the samples had a strong rhythm of *per3* with a peak at ZT3 and trough at ZT15, showing that both wt and *mbl* larvae were entrained to the LD cycle (Figure 78A and B, $p<0.001$, Two way ANOVA). The zebrafish opsins *exorh*, *rh1.1*, and *rgr1*, had a 24-hour rhythm in both wt and *mbl* larvae (Figure 78, Two way ANOVA). No rhythm was observed in *rgr2* expression (Two way ANOVA). A 24-hour rhythm of *rh1.2* was detectable in wt but not *mbl* larvae. The expression of *exorh*, *rh1.1* and *rh1.2* peaked at ZT15, whereas *rgr1* peaked at ZT9.

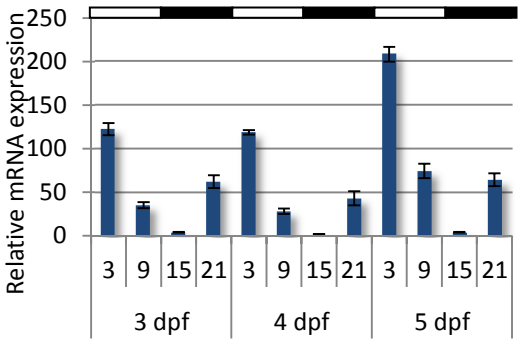
The expression of *exorh* was higher in the *mbl* larvae than wt (Figure 78 C and D, $p=0.0005$, Two way ANOVA). However, all other opsins gene showed a higher expression in the wt larvae than *mbl*. The expression of *rh1.1* was far higher in the wt larvae than *mbl* (Figure 78 E and F; $p<0.0001$, Two way ANOVA), this shows the rather high expression of *rh1.1* in the larvae eye. Wild type larvae had a higher opsin expression compared to the *mbl* in the expression of *rh1.2* (Figure 78 G and H; $p<0.0001$, Two way ANOVA), *rgr1* (Figure 78 J and K; $p<0.0001$, Two way ANOVA) and *rgr2* (Figure 78 L and M; $p<0.0001$, Two way ANOVA).

In the expression of *rh1.1* there was also a 6-hour phase difference between the peak and trough of wt (ZT 15 and ZT3 respectively) and mbl (peak at ZT21 and trough at ZT9). However, there was no phase difference in the expression of *exorh* or *rgr1* between wt and *mbl* larvae.

