2017

Localization of Zinc in the Circadian System and its Role in the Modulation of Circadian Responses to Light

Moshirpour, Mahtab

UNIVERSITY OF CALGARY

Localization of Zinc in the Circadian System and its Role in the Modulation of Circadian Responses to Light

by

Mahtab Moshirpour

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN PSYCHOLOGY

CALGARY, ALBERTA

JANUARY, 2017

Abstract

Zinc is found in multiple brain areas where it can act as a neuromodulator of postsynaptic receptors. There is evidence for the existence of zinc in the SCN as well as its vesicle transporter (ZnT3) in the retina. However, its presence in other circadian areas has not been examined. This study observed the anatomical distribution of zinc in the SCN and IGL as well as ZnT3 in the retinal ganglion cells that project to these areas. The role of zinc in photic entrainment was also examined. The IGL contained considerable amounts of zinc while ZnT3 was present in retinal cells that are able to project to the IGL. Nevertheless zinc in the IGL was not implicated in the photic entrainment pathway and the retina was shown not to be a necessary zinc input source. Together, these results present the first report of zinc in the IGL and circadian retinal projections.

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Michael Antle for his invaluable mentorship and support throughout my degree. I am very grateful to have had the opportunity to conduct research in his lab which has allowed me to gain knowledge and expertise in the field of circadian rhythms. I also want to thank Dr. Richard Dyck for allowing me to work in his lab, for his assistance on experiments and for serving on my defense committee. In addition, I wish to thank Dr. Vedran Lovic and Dr. Deborah Kurrasch for serving on my defense committee. I am also grateful for the help and guidance of my fellow colleagues, particularly Dr. Victoria Smith, Dr. Glenn Yamakawa, and Sarah Thackray as well as the university veterinarians Stefanie Anderson and Dean Brown. I would like to end by expressing my sincerest gratitude to my mom and dad for their lifelong support, my sister Mojgan for her irreplaceable friendship, love and guidance and my brother Mohammad for his unending support and for always encouraging me to pursue my goals.

To my mom for her incredible strength and tireless support

Table of Contents

List of Figures and Illustrations

List of Symbols, Abbreviations and Nomenclature

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptor

ANOVA: Analysis of variance

AP-3: Adaptor Protein 3

AVP: Arginine vasopressin

Brn3: Brain-specific homeobox/POU domain protein 3

CalB: Calbindin

CalR: Calretinin

CT: Circadian time

DD: Constant darkness

dLGN: Dorsolateral geniculate nucleus

DMSO: Dimethylsulfoxide

DR: Dorsal raphe

EAAT: Excitatory amino acid transporter

ENK: Enkephalin

GABA: γ-aminobutyric acid

GAD: Glutamic acid decarboxylase

GCL: Ganglion cell layer

GHT: Geniculohypothalamic tract

GPR: G-protein coupled receptor

GRP: Gastrin-releasing peptide

IGL: Intergeniculate leaflet

INL: Inner nuclear layer

i.p. : Intraperitoneal

IPL: Inner plexiform layer

ipRGCs: Intrinsically photosensitive retinal ganglion cells

KAR: Kainate receptor

KO: Knockout

MT- Metallothionein

Na_2SeO_3: Sodium Selenite

NMDAR: N-methyl-D-aspartate receptor

NPY: Neuropeptide Y

OPL: Outer plexiform layer

OPN: Olivary pretectal nucleus

PACAP: Pituitary adenylate cyclase-activating polypeptide

PBS: Phosphate buffered saline

PBSx: PBS with 0.1% Triton-X-100

P-ERK: Extracellular signal-regulated kinase I/II

PRC: Phase response curve

PVT: Paraventricular nucleus of the thalamus

RHT: Retinohypothalamic tract

SC: Superior colliculus

SCN: Suprachiasmatic nucleus

SEM: Standard error of the mean

SPZ: Subparaventricular zone

TPEN: N,N,N',N-tetrakis(2-pyridylmethyl) ethylenediamine

VGLUT1: Vesicular glutamate transporter 1

VIP: Vasoactive intestinal polypeptide

vLGN: Ventrolateral geniculate nucleus

VMH: Ventromedial hypothalamus

ZI: Zona Incerta

ZIP: ZRT/IRT-like proteins

$ZnCl_{2:}$ Zinc Chloride

ZnT: Zinc transporter

ZnT3: Zinc transporter 3

ZT: Zeitgeber time

Chapter One: General Introduction

1.1 Introduction

The essential transition element zinc (Zn^{2+}) is found throughout the brain and serves a plethora of functions, some of which are yet to be fully explored. As an important neuromodulator, synaptic zinc is known to exert its effects at the postsynaptic terminal by interacting with a diverse array of channels and receptors. Though the presence of zinc in certain brain areas such as the forebrain and its corresponding function in learning and memory have been the subject of much investigation, its role in other regions remains unclear. One such area is in the circadian system, the body's timekeeping mechanism. The presence of zinc in the circadian system suggests a role for the cation in the modulation of circadian rhythms. This study explored the role of synaptic zinc in the circadian system by investigating its presence in the circadian network, its source of input and its role in clock modulation. This chapter will begin with a review of zinc in the central nervous system, followed by a discussion of rhythms, culminating with current evidence of zinc in the circadian system as well as study overview and objectives.

1.2 Zinc Background

The divalent cation zinc is among the many essential elements found in biological tissue. Although zinc is not the most abundant element found in nature, it serves as one of the most widely used metals and is vital to the proper functioning of an organism (Vallee & Falchuk, 1993). As an essential mineral, zinc is found in many different food groups from meats and poultry to whole wheats and dairy products and can also be taken as a mineral supplement (Krebs, 2013). Zinc is mostly found in bone and muscle tissue while it is also abundant in the pancreas and brain (Smidt & Rungby, 2012). The majority of zinc serves a structural and catalytic function as a part of protein motifs and metalloenzymes (Palmiter, Cole, Quaife, &

Findley, 1996). In fact, zinc is found in all the different enzyme classes and participates as a cofactor in hundreds of different catalytic reactions. The abundance of zinc in the body and its participation in a wide array of chemical reactions is due in part to its electron configuration which allows it to form stable associations with enzymes and other protein molecules (Smidt & Rungby, 2012).

1.3 Zinc in the Central Nervous System

Less than 5% of zinc exists as loosely bound or histochemically stainable zinc mostly found in the presynaptic vesicles of a network of zinc-secreting neurons in the cerebral cortex (Frederickson, Suh, Silva, Frederickson, & Thompson, 2000). Zinc is most abundantly found in forebrain neuronal networks, most notably hippocampal mossy fibers, and elsewhere in the neocortex, striatum, olfactory bulbs and the amygdala (Toth, 2011). Efferent zinc fibers have been observed almost exclusively in cerebral output centers including the striatum, limbic system and the medial hypothalamus (Christensen & Frederickson, 1998; Howell, Perez-Clausell, & Frederickson, 1991; Long, Hardwick, & Frederickson, 1995). Zinc can act as a co-transmitter and neuromodulator at postsynaptic terminals (Palmiter et al., 1996; Smidt & Rungby, 2012). Given the abundance of zinc in the hippocampus, the cation is known to a play an important role in learning and memory through its effects on receptors mediating synaptic plasticity (Pan et al., 2011; Xie & Smart, 1994). Nonetheless, vesicular zinc interacts with a whole host of receptor molecules and its exact function is not fully understood. As a hydrophilic cation, zinc cannot pass across the cell membrane on its own and it relies on channel-mediated flux across the membrane and active uptake into vesicles and target cells (Redenti & Chappell, 2004).

1.3.1 Zinc homeostasis

Zinc that is taken up in the cell body is packaged into vesicles for release at the presynaptic terminal. At any time, the amount of cellular zinc is in a constant state of flux and its

availability is influenced by several factors including facilitated diffusion through membrane channels, active transport into the cell and export from the cell, and intracellular binding to cellular ligands.

1.3.1.1 Facilitated diffusion

Passive flux across the cell membrane occurs when levels of extracellular zinc are high such as when presynaptic terminals release their vesicular zinc stores (Frederickson et al., 2000). As the amount of extracellular zinc increases, more is free to diffuse across the membrane. This flux occurs across ion-gated channels including voltage-gated calcium channels, as well as ionotropic molecules such as N-methyl-D-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptors (AMPARS; Yin, Ha, Carriedo, & Weiss, 1998). These channels will be explained later in this thesis when discussing the effect of zinc on the postsynaptic neuron.

1.3.1.2 Zinc transporters

The amount of freely available zinc in neurons is tightly controlled by various transporters present in the cell and vesicle membranes (Frederickson et al., 2000). Zinc homeostasis is preserved through two main classes of transporters: The family of ZRT/IRT-like proteins (ZIP) and the family of ZnT proteins (Eide, 2000; Grotz et al., 1998). ZIP transporters promote an influx of extracellular zinc into cells while ZnT transporters decrease intracellular zinc and bring about an efflux from cells into organelles and vesicles for transport (Liuzzi & Cousins, 2004). In 1996, research on transporters led to the discovery of a vesicle-specific ZnT transporter, ZnT3 in murine tissue with the gene coding for the transmembrane protein sharing 87% homology with the human gene (Palmiter et al., 1996). At the time, ZnT3 was localized specifically to the testes and the brain, most notably in the cerebral cortex and hippocampus.

However its presence has now been documented in a range of cell populations including adipose cells, beta cells of the pancreas (Smidt et al., 2009) and retinal cells (Redenti & Chappell, 2004). In the brain, ZnT3 is responsible for the tight control of the pool of synaptic zinc and is required for its transport into vesicles for later release from presynaptic terminals (Salazar, Craige, Love, Kalman, & Faundez, 2005). The importance of ZnT3 for the transport of zinc has been documented in different animal models. Knockout (KO) mice that do not have the ZnT3 gene are not able to store and accumulate histochemical zinc in presynaptic vesicles and therefore do not show any synaptic zinc staining (Frederickson et al., 2000). Similarly, a strain of mutant mice referred to as AP-3-deficient or *mocha* mice, lacking a specific protein (adaptor protein 3) responsible for embedding the ZnT3 transporter in vesicles are unable to properly assemble vesicles and sequester zinc in neuronal terminals (Kantheti et al., 1998).

1.3.1.3 Metallothioneins

The intracellular binding of zinc can occur when molecules adhere to zinc and decrease its concentration in the cell. A group of small cellular proteins called metallothioneins (MTs) are capable of binding to metals such as zinc through their cysteine residues (Aschner et al., 1997). MTs can store free zinc, thereby decreasing cellular concentrations and can also act as zinc donors through rapid release or exchange with other proteins. However, MTs do not directly affect the transfer and storage of zinc in vesicles as do the main family of transporters previously discussed. Mice lacking the MT-3 gene coding for a metallothionein subtype expressed in the brain are still able to sequester zinc in vesicles (Erickson, Hollopeter, Thomas, Froelick, & Palmiter, 1997).

1.3.2 Synaptic zinc release

The release of zinc from presynaptic terminals is calcium-dependent and occurs via vesicular exocytosis (Howell, Welch, & Frederickson, 1984). Zinc is stored and released alongside the amino acid neurotransmitter glutamate. ZnT3 and vesicular glutamate transporter 1 (VGLUT1) have been shown to be co-targeted to the same vesicle populations and as such are responsible for the transport of both zinc and glutamate into the same vesicles (Salazar et al., 2005). In fact, most zinc-containing neurons observed in the brain have been shown to be glutamatergic (Frederickson, 1989). The amount of zinc released from vesicles is also dependent on the rate of action potentials. At higher rates of firing, the amount of zinc released increases in a non-linear fashion while a single depolarization event releases a unit quantity (Frederickson, 1989). Under certain circumstances such as excitotoxic injury, the total amount of zinc in vesicles can be released into the synaptic cleft resulting in greater than normal extracellular zinc concentrations (Frederickson, Hernandez, Goik, Morton, & McGinty, 1988).

After being released from presynaptic boutons, zinc diffuses across the synaptic cleft and makes its way towards postsynaptic neurons. The removal of zinc from the synaptic cleft must take place rapidly and can occur via diffusion into the extracellular fluid (Frederickson, 1989). There, various ligands can bind to zinc similar to the phenomenon seen inside the cell. This ensures that free zinc levels are kept at a consistently low level (Frederickson, 1989; Frederickson et al., 2000). Zinc can also be removed from the synaptic cleft through its reuptake by presynaptic boutons. This occurs due to the difference in concentrations of zinc between the synaptic cleft and the cytosol. Lower concentrations of zinc in the cell due to sequestering of the cation in vesicles can drive its influx from the outside (Frederickson et al., 2000). More specific

to its function, zinc can also exert its effects as a neuromodulator by acting on membrane proteins.

1.3.3 Zinc action at the postsynaptic terminal

Zinc has been shown to interact with a whole host of postsynaptic membrane proteins including voltage-gated ion channels, several ionotropic receptors for glutamate such as NMDARs, AMPARs, kainate receptors (KARs), metabotropic glutamate receptors, as well as GABAergic, glycinergic, serotonergic, and purinergic receptors, among others (Frederickson, Koh, & Bush, 2005). In addition, there are currently two known zinc-specific G-protein coupled receptors (GPR): GPR39 and GPR83 (Holst et al., 2007; Muller et al., 2013). Zinc can influence local receptors or those farther away through diffusion and its effects have been shown not only on neurons, but also glial cells (Spiridon, Kamm, Billups, Mobbs, & Attwell, 1998). Neuromodulation can depend on not only the type of receptor but also the concentration of available zinc, such that it can lead to receptor potentiation or inhibition (Nakashima & Dyck, 2009). Zinc's action at glutamate receptors will be discussed in the following section, given the importance of glutamate, both as the dominant zinc neurotransmitter and the major neurotransmitter of the circadian retinal pathway (Ebling, 1996).

1.3.3.1 Zinc and glutamate

The neurons that store and release zinc represent a subset of glutamatergic neurons and are referred to as gluzincergic due to the colocalization of zinc and glutamate within the same vesicles (Frederickson, 1989). Zinc aids in the storage of glutamate and is also involved in stabilizing and enhancing glutamate release (Palmiter et al., 1996). More importantly however, zinc is involved in modulating the activity of NMDARs for glutamate (Westbrook & Mayer, 1987). There are dual mechanisms by which zinc exerts its effects at the glutamate ionophore:

voltage-independent inhibition and voltage-dependent inhibition (Paoletti, Vergnano, Barbour, & Casado, 2009). The mechanism at play depends on the concentration of zinc and involves different NMDA subunits. The NMDAR molecule consists of two main subunit families, the N1 and N2 subunits (Paoletti & Neyton, 2007). Zinc binding of an allosteric site on the NR2A subunit takes place at extremely low concentrations and is therefore highly sensitive (Paoletti, Ascher, & Neyton, 1997). At resting levels, the zinc site on NR2A is occupied, resulting in inhibition of the receptor. Removal of zinc has been shown to potentiate postsynaptic responses (Paoletti et al., 1997). Voltage-dependent inhibition of NMDARs takes place at higher zinc concentrations and occurs via the cation directly blocking and inhibiting the receptor's activity (Paoletti et al., 1997). On the other hand, zinc can exert an inhibitory effect on excitatory amino acid transporters (EAAT) that transport glutamate into neurons, therefore causing an increase in the amount of glutamate and the subsequent potentiation of NMDARs and other glutamatergic receptors (Vandenberg, Mitrovic, & Johnston, 1998). The importance of NMDARs to learning and memory given their involvement in underlying mechanisms of long term potentiation have been well documented and zinc's role as an NMDAR modulator highlights its significance to mechanisms of synaptic plasticity (Pan et al., 2011; Xie & Smart, 1994).

Less is known about the impact of zinc at other glutamate receptors, namely, AMPARs and KARs. Similar to NMDAR modulation, zinc concentrations can influence the type of effect that the cation exerts on AMPARs but conflicting reports have been published. Zinc modulation can potentiate AMPAR activity at lower concentrations and can inhibit activity at higher concentrations (Mayer, Vyklicky, & Westbrook, 1989; Rassendren, Lory, Pin, & Nargeot, 1990). However there have also been reports of a lack of an effect on AMPARs in various cortical areas (Dreixler & Leonard, 1994). As is the case with NMDARs, zinc can exert its influence at

different AMPAR subunits. Sparse but similar findings have been reported for KARs. More specifically, zinc has been reported to both potentiate and inhibit KARs and seems to be concentration-dependent and specific to different subunits (Mayer et al., 1989).

1.3.4 Zinc neuropathology

The importance of zinc in neuropathology has been documented and the cation has been implicated in several neurological disorders such as Alzheimer's disease and stroke (Frederickson et al., 2000). For instance, the rapid and sudden release of zinc from presynaptic boutons has been shown to take place during a traumatic head injury or ischemic stroke. The accumulation of free zinc levels is toxic to cells and can lead to neuronal injury and death (Choi, 1998). Furthermore, excess zinc in the extracellular fluid has been shown to contribute to the accumulation of amyloid-β senile plaques while zinc chelation can prevent this from taking place (Bush et al., 1994; Regland et al., 2001). Aberrant changes in zinc metalloproteins including MTs have also been known to give rise to symptoms relating to amyotrophic lateral sclerosis (Smith & Lee, 2007; Smitt et al., 1994). As well, ZnT3 KO mice lacking synaptic zinc show a lower susceptibility to seizures involving GABAergic receptors but greater susceptibility to kainic acid-induced seizures (Cole, Robbins, Wenzel, Schwartzkroin, & Palmiter, 2000). The involvement of zinc in several acute and chronic diseases highlights the importance of maintaining zinc homeostasis.

1.4 Circadian Rhythms

Daily fluctuations in environmental conditions such as the external light-dark cycle, brought about by the earth's rotation around its own axis and the constant changes in sunlight availability present a challenge to living organisms who must anticipate and adapt to changes. In response to their fluctuating environmental surroundings, all organisms have evolved endogenous timekeeping systems known as circadian rhythms (Dunlap, Loros, & Decoursey,

2004). Evolutionarily, it is advantageous for an organism to be able to couple this endogenous cycle to that of the external environment in order to predict and anticipate changing conditions. This helps to increase the chances of survival by scheduling behaviors such as feeding and sleep around the most appropriate time points, increasing the likelihood of an organism persisting long enough to reproduce and pass along its genes to the next generation (Dunlap et al., 2004).

