# MIDKINES,
# IN THE NEURAL STEM CELL NICHE, DURING DEVELOPMENTAL AND REGENERATIVE NEUROGENESIS AND THEIR REGULATION BY THE CIRCADIAN CLOCK IN THE RETINA OF ZEBRAFISH

by

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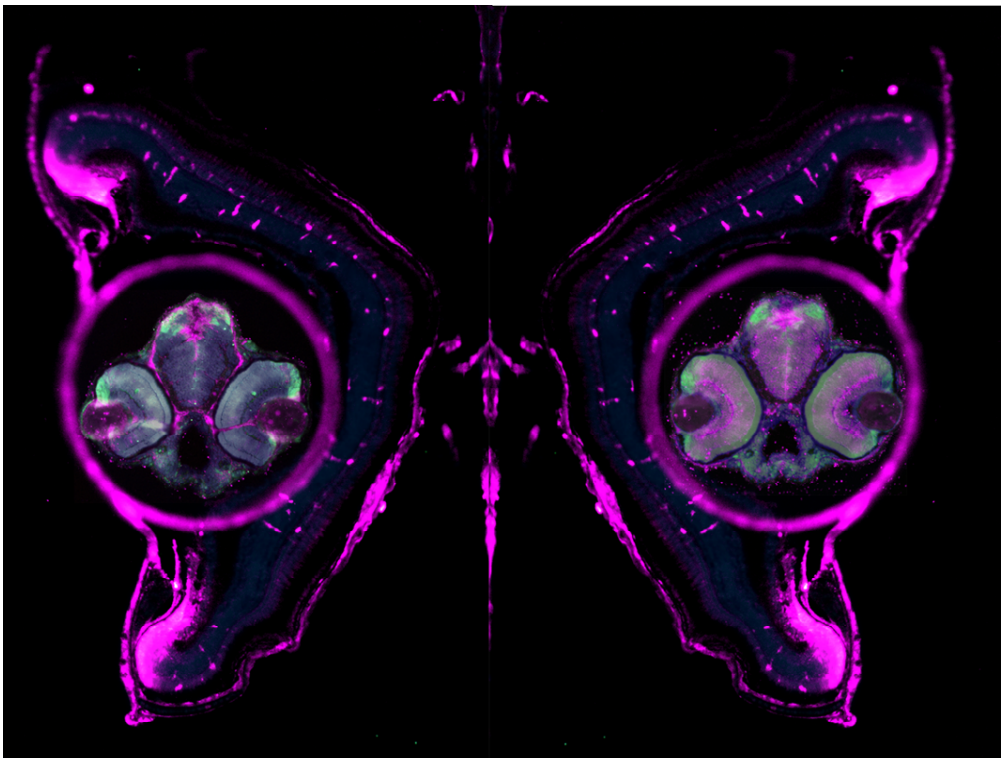

This is work is a tribute to my son, Oscar, for his uncanny wisdom and understanding, demonstrated at such a young age, for all the shortcomings I put him through, and for all the happiness he brings to all. His resilience and humor enable me to complete this dissertation and continue my scientific pursuit.

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ABSTRACT

In the retina of adult teleosts, stem cells are sustained in two specialized niches: the ciliary marginal zone (CMZ) and the microenvironment surrounding adult Müller glia. Recently, Müller glia were identified as retinal stem cells responsible for neuronal regeneration. In a screen to discover secreted molecules that regulate neuronal regeneration in the retina, we identified midkine-b (*mdkb*). Midkine is a highly conserved pleiotropic, heparin-binding growth-factor. The zebrafish genome encodes two midkine genes: *midkine-a* (*mdka*) and *mdkb.* Expression and function of Midkines in the vertebrate retina are largely unknown. My research shows that zebrafish *mdka* and *mdkb* are expressed in distinct patterns in developing, mature and regenerating retina, suggesting different functions for the two molecules. In the developing zebrafish retina, *mdka* is expressed in the CMZ and *mdkb* in newly postmitotic cells, suggesting these molecules may sequentially regulate aspects of retinal neurogenesis. In the juvenile/adult retina, *mdka* is expressed in presumptive Müller glia at the retinal margin, cells at the origin of the rod photoreceptor lineage, and in horizontal cells. Following selective death of photoreceptors in the adult retina, *mdka* and *mdkb* are co-expressed in horizontal cells, proliferating Müller glia and their neurogenic progeny.

The retina entrains the circadian clock to changes in the light/dark cycle and is characterized by numerous biological processes that follow a circadian rhythm. Expression of Mdka in horizontal cells is regulated by the circadian clock, with increased expression during subjective day. Expression of *mdkb* is weakly modulated by the circadian clock, increasing during subjective night in horizontal cells. The two midkin

es show therefore asynchronous circadian regulation, suggesting different biological activities at distinct circadian times. Expression of *mdkb* in horizontal cells during the subjective night, similar to the regenerating retina, suggests a role in persistent neurogenesis.