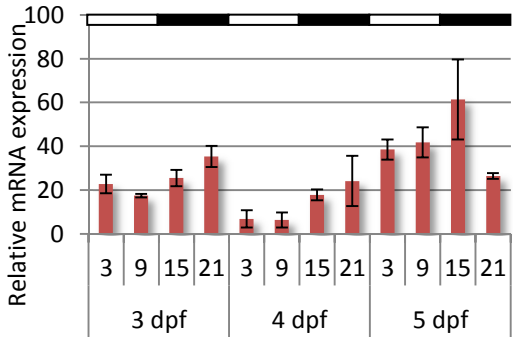
A. *Per3* wt



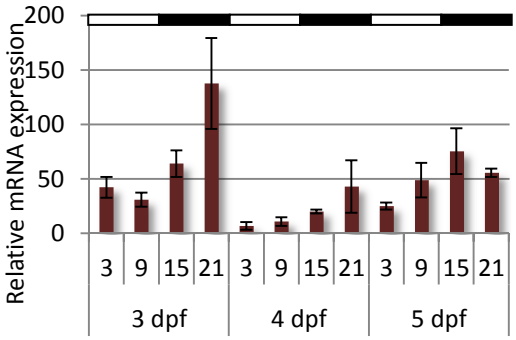
B. *Per3 mbl*



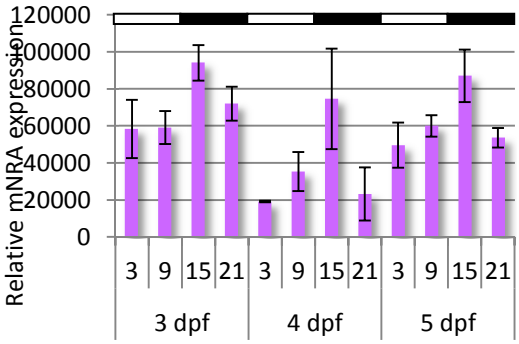
C. *Exorh* wt



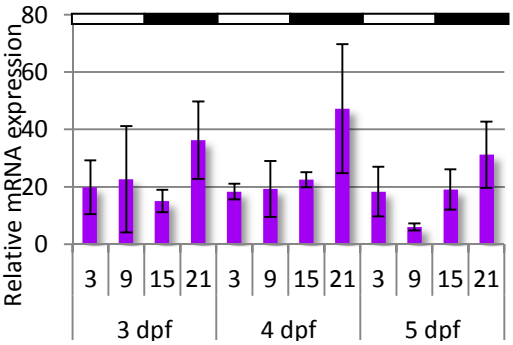
D. *Exorh mbl*



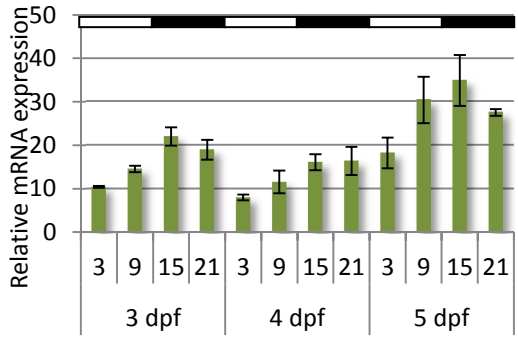
E. *Rh1.1* wt



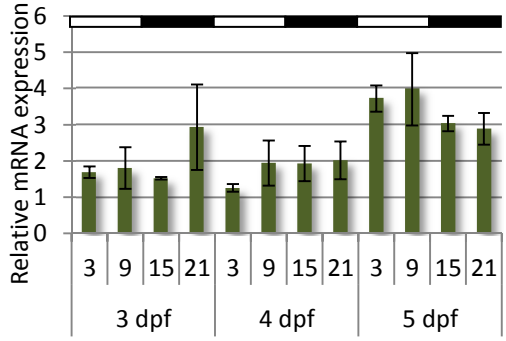
F. *Rh1.1 mbl*



G. *Rh1.2* wt



H. *Rh1.2 mbl*



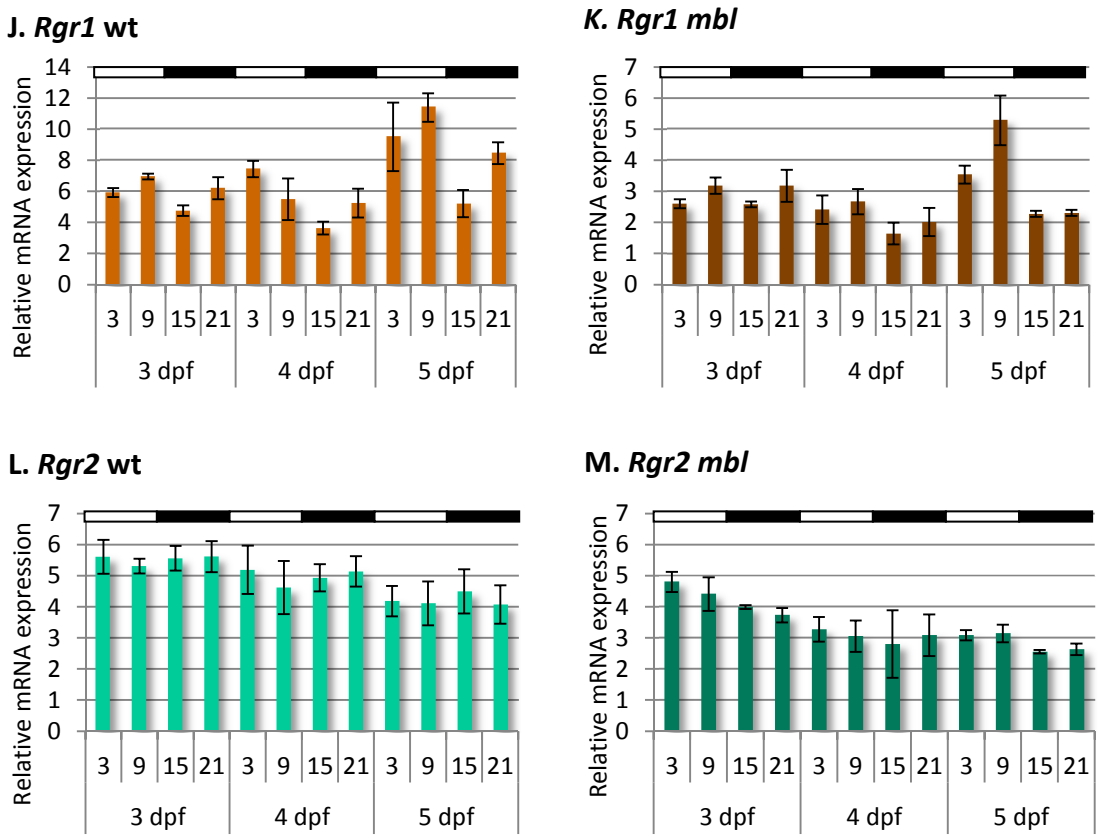


Figure 78 Rhythmicity of opsin expression in larvae and the effect of no eyes

Zebrafish wild type (wt) and *masterblind* (*mbl*) larvae on a 12:12LD were collected every six hours between 3-5 dpf. RNA was extracted. Expression of the core clock gene *period3* and opsin genes were examined by qPCR.

A & B Zebrafish larvae have an entrained clock during development; the 24-hour rhythm is shown by *per3* expression. This rhythmicity persists in mbl larvae lacking eyes with the same peak of ZT3 and trough at ZT15 (Two way ANOVA, $p<0.001$)

C & D *Exorh* has a 24-hour rhythmic expression in the zebrafish larvae, with a peak/trough on day 4 and 5 of ZT21/9 and ZT15/3 on day 6 in both wt (Two way ANOVA, $p<0.05$) and *mbl* (Two way ANOVA, $p<0.01$). Expression of *exorh* is higher in the mbl larvae than wild type (Two way ANOVA, $p<0.001$).

E & F *Rh1.1* has a 24-hour rhythmic expression in the zebrafish larvae, with a peak at ZT15 in wt (Two way ANOVA, $p<0.01$) and ZT21 in *mbl* (Two way ANOVA, $p<0.001$), with the amplitude of the rhythm increasing in the *mbl*. Expression of *rh1.1* is 1000 fold higher in the wild type than mbl larvae, signifying its high expression in the retina (Two way ANOVA, $p<0.001$).

G & H *Rh1.2* has a 24-hour rhythmic expression in wt zebrafish larvae, with a peak at ZT15 (Two way ANOVA, $p<0.01$), the expression in *mbl* is not rhythmic (Two way ANOVA, ns) and variable across the days (Two way ANOVA, $p<0.001$). Expression of *rh1.2* is 10 fold higher in the wt than *mbl* larvae (Two way ANOVA, $p<0.001$).

J & K *Rgr1* has a 24-hour rhythmic expression in wt with a peak at either ZT3 or 9 and trough at ZT15 (Two way ANOVA, $p<0.001$) and a peak at ZT9, trough at ZT15 in *mbl* zebrafish larvae (Two way ANOVA, $p<0.001$). The amplitude of the rhythm increases as the larvae develop (Two way ANOVA, $p<0.01$). Expression of *rgr1* is 2 fold higher in the wt than *mbl* larvae (Two way ANOVA, $p<0.001$).
L & M *Rgr2* is not rhythmic in either the wt or *mbl* larvae (Two way ANOVA, ns).
Each time point has 3 samples, each representing RNA from 10-15 larvae. Error bars represent the SEM. The 12:12LD condition is indicated by the white and black bars.

7.4.4 Opsin expression in larvae raised in constant light

Wild type zebrafish were either raised in 12:12LD or LL. Samples were then collected from 4 dpf larvae every six hours across a 24-hour period. RNA was extracted and the relative expression of *per3* and opsins was determined using qPCR.

Zebrafish larvae show a strong 24-hour rhythm in *per3* expression during LD (One way ANOVA, $p<0.0001$), and no rhythmicity in LL (One way ANOVA) (Figure 79A). The levels of *per3* mRNA remain around the LD mid-level during LL.

The expression of all the opsins was higher in LL conditions, suggesting that LL upregulates opsin expression (Figure 79B-F). Furthermore, in LL, there was no rhythmicity in *exorh*, *rh1.1*, *rh1.2*, or *rgr2* (One way ANOVA, ns). *Rgr1* expression was higher at CT21 than CT3 (One way ANOVA), however this is unlikely to be rhythmic as the peak/trough in LD conditions is ZT3/15. The loss of this 24-hour rhythm in LL suggests that *exorh*, *rh1.1*, *rh1.2*, and *rgr1*, could be circadian controlled genes during development.