Circadian rhythms (Latin for *Circa* "around" and *Diem* "day") are endogenous oscillations with a length of about a day and have been observed in virtually all organisms, including both eukaryotes and prokaryotes (Dunlap et al., 2004; Kondo et al., 1993). It has been proposed that early on in evolutionary history, metazoans evolved means of anticipating changing levels of sunlight, allowing them to protect their genetic material against the damaging effects of ultraviolet radiation by traveling deeper in the ocean during the day and coming back up at nighttime (Gehring & Rosbash, 2003). The length of each endogenous cycle is referred to as the intrinsic circadian period and follows close to 24 hours. However, there are species-specific differences in period length both in humans and other animals, with each having a species-specific mean ranging from slightly below to slightly above 24 hours (Pittendrigh & Daan, 1976). For synchronization to take place, the endogenous cycle must follow the exact environmental cycle length and therefore needs to either be delayed or advanced. For instance, an organism with a period of 23 hours needs a daily delay of 1 hour in order to keep up with the 24 hour external cycle.

1.4.1 Central pacemaker

Although the endogenous cycle can mimic the external cycle, it is also capable of maintaining itself in the absence of any outside cues such as in constant conditions. Under these circumstances, an organism's internal cycle maintains its own rhythmicity and is therefore said

to be free-running (Holzberg & Albrecht, 2003). Therefore this rhythmicity can operate independent from any outside influences and depends on some biological timekeeping system driven by an internal clock. Research in this area led to the discovery of a central pacemaker in the mammalian hypothalamus. Lesioning of a pair of nuclei in the anterior hypothalamus called the suprachiasmatic nuclei (SCN) eliminated sleep-wake cycles, drinking and locomotor activity (Ibuka & Kawamura, 1975; Stephan & Zucker, 1972). More evidence in favor of the SCN as the main clock came from the discovery of a single-gene mutation in a Syrian hamster animal model called the tau mutant hamster (Ralph & Menaker, 1988). In these animals, the circadian period is shortened from the usual 24 hours to approximately 22 hours in heterozygous and as short as 20 hours in homozygous animals (Ralph & Menaker, 1988). It was shown shortly after this discovery, that transplanted SCN from animals with normal circadian periods was sufficient to restore the period in the tau mutants to that of the donor animals (Ralph & Menaker, 1990). In other words, the mutants were able to attain 24-hour periodicity by virtue of the transplant. This result provided strong support for the role of the SCN as the central pacemaker.

1.4.1.1 SCN heterogeneity

The SCN are a pair of nuclei consisting of about 20,000 individually rhythmic cells (Reppert & Weaver, 2001). SCN cells express a range of different phases and periods and there is heterogeneity of function among cell populations (Antle & Silver, 2005; Welsh, Logothetis, Meister, & Reppert, 1995). Each SCN nucleus can be anatomically differentiated on the basis of a distinct dorsomedial shell of individually rhythmic cells driven by an endogenous autoregulatory transcription-translation loop of clock genes and a ventrolateral core composed of cells receiving retinal input (Antle & Silver, 2005; Holzberg & Albrecht, 2003). Cells in the core do not inherently express clock genes but rather act to relay the incoming photic information

from the retina to the rhythmic cell population residing in the shell (Antle & Silver, 2005). Photic entrainment takes place when non-rhythmic cells input the light signal to the rhythmic cells, causing a synchronization of the different phases and the output of a unified overt rhythm.

Cells in the core and shell are phenotypically distinct, with different cells expressing a variety of peptides. Generally, intrinsically rhythmic cells of the shell express arginine vasopressin (AVP) while cells in the core express vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP; Moore, 1996). This pattern is seen in different species including rodents and humans. However, there are a great deal of peptidergic cell types in the SCN, some of which are not yet fully understood, and differences exist between species (Morin & Allen, 2006). For instance, the hamster SCN core is further characterized by calbindin (CalB), and extracellular signal-regulated kinase I/II (P-ERK) while the mouse core also expresses CalB, calretinin (CalR) and γ-aminobutyric acid (GABA), among others (Abrahamson & Moore, 2001; Antle & Silver, 2005). The core region receives afferent input from the retina as well as other brain areas and communicates with the shell to synchronize the different cell oscillators.

1.4.2 Projections to the SCN

There are three main input pathways to the SCN. Photic information reaching the retina is relayed to the SCN via a direct axonal pathway known as the retinohypothalamic tract (RHT; Figure 1.1; Morse & Sassone-Corsi, 2002). The retinal cells that project to the SCN do so through the release of glutamate which acts on NMDARs present in SCN cells and eventually leads to the coordination of the clock's rhythm (Ebling, 1996; Morse & Sassone-Corsi, 2002). In addition, these cells contain pituitary adenylate cyclase-activating polypeptide (PACAP) which can also be involved in the photic pathway (Nielsen, Hannibal, Knudsen, & Fahrenkrug, 2001). The SCN also receives photic information indirectly from the intergeniculate leaflet (IGL) via

the geniculohypothalamic tract (GHT; Figure 1.1; Moore, 1996; Moore & Card, 1994). A third input pathway to the clock is the serotonergic projections from the raphe nuclei (Meyer-Bernstein & Morin, 1996). The first two pathways will be further discussed due to their relevance to this thesis.

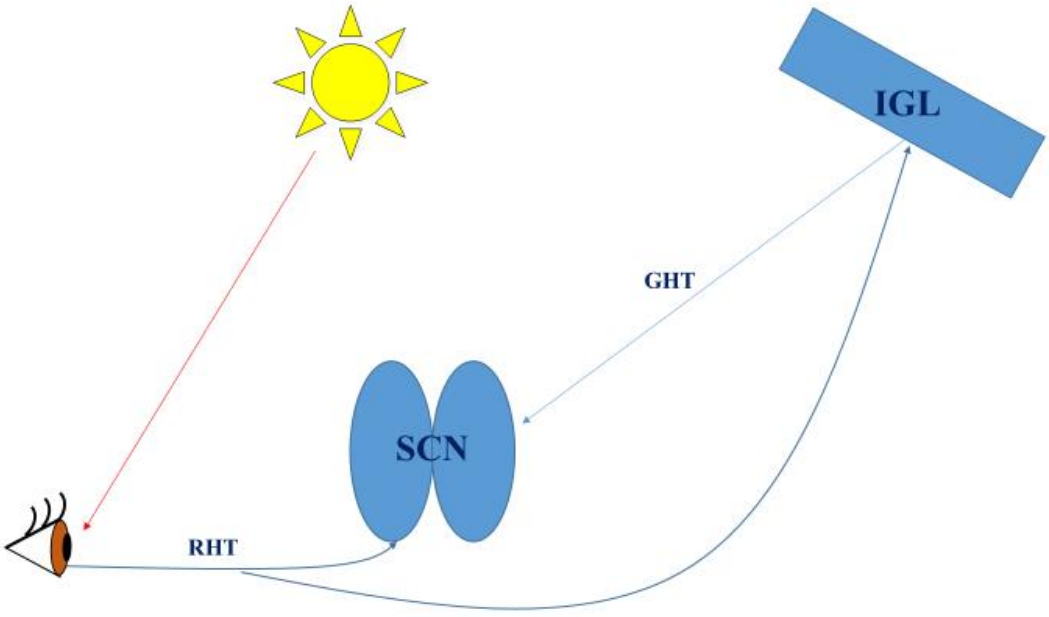

Figure 1.1. Schematic of the network underlying circadian responses to light.

1.4.3 The retinohypothalamic tract and photic entrainment

Entrainment or the rhythmic synchronization of the endogenous cycle from its free-running rhythm occurs via an external entraining cue or zeitgeber (Dunlap et al., 2004). Light is

the most common external cue that is capable of shifting the internal clock and synchronizing it to the external rhythm (Hirota & Fukada, 2004). However although the focus will from here on be placed on photic entrainment, it is important to note that there are a whole range of other zeitgebers such as temperature, feeding, exercise, and sleep deprivation, referred to as non-photic stimuli (Mistlberger & Antle, 2011). Photic input to the clock remains the dominant entraining agent among terrestrial organisms however and takes place when light enters the eye and is picked up and relayed to the SCN (Hirota & Fukada, 2004). Photic stimuli delivered during the subjective night (when an animal held in constant lighting conditions behaves as if it were night) can alter the phase of free-running circadian rhythms (Pittendrigh & Daan, 1976). On the other hand, light delivered during an animal's subjective day usually does not have an effect on shifting the timing of rhythms (Prosser, McArthur, & Gillette, 1989). A brief pulse of light in early subjective night causes a phase delay (the animal has a delayed activity onset on the subsequent day) while light delivered at late subjective night causes a phase advance (the animal has an earlier activity onset). The pattern of shifts in phase of rhythms in response to light can be plotted producing a photic phase response curve (PRC). Although there are some between-species differences, the shape of the photic PRC is quite stable and can provide information regarding the magnitude of phase shifts delivered during various times in the cycle.

1.4.3.1 Role of the retina in photic entrainment

When light enters the eye, it is first picked up and relayed to the SCN by a subset of SCN-projecting photoreceptors in the ganglion cell layer of the retina known as intrinsically photosensitive retinal ganglion cells (ipRGCs; Berson, Dunn, & Takao, 2002; Moore, Speh, & Card, 1995). These special photoreceptors represent only a very small portion (2-3%) of ganglion cells in the mammalian retina and are unique from the rods and cones that are primarily

involved in vision (Berson et al., 2002; Hattar, Liao, Takao, Berson, & Yau, 2002). Photic entrainment has been shown to persist in functionally blind mice lacking rods and cones, thereby illustrating the independence of the retinal entrainment pathway (Freedman et al., 1999). Furthermore, in response to light, ipRGCs are capable of depolarizing without any synaptic input from rods and cones (Berson et al., 2002). These cells stain positive for an opsin-derived photopigment known as melanopsin which was recognized as the pigment that gives the photoreceptors their photosensitive nature (Hattar et al., 2002; Provencio et al., 2000). Melanopsin was originally discovered in the skin melanophores of a species of frog, *Xenopus laevis,* where it was demonstrated that the photopigment was responsible for cell responsiveness to light (Provencio, Jiang, Grip, Hayes, & Rollag, 1998). Melanopsin messenger RNA was also found to be expressed in the SCN and eye of the same species. The photopigment was later localized to the mammalian retinal ganglion cell layer (Provencio et al., 2000). Further evidence in support of melanopsin as the major circadian photoreceptor as opposed to other photopigments was provided by Lucas et al. (2003) showing a loss of intrinsic photosensitivity in RGCs of melanopsin-KO mice.

Melanopsin-positive ipRGCs are characterized by a large soma and branching dendrites that extend out to the inner plexiform layer (IPL) of the retina (Hattar et al., 2002). The dendrites are recognizable by swellings across their length and extend out from neighboring cells creating a network of projections. Research on ipRGCs has unveiled considerable diversity in cell morphology, function and projections. Currently, at least five different subtypes have been identified that are named the M1-M5 cells (Ecker et al., 2010). The SCN is predominately innervated by the M1 cells, although M2 cell projections to the clock have also been identified (Baver, Pickard, Sollars, & Pickard, 2008). M1 cells are characterized by smaller cell bodies,

comparatively higher melanopsin levels, and dendritic arborization into the outer sublayer of the IPL (Ecker et al., 2010; Jain, Ravindran, & Dhingra, 2012). M1 cells also send projections to other areas including the IGL and the olivary pretectal nucleus (OPN), an area implicated in the pupillary light reflex (Lucas, Douglas, & Foster, 2001).

On the other hand, the non-M1 subtypes generally have projections that extend to the inner IPL sublayer (Hattar et al., 2002). The M3 subtype however is an exception in that it is characterized by cells with dendrites that stratify in both the inner and outer sublayers (Jain et al., 2012). M2 cells also project to the circadian system but to a lesser extent than the M1 subtype and show a decreased response to light owing to their lower melanopsin levels (Berson, Castrucci, & Provencio, 2010; Ecker et al., 2010). The M4 and M5 subtypes have been more recently described and are lower in melanopsin levels. Given their lower intrinsic photosensitivity, the non-M1 ipRGCs project to brain areas important for non-circadian functions such as vision, including the dorsolateral geniculate nucleus (dLGN) and the superior colliculus (SC; Ecker et al., 2010).

1.4.4. Intergeniculate leaflet

Photic information is indirectly relayed to the SCN via a secondary afferent pathway arising from the intergeniculate leaflet (IGL; Pickard, 1982). The IGL is a long bilateral component of the lateral geniculate complex that lies in between the dLGN and the ventrolateral geniculate nucleus (vLGN). Although the IGL was once thought to be a part of the vLGN, it is now differentiated as a distinct region and is characterized by a morphologically diverse population of cells (Hickey & Spear, 1976; Moore & Card, 1994). Moore & Card (1994) found dense bilateral axonal terminations of retinal ganglion cells from the optic tract throughout the IGL. However, retinal projections to the contralateral IGL were found to outnumber the

projections to the ipsilateral side. These projections were later identified as originating from melanopsin-containing ipRGCs (Hattar et al., 2002).

The IGL contains a subset of neurons that produce the chemical messenger neuropeptide Y (NPY), the majority of which project to the SCN via the GHT and are believed to be among the many cells that convey photic information received from the eyes to the circadian clock (Card & Moore, 1982; Pickard, Ralph, & Menaker, 1987). In addition, IGL neurons have been shown to express enkephalin (ENK), GABA and glutamic acid decarboxylase (GAD) with the distribution of different cell populations often overlapping (Moore & Card, 1994). Furthermore, both GABAergic and ENK-positive neurons send afferent projections to the SCN (Moore & Card, 1994; Moore, Weis, & Moga, 2000). The IGL also communicates with its contralateral counterpart through ENK-positive neurons (Moore et al., 2000). Other projections to brain areas that play a role in the circadian system and communicate with the SCN include the subparaventricular zone (SPZ) and the paraventricular nucleus of the thalamus (PVT). Subthalamic regions including the zona incerta (ZI), and midbrain areas important for visual functions such as the SC and pretectal area also receive IGL input (Moore et al., 2000). In addition to retinal projections, there are many other afferent projections to the IGL as evidenced by retrograde tracing studies and the presence of fibers that stain positive for a variety of neuromodulators including substance P, serotonin, and dopamine (Moore & Card, 1994). The IGL receives input from the SCN and other important circadian areas, including the ventromedial hypothalamus (VMH), an area implicated in food entrainment, and the dorsal raphe nuclei (DR). Other projections include the ZI, the SC, visual cortex, and pretectal nuclei. Afferent and efferent IGL projections have been explored in rodents, rats in particular, and are mostly preserved across species, despite some differences (Moore et al., 2000; Vrang, Mrosovsky, & Mikkelsen, 2003).

The IGL can be thought of as a backdoor to the circadian system and is involved in mediating the effects of light on circadian activity (Pickard et al., 1987). Nonetheless, the exact function of the GHT pathway remains unclear. The RHT alone seems to be sufficient for photic entrainment and studies looking at the effect of IGL destruction have reported little disruption to entrainment (Dark & Asdourian, 1975). Nonetheless, there is a consensus as to the significance of the IGL to the transmittance and integration of photic information to the master clock (Pickard et al., 1987). IGL lesions in the golden hamster give rise to disrupted information and decreased discriminatory abilities about light wavelength and intensity (Pickard et al., 1987). Furthermore, diminished phase advances in response to light pulses delivered at late subjective night have been observed, with similarly smaller advances in response to dark pulses given to animals housed in constant light (Harrington & Rusak, 1986). Although the IGL does not appear to be necessary for entrainment, lesioned animals take longer to re-entrain to a new light-dark cycle (Johnson, Moore, & Morin, 1989). Lesioning has also been shown to disrupt photic entrainment to a skeleton photoperiod, a lighting schedule that approximates the natural lighting conditions of nocturnal animals with brief light pulses at early and late subjective night (Edelstein & Amir, 1999).

1.5 Zinc in the Circadian System

With the discovery of ZnT3, it was initially thought that the presynaptic terminals of forebrain neurons represented one of only a few areas where the transporter is found. However, it has since been found in many other tissues including adipose, epithelial and pancreatic tissue (Smidt et al., 2009; Smidt et al., 2007; Wongdee, Teerapornpuntakit, Riengrojpitak, Krishnamra, & Charoenphandhu, 2009). More importantly, findings by Redenti & Chappell (2004) localized the transporter to the mouse retina. A band of ZnT3 transporters were visualized in the inner segment, outer plexiform and inner nuclear layers of the retina. In addition, ZnT3 reactivity was

observed in the inner plexiform and ganglion cell layers where melanopsin ganglion cells that play a vital role in the circadian system are also localized (Provencio et al., 2000). Although past studies had reported the presence of zinc in the rat retina with the aid of Timm's silver sulphide staining method (Ugarte & Osborne, 2001), the discovery of a zinc vesicle transporter was the first to propose a role of zinc in neuromodulation through its storage, transport and release from vesicles.

Histochemical zinc has also been directly observed in the rat SCN using the selenium silver staining method (Huang, Peng, & Yau, 1993). More specifically, zinc staining was observed throughout the SCN but was particularly stronger in the ventrolateral region which receives afferent projections including the RHT. The presynaptic terminal staining allowed the visualization of synaptic zinc and demonstrated its localization in vesicles where ZnT3 is also found (Palmiter et al., 1996). Functionally, zinc was shown to potentiate the voltage-gated potassium channels present in individual SCN neurons, even at very low concentrations (Huang et al., 1993). Histochemical zinc has also been observed in the lateral geniculate of young rats and anterograde labeling of axon terminals has shown zinc staining in the retinogeniculate pathway originating in the retina (Land & Shamalla-Hannah, 2001).

1.6 Current Study: Overview and Objectives

The direct observation of histochemical zinc in the SCN raises the possibility that the cation plays a role in the circadian system (Huang et al., 1993). However, it is not clear whether zinc in the SCN is present in glutamatergic neurons. Therefore despite the presence of the cation in the central pacemaker, its role and mechanism of action are not fully understood. Nonetheless, given that zinc is frequently colocalized with glutamate, the presumed neurotransmitter signal of the circadian photic entrainment pathway (Ebling, 1996), zinc can play a role in the modulation of the circadian photic response. Zinc staining in the SCN has been noted to be especially dense

in the ventrolateral core (Huang et al., 1993), which receives afferent projections, including retinal input providing photic information. The aims of the proposed project are to better understand the role of histochemical zinc in the circadian system. More specifically, to visualize zinc in circadian brain areas, to determine its source of input to the circadian system as well as examine the effect of zinc level modification on photic entrainment. To these ends, Chapter 2 will consist of experiments looking at the anatomical distribution of zinc in the SCN and IGL and explores the source of zinc input to the circadian network. It is hypothesized that zinc will be present in the circadian system and that its source of input is most likely retinal. Chapter 3 examines the distribution of the zinc vesicle transporter in the retina. Chapter 4 contains experiments exploring the role of zinc in photic entrainment as a consequence of zinc level modulation in the IGL. It is proposed that zinc acts to enhance the circadian photic response. A general discussion of findings and implications is found in Chapter 5.