In conclusion, Mdka and Mdkb are molecular components in the retinal stem cell compartments during developmental, regenerative and growth-associated neurogenesis suggesting they function as autocrine/paracrine signaling molecules and sequentially regulate different aspects of neurogenesis in the zebrafish retina. These data establish the foundation for future studies to investigate functional roles of these molecules in retinal neurogenesis.

# CHAPTER 1

## INTRODUCTION

Recent advances in biology point to a fascinating emerging concept, that fundamental processes such as cell division, migration, differentiation and tissue integration are guided by signaling pathways that employ the same molecular players during development and injury-induced regeneration and these same pathways, when disrupted, lead to cancer formation (Beachy et al., 2004). In mammals, regeneration following injury is possible in some organs like skin, bone or liver but is largely absent in critical organs like the central nervous system and the heart, where injury results in irreversible tissue degeneration, scar formation and loss of function. In lower vertebrates, examples of remarkable regenerative capacity are abundant: newts and salamanders can regenerate limbs and hearts, spinal cords and jaws, lenses, retinas; frogs can regenerate tails and limbs as larvae but not as adults; zebrafish can regenerate fins, hearts, nerves, and also retinas (Kumar et al., 2007, Nye et al., 2003a and 2003b, Neff et al., 1996, Chernoff et al., 2003, Butler and Ward, 1965, Mitasov, 1996, Slack et al., 2004, Poss et al., 2002, Poss et al., 2003, Bernhard, 1999, Vihtelic and Hyde, 2000). Amazingly, urodele salamanders, when exposed to carcinogens will generate ectopic limbs rather then develop tumors (Gardiner, 2005).

The zebrafish retina, having a simple, and extensively described structure, offers a wonderful model to uncover mechanisms of regeneration in the CNS, a tissue with complex cyto-architecture that poses challenges for analytical pursuit. Several groups of scientists are intensely studying regeneration in the zebrafish retina, and this effort has lead to remarkable advances in the field. The cellular players in retinal regeneration have been identified, and numerous molecules involved in retinal development have been shown to be re-expressed in injury-activated stem cells, suggesting that regenerative events largely recapitulate developmental steps. Still missing is knowledge on the initial triggers of regeneration, the first signal or combination of signals that initiate the regenerative cascade.

In the mammalian olfactory epithelium, it has been shown that olfactory bulb ablation (OBX) results in increased expression of leukemia inhibiting factor (LIF) in the injured neurons themselves. LIF is responsible for the proliferation of the neuronal progenitors that will regenerate the olfactory sensory neurons, since in LIF knock-out mice, OBX fails to elicit a proliferative response (Bauer et al, 2003). We have hypothesized that similar to the olfactory bulb, in the zebrafish retina, secreted molecules are critical for regulating the regenerative response.

To start the search for these critical signals in the retina, we took advantage of recent high-throughput gene-chip array technology, of the increasingly well characterized and annotated zebrafish genome, and also of an elegant, non-invasive light-injury paradigm in albino zebrafish, developed by Tom Vihtelic (University of Notre Dame), a paradigm that selectively kills

photoreceptors while leaving the rest of the retina intact. We analyzed gene expression changes between light-lesioned and control retinas at the time of maximum proliferation, and looked for secreted molecules that are differentially expressed between normal and lesioned retinas. This analysis identified seven secreted molecules, potential candidates that could satisfy our hypothesis. Midkine-b was one of the seven. My goal was to uncover its biological roles in the zebrafish retina. In zebrafish there are two midkine paralogs, *mdka* and *mdkb.* As a first step towards my goal, I described in detail the cellular expression of both *mdka* and *mdkb* during retinal development and also during photoreceptor regeneration, using a modified light-lesion paradigm that we developed for pigmented fish. Whereas we found *mdkb* in our unbiased gene-array screen, my expression analysis has shown Midkine-a is also an extremely intriguing candidate signaling factor to regulate retinal development and regeneration.

In the following introductory chapter I present a brief summary of current knowledge on retinal development and injury-induced retinal regeneration, and I expand on knowledge about Midkines, their expression patterns in different animal models and humans, known receptors and signaling pathways activated by them and, finally, brief perspectives on Midkines in cancers and injury-induced repair. In the second chapter I present data on expression of *mdka* and *mdkb* during two neurogenic events, retinal development and injury-induced regeneration. This chapter is a largely unabridged version of the manuscript submitted to the Journal of Comparative Neurology: “The Cellular Expression of

Midkine-a and Midkine-b During Retinal Development and Photoreceptor Regeneration" by Anda-Alexandra Calinescu, Thomas S. Vihtelic, David R. Hyde and Peter F. Hitchcock. In the third chapter I present data on the circadian regulation of Midkines. This chapter is a manuscript in preparation. Finally, in the fourth chapter I present concluding insights from my work on Midkines, summarize a few functional approaches I attempted, present several intriguing questions that result from my in depth expression analysis and propose a few possible projects that may help answer these questions.