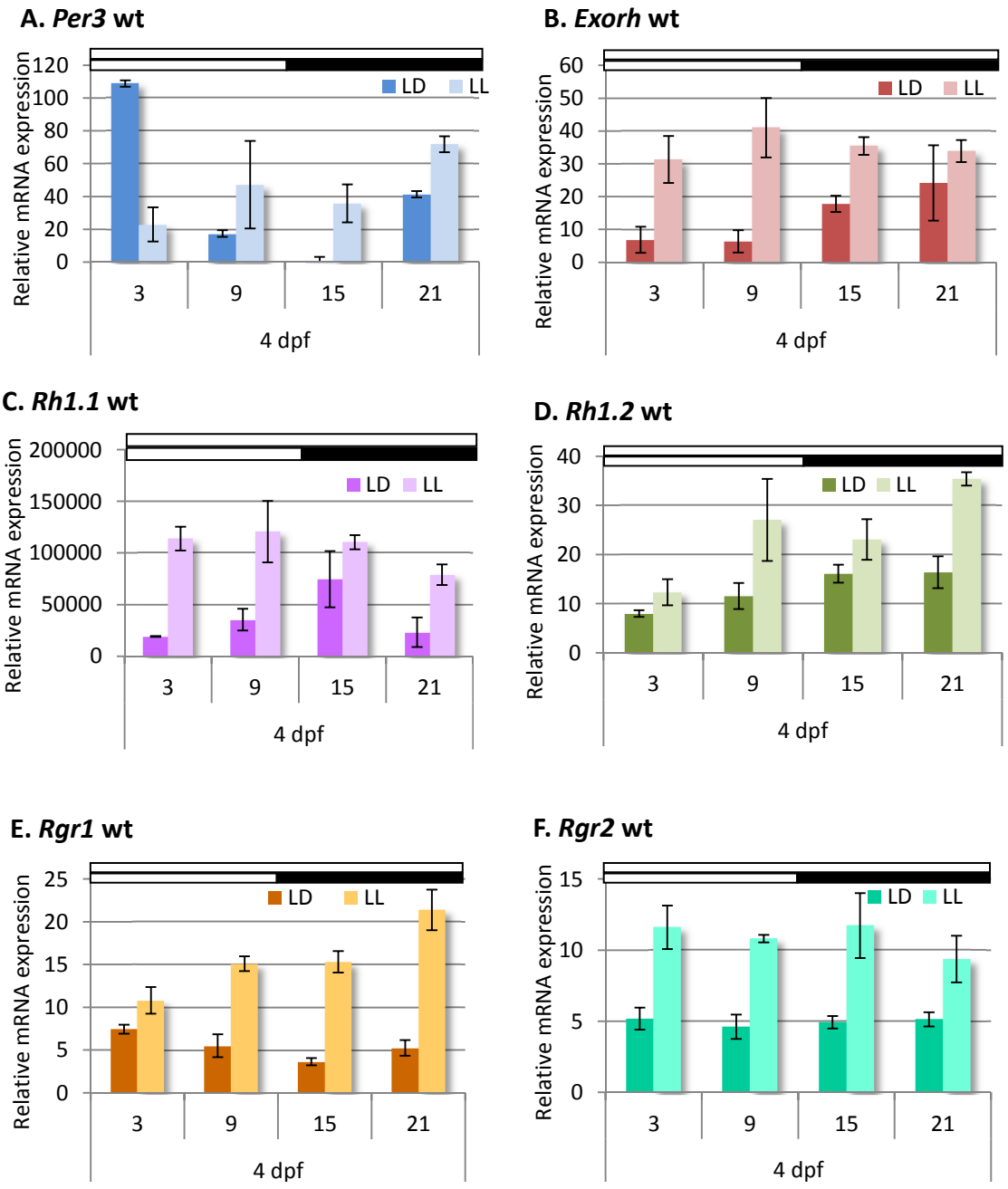


Figure 79 Suppression of rhythmicity of opsin expression in wt zebrafish larvae raised in LL

RNA was extracted from 4 dpf wild type larvae zebrafish after being raised in either 12:12LD or constant light.

A Zebrafish larvae show a strong 24-hour rhythm in *per3* expression during LD (One way ANOVA, $p<0.0001$), and no circadian rhythm in LL (One way ANOVA). The levels of *per3* mRNA remain around the LD mid-level during LL.

B – F In constant light there was no rhythmicity in *exorh*, *rh1.1*, *rh1.2*, or *rgr2* (One way ANOVA, ns). *Rgr1* expression was higher in CT21 than CT3 (One way ANOVA), however this is unlikely to be rhythmic as the peak/trough in LD conditions in ZT3/15. This suggests that *exorh*, *rh1.1*,

rh1.2, and *rgr1*, could be circadian controlled genes during development. The expression of all the opsins was higher in LL conditions.

Each time point includes 3 samples each containing 10-15 larvae. Error bars represent the SEM, the 12:12LD or LL regime is indicated by the white and black bars.

7.5 Discussion

The zebrafish genome contains a vast number of opsin photopigments. This may result from the various opsins having specialised roles to regulate the light sensitivity of the circadian clock, and this may even be tissue specific. However, the photoreceptors and phototransduction signals in the zebrafish have not yet been fully elucidated. The zebrafish brain is directly light sensitive (Whitmore, Foulkes, & Sassone-Corsi 2000) and has regions that highly express clock genes (chapters 3 and 4). Furthermore, different neural regions are activated by various changes in light conditions (chapter 5). Therefore, it has been hypothesised in this project that the zebrafish brain may show a region specific expression of the photosensitive opsins. Indeed, the strongly localised expression of *rgr1* supports this idea.

7.5.1 *Rgr1* expression is localised in the zebrafish brain

Rgr is an opsin involved with the generation of 11-*cis*-retinal as part of photoisomerase activity in the mammalian visual system (Wenzel et al. 2005). Here, CISH results show *rgr1* is highly expressed in many of the rhythmic and light responsive regions of the adult zebrafish brain, including the pineal, Vv, SCN, habenula, ventral thalamic nuclei, PG, Hv, Hd, PGZ and granular layers of the cerebellum. Also, *rgr1* was detected in many more regions that expressed only one or two of the core clock genes *cry1a*, *per2* and *per3* (see appendix for comparative table). However, there were also rhythmic and light responsive regions where *rgr1* was not detectable, such as the TL and LCa. It is therefore likely that other photopigments or pathways are involved. Also, there were regions that expressed *rgr1* but did not express *cry1a*, *per2* or *per3*, such as the tegmental NLL and NIn. Furthermore, *rgr1* could not be narrowed down to be the photopigment responsible for the induction of *c-fos* either at lights off or after a late subjective night light pulse. Although there was overlap of expression in many of the regions this was not absolute in either situation. These data do not exclude that *rgr1* is a photopigment in the light entrainment pathway in the rhythmic and light responsive regions; however there are likely be several other photopigments also involved.

There have been a limited number of studies evaluating the expression of opsins in the adult zebrafish brain, most of which have focussed solely on the pineal rather than the whole brain

(Forsell et al. 2001; Mano et al. 1999). However, using immunohistochemistry, the VAL opsin has been located exclusively to the central posterior thalamic nucleus (CP) in the zebrafish brain and horizontal retinal cells (Kojima et al. 2000). CISH data shown here shows *rgr1* is also expressed in the CP amongst other brain regions. The CP is a non-GABAergic nucleus that projects to the Dm, Dl, ventral thalamic nuclei and TS in zebrafish (Mueller 2012). This region also expresses hypocretin receptors, and altogether this suggests that the expression of numerous photopigments here may be important for the light entrainment and circadian regulation of sleep/wake cycles (Kaslin et al. 2004; Yokogawa et al. 2007).

What is the spatial expression of *rgr* in the brain of other species? *Rgr* was first identified in bovine retinal epithelial cells (Jiang, Pandey, & Fong 1993), and has since been found highly expressed in the mouse retina (Tao et al. 1998). However, few experiments have addressed its spatial localisation in the brain. Radioactive ISH has localised *rgr* in the pineal and eyes with further low non-localised expression throughout the brain of the chick (Bailey 2004). A homolog of *rgr*, *Ci-opsin3*, has also been found in larvae neural tissues of a sea squirt, *Ciona intestinalis* (Nakashima et al. 2003). Therefore the results presented here in zebrafish are the first showing regional localised expression of *rgr* throughout a vertebrate brain.

It remains plausible that highly expressed and localised opsins are involved in the light entrainment of circadian rhythms in the zebrafish. Further investigation of other opsins, such as *tmt*s, and other putative photopigments using CISH in the adult zebrafish brain would be invaluable to solve this conundrum. Such studies would then need to be followed up with functional analysis of these photopigments, in an attempt to prove which molecule(s) are required for zebrafish clock entrainment.