Chapter Two: Anatomical Zinc Distribution

2.1 Introduction

Zinc is a transition metal that is found in the body and serves many important functions. Although the cation is not synthesized endogenously, it is an essential component of living organisms and its wide array of functions extends to multiple areas of the body including bone and muscle tissue, the visual system and the central nervous system (Smidt & Rungby, 2012). Most of the zinc present in living tissues is bound to protein molecules including structural proteins as well as enzymes that are involved in vital biological reactions (Palmiter et al., 1996; Vallee & Falchuk, 1993). In this form, zinc is not free to bind to other molecules but instead is an incorporated substituent of the proteins it adheres to. Therefore the actual concentration of free zinc at any time is very small.

Free zinc stores are most commonly found in the central nervous system, where the cation is stored and released from vesicles containing the neurotransmitter glutamate (Frederickson, 1989; Paoletti et al., 2009; Salazar et al., 2005). Zinc is most profusely found in the neuronal networks of the cerebral cortex. However findings by Huang et al. (1993) have also shown the presence of the cation in the mammalian circadian pacemaker, the suprachiasmatic nucleus (SCN), a small paired structure housed in the anterior hypothalamus. This study is among the first and only reports of zinc distribution in the circadian system. Zinc staining was observed throughout the rat SCN but more so in the ventrolateral region which is also where photic information is relayed to the SCN, helping set the clock's rhythm (Huang et al., 1993). This finding is corroborated by reports of histochemical zinc and its vesicle transporter ZnT3 in the mammalian retina (Redenti & Chappell, 2004; Ugarte & Osborne, 2001). This suggests the involvement of the cation in the glutamatergic retinohypothalamic tract (RHT), a projection responsible for providing photic information to the clock (Ebling, 1996). While the method of

zinc staining in the SCN suggests localization in presynaptic terminals surrounding cell bodies (Huang et al., 1993), this result has not been replicated in the lab in the hamster SCN, although more precise sections of the SCN need to be obtained in order to confirm the presence or absence of any zinc (unpublished finding from the labs of Drs. Dyck and Antle).

In order to better understand the role of zinc in the circadian system, it is necessary to stain for the cation in the major circadian brain areas including the SCN and the intergeniculate leaflet (IGL) of the lateral geniculate complex which also receives retinal input and communicates with the clock (Harrington, Nance, & Rusak, 1985; Hattar et al., 2002). Research on zinc in the IGL is absent and therefore it is unclear whether or not this area contains any zinc. The presence zinc in the circadian areas can be examined by means of an autometallographic technique for zinc terminal staining, previously employed by Danscher (1982). This technique starts with an intraperitoneal injection of sodium selenite (Na_2SeO_3). Zinc has a high affinity for selenium and binds to it once it is absorbed in tissue forming a crystal. This process is allowed to take place within approximately an hour following the injection. Zinc-selenium complexes can then be visualized by the extraction of the brain and the development of brain slices in a silver lactate solution with the silver surrounding the complexes and allowing the visualization of the formed crystals (Danscher, 1982).

Given the observation of zinc in the SCN (Huang et al., 1993), past findings in the lab of a lack of zinc staining in this area may be due to changing zinc levels. More specifically, zinc levels in the SCN may follow an oscillatory pattern with peaks and falls at various time periods. To better inquire about the possible circadian nature of zinc levels in both the SCN and IGL, zinc terminal staining can be used to visualize zinc at two specific time points: the middle of the light period (defined as zeitgeber time, ZT6) and the middle of the dark period (ZT18) in hamsters

housed in constant darkness (DD) conditions for two days following housing in a 14:10 light dark cycle. The two-day period in constant darkness will not cause the animal's internal rhythm to drift significantly and can allow sufficient time for zinc to build up if the cation is involved in the circadian photic pathway. Given enough time in the absence of light, more zinc can become transported and sequestered in synaptic vesicles and it will begin to accumulate in the dark. Previous work in the lab employing staining techniques on the murine brain have not attempted to take this into account with brains having been collected under light and so the absence of zinc in the SCN may have been attributed to depletion with light exposure.

The present study looked at zinc distribution in the circadian system in a number of ways. First, the presence of zinc was examined in two important circadian brain areas, the SCN and IGL. Next, zinc was observed at different time points throughout the day in these areas to see if there is a circadian rhythm to zinc levels. Finally, the retinal contribution of zinc to the IGL was investigated. In order to do so, double enucleation surgery for the removal of the eyes in hamsters exposed to the same conditions as described above was conducted. It was proposed that histochemical zinc would be present in the SCN and IGL. As well, it was hypothesized that there would be a significant deficiency in zinc staining in the IGL of enucleated animals as compared to control animals with intact eyes.

2.2 Methods

2.2.1 Experiment 1: Visualization of zinc in the SCN and IGL

2.2.1.1 Animals

A total of 8 male Syrian hamsters (*Mesocricetus auratus*, 80-90g) were obtained from Charles River Laboratories (Kingston, NY, USA). Animals were initially housed in pairs in a temperature and humidity-controlled room with a 14:10 light dark cycle and provided with food and water *ad libitum*. Two weeks prior to the beginning of the experiment, the animals were

transferred to individual cages and kept under the same light dark cycle. Two days prior to receiving injections, all animals were moved to and kept in DD conditions. Four animals were used for each time point (ZT6 and ZT18). All procedures were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and adhered to the policies of the Canadian Council of Animal Care.

2.2.1.2 Sodium selenite injections

Hamsters received an intraperitoneal (i.p.) injection of 15mg/kg of sodium selenite dissolved in molecular grade water in DD conditions with the aid of night vision goggles (BG15Alista, Richmond Hill, Ontario, Canada) at two time points: ZT6 and ZT18 and were then allowed to survive up to a period of 1 hour, during which time they were periodically checked on every 15 minutes. Following the survival period, animals were given an overdose of sodium pentobarbital (~0.7 mL) (CEVA Santé Animale, France) and following the absence of withdrawal reflexes, their heads were covered in tinfoil to prevent light from reaching the eyes and immediately severed from the body.

2.2.1.3 Zinc staining

Brains were extracted and immediately frozen on dry ice and stored at -20°C for up to a week prior to sectioning. Brains were cut with a Leica cryostat set at a temperature of -19°C, at a thickness of 35 microns through the SCN and IGL and thawed/collected directly on gelatin-coated microscope slides stored at -20°C. All SCN and IGL sections separately underwent the staining procedure on consecutive days. The autometallographic zinc staining procedure mostly followed the technique previously employed by Danscher (1982). The tissue was allowed to thaw at room temperature and then rehydrated in an alcohol series (95% EtOH for 15 minutes, 70% for 2 minutes, 50% for 2 minutes, 3x2 minutes in dH2O). Slides were then dipped in gelatin

and transferred to a silver lactate solution for physical development. The developer solution was prepared by mixing the following solutions in order: 50% gum arabic from African Acacia trees (100 ml in 200 ml dH2O), citrate buffer (5.1g citric acid, 4.7 g sodium citrate, 20 ml dH2O), silver lactate (0.22g in 30 ml dH2O), and hydroquinone (1.7g in 20 ml dH2O). Slides were left in the developer anywhere from 120-150 minutes in a dark box and were checked on every 15 minutes. Slides were removed from the solution after the tissue had obtained a dark brown uniform stain. The tissue was then gently washed under running tap water at 37°C for 10 minutes and then in dH2O (2x3 minutes). Slides were then exposed to 5% sodium thiosulfate (10g in 200 ml dH2O) for 12 minutes and washed in dH2O (2x2 minutes). Sections underwent a final alcohol dehydration series, were cleared with xylene and coverslipped with Permount.

2.2.1.4 Quantification of zinc staining at the SCN and IGL

Brightfield images were captured with a digital camera mounted on an Olympus BX51 microscope. All images were captured with the same lighting conditions and exposure time. Densitometry analysis was completed on 16 bit images with the use of ImageJ software (ImageJ 1.42q; National Institutes of Health, Bethesda, MD). Zinc staining was quantified for the SCN by measuring the density of staining in each SCN nuclei and comparing the values to the density in the optic chiasm. The relative optical density (ROD) was calculated by taking the ratio of the SCN to optic chiasm density for the dorsolateral, dorsomedial and ventral regions, at ZT6 and ZT18. Density values were averaged across the SCN nuclei and ROD values were averaged across three consecutive SCN sections. ROD of IGL sections was determined by taking the ratio of staining in the IGL to staining in the dorsolateral geniculate nucleus (dLGN) for the left and right IGL at ZT6 and ZT18. Representative rostral, mid and caudal IGL sections were averaged for the left and right IGL. Statistical significance was set at $p<0.05$ for all tests. Separate two-

tailed independent samples t-tests were conducted to analyze the ROD between the two time points for the dorsomedial, dorsolateral and ventral SCN, and the left and right IGL.

2.2.2 Experiment 2: Testing retinal zinc input to the circadian system

2.2.2.1 Animals

A total of 6 male Syrian hamsters (*Mesocricetus auratus*, 80-90g) were obtained from Charles River Laboratories (Kingston, NY, USA) with 3 serving as controls and 3 animals undergoing enucleation surgery. Animals were initially housed in pairs in a temperature and humidity-controlled room with a 14:10 light dark cycle and provided with food and water *ad libitum.* Experimental animals then underwent double enucleation surgery for removal of the eyes. Following surgery, animals were transferred to individual cages and allowed to recover for a period of one week under the same conditions. Subsequently, animals were given sodium selenite injections, euthanized and their brains collected. All procedures were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and adhered to the policies of the Canadian Council of Animal Care.

2.2.2.2 Enucleation surgeries

All surgeries were performed by the university veterinarian, Dr. Stefanie Anderson. Hamsters were anesthetized with sodium pentobarbital (100-120 mg/kg) and provided with butorphanol as a pre-operative analgesic. The area around the eye was clipped and sterilely prepared for surgery. A local injection of 0.5% bupivacaine was administered subcutaneously into the area immediately lateral to the eye to block the ophthalmic nerve, and a line block was performed over each eyelid. The total dose of bupivacaine did not exceed 8mg/kg. Once the animal was placed in the surgical plane and there was an absence of withdrawal and palpebral reflexes, the globe of the eye was grasped with forceps and gently prolapsed from the orbit. While maintaining tension on the globe, the conjunctiva and extra orbital muscles were severed

near the limbus. The optic nerve was then severed, releasing the globe from the orbit. Hemostasis was provided to minimize bleeding from the retro-orbital sinus into the orbit. Finally, the eyelid margins were debrided with scissors, and sutured together to close the wound. Post-operative analgesics (butorphanol) was administered as needed.

2.2.2.3 Sodium selenite injections

After one week of recovery which also allowed time for the degeneration of remaining retinal projections, all hamsters received an i.p injection of 15mg/kg of sodium selenite dissolved in molecular grade water and were then allowed to survive up to a period of 1 hour, during which time they were periodically checked on every 15 minutes. Following the survival period, animals were given an overdose of sodium pentobarbital (~0.7 ml) (CEVA Santé Animale, France) and were then transcardially perfused with ~50ml of cold phosphate buffered saline (PBS) and subsequently, ~50ml of cold 4% paraformaldehyde in PBS. Although zinc staining is most ideal with fresh brains (experiment 1), it is more difficult to do fluorescent staining with fresh tissue and due to the need to obtain alternate NPY sections (see below), fixed rather than fresh brains were used. Brains were post-fixed at 4°C in 4% paraformaldehyde overnight and then transferred to 20% sucrose in PBS solution for 24 hours. Brains were cut with the use of a Leica cryostat set at a temperature of -19°C, at a thickness of 35 microns and thawed/collected directly on gelatin-coated microscope slides. Sections cut through the SCN and IGL were collected and kept frozen at -20°C before undergoing autometallographic staining for zinc.

2.2.2.4 Zinc staining and quantification

Given sparse zinc staining in the SCN (experiment 1), only IGL sections were collected and used for the analysis since the IGL showed considerable zinc staining. The IGL for all hamsters underwent the staining procedure for zinc on the same day. The autometallographic

zinc staining procedure followed the same protocol employed for experiment 1 (see section 2.2.1.3). Similarly, quantification of staining in the IGL for control and enucleated animals followed the same method used for the ZT6 and ZT18 time points (see section 2.2.1.4).

2.2.2.5 NPY immunohistochemistry

Alternate IGL sections were collected for NPY staining for the purpose of confirming zinc staining in the IGL by matching the region of zinc staining with the delineated area of NPY immunoreactivity (IGL region). Sections were taken out of the freezer and allowed to thaw at room temperature. Slides were soaked in 4% paraformaldehyde for 30 minutes and then exposed to three 10 minute washes in 0.3 M PBS with 0.1% Triton X-100 (PBSx), followed by a 90-minute incubation in blocking buffer (1% normal donkey serum in PBSx). Slides were then incubated for 48 h in the NPY primary antibody (rabbit anti-NPY, #22940, 1:10,000, ImmunoStar, Hudson, WI, USA) diluted in blocking buffer at 4°C. Sections again underwent three 10 minute washes in 0.3% PBSx and then protected from light and incubated for 2 h in the secondary antibody (CY-2 donkey anti-rabbit; 1:200 in PBSx; Jackson ImmunoResearch Laboratories Inc. West Grove, PA, USA). A final set of three 10 minute washes in 0.3% PBSx were performed after which slides were dehydrated in a series of alcohol rinses (70% EtOH to 95% to 100%) and coverslipped with Krystalon.

2.3 Results

2.3.1 Experiment 1: Zinc is present in the SCN and IGL

Quantification of zinc staining in SCN and IGL sections took place on seven brains, with one animal being excluded due to the inability to obtain representative sections. A small amount of zinc staining was observed throughout the SCN, which was more visible in the medial and ventral regions. However, there was no significant difference in zinc levels (as measured by

relative optical density) between the two time points, ZT6 and ZT18, for the dorsomedial ($t(5) = -1.879$, $p>0.05$), dorsolateral ($t(5) = -1.634$, $p>0.05$) or ventral ($t(5) = .875$, $p>0.05$) SCN areas (Figure 2.3 A and B, Figure 2.4). In contrast, a considerable amount of zinc was shown to be present in the IGL. Figure 2.1 shows the presence of zinc staining throughout the IGL and extending lower down in the ventrolateral geniculate nucleus (vLGN). A colored image was obtained to better illustrate the contrast of the zinc stain with the outside areas. Staining was also observed all throughout the IGL sections collected in the rostral to caudal plane (Figure 2.2). However zinc levels did not significantly differ at ZT6 or ZT18 for the left ($t(5) = 1.518$, $p>0.05$) or right ($t(5) = .999$, $p>0.05$) IGL (Figure 2.3 C and D, Figure 2.5).

2.3.2 Experiment 2: Zinc is present in the IGL with or without retinal input

Zinc staining was observed in both the enucleated and control animals and there was no significant difference in zinc staining between the two groups for both the left ($t(4) = 1.741$, $p>0.05$ and right IGL ($t(4) = .477$, $p>0.05$; Figure 2.6 A and B, Figure 2.7). The presence of zinc in the IGL was confirmed by NPY staining (Figure 2.6 C) of consecutive IGL sections.

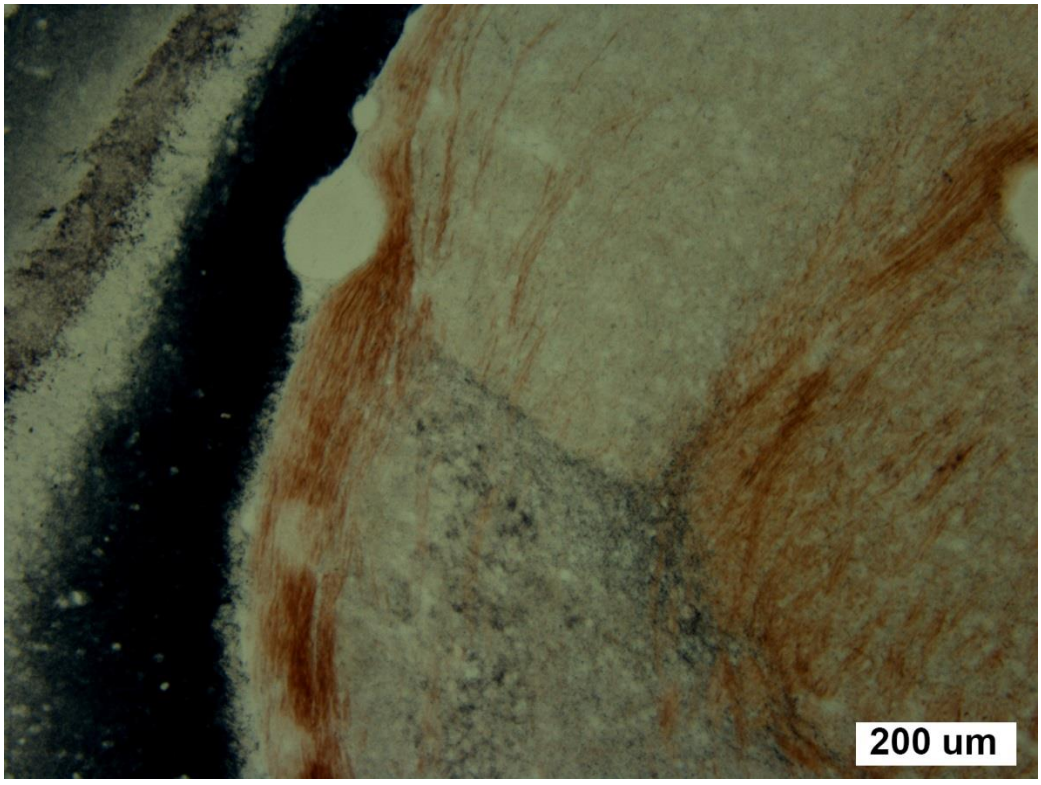

Figure 2.1. Autometallographic staining for histochemical zinc in the left intergeniculate leaflet (IGL; 10x magnification). IGL staining is visible as dark grey coloring that extends all the way down towards the ventrolateral geniculate nucleus (vLGN).