## 1.1 NEUROGENESIS IN THE DEVELOPING AND GROWING ZEBRAFISH RETINA

The neural retina in all vertebrates develops from a sheet of neuroepithelial cells that line the inside of the optic cup. The cells on the outside will differentiate to form the retinal pigmented epithelium (RPE). The space between the neural retina and the RPE, which initially communicates with the ventricular system in the CNS, collapses so that the RPE and neural retina are closely apposed early in development. The retinal polarity thus corresponds to the CNS polarity with the ventricular (apical) side facing the RPE and the pial (basal) side facing the vitreous cavity. The neuroepithelial cells of the primordium of the neural retina are fusiform and radially oriented with endfeet connected to the ventricular and pial surfaces. Retinal progenitor cells undergo mitosis at the ventricular side, similar to the cortex, and migrate radially, toward the basal side during the other stages of the cell cycle. As they leave the cell cycle, retinal neurons, except photoreceptors, lose contact with the ventricular side and migrate to their final laminar position (Dyer et al, 2003). Cellular birthdating experiments (labeling cells undergoing their last S phase with [$^3$H] thymidine) have established that retinal neurons and Müller glia, while originating from the same precursors, are generated sequencially in an order that is largely conserved between all species analyzed. Two "waves" of cell genesis have been described in mammals: an early phase when retinal ganglion cells are "born",

followed by horizontal, amacrine and cone photoreceptors, in a largely overlapping manner, and a late phase when rod photoreceptors, Müller glia and bipolar cells undergo their last mitosis (Cepko et al., 1996, Rapaport et al, 2004). The developmental time of the last mitosis is therefore a strong predictor of cellular identity of the newly born cell.

Similar to other vertebrates, the zebrafish retina develops at the end of gastrulation from anterior neural plate cells specified to form the eye fields, which are separated by diencephalic precursors into bilateral eye primordia that form the optic cups (Varga et. al.1999, Schmitt and Dowling, 1994). By 24 hours post fertilization (hpf) the eyecups are well developed and consist of proliferating neuroepithelial cells with apico-basal polarity. Neuronal differentiation in the retina begins at 28-32 hpf within a precocious patch, ventro-nasal to the optic stalk, where the first neurons born are retinal ganglion cells (Burrill and Easter, 1995, Hu and Easter, 1999, Schmitt and Dowling, 1994, Schmitt and Dowling, 1999). The subsequent differentiation and lamination proceed at a fast pace in a wave that emerges from the ventro-nasal patch and sweeps dorsally and then temporally, such that by 72hpf the retina has acquired most of its mature characteristics (Schmitt and Dowling, 1999, Easter and Nicola 1996, Hu and Easter, 1999). As in the cortex (Bystron et al, 2008), there is an inside to outside direction of differentiation, with retinal ganglion cells (RGC's) in the basal, inner or vitreal side of the retina born first, and rod photoreceptors, at the apical, outer or scleral side of the retina last. In the zebrafish retina there is also a ventral to dorsal gradient of differentiation, such that the dorsal retina develops later than

the ventral retina and continues to grow at a slightly faster rate after the zebrafish larvae have achieved vision. Therefore the emergence of the optic nerve is always closer to the ventral retinal margin.

The continual growth of the zebrafish retina provides an excellent model to study neurogenesis. Unlike terrestrial animals, which limit their growth to accommodate the effects of gravity, aquatic animals have the ability to continually grow, given availability of food and swimming habitat. The growth of the retina is yoked to the overall growth of the animal and, in order to maintain adequate visual sensitivity, new neurons are added to the retina continuously, at a higher rate in rapid-growing young animals. This addition of new neurons occurs at two different sites, at the retinal margin, the circumferential marginal zone (CMZ), where all retinal cell types are formed, with the exception of rods, and in the central, inner retina, where intrinsic stem cells generate exclusively rod photoreceptors (Johns, 1982, Raymond and Rivlin, 1987, Otteson et al., 2002, Hitchcock et al., 2004, Bernardos et al., 2007). Within the CMZ, developmental time is spatially recapitulated, such that pluripotent stem cells are located peripherally in the CMZ, adjacent to the iris, and progenitors with increasingly restricted competence are located more centrally. This spatial pattern is evidenced by a regionalized expression of genes that sequentially specify cellular identities (Raymond et. al. 2006, see also Harris and Perron, 1998). This remarkable system has enabled researchers to characterize the molecular makeup of retinal progenitors at different stages in their development, and even

more remarkable is that these molecular components are largely the same in different vertebrates.