7.5.2 Pineal opsin expression

The results presented here confirm previous reports that *exorh* is expressed abundantly in the pineal (Asaoka et al. 2002; Mano et al. 1999; Pierce et al. 2008). Using a sensitive technique, qPCR, reveals that *exorh* is also expressed in other regions of the brain but at an extremely low level, indeed ten-fold lower than basal *per1b*, and therefore it was undetectable using CISH. The inconsistency of low relative expression of *exorh* in the qPCR results compared with the abundant expression found using the NanoString technique is likely due to inclusion of the

exorh-rich pineal in the samples used in the NanoString technique, as qPCR on brain samples that did not remove the pineal were typically 400-fold higher (data not shown).

The first report localising *exorh* in the pineal also examined the expression of *rh1.1* and did not find it in the pineal (Mano et al. 1999). However, recent studies have reported that RH1.1 was expressed in the pineal (Magnoli et al. 2012). Although, the authors state that the technique used immunohistochemistry with an antibody that may not have distinguished between RH1.1 and EXORH. Here, CISH reveals that *rh1.1* is not expressed in the pineal, or is expressed in a very low level that was not detectable by the methods used. This confirms the earlier report and clarifies that these different rhodopsins, *exorh* and *rh1.1* are solely prominent in either the pineal or retina, respectively.

7.5.3 Rhythmic opsin expression in adult *vs* larvae

Opsin expression has found to be rhythmic in many species. In albino rat retina *melanopsin* is expressed with a peak in the early subjective night (Hannibal et al. 2005). The chick pineal has a 24-hour rhythm of *rgr* expression on a LD cycle (Bailey 2004) and the expression of the red-sensitive cone pigment, *iodopsin*, fluctuates with a circadian rhythm in chick retina (Pierce et al. 1993). In the brain and retina of another teleost, the African cichlid, *Astatotilapia burtoni*, the opsins melanopsin, rhodopsin and VA-opsin were all rhythmically expressed on a LD cycle (Grone et al. 2007).

Zebrafish larvae on 12:12LD show rhythmicity of *exorh*, *rh1.1*, *rh1.2* and *rgr1*. This is consistent with findings that other opsins are rhythmic in zebrafish larvae on a LD cycle; including *exorh* in the pineal (Pierce et al. 2008), and the melanopsin genes *opn4m1* and *opn4m2* in the retina (Matos-Cruz et al. 2011). This is also in agreement with functional studies showing larvae have a circadian rhythm in visual sensitivity (Emran et al. 2010).

However, although the opsins *exorh*, *rh1.1*, *rh1.2*, *rgr1* and *rgr2* were all expressed in the adult zebrafish brain on a 14:10LD, there was no apparent 24-hour rhythmicity. This is in contrast with other studies that show adult zebrafish retain a rhythmic light sensitivity threshold in the rods and cones (Li & Dowling 1998). Furthermore, there is a correlation between the 24-hour fluctuation of the expression of *lws1* opsin and behavioural sensitivity to red light (Li et al. 2005).

In fact, circadian rhythms of *rh1.1* has been found in isolated adult zebrafish retinas, however individual cells were not synchronised (Yu et al. 2007). Perhaps opsin rhythmicity was not detected in whole zebrafish brain samples due to localised expression of opsins and masking from different regions.

It is also plausible that zebrafish adult brains may have lost rhythmicity of opsin genes, as changes in both the temporal and spatial distribution of opsin genes during development have been previously reported. The four *rh2* genes change in their spatial distribution in the zebrafish retina when examined by CISH at 3 days, 1 week, 1 month, and 2 years (Takechi & Kawamura 2005). In another teleost fish, the Nile tilapia*, Oreochromis niloticus*, a different subset of cone opsins are expressed between larvae, juvenile and adult developmental stages (Carleton et al. 2008). The teleost, eel, contains rod photoreceptors that express alternate *rh1* genes during development; this change coincides with the transition as a juvenile in a freshwater habitat to an adult in a deep-sea habitat (Archer, Hope, & Partridge 1995). Therefore, further investigation using a spatial as well as temporal technique will be required to resolve the question of opsin rhythmicity in the adult zebrafish.

7.5.4 *Rh1.2* is expressed in the adult brain and larvae

Rh1.2 is a recently discovered rhodopsin gene, and its expression alongside *rh1.1* has been investigated in adult and larvae zebrafish (Morrow et al. 2011). Using rt-PCR the authors showed *rh1.2* expression in the retina, however were unable to detect *rh1.2* in the 4dpf larvae, or adult brain. Data presented here, using a more sensitive technique, qPCR, show both larvae from 3dpf to 5dpf and the adult brain do express *rh1.2*, but at a far lower level than *rh1.1*.

Rh1.2 was not tested for spatial expression in the adult brain due to not having a probe available; however, it's extremely low level of expression would likely have meant it was undetectable in the adult zebrafish brain using CISH, unless it had a highly specific localised signal.

7.5.5 Effect of lack of eyes in larvae opsin expression

Most examinations of the opsin gene have focussed on the expression in the retina (Morrow et al. 2011; Robinson, Schmitt, & Dowling 1995; Takechi & Kawamura 2005). However, can opsin

expression in the rest of the body persist without retinal inputs? The results here show that there are differences in the temporal and/or level of gene expression between wt and eyeless *mbl* mutant in all the opsins tested, despite the expression of the rhythmic core clock gene *per3* being unaltered. However, comparing gene expression levels in *mbl* and wt larvae does not distinguish whether these opsins are expressed differently because of a lack of retinal input, or whether the *mbl* is simply showing the normal gene expression in the rest of the body, which is otherwise masked in the presence of the eyes. Further investigation with CISH would be very useful to distinguish these two explanations. However, it can be concluded that opsin expression persists in eyeless larvae and although the eyes may be involved in opsin expression, they are not necessary for its presence in the periphery.

Furthermore, there is evidence that retinal inputs are neither required nor involved in the expression of some opsins in the larvae brain. Recent evidence shows that *opn4m1* expressing cells in the hypothalamus are responsible for detecting the changes of light that allow for the visual motor response, and this is unaffected by loss of eyes in larvae (Fernandes et al. 2012).

Only one of the opsins was expressed higher in the *mbl* than wt, *exorh*. There are a number of plausible explanations for this. The eyes might normally be signalling to the pineal to suppress *exorh* expression. Perhaps as *exorh* is not expressed in the eyes (Mano et al. 1999), the removal of the eyes raises the overall relative level of *exorh*. Or, intriguingly, perhaps in the absence of eyes, the pineal compensates as the primary photosensitive tissue, and upregulates *exorh* expression. The most likely explanation, however, is that the pineal gland itself becomes enlarged in the *mbl* mutant, and so more *exorh* expressing cells would contribute to this structure.

Rh1.1 was by far the most highly expressed gene tested in the larvae, and its level was dramatically reduced in the eyeless mutants. This is in agreement with many previous studies that have shown the strong expression of *rh1.1* in the retina (Mano et al. 1999). However, *rh1.1* was still present in the eyeless larvae, suggesting that it also present outside the larval eye, albeit in very low levels.