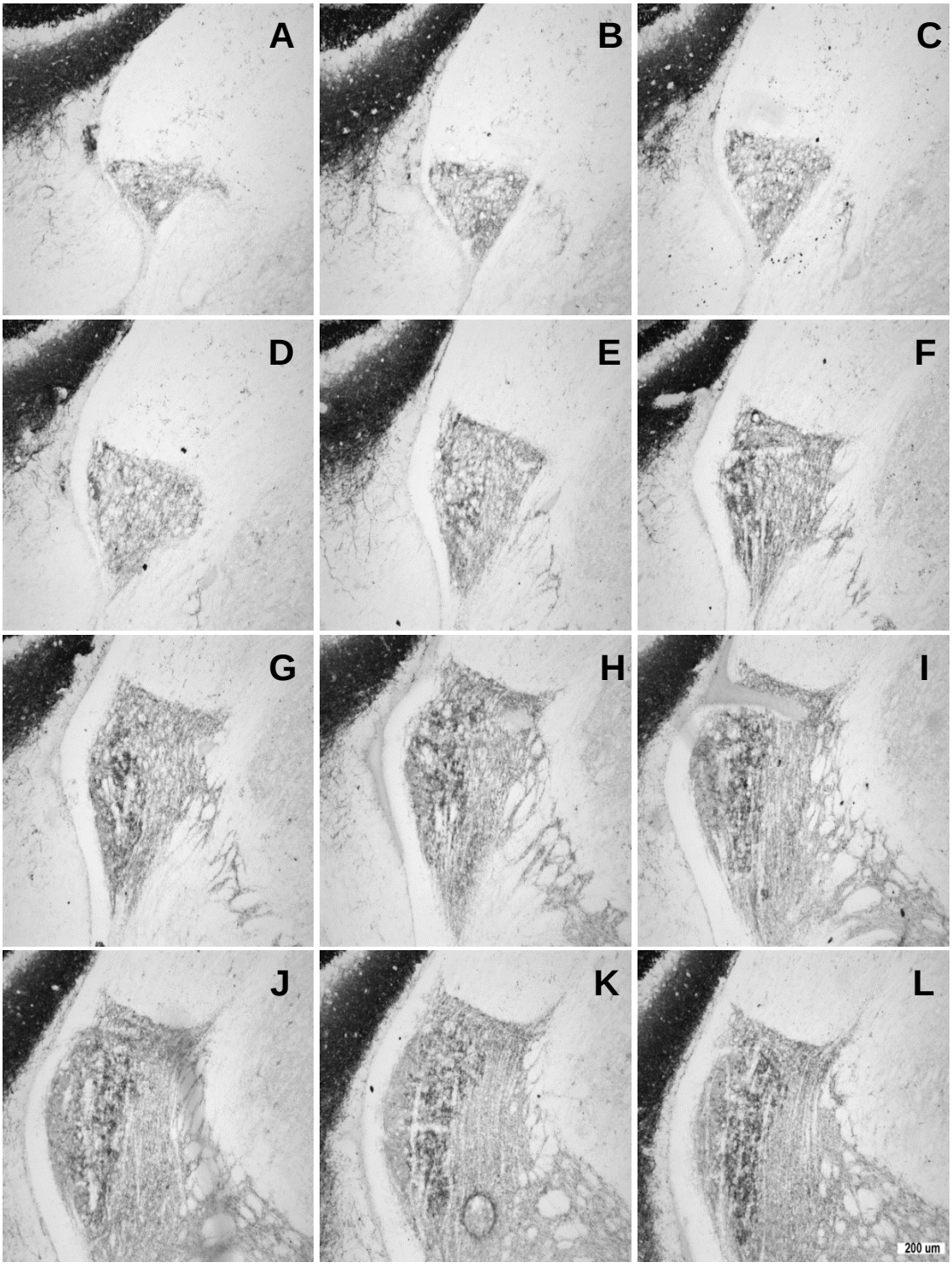

Figure 2.2. Autometallographic staining for histochemical zinc in the left intergeniculate leaflet (IGL). Panelized photomicrographs (10x magnification) of representative hamster coronal slices in a rostral to caudal direction (A to L).

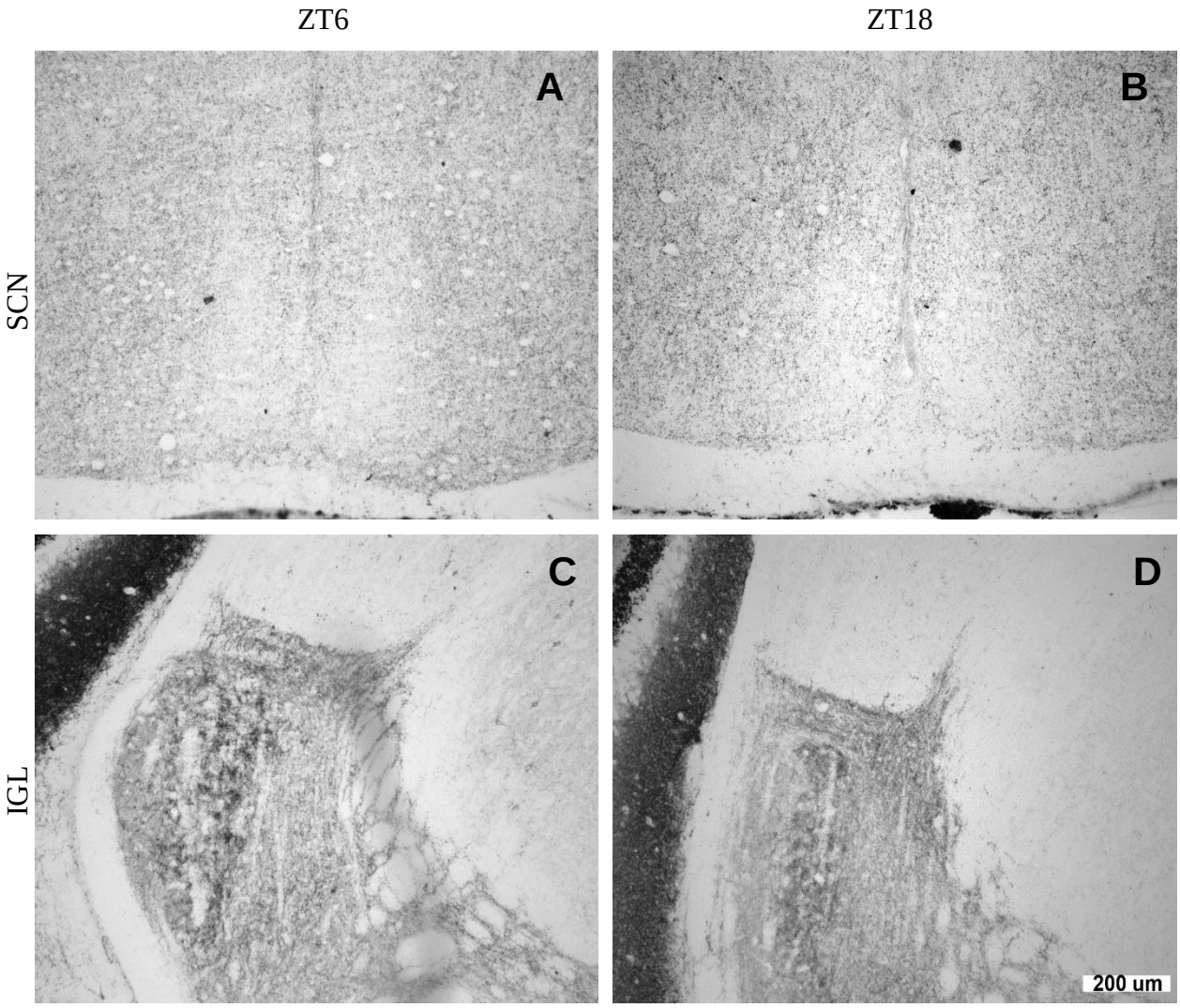

Figure 2.3**.** Autometallographic staining for histochemical zinc in the suprachiasmatic nucleus (SCN) and intergeniculate leaflet (IGL). Photomicrographs (10x magnification) of representative hamster coronal slices showing levels of zinc staining in the SCN at (A) ZT6 and (B) ZT18 and in the IGL at (C) ZT6 and (D) ZT18. No significant differences were found between the time points, $p > 0.05$.

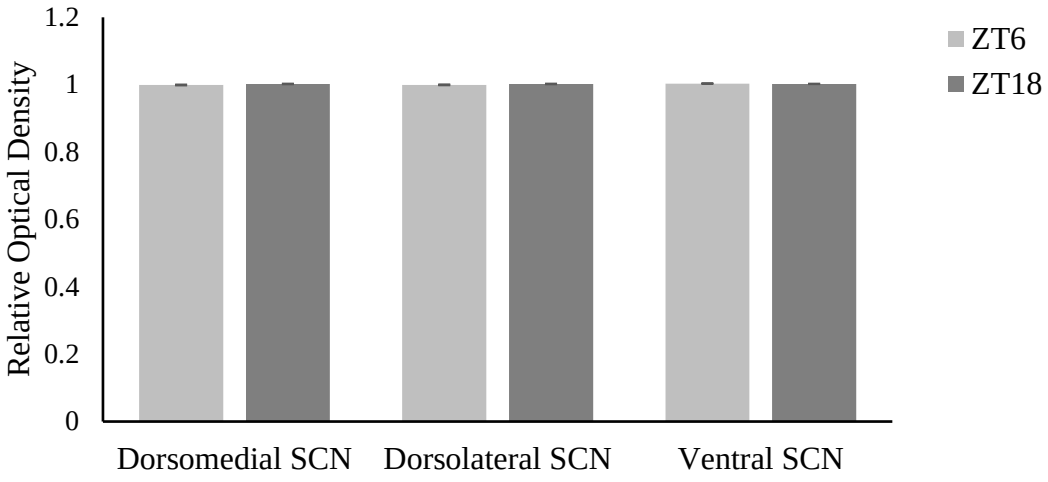

Figure 2.4. Mean relative optical density (±SEM) of the dorsomedial, dorsolateral and ventral SCN regions, at ZT6, and ZT18. Relative optical density (ROD) is measured as the ratio of the level of autometallographic zinc staining of the SCN to staining in the optic chiasm. No significant difference in ROD was found between the two time points, $p>0.05$.

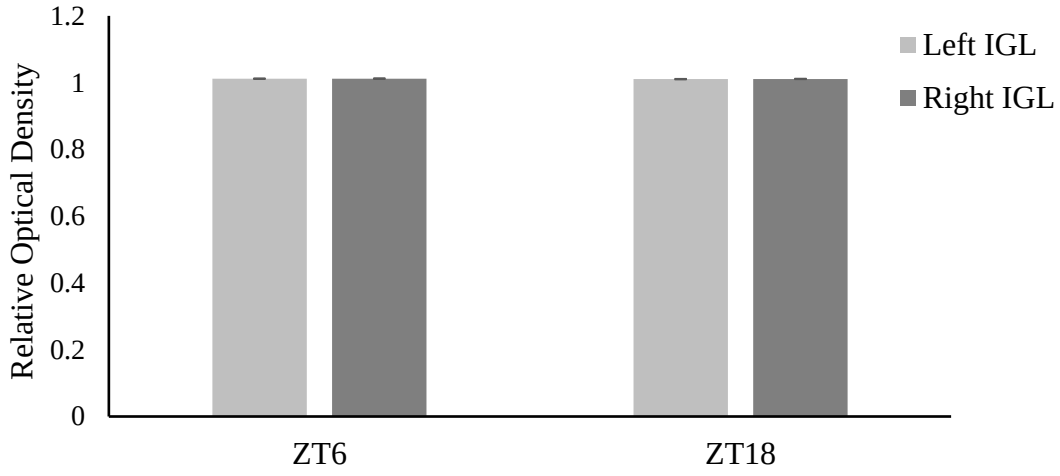

Figure 2.5. Mean relative optical density (±SEM) of the left and right IGL at ZT6, and ZT18. Relative optical density (ROD) is measured as the ratio of the level of autometallographic zinc staining of the IGL to staining in the dorsolateral geniculate nucleus (dLGN). No significant difference in ROD was found between the two time points, $p>0.05$.

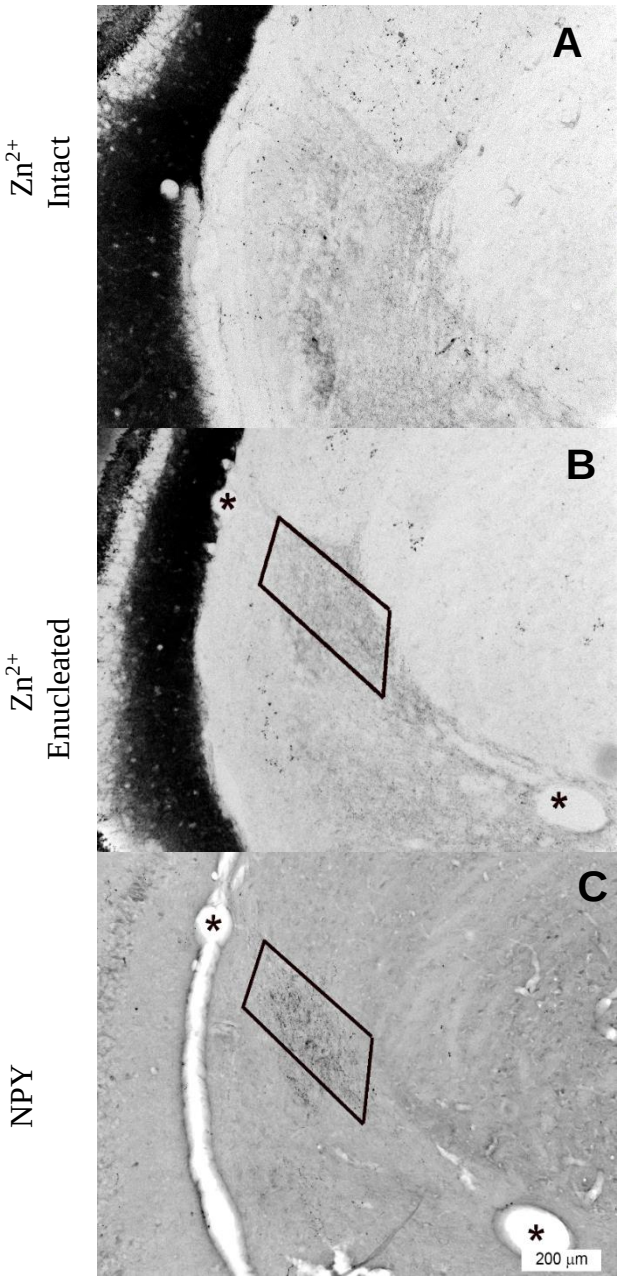

Figure 2.6. Photomicrographs (10x magnification) of representative hamster coronal slices showing zinc staining in (A) animals with intact retinas and (B) enucleated animals. No significant difference was found between the two conditions, $p > 0.05$. IGL staining was confirmed by matching an adjacent delineated NPY region (C) to that of the presumed IGL region (B; boxed area is the IGL). Matching landmarks (such as blood vessels) are denoted by (*) for comparison and matching purposes.

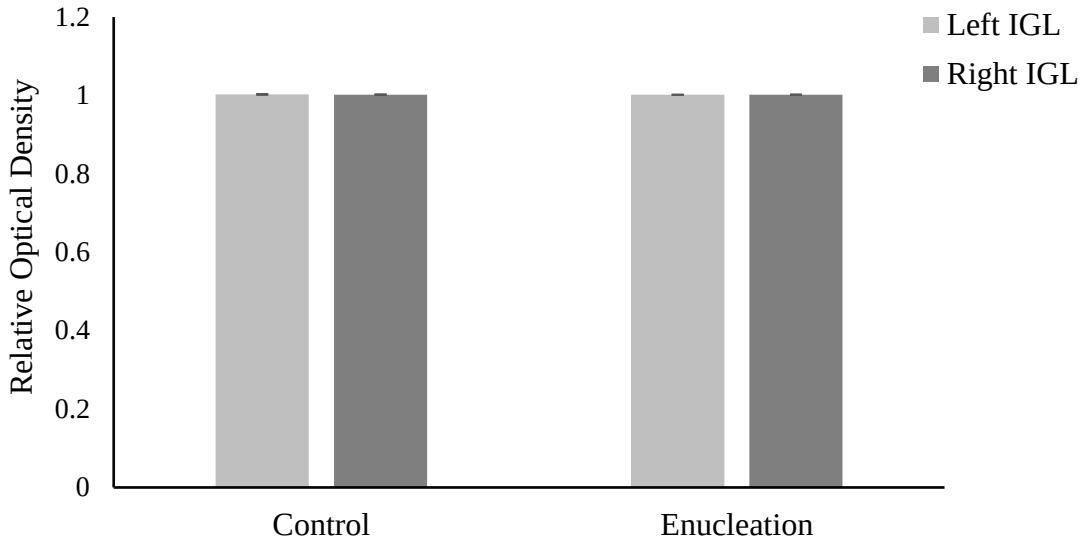

Figure 2.7. Mean relative optical density (±SEM) of the left and right IGL of control and enucleated hamsters. Relative optical density (ROD) is measured as the ratio of the level of autometallographic zinc staining of the IGL to staining in the dorsolateral geniculate nucleus (dLGN). No significant difference in ROD was found between the two time points, $p>0.05$.

2.4 Discussion

This study explored the distribution of histochemical zinc as well as its source of input to the mammalian circadian system. To these ends, the study consisted of experiments that first sought to stain for zinc in the major circadian brain areas, the SCN and IGL and then to test the hypothesis that zinc is being relayed to the circadian circuitry by the retina. While zinc staining was scarce in the SCN, the IGL was shown to contain a sizable amount of zinc throughout the entire structure. As well, zinc staining was observed throughout the vLGN. However there was no difference seen in zinc levels in either structure at different times in the day. This study is the first to report on the presence of histochemical zinc in the IGL, suggesting a possible role of the cation in the circadian system. However the function of zinc in this area remains unknown. The IGL is an important component of the circadian system that also receives retinal input and conveys photic information to the circadian clock (Card & Moore, 1982; Pickard et al., 1987). Therefore it is possible that zinc plays a role in sending photic information to the IGL which is sent to the SCN and ultimately results in clock modulation.

The limited amount of zinc observed in the SCN in this study is in contrast to previous findings by Huang et al. (1993) which have demonstrated a sizable amount of histochemical zinc in the SCN, especially in the ventrolateral regions. Although a small amount of zinc staining was observed along the ventral and medial areas (experiment 1), this was nowhere near the amount reported in the literature. However the differences in the methodology used in that study and the present one should be taken into consideration. For instance, although the zinc staining protocol was quite similar for both, the experiment by Huang et al. (1993) used different concentrations of some developer ingredients and brains were collected and fixed in glutaraldehyde while fresh brains were used in this experiment. More importantly however, this study attempted zinc staining in hamster brains which has not been reported in the literature while Huang et al. (1993)

stained rat SCN sections. Therefore the differences observed in staining could be the result of species-specific differences. As a result of sparse and considerable staining in the SCN and IGL respectively, investigation of the source of zinc input was followed up in experiment 2 in the IGL only.