The CMZ can be divided into three rather distinct zones, based on the classes of genes expressed, as elegantly described for the frog retina (Harris and Perron, 1998) and recently comprehensively detailed for the zebrafish retina (Raymond et al., 2006). At the distal periphery of the retina lies the zone of proliferation and retinal specification, harboring undifferentiated retinal stem cells which co-express pax6, *vsx2(chx10), rx1, six3,* Ncadherin, *ascl1a* ( the zebrafish ash homologue) and members of the Notch/Delta family and their downstream molecular targets. The middle or proneural and neurogenic region represents the location of cells that express proneural genes is found (*delta, notch, ascl1a, pax6a*). The central, cellular determination region is where cells express markers according to their specific cell type committment (*rx1, rx2, neurod*).

This detailed characterization of the cellular and molecular components of the retinal stem niche yields an understanding of the sequence of events in retinal neurogenesis and also has potential therapeutic implications. Recently, retinal repair by means of stem cell transplantation has been shown to be a possible therapeutic direction for treating retinal blinding diseases (MacLaren et al., 2006). However functional integration of transplanted cells is possible only with donor retinal precursor cells at a very specific stage in their development (*i.e*. immature rod progenitors characterized by expression of Nrl, a b-ZIP transcription factor).

In central retina, intrinsic stem cells in the inner nuclear layer, recently identified as Müller cells (Bernardos et al., 2007, Fimbel et al., 2007, Fausett & Goldman, 2007), give rise to a lineage of cells that proliferate, migrate to the outer nuclear layer (ONL) where they proliferate a few more times, and differentiate exclusively into new rod photoreceptors (Johns, 1982, Raymond and Rivlin, 1987, Otteson and Hitchock, 2003, Hitchcock et al, 2004, Bernardos et al., 2007). These new rods are integrated in the retinal circuitry such that, as the fish grows, visual sensitivity is maintained (Powers et al, 1988).

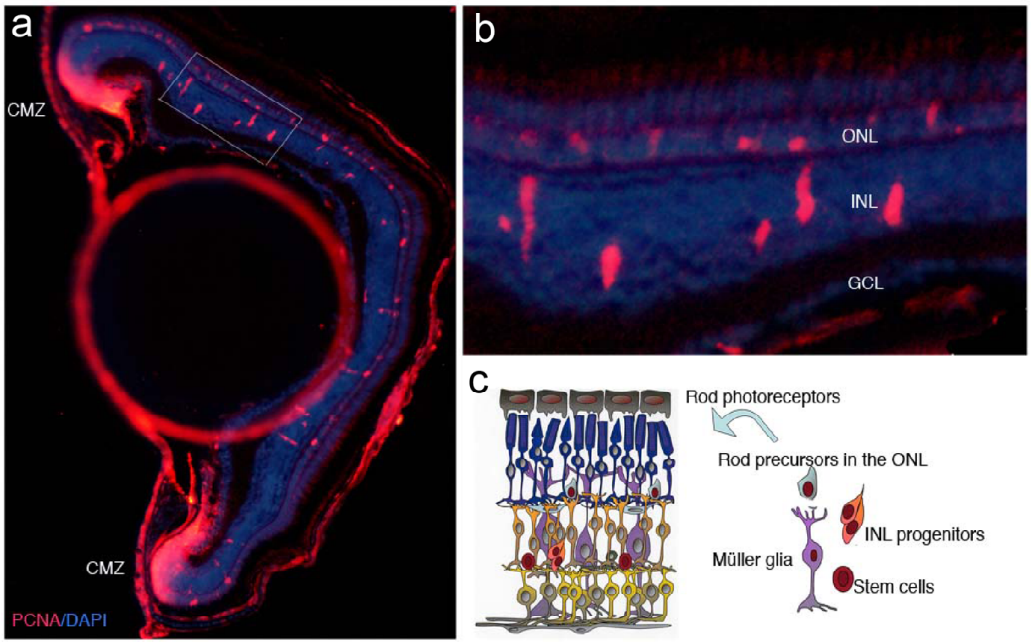


**Fig. 1.1 Growth-associated neurogenesis in the zebrafish retina**

In the retina of zebrafish neurogenesis persists in two regions, the circumferential marginal zone (CMZ), at the boundary between the retina and iris, region where all retinal neurons are born, except rod photoreceptors, and in central retina, where intrinsic stem cells are at the origin of the rod photoreceptor lineage. Panel a illustrates a cross-section through the eye of a zebrafish (1Mo old), dorsal side up, immunostained with antibodies against PCNA and counterstained with DAPI, to visualize the retinal laminae. Panel b is a magnified view of the central retina (white rectangular insert in panel a). Note the clusters of PCNA positive, fusiform cells in the INL, representing stem cells and INL progenitors, and the numerous proliferating cells in the ONL, representing rod precursors. CMZ, circumferential marginal zone, ONL, outer nuclear layer, INL, inner nuclear layer, GCL, ganglion cell layer, PCNA, proliferating cell nuclear antigen, DAPI, nuclear stain 4,6-diamidino-2-phenylindole dihydrochloride.