7.5.6 Light upregulation of opsins during development

The expression of *exorh*, *rh1.1*, *rh1.2*, *rgr1* and *rgr2* was higher in LL conditions. This is in contrast to one study that show melanopsin gene expression is decreased in LL, and almost undetectable after five days, in the retina of adult albino Wistar rats (Hannibal et al. 2005). However, LL has no effect on retinal melanopsin expression in albino Wistar rats during postnatal development (González-Menéndez et al. 2010), and therefore this effect may be dependent on developmental stage. Furthermore, light will increase the demand for 11-*cis*-retinal in photoreceptors such as cones, and therefore the upregulation of opsins such as rgr, which regenerate 11-*cis*-retinal may be advantageous.

All of the opsins tested lost rhythmicity in larvae, which supports the idea that the expression of the opsin genes *exorh*, *rh1.1*, *rh1.2*, and *rgr1*, are under control of the circadian clock. Future experiments to monitor expression under constant dark conditions will be necessary to determine whether these are true clock controlled genes.

7.5.7 Are opsins the circadian photopigments?

Opsins are a diverse group of photopigments. Many are good candidates for detecting light for the entrainment of the zebrafish molecular clock because they are expressed in many tissues and detect different wavelengths of light (Moutsaki et al. 2003). However, other light responsive components to the clock could also be involved in the light sensitive pathway. *Cry4* has been suggested as a strong candidate as it is not rhythmically expressed and due its strong homology with *Drosophila cry*, which acts as a photopigment for entraining the *Drosophila* circadian clock (Kobayashi et al. 2000). In addition, flavin-containing oxidases have also been suggested (Hirayama, Cho, & Sassone-Corsi 2007). Hydrogen peroxide is implicated as a secondary messenger in the zebrafish light entrainment pathway (Hirayama et al. 2007), and flavin-containing oxidases, which can increase hydrogen peroxide levels, have been shown to absorb near violet–blue light (Setoyama et al. 1995). Furthermore, in zebrafish the upregulation of *per2* by light involves both blue light wavelengths and retinol independent photopigments (Cermakian et al. 2002). This would be consistent with the involvement of a Cry photoreceptor or flavin-containing oxidase, but not with in an opsin. Therefore, it is likely that a range of photoreceptor types are active for the entrainment of the circadian system.

Overall, this chapter has explored the expression of opsin genes in both the adult brain and larvae. Two highly expressed opsins, *exorh* and *rgr1*, were found in specific regions of the brain. Notably, *rgr1* is expressed in many regions which have considerable overlap with *cry1a*, *per2* and *per3* expression, which suggests its importance as an opsin in the light entrainment of the circadian clock. The expression of opsins in larvae appears to be more sensitive to lighting condition than in adults as larvae display a rhythmic expression of opsins *exorh*, *rh1.1, rh1.2* and *rgr1* in LD, and constant light removes this rhythmicity and increases expression. As mentioned in the introduction to this chapter, the work on these selected opsins is part of a much larger collaborative effort to study all 43 opsins in the fish. However, these results themselves add to an increased understanding of the light responsive nature of the zebrafish brain.

7.6 Conclusions

- In the adult brain there is a high expression of *exorh* and *rgr1*. These genes are also regionally expressed; *exorh* is only localised in the pineal, whereas *rgr1* is found in many nuclei including the pineal, SCN, Dm, ventral thalamic nuclei, hypothalamus, PGZ and granular layers of the cerebellum.
- *Rh1.1* and *rgr2* are not detectable in specific regions of the adult brain.
- The adult zebrafish brain does not show overall rhythmicity of *exorh*, *rh1.1*, *rh1.2*, *rgr1* or *rgr2*.
- However, larvae zebrafish display rhythmicity in expression of opsins *exorh*, *rh1.1, rh1.2* and *rgr1*, but not *rgr2*, which is present but not rhythmic.
- *Mbl* mutant larvae, lacking eyes, have an altered phase and/or level of expression of *exorh*, *rh1.1, rh1.2, rgr1* and *rgr2*, despite having an unchanged rhythm of *per3*.
- In larvae, *exorh*, *rh1.1, rh1.2, rgr1* and *rgr2* expression is upregulated by constant light.

Chapter 8

General discussion

This general discussion discusses the significance of this thesis in the wider scientific field, more specific discussions of the data can be found near the end of each results chapter (Chapters 3-6).

8.1 General discussion

Circadian clocks are a fundamental adaptive feature of most organisms, which regulate a wide range of physiological and behavioural processes. It is therefore of great interest to gain an understanding of how circadian rhythmicity itself is regulated. Furthermore, as light is one of the most prominent environmental time-cues, the investigation of light sensitivity is important to understand the regulation of the circadian clock. The zebrafish poses a useful vertebrate model for furthering this understanding as the molecular clock in their peripheral tissues can be directly reset by light (Whitmore, Foulkes, & Sassone-Corsi 2000).

Moreover, zebrafish are now particularly popular models in a vast array of areas, including developmental biology, cancer, pharmacology, toxicology, disease modelling, tissue regeneration, and neuroscience (Ullmann et al. 2010). The Home Office have reported that fish are the second most common animal model in the UK, and this is due to the rising number of studies performed using zebrafish (*Statistics of Scientific Procedures on Living Animals, Great Britain* 2011). Some of the advantages of zebrafish over other vertebrate lab models are the low-cost, fast generation time, ease of genetic manipulation, and the fact that they are diurnal. With the recent completion of the zebrafish genome sequencing it is likely their popularity will only increase.

As many of the biological areas using the zebrafish model are impacted by the effects of circadian rhythms, there is a definite need to have a more thorough understanding of the regulation of circadian rhythms in zebrafish. This will enable the better conduct of scientific studies and understanding of the results and implications. However, there are many unresolved questions, especially concerning the neurobiological basis of the zebrafish clock. This study set out with the aim of examining the circadian regulation and light sensitivity of the zebrafish brain. In this final chapter, the key novel finding of this thesis will be discussed in the context of the present literature and future work.

8.1.1 Putative neuronal pacemaker regions

The first experiments to use zebrafish as a model for clock function showed the pineal has endogenous rhythmicity *in vitro* (Cahill 1996). As the pineal is also a neuronal pacemaker region

in other species (Zimmerman & Menaker 1979) it has been speculated that the pineal was a neuronal pacemaker in zebrafish (Vatine et al. 2011). However, endogenous rhythmicity is only one characteristic of a neuronal pacemaker and it has only recently been shown that removal of pineal photoreceptors disrupts locomotor activity (Li et al. 2012). A large amount of the focus of study has been on the directly light responsive peripheral tissues in the zebrafish (Whitmore et al. 2000), which have been unravelling the details of the molecular clock (Tamai, Young, & Whitmore 2007). Therefore less attention has been paid to the details of how the central nervous system could be regulating circadian rhythms. At the beginning of this project the brain was treated as a uniform, rhythmic structure. The wider aim of this thesis was to explore for the first time whether this was actually true.

This project first set out to address whether there are any other regions in the zebrafish brain that display robust rhythmicity, as this is usually one of the first identifiable characteristics of neuronal pacemakers (Herzog 2007). The results from this project provide strong indication that there are multiple neuronal pacemakers throughout the zebrafish brain, in addition to the pineal. CISH and bioluminescent imaging has shown there are indeed localised regions that display robust rhythmicity (chapter 3). Alongside this there are regions that rapidly dampen in DD *in vitro*, suggesting that these regions require entraining signals from neuronal pacemakers *in vivo*.