The presence of ZnT3 in the retina (Redenti & Chappell, 2004) also raises the possibility that zincergic terminals from the eye may innervate the IGL. However the findings of this study do not support the retina as the source of zinc input to this area as there was no significant difference in zinc staining between animals with an intact retina and enucleated animals. Zinc staining was still present in the IGL of these animals, suggesting that the zinc present in this area is not exclusively coming from the eyes. However it cannot be said with certainty that the retina is not a source of zinc input to the IGL. Rather it can be concluded that if the retina is indeed providing zinc to this area, it is not the only source and there are likely other sources of zinc input from other brain areas. The IGL receives considerable afferent projections from several different brain areas including visual centers such as the superior colliculus (SC), visual cortex and dLGN (Moore et al., 2000). Therefore it could be possible that zincergic fibers observed in the IGL also arise from one of these areas and may have visual functions. However, the IGL receives input from some other areas serving circadian functions including the dorsal raphe and it could be involved in these pathways. A follow-up experiment employing retrograde tracing methods could potentially aid in a better understanding of where the zinc in the IGL is coming from. It should also be noted that the type of zinc staining employed is a terminal staining technique which shows synaptic zinc and if zinc is present in cells in the IGL, it cannot be differentiated from the synaptic zinc. Therefore retrograde tracing could help label cell bodies

and allow the visualization of the cells where the zinc is coming from. This could help uncover the source of zinc and also reveal whether or not any of the cells originate in the IGL itself.

The findings of this study do not support the presence of a circadian rhythm to zinc levels in the SCN or IGL. There were no changes seen with respect to zinc levels in the middle of the light period and the middle of the dark period. This likely means that zinc levels remain constant throughout the day. Nonetheless, a future study incorporating more than two points could be conducted in order to get a better sense of any oscillatory pattern that may exist and has not been revealed by the two time points designated in this experiment.

In summary, this study has shown the presence of histochemical zinc in the thalamic backdoor to the circadian system, the IGL. However the origin of zinc in this area remains unclear. While zinc could be produced by IGL cells, it could also be reaching this area from a wide array of brain targets as well as the retina, even though the latter was shown not to be necessary or solely responsible for zinc input. Following up the results of this experiment with an inquiry into the different sources of zinc input to the IGL could prove difficult but could nonetheless help build on the current knowledge of the distribution of zinc in the brain, particularly in subcortical areas which have been largely overlooked in the literature.

Chapter Three: Distribution of Zinc in the Retina

3.1 Introduction

The element zinc is found abundantly in living organisms where it exerts its effects as a component of structural proteins and metalloenzymes, as well as a neuromodulator of postsynaptic receptors in the brain (Frederickson et al., 2000; Palmiter et al., 1996). A small quantity of zinc exists as a freely available ion that is sequestered and later released from the presynaptic vesicles of a subset of glutamate-secreting neurons (Paoletti et al., 2009). A vesicle-specific transporter, zinc transporter 3 (ZnT3) is responsible for transferring zinc into vesicles for later release (Palmiter et al., 1996). Since its discovery, ZnT3 has been localized to various areas outside the brain including in the ganglion cell layer of the retina suggesting the presence of reactive zinc in the eye (Redenti & Chappell, 2004). Zinc itself has also been visualized in the rat retina with autometallographic staining, yet research is lacking as to the distribution of zinc in retinal ganglion cells (Ugarte & Osborne, 2001).

Given the almost exclusive presence of zinc in glutamatergic neurons, the presumed excitatory neurotransmitter signal of the circadian photic entrainment pathway, it is possible that the cation is released from the intrinsically photosensitive retinal ganglion cells (ipRGCs) that project to the mammalian master circadian clock, the suprachiasmatic nucleus (SCN) in the hypothalamus (Ebling, 1996; Moore et al., 1995). These cells can be differentiated from other ganglion cells by the presence of melanopsin, an opsin photopigment (Provencio et al., 1998). Melanopsin is a type of G-protein coupled receptor (GPR) that is responsible for the inherent light sensitivity of ipRGCs (Provencio et al., 2000). Although it is similar to other opsins such as the rhodopsin found in rods, melanopsin has a unique depolarizing light response (Berson et al., 2002; Hattar et al., 2002). Whereas light exposure leads to hyperpolarization in rods, it leads to a series of conformational changes in the melanopsin molecule that brings about an influx of

sodium and other cations, resulting in depolarization (Hankins, Peirson, & Foster, 2008; Hardie & Raghu, 2001).

The prospect of zinc input to the circadian clock from the retina is supported by the observation of the cation in areas of the SCN which receive the most photic input from the eyes (Huang et al., 1993). However, ipRGCs have not been examined for zinc and therefore it is not known whether zinc is present in these cells. The examination of ZnT3 in these cells could help confirm the presence of zinc in the photic entrainment pathway, given that the transporter is necessary for the packaging of the cation in vesicles inside the cell (Palmiter et al., 1996). However the findings of Chapter 2 showed the presence of zinc in the IGL but not in the SCN. As a result, it is expected that ZnT3 will be mostly present in cells that project to the IGL and not the SCN.

The purpose of the present study is to confirm the presence of ZnT3 in melanopsin-containing retinal ganglion cells that project to the IGL. Immunofluorescent labelling for melanopsin-containing cells, as well as ZnT3 will allow the visualization and identification of ZnT3 in cell populations that project to the circadian centers. In this study, fluorescent labelling was also undertaken for Brn3, a POU domain transcription factor that is expressed in some ipRGC subtypes and is important to the development of these cells (Jain et al., 2012). This was done in order to examine if ZnT3 might be restricted to certain subclasses of ipRGCs. Brn3-positive melanopsin ipRGCs have been shown to project to the IGL and therefore the expression of the protein could help differentiate ipRGCs projecting to this circadian area. If zincergic ipRGCs preferentially innervate the IGL, then it is expected that ZnT3 should be mostly observed in Brn3-positive melanopsin containing ipRGCs. Therefore, it is proposed that fluorescent labeling should yield a number of melanopsin single-labeled cells along with a subset

of melanopsin, ZnT3, and Brn3 triple-labelled cells but few melanopsin ZnT3 double-labelled cells that could project to the SCN or other brain areas.

3.2 Methods

3.2.1 Animals

Eyes taken from a total of four male C57BL/6J wild type adult mice were used for the purpose of antibody quantification. All procedures were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and adhered to the policies of the Canadian Council of Animal Care.

3.2.2 Retinal slice preparations

Mice received an overdose of sodium pentobarbital (CEVA Santé Animale, France) and were then transcardially perfused with ~50ml of cold phosphate buffered saline (PBS) and subsequently, ~50ml of cold 4% paraformaldehyde in PBS. Eyes were extracted by severing the optic nerve and were post-fixed at 4°C in 4% paraformaldehyde overnight and then transferred to 20% sucrose in PBS solution for 24 hours. Storage in sucrose served the purpose of cryoprotecting the tissues until they were ready to be sliced. A base of Tissue Tek Optimal Cutting Temperature mounting media was created on a mounting disk and left to freeze. Eyes were placed on top of the frozen mounting media and subsequently covered in additional media and left to freeze. Sagittal retinal sections were cut with the use of a Leica cryostat set at a temperature of -19°C, at a thickness of 20 microns and thawed/collected directly on gelatin-coated microscope slides.

3.2.3 Immunohistochemistry

In order to confirm the colocalization of ZnT3, Brn3 and melanopsin in retinal ganglion cells, retinal slices were triple-labelled for melanopsin (anti-rabbit 1:5000, Cedarlane, Ontario, Canada), ZnT3 (anti-mouse 1:800, Synaptic Systems, Goettingren, Germany), and Brn3 (anti-

goat, 1:250, Santa Cruz Biotechnology, Texas, US). The secondary antibodies used included CY-3 donkey anti-rabbit, CY-2 donkey anti-mouse, and CY-5 donkey anti-goat (Jackson ImmunoResearch Laboratories Inc. West Grove, PA, USA), all in 0.3% PBSx. Retinal sections were soaked in 4% paraformaldehyde for 20 minutes and then exposed to three 10 minute washes in 0.3 M PBS with 0.1% Triton-X-100 (PBSx), followed by a 60 minute incubation in blocking buffer (10% normal donkey serum in PBSx). Subsequently, slides were incubated for 48 h in the primary antibodies diluted in blocking buffer at 4°C. Sections were then exposed to six 10 minute washes in 0.3% PBSx and then protected from light and incubated for 2 h in the secondary antibodies. A final set of three 10 minute washes in 0.3% PBSx were performed after which slides were immediately cleared with xylene and coverslipped with Krystalon.

3.2.4 Quantification of melanopsin, ZnT3, and Brn3 colocalization

Images of the immunofluorescent retinal slices were captured with a digital camera mounted on an Olympus BX51 microscope equipped with filters for the different cyanine dyes used in the immunohistochemistry protocol. Colocalization was determined by collecting and superimposing separate computerized images using imaging software (ImagePro Plus 5.1.2.59; Media Cybernetics, Inc.). Initially, a melanopsin cell was identified in the retinal ganglion cell layer (on the CY-3 filter channel) by its characteristic shape and extensive dendritic networks. Colocalization was then determined by the presence of a ZnT3 and Brn3 protein in the same position as the melanopsin cell (on their respective channels). Colocalization was identified as the presence of each signal in a cell of the same shape and size.

3.3 Results

Figure 3.1 shows the immunofluorescent retinal slices obtained with the staining of melanopsin-containing ipRGCs, ZnT3 and Brn3. A small subset of ipRGCs that contain ZnT3 project to the circadian system. Images of positive staining for melanopsin show the entire

ipRGC cell with its large cell body and extensive dendritic arbor visible in the ganglion cell layer. Positive ZnT3 staining was identified as a speckle formation outlining a cell along its perimeter. Similarly, positive Brn3 staining was identified by a hollow speckle formation in the shape of a nucleus within the cell perimeter. As shown in Figure 3.1, the merged image of a cell is a result of the superimposing of separate images for each signal. The majority of ipRGCs were identified as melanopsin single-labelled cells (47.5%). There was also a subset of melanopsin ZnT3 double-labelled cells (38.3 %) and a minority of melanopsin ZnT3 Brn3 triple-labelled cells (14.2%). Figure 3.2 shows the frequency distribution of the different types of ipRGCs identified.

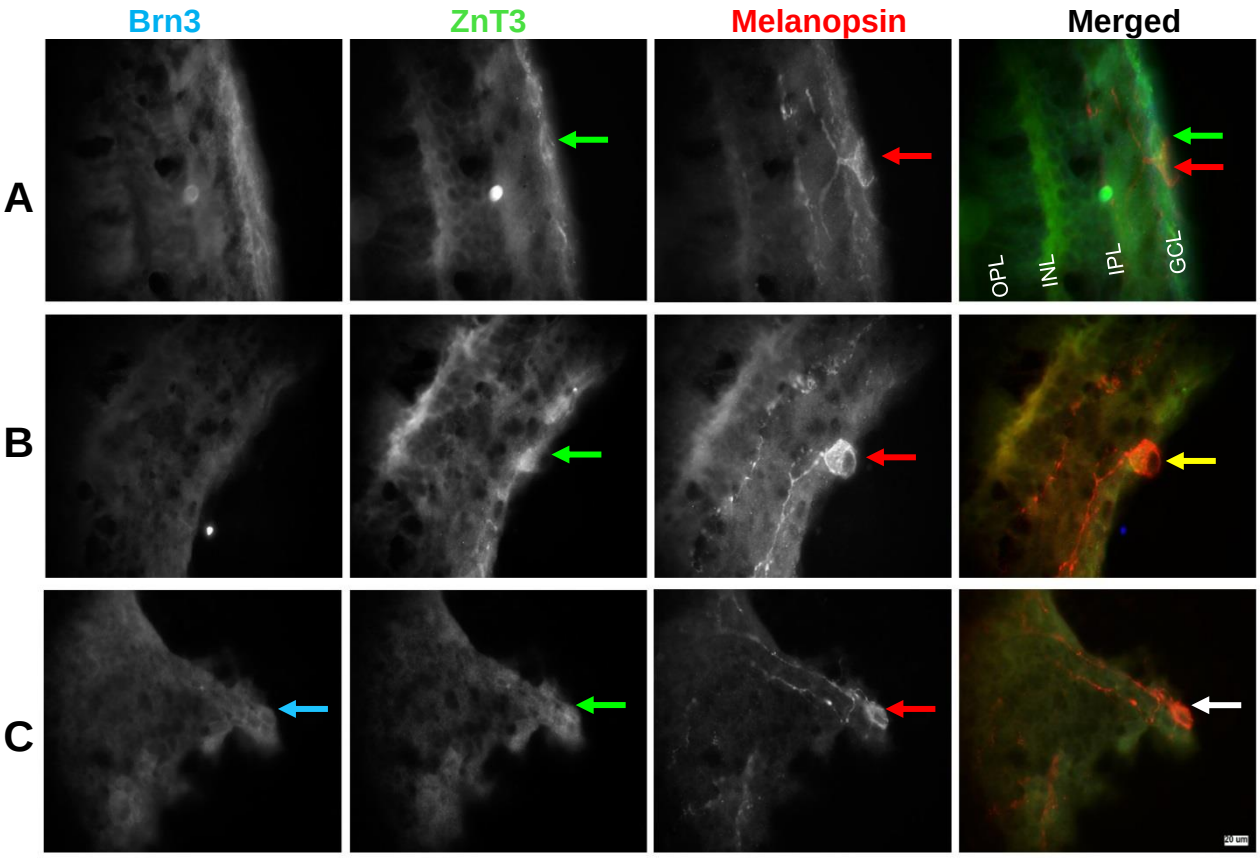

Figure 3.1. Immunofluorescent labelling for melanopsin, ZnT3 and Brn3. Panelized images (100x magnification) of representative mouse retinal slices showing staining for Brn3 (first column, blue arrows), ZnT3 (second column, green arrows) and melanopsin cells (third column, red arrows) in the ganglion cell layer (GCL). The fourth column shows a merged image from which colocalization can be deciphered. Rows depict: (A) A melanopsin single-labelled cell not colocalized with a neighboring ZnT3 (B) A melanopsin ZnT3 double-labelled cell (C) A melanopsin ZnT3 Brn3 triple-labelled cell. Inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL).

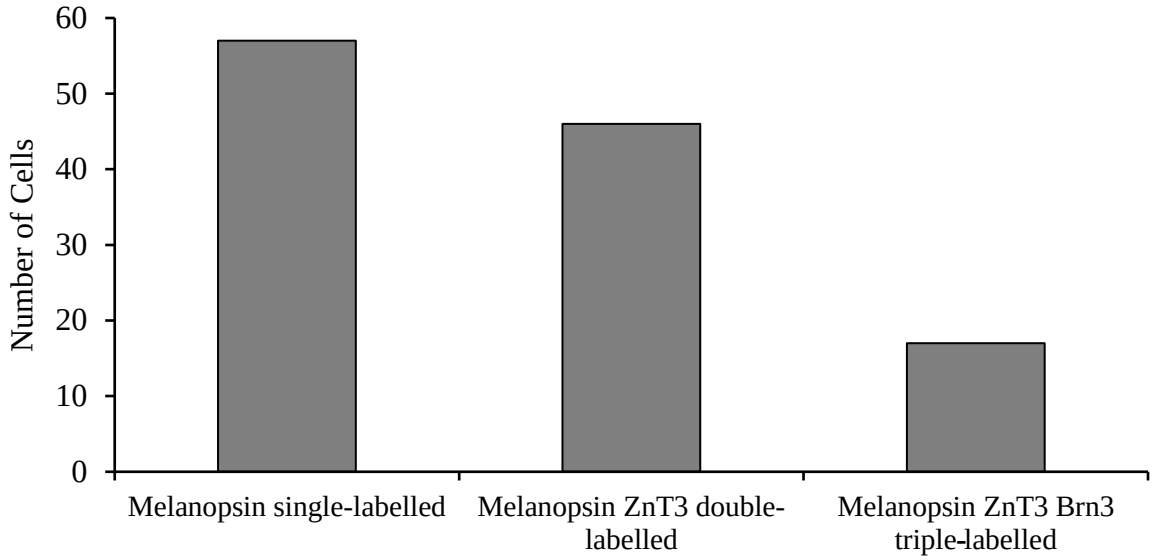

Figure 3.2. Frequency distribution of single-labelled, double-labelled, and triple-labelled ipRGCs found in the ganglion cell layer of the mouse retina. Approximately 120 cells were identified: 47.5% single-labelled, 38.3% double-labelled, 14.2% triple-labelled cells.

3.4 Discussion

This study explored the distribution of the zinc transporter ZnT3 in ipRGCs of the mammalian retina. More specifically, the presence of ZnT3 in cells capable of projecting to the IGL was investigated through immunohistochemistry. The majority of ipRGCs found in the ganglion cell layer were single-labelled melanopsin cells while a smaller proportion of melanopsin ZnT3 double-labelled cells were also identified. As well, a subset of melanopsin ZnT3 Brn3 triple-labelled cells were also found. This finding suggests that zinc is present in the cells that can project to the IGL. This result is the first report of zinc in ipRGCs and would be best followed up with an examination of the possible role of zinc in the circadian system.

Despite the observation of zinc in cells that communicate with the circadian system, a greater proportion of cells were identified as double-labelled and therefore containing ZnT3 but not Brn3. However it cannot be said with certainty whether or not these cells also project to the circadian system or if they are communicating with other areas. This is because it is not yet known whether all cells that project to the IGL via the GHT express Brn3 and whether or not the presence of this transcription factor is a determining factor in differentiating the different ipRGCs and their projections. Currently up to five different ipRGC morphological subtypes have been identified and among the cell populations, the circadian areas have been shown to receive input almost exclusively by the so-called M1 cells (Chen, Badea, & Hattar, 2011; Ecker et al., 2010). Within this subtype, SCN-projecting cells appear to be differentiated by the absence of Brn3 while Brn3-containing cells can communicate with the IGL. Although this suggests that Brn3-positive cells are likely the cells that provide photic input to the IGL, more research needs to be conducted in this area. For instance, it could be the case that the cells are inputting information to another area such as the olivary pretectal nucleus (OPN) which also has a

nonvisual function. At least one conflicting study has also been published that argues against the presence of Brn3 in ipRGCs involved in the circadian system, including the IGL (Jain et al., 2012).

Therefore although the findings of this study provide evidence for the presence of zinc in ipRGCs that can project to circadian areas such as the IGL, it does not altogether eliminate the possibility that the triple-labelled cells could also be projecting elsewhere in the brain and participating in other non-visual functions other than circadian rhythms (Chen et al., 2011). Furthermore, the identity and projections of the double-labelled cells observed remains unclear. Therefore, a limitation present in the study is the inability to isolate ipRGC projections to the circadian system as this has not yet been undertaken and there is currently no way of differentiating between all the possible projections. Remarkably, it has recently been shown that a single ipRGC is capable of sending projections to several brain areas at a time and so this makes it ever more difficult to isolate input targets (Fernandez, Chang, Hattar, & Chen, 2016). However the findings from Chapter 2 support the findings of this study in that the IGL was shown to contain a sizable amount of zinc while the SCN was not, suggesting the presence of zinc in IGL-projecting ipRGCs.