The addition of new rods into the retina is under the control of growth-associated endocrine factors, such as members of the growth hormone/insulin-like growth factor family (GH/IGF-1) (Otteson and Hitchcock, 2002, Mack and Fernald, 1993, Zygar et al., 2005). IGF-1 production by cone photoreceptors in the cichlid *H.burtoni* has been shown to regulate the proliferation of rod precursor cells (Zygar et al., 2005). In some rapidly growing fish, like cichlids and rainbow trout, the proliferation of rod precursors in the ONL has been shown to follow a diurnal rhythm, with increased proliferation during the night compared to the day (Julian et al, 1998, Chiu and Fernald, 1995). Also, IGF-I production in H.Burtoni has been shown to increase during the night (Zygar et al., 2005). However, administration of exogenous IGF-I to organotypic cultures of goldfish retinas increases proliferation only in the CMZ, and does not significantly change the size of the rod precursor population (Boucher et al, 1998a). Radio-labeled ligand binding of IGF-I has shown that IGF-I, in the goldfish retina, binds only to the CMZ and inner plexiform layer (Boucher et al, 1998b). Whether these data reflect differences between different teleost species, or whether alternative explanations can reconcile these findings is not yet known. It is however indisputable, that young, healthy, fast growing fish will have a high number of rod progenitors in the retina, and growth is yoked to the GH/IGF-I axis.

Within the rod lineage, Müller cells and their neurogenic offspring in the INL express some of the same molecular markers as cells the CMZ : *pax6* (Otteson and Hitchcock, 2003), members of the Delta/Notch family and their

downstream targets *hes1, 5* (Raymond et al., 2006). As these cells migrate to the ONL they express genes characteristic of their rod cell fate: *crx, rx1*, *neuroD*, Nr2E3 (Raymond et al, 2006, Bernardos et al, 2007, Hitchcock and Kakuk-Atkins, 2004, Ochocinska and Hitchcock, 2007, Morris et al, 2008). Thus, within the growing, adult zebrafish retina, the peripheral to central gradient of neurogenesis and differentiation found in the CMZ transforms into an inside-out pattern, similar to the developing retina and cortex.

## 1.2 INJURY INDUCED NEUROGENESIS IN THE ZEBRAFISH RETINA

The retina of teleosts can regenerate neurons following a wide variety of injuries. Surgical excision of an entire segment of the retina leads to complete regeneration of the retinal histo-architecture and regeneration of all retinal types (Hitchcock et al., 1992). Other methods of lesion: laser ablation (Braisted et al, 1994, Wu et al., 2001), metabolic poisons or neurotoxins (Raymond et al., 1988, Braisted and Raymond, 1992, Fimbel et al, 2007), thermal injury (Raymond et al, 2006), surgical injury (Yurco and Cameron, 2005, Fausett and Goldman, 2006) or, less invasive, selective photoreceptor damage with intense light (Vihtelic and Hyde, 2000, Bernardos et al, 2007) have shown that the teleost retina has robust mechanisms that respond to injury by engaging intrinsic stem cells to regenerate lost neurons. Recent studies have identified the stem cells at the origin of both growth-associated and injury-induced neurogenesis to be the Müller glia (Bernardos et. al, 2007, Yurko and Cameron, 2005, Raymond et al., 2006, Fausett and Goldman, 2006).

Following the death of extant retinal neurons, Müller glia de-differentiate, reenter the cell cycle and give rise to multipotent progenitors that proliferate, migrate, differentiate and replace the missing neurons. Genes that guide retinal development and growth, such *as rx1, vsx/Chx10, pax6*, members of the Notch/Delta signaling pathway and downstream molecules, apolipoprotein E (*apoE)*, brain lipid binding protein (BLBP), *ascl1a,* Ncadherin are re-expressed in these Müller cells and progenitors (Raymond et. al., 2006 and the references therein). The microenvironment surrounding Müller glia thus constitutes an active stem cell niche where mechanisms that regulate development and growth at the CMZ are re-employed during regeneration. Little is known, however, about secreted signaling factors that regulate these regenerative events. In a microarray screen for genes regulated by the selective death of photoreceptors, we uncovered *midkine-b,* a member of the heparin-binding growth-factor family.