The use of a spatial bioluminescent imaging technique has provided pilot data showing strong endogenous rhythms from regions such as the PGZ. This is advantageous to bioluminescent recording from a whole tissue, which can show rhythmicity and light sensitivity, but cannot distinguish whether this response is coming from the entire tissue or particular regions. Furthermore it complements techniques such as CISH, which show detailed expression, by providing high temporal resolution and dynamic monitoring of gene expression. Historically, the limited number of circadian studies in teleosts have focussed on the neuroanatomy of larger fish, and the zebrafish brain, due to its small size, has largely been overlooked (Vallone et al. 2005). Hopefully, this successful demonstration using spatial bioluminescent recordings of a *per3-luc* zebrafish, coupled with the new brain culturing technique, will make a vital contribution

to improve access to studying rhythmicity in this small fish brain more straightforward in the future.

Investigation into the circadian regulation of many species shows that many have multiple neuronal pacemaker structures (section 1.1.3). Indeed, the circadian system may benefit from multiple brain regions "driving" different tissues and downstream responses, as different regions of the brain can integrate various environmental and internal signals to produce a driving output signal. This can provide a means to differentiate the regulation of different tissues. Even decentralised clock models, such as *Drosophila*, which have directly entrainable light sensitivities peripheral tissues, have neuronal pacemakers that regulate some behavioural and hormonal rhythms (section 1.1.7). Therefore these localised regions in zebrafish hold promise to be implicated in neuronal circadian regulation, and the results have begun to narrow down the putative neuronal pacemaker regions.

It is difficult to speculate which rhythmic activities these individual regions might drive; this would be answered by ablation experiments. However, a little is already known about the roles of some of these regions in normal zebrafish physiology and this can provide us hints as to the potential function they may play in the regulation of circadian biology. The tectum is known to play major roles in sensory-motor coupling and fine motor controls (Bally-Cuif & Vernier 2010), and therefore the PGZ might also be involved in locomotor behaviour rhythms. Additionally the cerebellum is also involved in motor control (Bae et al. 2009) and high expression of rhythmic clock genes here may also regulate locomotor behavioural rhythms. Although, novel data suggests that the mammalian cerebellum contains a circadian oscillator sensitive to feeding cues, which may regulate food-anticipatory activity under restricted feeding (Mendoza et al. 2010). However, it is too soon to tell whether this will also be relevant in zebrafish. The vagal lobe (X) innervates the gut (Olsson, Holmberg, & Holmgren 2008) and therefore might be involved in metabolic rhythms involving digestion. The zebrafish Dm is likely to be homologous to the amygdala (Mueller 2012; Wullimann & Mueller 2004), therefore rhythmic expression in this region may be important for controlling levels of anxiety during the 24-hour period, heightening at times of potential increased predator interactions. The zebrafish SCN expresses rhythmic clock genes and light sensitive genes, and responds with *c-fos* induction at both lights

off and when a light pulse is given in the late subjective night, however none of these experiments have yet distinguished the SCN from other regions that also display these characteristics. One of the earlier experiments that distinguished the mammalian SCN was the rhythmic uptake of radioactive 2-deoxy-D-[^{14}C]glucose reflecting glucose utilisation (Schwartz, Davidsen, & Smith 1980). Further experiments in the zebrafish using similar approach may be useful in determining the role of the SCN in teleost.

The next steps taken in this work were to further understand the light sensitivity of these regions. However, these neuronal pacemaker candidates must be further tested. Many key questions remain: How well do they retain rhythmicity? For how long? Long-term mammalian SCN cultures have been shown to retain a robust *per1* rhythmicity after 32 days in culture (Yamazaki et al. 2000). If the regions are removed, is a rhythm in another region disrupted? This has been shown with the zebrafish pineal (Li et al. 2012), and may also be applied to other areas. Can a brain region carry host endogenous rhythmicity to a donor? The success of the brain culturing technique in this work and the capacity for neurogenesis during adulthood (Adolf et al. 2006; Zupanc, Hinsch, & Gage 2005) suggests that zebrafish brain transplants might be viable in the future; this would be a key step in showing a brain region could drive donor rhythmicity in a host.

8.1.2 Light sensitivity

The second aim of the project was to understand the light sensitivity of the zebrafish brain. As light sensitivity is often coupled with highly rhythmic regions it was encouraging to discover that two light responsive core clock genes, *cry1a* and *per2* also show localised expression. This provides further evidence towards zebrafish having multiple discrete neuronal pacemakers.

However, not all cells in the brain appeared to be expressing either rhythmic or light sensitive clock genes as the mRNA could not be detected using CISH. This has also been reported using single-cell RT-PCR examining *clock* and *per3* in zebrafish retinal cells (Li et al. 2008). It is possible that these cells are expressing low levels of these genes but are not detectable with the techniques used. Indeed one of the limitations of CISH is that the technique has difficulties detecting low levels of transcripts. This is highlighted by later experiments that use qPCR, a highly sensitive technique, which detected low levels of opsin genes in the adult zebrafish brain

that could not be detected using CISH. However, CISH is the most sensitive technique for detecting mRNA spatially, and the use of sense probes to show the inevitable background staining is a useful control, as is using a control tissue, which should show no staining (if one exists). It is also possible that these regions express different clock genes. However it is also plausible that these regions do not express clock genes. Alongside the findings from *per3-luc* tissues that many regions lose rhythmicity rapidly in culture, this suggests that some regions may require entraining cues from pacemakers *in vivo*.

One of the more significant findings to emerge from this project is *c-fos* is upregulated in at least four different responses to light conditions in zebrafish. *C-fos,* a marker for neuronal activity, shows different types of light induction in different mammalian photoreceptors (Onodera et al. 1999; Rusak et al. 1990; Schwartz et al. 1994; Sumová et al. 1998). Further investigation of some of these responses showed *c-fos* induction occurring in different zebrafish brain nuclei. This is highly indicative of the brain containing several types of photosensitive cells, which respond to different changes in lighting conditions. Perhaps this should not come as much of a surprise considering the number of different photosensitive pigments found in the zebrafish genome. To further answer how the clock is functioning in different neural regions, electrophysiological examination will be important in the future. Taken together, this thesis has begun to reveal the complexities that need to be unravelled in understanding the neurobiological basis of the clock.

The studies in chapter 7 set out to explore whether some of the highly expressed opsins could be accountable for the diversity in photoreceptor cells that might be responsible for the entrainment of light in the different regions of the adult brain. Indeed, the expression of *rgr1* appears consistent with many of the regions that show expression of rhythmic and light sensitive core clock genes. A limit to only studying the mRNA expression using qPCR and CISH is that it doesn't show whether the protein is functional or what post-translational modifications may occur. The availability of specific antibodies to zebrafish clock genes and photoreceptors would be necessary to study the function of the protein.

Circadian regulation has been implicated in the rhythmic expression of some opsins in zebrafish. A rhythm *of rh1.1* in zebrafish retinas is abolished when treated with anti-*clock*

morphilinos (Yu et al. 2007). Furthermore, individual cells in these retinal cultures do not display synchronicity, suggesting that they may receive timing cues from neuronal pacemakers.