In summary, this study has found ZnT3 in a subset of ipRGCs. However, it is not completely known if these cells are indeed IGL-projecting and therefore involved in the modulation of photic signals or if they project to other areas of the brain where zinc could be involved as well. Nonetheless, the results do shed some light on the potential involvement of zinc in the circadian system.

Chapter Four: Zinc in Photic Entrainment

4.1 Introduction

The transition metal zinc is among the many elements and molecules that are necessary for the proper functioning of the central nervous system (Toth, 2011). Zinc exists as a freely available cation in many brain areas, in particular in the presynaptic boutons of forebrain neurons (Frederickson, 1989). This pool of synaptic zinc is freely available and is known as histochemically reactive due to its ability to bind to other molecules including those used to stain tissues for zinc (Danscher, 1982; Frederickson, 1989). The concentration of synaptic zinc is tightly controlled by many different proteins and ligands (Grotz et al., 1998). The cation's sequestering and release from presynaptic vesicles is dependent on zinc transporter 3 (ZnT3) which has also been found in other tissues, including the retina (Palmiter et al., 1996; Redenti & Chappell, 2004; Smidt & Rungby, 2012). Almost all zinc-containing neurons observed to date have been identified as also containing the neurotransmitter glutamate (Frederickson, 1989; Paoletti et al., 2009). Zinc aids in glutamate release but more importantly, it acts as a neuromodulator of various glutamate receptors at postsynaptic neurons (Paoletti et al., 2009).

The role of zinc at glutamatergic receptors has been studied in the forebrain (Pan et al., 2011) yet research is lacking on the cation's function in other brain areas, particularly the subcortical regions. One such area is in the circadian system, which involves many different brain regions but is governed by the central clock, the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Ibuka & Kawamura, 1975; Reppert & Weaver, 2001). Glutamate is an important player in the circadian system and acts as an excitatory signal to the clock from the retinohypothalamic tract (RHT; Ebling, 1996). This pathway relays photic information from the eye to the clock, synchronizing or entraining the clock to the exogenous light-dark cycle (Moore et al., 1995).

The colocalization of zinc with glutamate and its influence on glutamatergic receptors such as NMDARs in cortical brain areas (Paoletti et al., 2009), raises the possibility that the cation may be exerting its effects on regions with glutamatergic input such as the SCN. Evidence for a role of zinc in the circadian system comes from the discovery of ZnT3 in the ganglion cell layer of the mouse retina where clock-projecting melanopsin ganglion cells are localized (Redenti & Chappell, 2004), and our observation that melanopsin cells themselves can contain ZnT3 (Chapter 3). As well, histochemical zinc staining has been shown in the ventrolateral region of the rat SCN where most input pathways meet, including the RHT (Huang et al., 1993). However, the addition or removal of zinc directly in the SCN of Syrian hamsters housed in complete darkness, by means of a zinc donor and a metal ion chelator such as N,N,N',N-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) prior to a light pulse late in the animal's subjective night (circadian time or CT18) has not resulted in a significant photic phase shift (unpublished data from Dr. Antle's lab). Given our observation that zinc levels in the hamster SCN are quite low (Chapter 2), this negative finding may not be that surprising. Although modulation of zinc levels in the SCN did not produce a significant photic response, this does not altogether undermine the possible role of zinc in photic entrainment. This is because photic information is also indirectly relayed to the SCN through a thalamic backdoor to the circadian system, the intergeniculate leaflet (IGL) via the geniculohypothalamic tract (GHT; Harrington & Rusak, 1986). Given the strong zinc staining that we've observed in the hamster IGL (Chapter 2), it is possible that zinc may be modulating circadian responses to light through activity at the IGL.

This study examines the effect of zinc level modifications in the IGL, on photic entrainment. If the IGL is the site of zinc modulation of photic information, then alterations in zinc levels in the IGL should produce a marked photic response. Zinc levels can be modified by

supplying zinc to the IGL by means of a zinc donor such as $ZnCl_2$ and removing zinc by means of a metal ion chelator such as TPEN which has a high affinity for zinc (Cuajungco & Lees, 1996). It is proposed that zinc acts at the level of the IGL, to enhance the photic response and the SCN's sensitivity to photic signals. Therefore it is hypothesized that adding zinc to the IGL will increase circadian responses to light while zinc chelation will result in decreased responses.

4.2 Methods

4.2.1 Animals

A total of 14 male Syrian hamsters (*Mesocricetus auratus*, 80-90g) were obtained from Charles River Laboratories (Kingston, NY, USA). Animals were placed in pairs in a temperature and humidity-controlled room with a 14:10 light dark cycle and provided with food and water *ad libitum*. Following cannula implantation, each animal was transferred to an individual cage and allowed to recover for a period of one week under the same conditions. Subsequent to recovery, animals were transferred to individual polycarbonate cages (20x45x22 cm) equipped with a running wheel (14 cm in diameter), and maintained in constant darkness (DD) for the duration of the experiment. Periodic cage changes took place seven days prior to the day of a manipulation.

4.2.2 Surgeries

Stereotaxic surgery was conducted for double cannula implantation in the left and right IGL of each animal. The surgical procedure for cannula implantation followed that of a previously outlined protocol aimed at the SCN (refer to Sterniczuk, Stepkowski, Jones, & Antle (2008)) with the exception that double cannula implantation was required owing to the bilateral nature of the IGL. Coordinates were adapted from previous work by Mintz, Gillespie, Marvel, Huhman, & Albers (1997; coordinates 1.9 mm posterior to bregma, 3.3 mm lateral to the midline, and the average of 3.3 mm and 4.8 mm ventral to the skull and dura, respectively).

4.2.3 Drugs

Zinc chloride ($ZnCl_2$; 208086, Sigma) dissolved in 0.9% saline to a final concentration of 50µm (Takeda, Minami, Seki, & Oku, 2003) was used as a Zn^{2+} donor while TPEN (P4413, Sigma) dissolved in 10% dimethylsulfoxide (DMSO) to a final concentration of 5mM (Cuajungco & Lees, 1996) was used as a zinc chelator. A vehicle control of 0.9% sterile saline and 10% DMSO were included for the zinc donor and chelator, respectively. Drugs were administered to animals based on a counterbalanced design throughout the course of the experiment. Bilateral intracranial injections of 0.5µL of drugs were administered over a period of 30 seconds during each manipulation by means of a 1µL Hamilton syringe attached to polyethylene 20 tubing with an injector tip which was attached to the guide cannulas. The injector was left in place for an additional 30 seconds following injections. All injections were made in DD conditions with the aid of night-vision goggles.

4.2.4 Photic phase shifts

Ten minutes following drug administration, animals were placed in light boxes (40 lux illuminance) and exposed to a 15 minute light pulse at late subjective night (CT18). The experiment was timed relative to activity onset in DD conditions which is defined as circadian time or CT12. The light pulse was given 6 hours following activity onset at CT18 as this time point elicits maximum phase advances in Syrian hamsters (Daan & Pittendrigh, 1976). The wheel running activity of the animals was detected by means of a magnetic switch fastened to each wheel and monitored using the Clocklab program (Actimetrics, Wilmette, IL). Photic phase shifts for each manipulation were calculated by fitting a line of best fit to activity onsets, following a previously described method of calculation (Sterniczuk et al., 2008).

4.2.5 Histology/perfusion

In order to confirm the bilateral placement of the cannulas in the IGL, the brains of animals were collected for histology at the end of the experiment. All animals received an overdose of sodium pentobarbital and were then transcardially perfused with ~50ml of cold phosphate buffered saline (PBS) and subsequently, ~50ml of cold 4% paraformaldehyde in PBS. Brains were post-fixed at 4°C in 4% paraformaldehyde overnight and then transferred to 20% sucrose in PBS solution for 24 hours. Brains were cut with the use of a Leica cryostat set at a temperature of -19°C, at a thickness of 35 microns and thawed/collected directly on gelatin-coated microscope slides. Brain sections were subsequently stained using a cresyl violet Nissl stain and coverslipped in Permount. The slides were then viewed under an Olympus BX51 microscope to confirm cannula placements. Animals were excluded if the tip of either one of the two cannulas was more than 600µm away from the margin of the left and right IGL.

4.2.6 Statistical analyses

All comparisons were made using SigmaPlot (Systat Software, Inc.; San Jose, CA). Statistical significance was set at $p<0.05$ for all tests. A one-way repeated measures ANOVA was used to determine whether there is a statistically significant difference in the photic phase shifts between the conditions (zinc donor, zinc chelator, vehicle control). Furthermore, a two-tailed independent samples t-test was also conducted to analyze the photic phase shifts between the different vehicles (saline and DMSO). All means are reported as ± standard error of the mean (SEM) in the figures and as ±standard deviation in the text.

4.3 Results

Data from a total of eight hamsters were used in the analysis. Histological examination led to three animals being excluded due to missed placements for one of the two cannulas aimed at the left or right IGL. Another animal was excluded after losing its headcap during the first

manipulation. Two more animals were excluded due to near zero phase shifts following light exposure in the vehicle control condition. All but two animals used in the analysis received all treatments once. The other two animals lost their headcaps at some point during the experiment, but each received at least one drug treatment and a vehicle control treatment. The results of an independent samples t-test showed no significant difference in the mean photic phase shift between the two vehicle treatment conditions (saline: 1.854±0.640; DMSO: 1.924±0.722), $t(12) = -0.192$, $p=0.851$. As a result, the saline and DMSO data was combined into one vehicle group for both the $ZnCl_2$ and the TPEN conditions. There was no significant difference in mean phase shift between the vehicle (1.843±0. 432), $ZnCl_2$ (1.619±0.743), or TPEN (1. 571±0.800) conditions, $F(7, 13) =0.752$, $p =0.491$ (Figure 4.2). Therefore, the animals exhibited a similar magnitude in their phase shift following light exposure at CT18 as seen in the actograms of representative animals (Figure 4.1). A histology image showing bilateral cannula placement in the left and right IGL is shown in Figure 4.3.

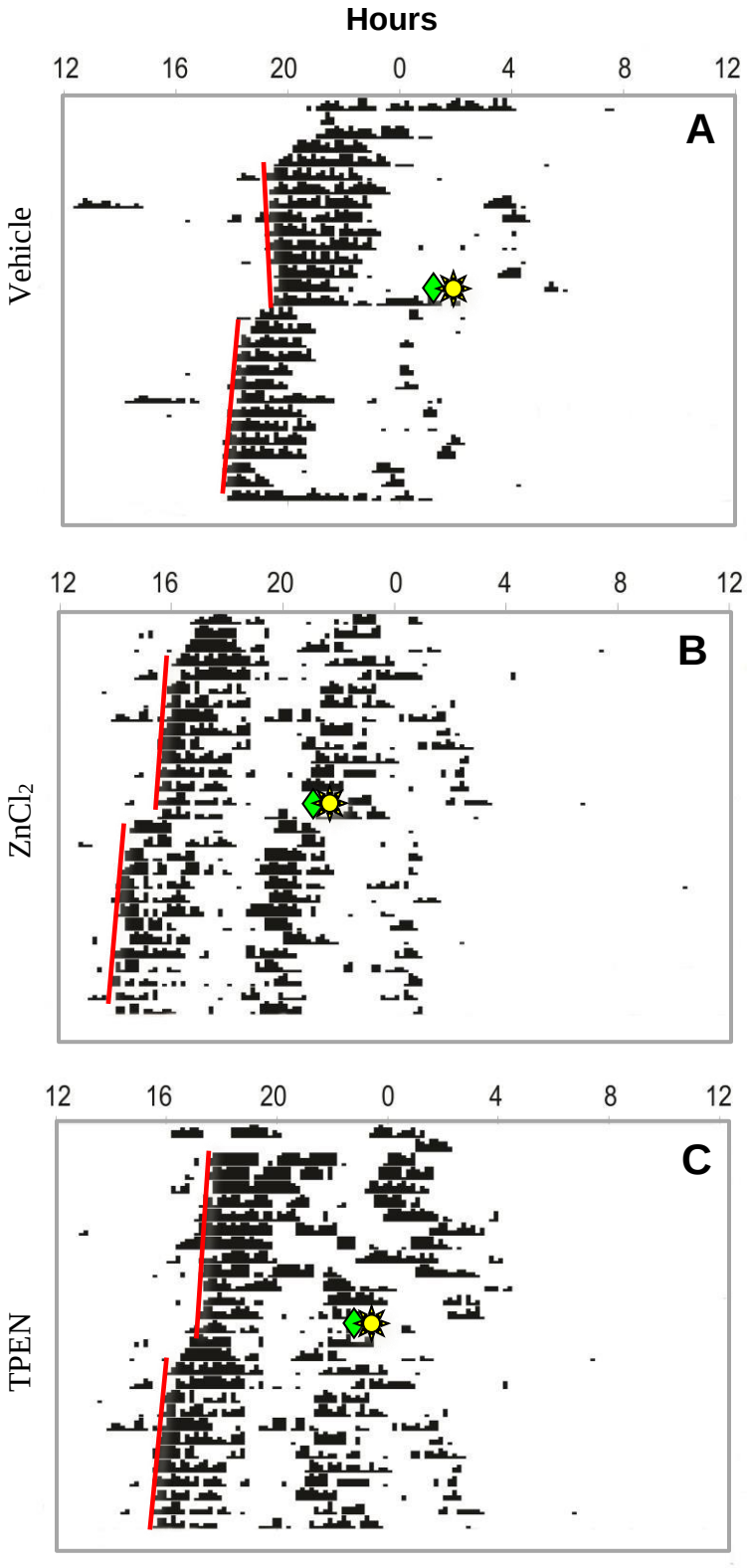
Hours
12
16
20
0
4
8
12
A
Vehicle
B
ZnCl2
C
TPEN

Figure 4.1. Actograms from representative animals depicting the photic phase shift after treatment with (A) vehicle (10% DMSO shown) (B) $ZnCl_2$ and (C) TPEN, denoted by a diamond (◊)10min prior to a 15min light pulse (☼; 40lux) at CT18. Each horizontal line represents a day of wheel-running as shown by the black vertical bars with subsequent days plotted below. The height of the bars is proportional to the number of wheel revolutions. The red diagonal lines are regression lines fitted to activity onset before and after the manipulation.

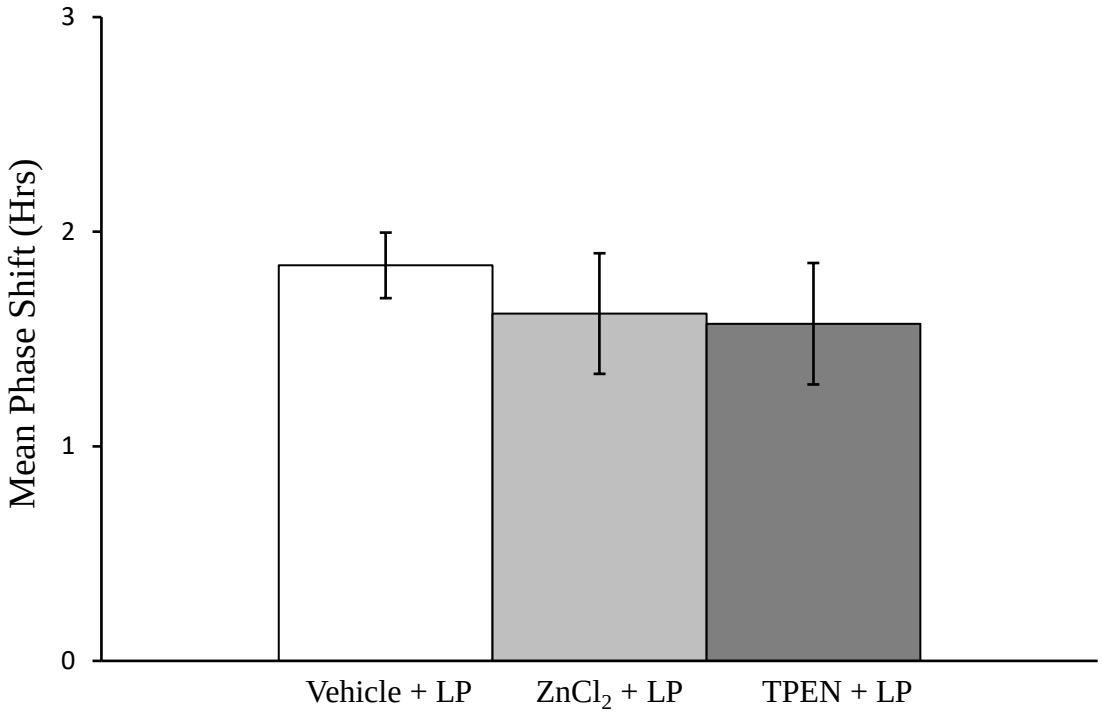

Figure 4.2. Mean phase shift (±SEM) of hamsters exposed to vehicle (averaged data of saline and DMSO for each animal), $ZnCl_2$ and TPEN (n=8) conditions prior to a 15 minute light pulse (LP) at late subjective night (CT18). There was no significant difference in photic phase shifts between the treatments, $p>0.05$.

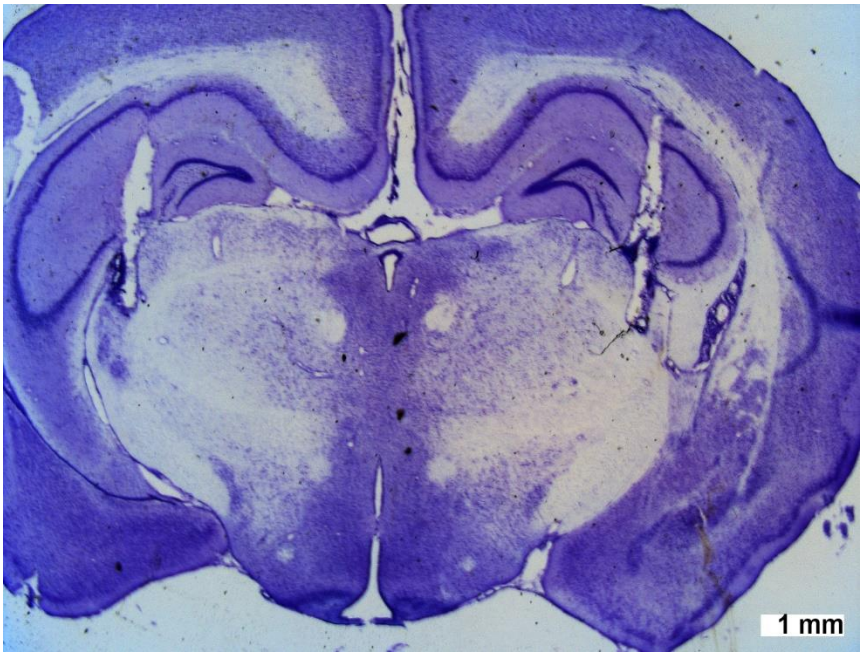

Figure 4.3. Photomicrograph (1.25x magnification) of bilateral cannula placement in the left and right IGL of a representative hamster coronal slice stained with cresyl violet. Cannula placements are visible as two white narrow tracts running down from the hippocampus and terminating on the left and right IGL.