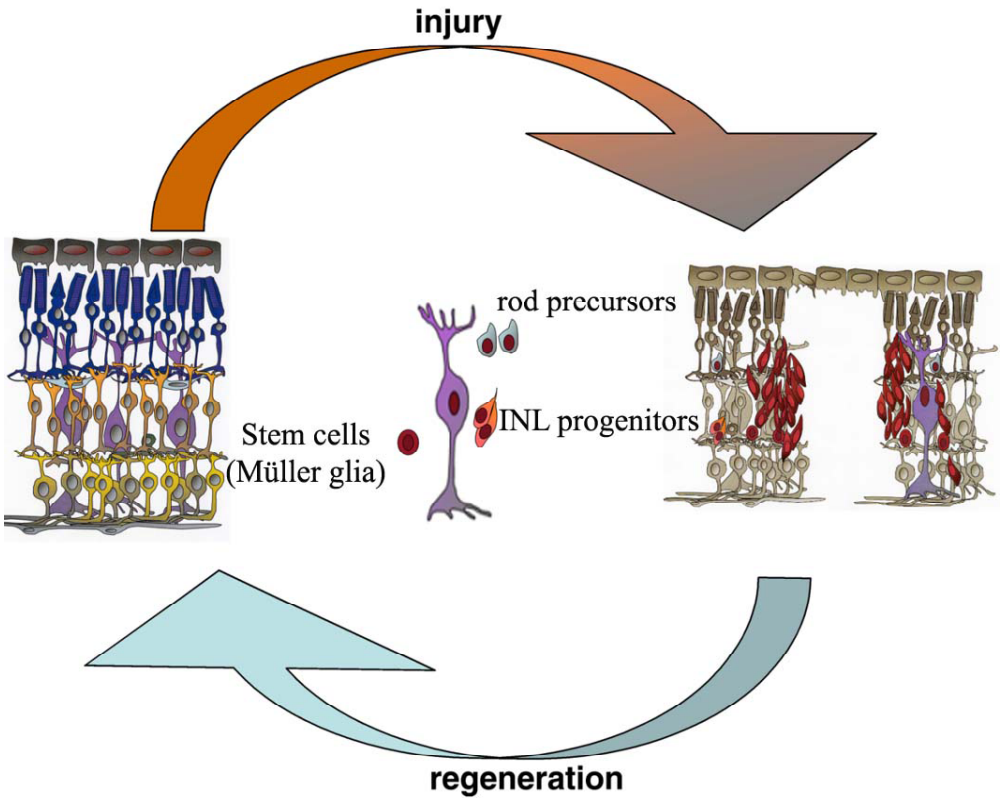


**Fig.1.2 Injury-induced regeneration in the zebrafish retina.**

Cellular players of injury-induced regeneration originate with Müller glia, the stem cells that give rise to the rod lineage during growth-associated neurogenesis.Müller cells de-differentiate, proliferate and generate inner nuclear layer (INL) progenitors, which migrate into the outer nuclear layer where they divide a few more times and generate photoreceptors in the intact retina, or regenerate all retinal cell types following injury. Still unknown are signaling molecules that trigger regeneration.
(adapted from Hitchcock et al., 2004)

## 1.3 MIDKINES

### 1.3.1 EXPRESSION PATTERNS AND FUNCTIONAL CORRELATES

Midkines and pleiotrophin are members of a distinct family of heparin-binding growth/differentiation factors, highly conserved in the animal kingdom with numerous biological activities (Winkler et. al, 2003, Kadomatsu and Muramatsu, 2004). Midkine (MK) was identified in a screen of retinoic acid-inducible genes in embryonic carcinoma cells (Kadomatsu et al. 1988, Kadomatsu et. al. 1990). It is a small protein (13kDa), rich in basic amino acids, with two distinct domains C-terminal and N-terminal, joined by a hinge region (Iwasaki et al., 1997). The C-terminus is highly conserved between various species, harbors the two amino-acid clusters important for heparin binding, and represents the most important functional domain (Muramatsu 1994, Kojima 1995).

In mammals, MK is widely expressed during embryonic development in numerous tissues, most prominently in the developing neural tube and at sites of epithelial–mesenchymal boundaries (Kadomatsu et al., 1990, Muramatsu et al, 1993, Mitsiadis et al., 1995, Fan et al., 2000). Expression strongly decreases in the adult where it is found only at low levels in the kidney and brain (see also Table 1.1). In chicks, Midkine is expressed in the tail-bud at the time of mesenchymal-epithelial conversion during the formation of the secondary neural tube. This expression can be inhibited by exogenous administration of retinoic acid, in contrast to the mammalian MK which is induced by retinoic acid (Griffith and Zile, 2000). In Xenopus, XMK is expressed in the neural tube and its

derivatives, neural crest derivatives and at intersomitic junctions (Sekiguchi et al, 1995). XMK is strongly neurogenic: ectopic XMK induces the formation of hypertrophic neural tissue, increases expression of head specific neural markers (XANF-1 and Xotx2), and reduces expression of activin-induced mesodermal markers (Yokota et al, 1998).

The zebrafish genome encodes two distinct midkine genes: *mdka* and *mdkb*, translated into the secreted heparin-binding proteins: Midkine-a (Mdka) and Midkine-b (Mdkb), which share 68% identity at the amino acid level (Winkler et. al., 2003). Chromosomal localization studies by Winkler and collegues (Winkler et al, 2003) have shown that the two zebrafish midkine paralogs map to different linkage groups, on different chromosomes, *mdka* on LG7, closely linked to *eng2a* and *shh*, and *mdkb* on LG25. Both linkage groups display extensive synteny to human chromosome 11, where the human midkine ortholog is encoded. In addition, the ratio of synonymous to nonsynonymous substitutions shows that the two genes have evolved separately, under purifying selection (Winkler et. al., 2003). This is underscored by the distinctly different expression patterns of the two genes and, as so far described, different biological activities.