8.1.2.1 Lab vs natural lighting conditions

All the experiments in this project were carried out using a LD cycle where the light intensity and spectrum remained constant during the light phase, and transition between the phases were simply lights on or lights off. A recent study in teleost has shown that rhythms in light spectral quality, which mimic natural daylight and keeping light intensity constant, could entrain circadian activity (Pauers et al. 2012). Zebrafish tissues express visual opsins which span the light spectrum from UV part of the spectrum (355nm) to the red end (558nm) (Chinen et al. 2003). Circadian rhythms can be entrained by light from wavelengths across the visual spectrum into UV, PAC2 zebrafish cell lines are responsive to wavelengths restricted in 50nm steps from 400-700nm (Whitmore, personal communication). Therefore it will be important and of great interest to assess the function of opsins in zebrafish kept in more natural lighting conditions where the colour of light changes during the day, rather than LD.

Furthermore, as illustrated by a study in gerbils (Cooper et al. 1998), *c-fos* induction can be altered in animals kept in twilight as opposed to an artificial lights on and off. Twilight involves rapid changes of light intensity, spectral and polarization properties, and it has been shown to enhance the synchronisation of circadian rhythms in rodents (Boulos, Macchi, & Terman 1996), arthropods (Fleissner & Fleissner 2003), and humans (Danilenko et al. 2000). Therefore, a further study could assess the effect of twilight conditions on the regulation of the zebrafish circadian system.

8.1.3 Developmental changes in circadian regulation

In chapter 6 zebrafish larvae were used to investigate the importance of the classical photosensitive structures, the eyes and pineal, due to the availability of mutants lacking these structures. The results revealed that light entrainment and circadian rhythmicity could persist without these tissues. Moreover, the results also revealed developmental differences in the regulation of circadian rhythms in zebrafish. CISH reveals for the first time that *per3* is expressed in all larvae brain regions, and qPCR indicates *c-fos* shows a more limited response

to changing light conditions. Furthermore, opsin expression was rhythmic in larvae but not in adult.

However, there were some similarities between adult and larvae. The phase of *per3* expression, with a peak at ZT3, was consistent. This agrees with studies of locomotor activity that show that high activity during the day is conserved from larvae (Cahill, Hurd, & Batchelor 1998) to adult (Hurd et al. 1998).

8.1.4 Conclusion

In conclusion, this thesis has provided a detailed examination of rhythmic and light sensitive clock gene expression in the zebrafish brain, showing it has the potential to contain numerous neuronal pacemakers. Furthermore evidence has been provided that there are likely to be several photoreceptors involved in the photodetection for the entrainment of the circadian clock. This study has gone some way towards enhancing our understanding of the regulation of circadian rhythms in the zebrafish brain.

Appendix: Spatial gene expression in the adult zebrafish brain

Table 8 describes the spatial mRNA expression of genes studied in this thesis, collated for ease of comparison. Spatial expression is shown in various brain nuclei of adult wild type zebrafish as shown using chromogenic *in situ* hybridization. Zebrafish were kept on a 14:10LD cycle (ZT) or DD (CT). Light pulse (LP) was 30-min for *c-fos*, or 3-hour for *cry1a* and *per2*.

[n.d., not determined; -, no detectable signal; +, signal found in some cells in the region; ++, signal found in many cells in the region]

Zebrafish brain nuclei	Neuronal activity				Circadian genes						Opsin			
	C-fos				Light Responsive (CT22)				Rhythmic		ZT3			
	ZT3 (L)	ZT15 (D)	ZT21 (D)	ZT21 (LP)	*Cry1a* (D)	*Cry1a* (LP)	*Per2* (D)	*Per2* (LP)	*Per3* (ZT15)	*Per3* (ZT3)	*Exorh*	*Rh1.1*	*Rgr1*	*Rgr2*
Telencephalon														
OB, Olfactory Bulbs														
ICL, internal cellular layer	-	++	-	-	-	-	-	++	-	++	-	-	-	-
ECL, external cellular layer	-	-	-	++	-	++	+	+	-	++	-	-	-	-
GL, glomerular layer	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D, Dorsal telencephalic area														
Dc, central zone of D	-	-	-	+	-	-	-	+	-	-	-	-	+	-
Dd, dorsal zone of D	-	-	-	+	-	-	-	-	-	++	-	-	+	-
Dl, lateral zone of D	-	++	-	+	+	+	+	++	-	+	-	-	+	-
Dm, medial zone of D	-	++	+	+	+	+	+	++	-	-	-	-	++	-
Dp, posterior zone of D	-	-	-	-	-	++	-	+	-	-	-	-	+	-
V, Ventral telencephalic area														
Cv, commissural nucleus of V	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vd, dorsal nucleus of V	+	++	+	++	-	++	-	++	-	++	-	-	-	-
Vv, ventral nucleus of V	-	++	-	++	-	++	-	++	-	++	-	-	++	-
Vp, postcommissural nucleus of V	+	++	-	++	-	++	-	++	-	++	-	-	+	-
Vs, supracommissural nucleus of V	-	++	-	++	-	++	-	++	-	++	-	-	-	-
Vc, central nuclei of V	-	++	-	++	-	++	-	++	-	++	-	-	-	-
Vl, lateral nuclei of V	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EN, entopeduncular nucleus	-	++	-	+	-	+	-	-	-	++	-	-	-	-
NT, nucleus taeniae	-	++	-	-	-	++	-	-	-	++	-	-	++	-

Zebrafish brain nuclei	Neuronal activity				Circadian genes						Opsin			
	C-fos				Light Responsive (CT22)				Rhythmic		ZT3			
	ZT3 (L)	ZT15 (D)	ZT21 (D)	ZT21 (LP)	*Cry1a* (D)	*Cry1a* (LP)	*Per2* (D)	*Per2* (LP)	*Per3* (ZT15)	*Per3* (ZT3)	*Exorh*	*Rh1.1*	*Rgr1*	*Rgr2*
Diencephalon														
Area preoptica														
CO, optic chiasm	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OT, optic tract	-	-	-	+	-	-	-	-	-	-	-	-	-	-
PPa, parvocellular preoptic nucleus, anterior part	-	++	-	+	-	++	-	++	-	++	-	-	++	-
PPd, dorsal part of the pretectal diencephalic cluster	-	++	-	-	-	++	-	++	-	++	-	-	-	-
PPp, parvocellular preoptic nucleus, posterior part	-	++	-	-	+	++	-	++	-	++	-	-	++	-
SCN, suprachiasmatic nucleus;	-	++	-	+	-	++	-	++	-	++	-	-	++	-
Epithalamus														
Ha, ventral and dorsal habenula	-	++	-	+	-	++	-	++	-	++	-	-	++	-
Pineal	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	++	-	++	-
Thalamus														
VM, ventromedial thalamic nucleus	+	++	-	++	-	++	-	++	-	++	-	-	++	-
VL, ventrolateral thalamic nucleus	-	++	-	++	-	++	-	++	-	++	-	-	++	-
A, anterior thalamic nucleus	-	++	-	++	-	++	-	++	-	++	-	-	++	-
DP, dorsal posterior thalamic nucleus	-	++	-	++	-	++	-	++	-	-	-	-	++	-
CP, central posterior thalamic nucleus	-	++	-	++	-	++	-	++	-	-	-	-	++	-
ZL, zona limitans	-	++	-	-	-	++	-	++	-	++	-	-	++	-