4.4 Discussion

The present study examined the role of histochemical zinc in modulation of the circadian photic phase response. Direct modulation of zinc levels in the IGL by use of a zinc donor and chelator did not significantly alter light-induced phase shifts as was hypothesized. Therefore, it remains unclear whether or not zinc plays a role in modulation of incoming light information supplied to the master clock by the IGL. The outcome of this experiment is comparable to past findings in the lab of a lack of a significant photic response as a result of changing zinc levels in the SCN itself. Taken together, these findings do not support a role for zinc in photic entrainment, though the absence of a significant effect does not completely rule out a possible zinc entraining function.

Although the lack of alterations in photic phase shifts may be attributed to the absence of zinc in the IGL, this is likely not the case given the findings discussed in Chapter 2. More specifically, histochemical zinc staining was observed throughout the IGL. Therefore, it is unclear as to why there is an absence of a zinc-modulated response in a zinc-containing region. One possible explanation for the results is that the method of zinc application may not have been ideal to modify photic phase shifts. A zinc donor such as $ZnCl_2$ applies extracellular zinc in the area but this may not have any effect on the modulation of endogenous zinc that is present in synaptic vesicles and participates in the photic entrainment pathway. If so, the application of extracellular zinc would do little to change the internal dynamics of zinc activity. Several findings in the literature seem to support this possibility. For instance extracellular zinc application has been shown to be ineffective at modulating the activity of different glutamate receptors owing to tonic zinc modulation (Paoletti et al., 1997). More specifically, zinc can modulate the activity of some receptors such as NMDARs by binding to an allosteric site on different subunits (Paoletti & Neyton, 2007). At resting levels, the zinc site would remain

occupied and modulation would depend on the number of zinc ions accumulated over time (Kay, 2003). In this case, very little zinc would actually be present in the extracellular space (Toth, 2011). Therefore receptor modulation may be influenced mainly by endogenous zinc saturating receptor sites rather than extracellularly applied zinc (Kay, 2003). This can make it harder to understand the effect of zinc at glutamatergic receptors and transporters and by extension, the role of zinc in the photic entrainment pathways. In order to get a clearer picture of the mechanisms involved, it may be more appropriate to directly influence the amount of endogenous zinc. Although zinc chelation did this in the experiment, it presents its own set of problems. For instance, chelators cannot distinguish between zinc that is loosely bound to receptors and zinc that may be present in vesicles and released in the synapse (Toth, 2011). As a result, neither the exact site of zinc activity nor its mechanism of action can be clearly understood.

The lack of change to the photic phase shifts with zinc modulation in the IGL can also be explained by the fact that the IGL sends projections to many other brain areas, both those that communicate with the SCN and those that lie outside the circadian network and serve various non-circadian functions (Moore et al., 2000). For instance, IGL efferent projections also include several thalamic and subthalamic regions including its contralateral counterpart, the paraventricular nucleus of the thalamus (PVT), and the zona incerta (ZI). Therefore, the lack of a photic response may not be attributed to the absence of zinc in the IGL but rather its involvement and input to brain regions other than the SCN. The exact role of synaptic zinc remains unclear but the cation may very well be involved in modulating the effects of other regions, including the contralateral IGL. This would serve as support for the observance of zinc in the IGL (Chapter 2). Anterograde tracing of zinc in the IGL could aid in the observation of zincergic projections and

shed more light on the cation's role in entrainment. Conversely, zinc present in the IGL could be coming from another brain area rather than being produced by IGL neurons. The method of staining for histochemical zinc (Danscher, 1982) generally reveals zinc in presynaptic terminals rather than cell bodies (or if it does, it is hard to distinguish them from one another). Therefore, it is unclear whether the zinc is indeed stored and released from IGL neurons or is coming from somewhere else. Given that there are various afferent projections to the IGL, this could mean that zinc is coming from other brain regions (Moore & Card, 1994). Retrograde tract tracing of zinc in the IGL could help identify the origin and possibly, the function of the synaptic zinc that is observed there.

Furthermore, this study specifically examined the effect of zinc modulation prior to a light pulse at late subjective night which elicits phase advances. However zinc in the IGL may modulate delays instead. If so, the absence of a photic response may be attributed to the functional relevance of zinc in eliciting phase delays. As a follow-up, this experiment could be repeated with the exception of delivering light pulses at early subjective night. Given the contribution of the IGL to the phasic effects of light (Pickard et al., 1987), manipulation of zinc in this area could potentially influence entrainment through phase delays.

In addition to its involvement in photic responses, the IGL is also implicated in non-photic responses which were not examined in this study. For instance, IGL lesions have resulted in the attenuation of activity-induced phase shifts (Wickland & Turek, 1994) while NPY has also been shown to be involved in non-photic responses (Biello, Janik, & Mrosovsky, 1994). Given these findings, zinc in the IGL may be implicated in non-photic entrainment. In order to better understand the cation's functional contribution in the IGL, it may be appropriate to design an experiment with the use of cues other than light.

In conclusion, this study has shown that changing zinc levels in the IGL does not significantly diminish or enhance the photic phase response. Future experiments directly targeting internal zinc levels could help in better understanding the role of zinc in clock entrainment. For instance, experiments involving the direct manipulation of a zinc receptor binding site and its affinity for zinc through pharmacological and genetic approaches could be undertaken. Furthermore, tract tracing studies will need to be conducted to fully understand where zinc is coming from and traveling to from the IGL. Although this will likely be more difficult, a successful approach could more clearly expose the mechanisms at play in zinc modulation.

Chapter Five: General Discussion

5.1 Summary and Conclusions

The purpose of the experiments outlined in this thesis was to expand the current knowledge of the contribution of synaptic zinc to the mammalian circadian system. Though there has been ample research on zinc in recent years with a growing awareness of its importance to the proper functioning of the central nervous system, knowledge on its distribution and mechanism of action in the brain are only just emerging and there is much more left to observe and understand. In particular, the cation's involvement in subcortical brain areas such as the circadian clock and its afferent projections has been acknowledged yet remains largely unexplored. Therefore, the aims of the experiments were to expand this body of knowledge by exploring zinc distribution in the circadian circuitry and investigate a possible role for zinc in photic entrainment of the circadian clock.

The distribution of zinc in the two major circadian regions, the SCN and IGL was studied in Chapter 2. More specifically, an autometallographic staining technique allowed the visualization of histochemical zinc in these areas. While the SCN did not reveal a sizable amount of zinc, with only minor terminal staining observed in the medial and ventral areas of both nuclei, the IGL showed abundant staining. The amount of zinc present did not show a significant rhythm when observed at two separate time points in the IGL, with staining being comparatively the same for both times in the left and right IGL regions. Chapter 2 also investigated the hypothesis that the zinc present in the IGL originates in retinal cells that input to the circadian system. However contrary to predictions, the removal of the eyes and therefore the supposed source of zinc did not significantly attenuate the amount of zinc in the IGL, as compared to the control condition.

Chapter 3 examined the distribution of zinc in the retinal ganglion cell layer. Specifically, the colocalization of the zinc vesicle transporter ZnT3 with melanopsin in a subset of ganglion cells capable of projecting to the IGL was observed through fluorescent immunohistochemistry. Most of the cells that were examined were single-labelled for melanopsin and therefore did not possess ZnT3. However, a subset of melanopsin-containing cells with the ability to project to the IGL (as evidenced by the presence of the Brn3 transcription factor) were shown to also contain ZnT3. However, a smaller proportion of triple-labelled cells were observed than those cells that stained negative for Brn3 but still contained ZnT3.

A possible function of zinc as a modulator of the circadian photic response was investigated in Chapter 4. Zinc levels were manipulated with the use of a zinc donor and high-affinity chelator in the IGL prior to light exposure and the resulting photic phase response was observed. Contrary to predictions, neither adding nor removing zinc resulted in a significant change in the light-induced phase response. This finding was similar to past findings in the lab employing similar procedures to look at photic entrainment. However, those experiments sought to change levels of zinc directly in the SCN rather than looking at the IGL. Nonetheless, when taken together, both studies reported no significant attenuation or enhancement of photic phase shifts through zinc modulation.

Given the present findings, some conclusions can be made about zinc in the mammalian circadian system. Firstly, zinc is present in the IGL which is one of the major brain areas involved in the functioning of the circadian system and the clock's response to light. Secondly, zinc is present in retinal cells that are capable of conveying photic information to the IGL. Thirdly, zinc in the IGL could come from many different sources including the retina and the various afferent projections to the IGL. Importantly however, it can be concluded that the retina

is not necessary for supplying zinc to the IGL. Therefore, even if retinal cells do send zincergic projections to the IGL, zinc is present in this area, owing to either its production in the IGL itself, and/or it is coming from one or more of its afferent projections. Finally, zinc does not play a role in attenuating or potentiating phase advances in response to light through the effects of the IGL on the SCN although a potential role for zinc entrainment of the circadian system cannot be ruled out entirely.

5.2 Limitations and Future Directions

There are several important limitations to the present study that should be kept in mind when reflecting upon findings and taken into consideration for future research in this area. Firstly, while the experiments outlined in Chapter 2 looking at the distribution of zinc in the brain as well as the investigation of zinc's role in photic entrainment in Chapter 4 were all undertaken with the Syrian hamster as the animal model, the observation of ZnT3 distribution in the retina was done on C57BL/6J mice. This was done due to the inability of obtaining representative fluorescent labelling of melanopsin cells in the hamster retina. Past attempts in the lab of doing so did not result in discernable staining and therefore mouse retinas were used instead. However behavioral studies exploring the photic phase response similar to that discussed in Chapter 4 are most often carried out with the hamster model due to the precision of its activity rhythms such as wheel-running. This allows them to be the most ideal animal model of studying entrainment. Additionally, the double cannula implant necessary for this experiment was facilitated in the hamster owing to its larger skull. As a result of these issues, it became difficult to use one animal model in all of the experiments. However, the ability to do so would be favorable as although the two rodent models are quite similar, species-specific differences can exist. For instance, there are not only differences in cell populations in the SCN, but also

differences in afferent and efferent IGL projections and likely other differences that are overlooked.

Another limitation of the study which is particularly relevant to the experiments outlined in Chapter 2 is the use of an autometallographic staining technique which allowed for the visualization of terminal zinc staining in the brain but could not stain for cell bodies which would be very useful in understanding where zinc in the IGL is coming from. While the zinc staining was successful in highlighting the presence of zinc in the IGL which was the purpose of experiment 1 in Chapter 2, there are many questions that remain as to the origin of the zinc in this area. Findings reported in Chapter 2 showed that the retina is not a necessary source of zinc input to the IGL. A way to uncover any of the possible sources of zinc input to the IGL would be to conduct a retrograde transport study through intracranial injections of sodium selenite in the IGL (Howell & Frederickson, 1990). However, this could prove a very ambitious endeavor given that the zinc staining will only be present in the cells that contain zinc and finding the stain signal will be very difficult even with the use of a counterstain on brain sections.

Retrograde labelling can also be done via intraperitoneal injections similar to what was done in Chapter 2, with the exception of waiting longer for the zinc to travel back to cell bodies before collecting and staining brains (Slomianka, Danscher, & Frederickson, 1990). Exposure of the tissue to hydrogen peroxide during staining can then allow for the visualization of cell bodies and not terminal staining (Brown & Dyck, 2003). However it should be noted that this experiment was carried out on mice and as was previously discussed, there could be species differences in staining effectiveness. Despite the apparent challenges, a retrograde tracing study would be very useful in uncovering the source of zinc input in the IGL and possibly uncover its functional importance in this area.

Another important limitation of this study pertaining to the experiment outlined in Chapter 3 is the inability to distinguish between ipRGC projection sites given the diversity of these cells as well as their capacity to send projections to multiple brain areas at a time (Ecker et al., 2010; Fernandez et al., 2016). Although fluorescent labeling of Brn3 aided in the identification of ipRGCs that can project to the IGL, this is not decisive enough to rule out the possibility that these cells project to other areas, some of which are important visual centers, therefore implicating zinc more so as a modulator of visual rather than circadian functions. However, there is no simple and decisive way of tracking zinc in these cells.

Furthermore, fluorescent labeling in the present study helped identify ZnT3, but not zinc directly. Although the presence of zinc is assumed given the presence of its vesicle transporter, direct zinc visualization in the retina is preferred. This has been reported in the literature using a modified silver sulphide staining technique (Ugarte & Osborne, 1999) but it will be more difficult to identify cells that stain for zinc in the ganglion cell layer as ipRGCs. A future study employing zinc staining on retinal flat mounts would prove useful in observing zinc in the retina. In addition, the same approach can be taken on eyes from animals that have been used in an IGL retrograde tracing study as discussed above. This could help support the retina as a source of zinc input (if any staining is observed) and could allow the visualization of actual zinc in ipRGC cell bodies.

Lastly, the ability to clearly study the effects of zinc level modulation on photic entrainment (Chapter 4) was hindered by the use of a zinc donor and chelator which can alter the extracellular zinc levels but may have little or no effect on manipulating endogenous zinc (Paoletti et al., 1997) which may be the actual player in the circadian clock's entrainment pathways. However, the lack of research on a feasible alternative approach towards zinc level

modulation made it difficult to approach this experiment in a different way. Certainly the use of a high-affinity zinc chelator (TPEN) was a reasonable approach towards studying the effects following zinc removal from the IGL. Nonetheless, it is unknown whether chelators are actually binding to vesicular zinc and not another source of zinc (Toth, 2011). Additionally, the zinc donor may not have contributed significantly towards changing endogenous zinc levels, which could have resulted in the inability to observe a significant photic response. In order to fully understand the functional significance of zinc to entrainment, it will be important for manipulations to actually target endogenous zinc levels which is easier said than done and could be done by finding ways to somehow affect the affinity of a zinc receptor or the release of zinc from terminals.

In conclusion, this study has found synaptic zinc in retinal ganglion cells and has shown the presence of zinc in the IGL which serves as one of the main circadian areas sending afferent projections to the master clock. However, synaptic zinc was mostly absent from the clock itself. As well, a role for zinc in modulating photic phase shifts was not determined as modification of zinc levels in the IGL were not shown to affect the circadian photic response.

References

Abrahamson, E. E., & Moore, R. Y. (2001). Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections. *Brain Research, 916*, 172-191.

Antle, M. C., & Silver, R. (2005). Orchestrating time: Arrrangements of the brain circadian clock. *Trends in Neurosciences, 28*, 145-151.

Aschner, M., Cherian, M. G., Klaassen, C. D., Palmiter, R. D., Erickson, J. C., & Bush, A. I. (1997). Metallothioneins in brain-the role in physiology and pathology. *Toxicology and Applied Pharmacology, 142*, 229-242.

Baver, S. B., Pickard, G. E., Sollars, P. J., & Pickard, G. E. (2008). Two types of melanopsin retinal ganglion cell differentially innervate the hypothalamic suprachiasmatic nucleus and the olivary pretectal nucleus. *European Journal of Neuroscience, 27*, 1763-1770.

Berson, D. M., Castrucci, A. M., & Provencio, I. (2010). Morphology and mosaics of melanopsin-expressing retinal ganglion cell types in mice. *The Journal of Comparative Neurology, 518*, 2405-2422.

Berson, D. M., Dunn, F. A., & Takao, M. (2002). Phototransduction by retinal ganglion cells that set the circadian clock. *Science, 295*, 1070-1072.

Biello, S. M., Janik, D., & Mrosovsky, N. (1994). Neuropeptide Y and behaviorally induced phase shifts. *Neuroscience, 62*, 273-279.

Brown, C. E., & Dyck, R. H. (2003). An improved method for visualizing the cell bodies of zincergic neurons. *Journal of Neuroscience, 129*, 41-47.

Bush, A. I., Pettingell, W. H., Multhaup, G., Paradis, M. D., Vonsattel, J. P., Gusella, J. F. (1994). Rapid induction of alzheimer Aβ amyloid formation by zinc. *Science, 265*, 1464-1467.

Card, J. P., & Moore, R. Y. (1982). Ventral lateral geniculate nucleus efferents to the rat suprachiasmatic nucleus ehibit avian pancreatic polypeptide-like immunoreactivity. *The Journal of Comparative Neurology, 206,* 390-396.

Chen, S. K., Badea, T. C., & Hattar, S. (2011). Photoentrainment and pupillary light reflex are mediated by distinct populations of ipRGCs. *Nature, 476*, 92-96.

Choi, D. W. (1998). Zinc and brain injury. *Annual Review of Neuroscience, 21*, 347-375.

Christensen, M. K., & Frederickson, C. J. (1998). Zinc containing projections of the amygdala. *The Journal of Comparative Neurology, 400,* 375-390.

Cole, T. B., Robbins, C. A., Wenzel, H. J., Schwartzkroin, P. A., & Palmiter, R. D. (2000). Seizures and neuronal damage in mice lacking vesicular zinc. *Epilepsy Research, 39*, 153-169.

Cuajungco, M. P., & Lees, G. J. (1996). Prevention of zinc neurotoxicity in vivo by N,N,N',N-tetrakis(2- pyridylmethyl) ethylenediamine (TPEN). *Neuroreport, 7,* 1301-1304.

Daan, S., & Pittendrigh, C. S. (1976). A functional analysis of circadian pacemakers in nocturnal rodents. II. The variability of phase response curves. *Journal of Comparative Physiology, 106*, 253-266.

Danscher, G. (1982). Exogenous selenium in the brain: A histochemical technique for light and electron microscopical localization of catalytic selenium bonds. *Histochemistry, 76*, 281-293.

Dark, J. G., & Asdourian, D. (1975). Entrainment of the rat's activity rhythm by cyclic light following lateral geniculate nucleus lesions. *Physiology & Behavior, 15*, 295-301.

Dreixler, J. C., & Leonard, J. P. (1994). Subunit-specific enhancement of glutamate receptor responses by zinc. *Molecular Brain Research, 22*, 144-150.

Dunlap, J. C., Loros, J. J., & Decoursey, P. J. (2004). *Chronobiology: Biological Timekeeping* (First ed.). Sunderland, MA: Sinauer Associates, Inc.