In zebrafish, *mdkb* is expressed shortly after the onset of gastrulation in the presumptive neural plate, primarily in the dorsal regions of the developing nervous system. This expression is modulated by retinoic acid (RA) in a dose-dependent manner, inhibited by BMP and stimulated by FGF and canonical Wnt signaling (Winkler and Moon, 2001, Winkler et al, 2003, Liedtke and Winkler, 2008). Ectopic expression of *mdkb* promotes dorsal neural fates (increased

expression of *hoxC10*) and represses formation of anterior neural structures (decreased expression domain of *emx1*), which is in contrast to XMK. *mdkb* defines the neural plate border, regulates specification of premigratory neural crest cells (increases expression of *pax3, foxd3, snail1b, sox10, sox9b*), and is required for the formation of Rohan-Beard sensory neurons (Winkler and Moon, 2001, Winkler et al., 2003, Liedtke and Winkler, 2008). Ectopic expression of a C-terminal truncated form of Mdkb can neutralize the gain-of-function effects of ectopic full length Mdkb, suggesting that the C-terminal half of Mdkb represents the active part of the molecule, and that the truncated form acts as dominant negative molecule (Winkler and Moon, 2001).

Expression of *mdka* begins later than *mdkb*, at the tailbud stage, in the paraxial mesoderm, and is expressed later in somites and the central part of the neural tube. *mdka* is excluded from the dorsal neural tube, where *mdkb* is expressed. Ectopic expression of *mdka* has no effect on head development, in stark contrast to *mdkb*, but specifically promotes medial floorplate formation and blocks somitogenesis (Winkler et. al. 2003, Schäfer et. al. 2005). Both midkines are strongly expressed in adjacent domains at the forebrain/midbrain and midbrain/hindbrain boundary, important organizing centers in the developing head (Winkler et. al. 2003). Taken together these findings show that the two zebrafish Midkines serve different functions, possibly through different receptors and likely act as molecular determinants of neural induction and patterning. Persistence of *mdka* and *mdkb* expression in the adult brain of the zebrafish, specifically of *mdka* in the subventricular zone of the optic tectum (Winkler et. al.

2003), a region where neurogenesis continues in the adult (Marcus et. al., 1999) suggests additional roles for this secreted molecule, possibly in regulating the neural stem cell pool.

### 1.3.2 MIDKINE RECEPTORS AND DOWNSTREAM SIGNALING FACTORS

Whereas there are numerous publications reporting *in vitro* binding of MK to several receptors and activation of several signaling pathways upon MK administration in tissue culture conditions, a comprehensive and clear understanding of the cellular and molecular mechanisms of MK function *in vivo* is largely lacking.

One of the MK receptors more extensively studied is the chondroitin-sulfate proteoglycan receptor-type protein-tyrosine-phosphatase ζ (PTPζ). Midkine can bind with high affinity to PTPζ. (Kd=56nM). This affinity is decreased after chondroitinase digestion or after mutating $Arg^{78}$, one of the basic amino acids in the first heparin-binding domain of the C-terminal half of MK (Maeda et al, 1999). Also, migration of cortical neurons is decreased when neurons are grown on membranes coated with MK in which $Arg^{78}$ has been mutated (Maeda et al, 1999). Migration of osteoblasts in culture is also dependent on MK activation of PTPζ , and is decreased by pharmacologic inhibitors of the PI3K and MAPK pathways (Qi et al, 2001). Binding of MK to PTPζ increases survival of embryonic neurons in culture (Sakaguchi et al., 2003) and MK-induced inhibition of caspase-dependent apoptosis is downstream of the PI3K and MAPK pathways

(Owada et al, 1999). Recently, Neuroglycan-C, known as a part-time transmembrane proteoglycan, has been shown to bind MK as well, and this interaction is important in cell attachment and process extension of oligodendroglial precursor cells (Ichihara-Tanaka et al, 2006).

Syndecans are a family of cell surface heparan-sulfate proteoglycans that can bind, by means of their heparan-sulfate chains, a variety of molecules like growth factors, cytokines, proteinases and extracellular matrix components. These interactions are important in regulating events in embryogenesis, tissue injury and inflammation (Bartlett et al. 2007). During development, an important mechanism of organ formation is represented by interactions between different types of tissues, particularly epithelial and mesenchymal tissues (Gurdon, 1992). In the developing mouse embryo, MK and syndecan-1 are localized at epithelial-mesenchymal boundaries and MK can bind syndecan-1 in a heparan-sulfate dependent manner (Mitsiadis et al., 1995a). Also syndecan-1 and syndecan-3 isolated from the brain and spinal cord of developing rats can bind MK, suggesting a functional role of MK-syndecan interactions during CNS development (Nakanishi et al., 1997).