Zebrafish brain nuclei	Neuronal activity				Circadian genes						Opsin			
	C-fos				Light Responsive (CT22)				Rhythmic		ZT3			
	ZT3 (L)	ZT15 (D)	ZT21 (D)	ZT21 (LP)	*Cry1a* (D)	*Cry1a* (LP)	*Per2* (D)	*Per2* (LP)	*Per3* (ZT15)	*Per3* (ZT3)	*Exorh*	*Rh1.1*	*Rgr1*	*Rgr2*
Pretectum														
PS, superficial pretectal nuclei	-	-	-	+	-	-	-	-	-	-	-	-	-	-
CPN, central pretectal nucleus	-	-	-	+	-	-	-	-	-	-	-	-	-	-
APN, accessory pretectal nucleus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PO, posterior pretectal nucleus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DAO/VAO, accessory optic nuclei	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PP, periventricular pretectal nucleus	-	-	-	+	-	+	-	+	-	-	-	-	++	-
Posterior tuberculum														
Tpp, periventricular nucleus of the posterior tuberculum	-	++		+	-	++	-	-	-	-	-	-	++	-
TLa, torus lateralis	-	-	-	-	-	-	-	-	-	++	-	-	-	-
CM, corpus mammilare	-	++	-	+	-	++	-	-	+	++	-	-	-	-
PTN, posterior tuberal nucleus	-	++	-	++	-	++	-	++	-	++	-	-	++	-
PVO, paraventricular organ	-	++	-	-	-	++	-	++	-	++	-	-	++	-
PGl/a, lateral/ anterior preglomerular nucleus	-	++	-	-	-	++	-	++	-	++	-	-	++	-
PGm, medial preglomerular nucleus	+	++	-	+	-	++	-	++	-	++	-	-	++	-

Zebrafish brain nuclei	Neuronal activity				Circadian genes						Opsin			
	C-fos				Light Responsive (CT22)				Rhythmic		ZT3			
	ZT3 (L)	ZT15 (D)	ZT21 (D)	ZT21 (LP)	*Cry1a* (D)	*Cry1a* (LP)	*Per2* (D)	*Per2* (LP)	*Per3* (ZT15)	*Per3* (ZT3)	*Exorh*	*Rh1.1*	*Rgr1*	*Rgr2*
Hypothalamus														
ATN, anterior tuberal nucleus	-	++	-	-	-	-	-	-	-	++	-	-	+	-
CIL, central nucleus of the inferior lobe	-	-	-	-	-	-	-	++	-	++	-	-	-	-
DIL, diffuse nucleus of the inferior lobe	+	++	+	++	-	-	-	++	-	++	-	-	-	-
Hc, caudal zone of periventricular hypothalamus	-	-	-	++	-	++	-	++	+	++	-	-	-	-
Hd, dorsal zone of periventricular hypothalamus	-	+		+	-	++	+	++	+	++	-	-	++	-
Hv, ventral zone of periventricular hypothalamus	+	++	+	++	-	++	-	++	-	++	-	-	++	-
LH, lateral hypothalamic nucleus	-	-	-	-	-	++	-	++	-	++	-	-	-	-
Superior and inferior colliculi														
TeO, optic tectum	-	-	-	+	-	-	-	+	-	+	-	-	+	-
PGZ, periventricular grey zone	+	++	+	++	-	++	-	++	-	++	-	-	++	-
TL, torus longitudinalis	-	++	-	-	-	++	-	++	-	++	-	-	-	-
TS, torus semicircularis	+	++	-	-	-	-	-	-	-	+	-	-	-	-
LLF, lateral longitudinal fascicle	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MNV, mesencephalic nucleus of trigeminal nucleus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nmlf, nucleus of the medial longitudinal fascicle	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PCN, paracommissural nucleus	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Zebrafish brain nuclei	Neuronal activity				Circadian genes						Opsin			
	C-fos				Light Responsive (CT22)				Rhythmic		ZT3			
	ZT3 (L)	ZT15 (D)	ZT21 (D)	ZT21 (LP)	*Cry1a* (D)	*Cry1a* (LP)	*Per2* (D)	*Per2* (LP)	*Per3* (ZT15)	*Per3* (ZT3)	*Exorh*	*Rh1.1*	*Rgr1*	*Rgr2*
Tegmentum														
DTN, dorsal tegmental nucleus	-	++		++	-	-	-	-	-	-	-	-	-	-
EW, Edinger-Westphal nucleus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NLV, nucleus lateralis valvulae	-	+		+	-	-	-	-	-	++	-	-	++	-
NIII, Oculomotor nucleus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NLL, nucleus of lateral lemniscus	-	-	-	-	-	-	-	-	-	-	-	-	++	-
PL, perilemniscal nucleus	-	++	-	-	-	++	-	++	-	++	-	-	-	-
SR, superior raphe nucleus;	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SRF, superior reticular formation	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NIn, nucleus interpeduncularis	-	-	-	-	-	-	-	-	-	-	-	-	++	-
NI, nucleus isthmus	-	+	-	-	-	-	-	-	-	-	-	-	-	-
LC, Locus coeruleus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SGN, secondary gustatory nucleus	-	++	-	-	-	-	-	-	-	-	-	-	-	-
Rhombencephalon														
Cerebellum														
$Val_{gra,}$ granular layer of lateral division of valvula cerebelli	-	++	-	-	-	++	+	++	+	++	-	-	++	-
$Val_{mol,}$ molecular layer of lateral division of valvula cerebelli	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$Vam_{gra,}$ granular layer of medial division of valvula cerebelli	-	++	-	-	-	++	+	++	-	++	-	-	++	-
$Vam_{mol,}$ molecular layer of medial division of valvula cerebelli	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Zebrafish brain nuclei	Neuronal activity				Circadian genes						Opsin			
	C-fos				Light Responsive (CT22)				Rhythmic		ZT3			
	ZT3 (L)	ZT15 (D)	ZT21 (D)	ZT21 (LP)	*Cry1a* (D)	*Cry1a* (LP)	*Per2* (D)	*Per2* (LP)	*Per3* (ZT15)	*Per3* (ZT3)	*Exorh*	*Rh1.1*	*Rgr1*	*Rgr2*
CCe_{gra}, granular layer of corpus cerebellum	-	++	-	-	-	+	-	+	-	++	-	-	+	-
CCe_{mol}, molecular layer of corpus cerebellum	-	-	-	-	-	-	-	-	-	-	-	-	+	-
EG, eminentia granularis	-	++	-	-	-	++	-	++	-	++	-	-	+	-
LCa_{gra}, granular layer of lobus caudalis cerebelli	-	++	-	-	-	++	-	++	-	++	-	-	-	-
LCa_{mol}, molecular layer of lobus caudalis cerebelli	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Medulla oblongata														
brainstem cranial nuclei (except LX)	-	-	-	-	-	-	-	-	-	-	-	-	+	-
LX, vagal lobe	-	-	-	-	-	++	-	++	-	++	-	-	+	-
Gc, griseum centrale	-	-	-	-	-	-	-	-	-	-	-	-	+	-

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