Ebling, F. J. P. (1996). The role of glutamate in the photic regulation of the suprachiasmatic nucleus. *Progress in Neurobiology, 50*, 109-132.

Ecker, J. L., Dumitrescu, O. N., Wong, K. Y., Alam, N. M., Chen, S. K., LeGates, T. (2010). Melanopsin-expressing retinal ganglion-cell photoreceptors: Cellular diversity and role in pattern vision. *Neuron, 67*, 49-60.

Edelstein, K., & Amir, S. (1999). The role of the intergeniculate leaflet in entrainment of circadian rhythms to a skeleton photoperiod. *Journal of Neuroscience, 19*, 372-380.

Eide, D. J. (2000). Metal ion transport in eukaryotic microorganisms: Insights from *Saccharomyces cerevisiae. Advances in Microbial Physiology, 43*, 1-38.

Erickson, J. C., Hollopeter, G., Thomas, S. A., Froelick, G. J., & Palmiter, R. D. (1997). Disuption of the metallothionein-III gene in mice: analysis of brain zinc, behavior, and neuron vulnerability to metals, aging, and seizures. *Journal of Neuroscience, 17*, 1271-1281.

Fernandez, D. C., Chang, Y. T., Hattar, S., & Chen, S. K. (2016). Architecture of retinal projections to the central circadian pacemaker. *Proceedings of the National Academy of Sciences of the United States of America, 113*, 6047-6052.

Frederickson, C. J. (1989). Neurobiology of zinc and zinc-containing neurons. *International Review of Neurobiology, 31,* 145-238.

Frederickson, C. J., Hernandez, M. D., Goik, S. A., Morton, J. D., & McGinty, J. F. (1988). Loss of zinc staining from the hippocampal mossy fivers during kainic acid induced seizures: a histofluorescence study. *Brain Research, 446,* 383-386.

Frederickson, C. J., Koh, J. Y., & Bush, A. I. (2005). The nuerobiology of zinc in health and disease. *Nature Reviews, 6,* 449-462.

Frederickson, C. J., Suh, S. W., Silva, D., Frederickson, C. J., & Thompson, R. B. (2000). Importance of zinc in the central nervous: The zinc-containing neuron. *The journal of nutrition, 130,* 1471S-1483S.

Freedman, M. S., Lucas, R. J., Soni, B., Schantz, M. v., Munoz, M., David-Gray, Z. (1999). Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors. *Science, 284,* 502-504.

Gehring, W., & Rosbash, M. (2003). The coevolution of blue-light photoreeption and circadian rhythms. *Journal of Molecular Evolution, 57,* S286-S289.

Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M. L., & Eide, D. (1998). Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. *Proceedings of the National Academy of Sciences of the United States of America, 95,* 7220-7224.

Hankins, M. W., Peirson, S. N., & Foster, R. G. (2008). Melanopsin: an exciting photopigment. *Trends in Neurosciences, 31,* 27-36.

Hardie, R. C., & Raghu, P. (2001). Visual transduction in *Drosophila*. *Nature, 413,* 186-193.

Harrington, M. E., Nance, D. M., & Rusak, B. (1985). Neuropeptide Y immunoreactivity in the hamster geniculo-suprachiasmatic tract. *Brain Research Bulletin, 15*, 465-472.

Harrington, M. E., & Rusak, B. (1986). Lesions of the thalamic intergeniculate leaflet alter hamster circadian rhythms. *Journal of Biological Rhythms, 1*, 309-325.

Hattar, S., Liao, H. W., Takao, M., Berson, D. M., & Yau, K. W. (2002). Melanopsin-containing retinal ganglion cells: Architecture, projections, and intrinsic photosensitivity. *Science, 295*, 1065-1070.

Hickey, T. L., & Spear, P. D. (1976). Retinogeniculate projections in hooded and albino rats: an autoradiographic study. *Experimental Brain Research, 24*, 523-529.

Hirota, T., & Fukada, Y. (2004). Resetting mechanism of central and peripheral circadian clocks in mammals. *Zoological Science, 21*, 359-368.

Holst, B., Egerod, K.L., Schild, E., Vickers, S.P., Cheetham, S., Gerlach, L.O., … Schwartz, T.W. (2007). GPR39 signaling is stimulated by zinc ions but not by obestatin. *Endocrinology, 148*, 13-20.

Holzberg, D., & Albrecht, U. (2003). The circadian clock: A manager of biochemical processes within the organism. *Journal of Neuroendocrinology, 15*, 339-343.

Howell, G. A., & Frederickson, C. J. (1990). A retrograde transport method for mapping zinc-containing fiber systems in the brain. *Brain Research, 515*, 277-286.

Howell, G. A., Perez-Clausell, J., & Frederickson, C. J. (1991). Zinc containing projections to the bed nucleus of the stria terminalis. *Brain Research, 562*, 181-189.

Howell, G. A., Welch, M. G., & Frederickson, C. J. (1984). Stimulation-induced uptake and release of zinc in hippocampal slices. *Nature, 308*, 736-738.

Huang, R.-C., Peng, Y.-W., & Yau, K.-W. (1993). Zinc modulation of a transient potassium current and histochemical localization of the metal in neurons of the suprachiasmatic nucleus. *Proceedings of the National Academy of Sciences of the United States of America, 90*, 11806-11810.

Ibuka, N., & Kawamura, H. (1975). Loss of circadian rhythm in sleep-wakefulness cycle in the rat by suprachiasmatic nucleus lesions. *Brain Research, 96*, 76-81.

Jain, V., Ravindran, E., & Dhingra, N. K. (2012). Differential expression of Brn3 transcription factors in intrinsically photosensitive retinal ganglion cells in mouse. *The Journal of Comparative Neurology, 520*, 742-755.

Johnson, R. F., Moore, R. Y., & Morin, L. P. (1989). Lateral geniculate lesions alter circadian activity rhythms in the hamster. *Brain Research Bulletin, 22*, 411-422.

Kantheti, P., Qiao, X., Diaz, M. E., Peden, A. A., Meyer, G. E., Carskadon, S. L. (1998). Mutation in AP-3 δ in the mocha mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles. *Neuron, 21*, 111-122.

Kay, A. R. (2003). Evidence for chelatable zinc in the extracellular space of the hippocampus, but little evidence for synaptic release of Zn. *Journal of Neuroscience, 23*, 6847-6855.

Kondo, T., Strayer, C. A., Kulkarni, R. D., Taylor, W., Ishiura, M., Golden, S. S. (1993). Circadian rhyhms in prokaryotes: Luciferase as a reporter of circadian gene expression in cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America, 90*, 5672-5676.

Krebs, N. F. (2013). Zinc homeostasis, whole body. In R. H. Kretsinger, V. N. Uversky, & E. A. Permyakov (Eds.), *Encyclopedia of Metalloproteins* (pp. 2428-2432). New York, USA: Springer.

Land, P. W., & Shamalla-Hannah, L. (2001). Transient expression of synaptic zinc during development of uncrossed retinogeniculate projections. *Journal of Comparative Neurology, 433*, 515-525.

Liuzzi, J. P., & Cousins, R. J. (2004). Mammalian zinc transporters *Annual Review of Nutrition, 24*, 151-172.

Long, Y. Y., Hardwick, A., & Frederickson, C. J. (1995). Zinc-containing innervation of the subicular region in the rat. *Neurochemistry International, 27*, 95-103.

Lucas, R. J., Douglas, R. H., & Foster, R. G. (2001). Characterization of an ocular photopigment capable of driving pupillary constriction in mice. *Nature Neuroscience, 4*, 621-626.

Lucas, R. J., Hattar, S., Takao, M., Berson, D. M., Foster, R. G., & Yau, K. W. (2003). Diminished pupillary light reflex at high irradiances in melanopsin-knockout mice. *Science, 299*, 245-247.

Mayer, M. L., Vyklicky, L. J., & Westbrook, G. L. (1989). Modulation of excitatory amino acid receptors by group IIB metal cations in cultured mouse hippocampal neurons. *The Journal of Physiology, 415*, 329-350.

Meyer-Bernstein, E. L., & Morin, L. P. (1996). Differential serotonergic innervation of the suprachiasmatic nucleus and the intergeniculate leaflet and its role in circadian rhythm modulation. *Journal of Neuroscience, 16*, 2097-2111.

Mintz, E. M., Gillespie, C. F., Marvel, C. L., Huhman, K. L., & Albers, H. E. (1997). Serotonergic regulation of circadian rhythms in Syrian hamsters. *Neuroscience, 79*, 563-569.

Mistlberger, R. E., & Antle, M. C. (2011). Entrainment of circadian clocks in mammals by arousal and food. *Essays in Biochemistry, 49*, 119-136.

Moore, R. Y. (1996). Entrainment pathways and the functional organization of the circadian system. *Progress in Brain Research, 111,* 103-119.

Moore, R. Y., & Card, P. (1994). Intergeniculate leaflet: an anatomically and functionally distinct subdivision of the lateral geniculate complex. *The Journal of Comparative Neurology, 344*, 403-430.

Moore, R. Y., Speh, J. C., & Card, J. P. (1995). The retinohypothalamic tract originates from a distinct subset of retinal ganglion cells. *The Journal of Comparative Neurology, 352,* 351-366.

Moore, R. Y., Weis, R., & Moga, M. M. (2000). Efferent projections of the intergeniculate leaflet and the ventral lateral geniculate nucleus in the rat. *The Journal of Comparative Neurology, 420*, 398-418.

Morin, L. P., & Allen, C. N. (2006). The circadian visual system. *Brain Research Reviews, 51,* 1-60.

Morse, D., & Sassone-Corsi, P. (2002). Time after time: inputs to and outputs from the mammalian circadian oscillators. *Trends in Neurosciences, 25,* 632-637.

Muller, A., Kleinau, G., Piechowski, C.L., Muller, T.D., Finan, B., Pratzka, J., … Biebermann, H. (2013). G-protein coupled receptor 83 (GPR83) signaling determined by constitutive and zinc(II)-induced activity. *PLoS ONE, 8,* e53347.

Nakashima, A. S., & Dyck, R. H. (2009). Zinc and cortical plasticity. *Brain Research Reviews, 59*, 347-373.

Nielsen, H. S., Hannibal, J., Knudsen, S. M., & Fahrenkrug, J. (2001). Pituitary adenylate cyclase-activating polypeptide induces *period1* and *period2* gene expression in the rat suprachiasmatic nucleus during late night. *Neuroscience, 103*, 433-441.

Palmiter, R. D., Cole, T. B., Quaife, C. J., & Findley, S. D. (1996). ZnT-3, a putative transporter of zinc into synaptic vesicles. *Proceedings of the National Academy of Sciences of the United States of America, 93*, 14934-14939.

Pan, E., Zhang, X. A., Huang, Z., Krezel, A., Zhao, M., Tinberg, C. E. (2011). Vesicular zinc promotes presynaptic and inhibits postsynaptic long-term potentiation of mossy fiber-CA3 synapse. *Neuron, 71*, 1116-1126.

Paoletti, P., Ascher, P., & Neyton, J. (1997). High-affinity zinc inhibition of NMDA NR1-NR2A receptors. *Journal of Neuroscience, 17*, 5711-5725.

Paoletti, P., & Neyton, J. (2007). NMDA receptor subunits: function and pharmacology. *Current Opinion in pharmacology, 7*, 39-47.

Paoletti, P., Vergnano, A. M., Barbour, B., & Casado, M. (2009). Zinc at Glutamatergic Synapses. *Neuroscience, 158*, 126-136.

Pickard, G. E. (1982). The afferent connections of the suprachiasmatic nucleus of the golden hamster with emphasis on the retinohypothalamic projection. *The Journal of Comparative Neurology, 211*, 65-83.

Pickard, G. E., Ralph, M. R., & Menaker, M. (1987). The intergeniculate leaflet partially mediates effects of light on circadian rhythms. *Journal of Biological Rhythms, 2*, 35-56.

Pittendrigh, C. S., & Daan, S. (1976). A functional analysis of circadian pacemakers in nocturnal rodents IV. Entrainment: pacemaker as clock. *Journal of Comparative Physiology, 106*, 291-331.

Prosser, R. A., McArthur, A. J., & Gillette, M. U. (1989). cGMP induces phase shifts of a mammalian circadian pacemaker at night, in antiphase to cAMP effects. *Proceedings of the National Academy of Sciences of the United States of America, 86*, 6812-6815.

Provencio, I., Jiang, G., Grip, W. J. D., Hayes, W. P., & Rollag, M. D. (1998). Melanopsin: an opsin in melanophores, brain and eye. *Proceedings of the National Academy of Sciences of the United States of America, 95*, 340-345.

Provencio, I., Rodriguez, I. R., Jiang, G., Hayes, W. P., Moreira, E. F., & Rollag, M. D. (2000). A novel human opsin in the inner retina. *The Journal of Neuroscience, 20*, 600-605.

Ralph, M. R., & Menaker, M. (1988). A mutation of the circadian system in golden hamsters. *Science, 241*, 1225-1227.

Ralph, M. R., & Menaker, M. (1990). Transplanted suprachiasmatic nucleus determine circadian period. *Science, 247*, 975-978.

Rassendren, F. A., Lory, P., Pin, J. P., & Nargeot, J. (1990). Zinc has opposite effects on NMDA and non-NMDA receptors expressed in Xenopus oocytes. *Neuron, 4*, 733-740.

Redenti, S., & Chappell, R. L. (2004). Localization of zinc transporter-3 (ZnT-3) in mouse retina. *Vision Research, 44*, 3317-3321.

Regland, B., Lehmann, W., Abedini, I., Blennow, K., Jonsson, M., Karlsson, I. (2001). Treatment of alzheimer's disease with clioquinol. *Dementia and Geriatric Cognitive Disorders, 12*, 408-414.

Reppert, S. M., & Weaver, D. R. (2001). Molecular analysis of mammalian circadian rhythms. *Annual Review of Physiology, 63*, 647-676.

Salazar, G., Craige, B., Love, R., Kalman, D., & Faundez, V. (2005). Vglut1 and ZnT3 co-targeting mechanisms regulate vesicular zinc stores in PC12 cells. *Journal of Cell Science, 118*, 1911-1921.

Slomianka, L., Danscher, G., & Frederickson, C. J. (1990). Labeling of the neurons of origin of zinc-containing pathways by intraperitoneal injections of sodium selenite. *Neuroscience, 38*, 843-854.

Smidt, K., Jessen, N., Petersen, A. B., Larsen, A., Magnusson, N., Jeppesen, J. B. (2009). SLC30A3 responds to glucose -and zinc variations in ß-cells and is critical for insulin production and *in vivo* glucose-metabolism during ß-cell stress. *PLoS ONE, 4*, 1-12.

Smidt, K., Pedersen, S. B., Brock, B., Schmitz, O., Fisker, S., Bendix, J. (2007). Zinc-transporter genes in human visceral and subcutaneous adipocytes: lean versus obese. *Molecular and Cellular Endocrinology, 264*, 68-73.

Smidt, K., & Rungby, J. (2012). ZnT3: a zinc transporter active in several organs. *Biometals, 25*, 1-8.

Smith, A. P., & Lee, N. M. (2007). Role of zinc in ALS. *Amyotrophic Lateral Sclerosis, 8*, 131-143.

Smitt, P. A. E. S., Mulder, T. P. J., Verspaget, H. W., Blaauwgeers, H. G. T., Troost, D., & Jong, J. M. B. V. d. (1994). Metallothionein in amyotrophic lateral sclerosis. *Neurosignals, 3*, 193-197.

Spiridon, M., Kamm, D., Billups, B., Mobbs, P., & Attwell, D. (1998). Modulation by zinc of the glutamate transporters in glial cells and cones isolated from the tiger salamander retina. *The Journal of Physiology, 506*, 363-376.

Stephan, F. K., & Zucker, I. (1972). Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proceedings of the National Academy of Sciences of the United States of America, 69*, 1583-1586.

Sterniczuk, R., Stepkowski, A., Jones, M., & Antle, M. C. (2008). Enhancement of photic shifts with the 5-HT_{1A} mixed agonist/antagonist NAN-190: Intra-suprachiasmatic nucleus pathway. *Neuroscience, 153*, 571-580.

Takeda, A., Minami, A., Seki, Y., & Oku, N. (2003). Inhibitory function of zinc against excitation of hippocampal glutamatergic neurons. *Epilepsy Research, 57*, 169-174.

Toth, K. (2011). Zinc in neurotransmission. *Annual Review of Nutrition, 31*, 139-153.

Ugarte, M., & Osborne, N. N. (1999). The localization of zinc in rat photoreceptors varies during light and dark adaptation. *Experimental Eye Research, 69*, 459-461.

Ugarte, M., & Osborne, N. N. (2001). Zinc in the retina. *Progress in Neurobiology, 64*, 219-249.

Vallee, B. L., & Falchuk, K. H. (1993). The biochemical basis of zinc physiology. *Physiological Reviews, 73*, 27.

Vandenberg, R. J., Mitrovic, A. D., & Johnston, G. A. (1998). Molecular basis for differential inhibition of glutamate transporter subtypes by zinc ions. *Molecular Pharmacology, 54*, 189-196.

Vrang, N., Mrosovsky, N., & Mikkelsen, J. D. (2003). Afferent projections to the hamster intergeniculate leaflet demonstrated by retrograde and anterograde tracing. *Brain Research Bulletin, 59*, 267-288.

Welsh, D. K., Logothetis, D. E., Meister, M., & Reppert, S. M. (1995). Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron, 14*, 697-706.

Westbrook, G. L., & Mayer, M. L. (1987). Micromolar concentrations of Zn^{2+} antagonize NMDA and GABA responses of hippocampal neurons. *Nature, 328*, 640-643.

Wickland, C., & Turek, F. W. (1994). Lesions of the thalamic intergeniculate leaflet block activity-induced phase shifts in the circadian activity rhythm of the golden hamster. *Brain Research, 660,* 293-300.

Wongdee, K., Teerapornpuntakit, J., Riengrojpitak, S., Krishnamra, N., & Charoenphandhu, N. (2009). Gene expression profile of duodenal epithelial cells in response to chronic metabolic acidosis. *Molecular and Cellular Endocrinology, 321,* 173-188.

Xie, X., & Smart, T. G. (1994). Modulation of long-term potentiation in rat hippocampal pyramidal neurons by zinc. *Pflügers Archiv - European Journal of Physiology, 427,* 481-486.

Yin, H. Z., Ha, D. H., Carriedo, S. G., & Weiss, J. H. (1998). Kainate-stimulated Zn^{2+} uptake labels cortical neurons with Ca^{2+}-permeable AMPA/kainate channels. *Brain Research, 781*, 45-56.