## Table 1.1 Expression of Midkine genes and their functional correlates

| | Embryonic | Adult | Functional correlates | Citation |
|---|---|---|---|---|
| **Humans** | -fetal liver and kidney<br>-fetal astrocytes<br>-Oligodendrocyte progenitor cells derived from human ES cells | -Kidney, low levels in the brain<br>-increased following injury in numerous tissues<br>-hallmark of various carcinomas | -Malignant progression of astrocytomas<br>-Stimulates neurite growth in adult rat sensory neurons | Mishima et al., 1997<br>Zhang et al., 2006<br>Kato et al., 2000<br>Kadomatsu and Muramatsu, 2004 |
| **Mouse** | -ubiquitous expression in the 7-13d embryo, progressively restricted to brain and ventricular neuroepithelium, anterior pituitary, lung, gastro-intestinal epithelia, metanephros. In the 15d embryo only in the kidney.<br>-Interface between developing epithelium and mesenchyme as well as in proliferating mesenchymal cells (truncated form of MK) | Low levels in the kidney and brain | -role in secondary embryonic induction and epithelial-mesenchymal interactions (inferred from expression pattern)<br>-epithelial–mesenchymal interactions, blood vessel signaling, the decision of proliferation vs differentiation (functions inferred from expression) | Kadomatsu et al., 1990<br>Muramatsu et al, 1993<br>Mitsiadis et al., 1995<br>Fan et al., 2000<br><br>Chen et al., 2005 |
| **Chick** | tailbud,<br>lateral plate mesoderm. | | Mesenchymal-epithelial conversion during secondary neurulation | Griffith and Zile, 2000 |
| **Xenopus** | Brain, spinal cord, branchial arches, optic and otic vesicles, intersomitic junc-<br>tional area between adjacent myotomes | | -Ectopic expression induces expression of head specific neural markers (*XANF-1, Xotx2*) and reduces trunc/tail neural markers (*XlHbox6 and F-spondin*) and mesodermal markers<br>-Binds agrin and modulates clustering of Ach at the NMJ | Sekiguchi et al., 1995<br>Yokota et al, 1998<br>Zhou et al, 1998 |
| **Gibel carp** | Neural keel, midbrain/hindbrain boundary, hindbrain,diencephalons, spinal cord | | | Yin et al., 2007 |
| **Zebrafish *mdka*** | Paraxial mesoderm and somites, central neural tube, midbrain/hindbrain boundary, forebrain/midbrain boundary | Telencephalon, Hypothalamus, subventricular zone of the optic tectum, lobus vagi | required for the formation of the medial floorplate (*shh*) | Winkler et al, 2003<br>Schäfer et al., 2005 |
| **Zebrafish *mdkb*** | Presumptive neural plate, presumptive neural crest and sensory neuron domain, midbrain/hindbrain boundary, forebrain/midbrain boundary | Telencephalon, hypothalamus, Crista cerebellaris (Purkinje cells) | Ectopic expression enhances neural crest cell fates (*fox3d, pax3, sox10, sox9b*) and represses anterior structures (enhances *emx1*, decreases *hoxC10*), promotes formation of Rohan-Beard sensory neurons | Winkler et al, 2003<br>Winkler and Moon, 2001<br>Liedtke and Winkler, 2008 |
| **Drosophila** | **miple1 and miple2**<br>(MK and pleiotrophin homologues)<br>*miple*1 in the CNS, miple2 in the midgut endoderm | | | Englund et al., 2006 |

Low density lipoprotein (LDL) receptor-related protein (LRP) is a member of a family of related transmembrane proteins that includes, among others, Brushin/megalin, LRP6 and ApoE receptor2 (ApoER2), with primary role in regulation of lipid homeostasis via receptor-mediated endocytosis and also in signal transduction during neurodevelopment (May et al., 2007). MK binds LRP from mouse embryonic homogenates with high affinity, and this affinity is decreased by receptor-associated protein (RAP), a protein known to interfere with the action of LRP (Muramatsu et al., 2000, Hertz et al., 1991). Moreover, RAP inhibits MK-induced survival of embryonic neurons, suggesting that the MK activation of the LRP receptor complex is needed for this function (Muramatsu, et al., 2000). Also, the binding of MK to LRP has been shown to mediate nuclear targeting of MK, via nucleolin, a nucleo-cytoplasmic shuttle protein that strongly binds MK (Take et al., 1994). Nuclear targeting of MK is important for survival of embryonic neurons in culture (Shibata et al., 2002). Other members of the LRP family, specifically apoER2 and LRP6, components of the Reelin receptor complex, have been shown to bind MK as well, yet with lower affinity than LRP (Sagakuchi et al., 2003), but the functional importance of this interaction is not known.

Anaplastic lymphoma kinase (ALK) is a receptor-type protein kinase in the insulin receptor superfamily, first identified in chromosomal translocations in anaplastic large cell lymphomas (Morris et al, 1994). ALK is considered a bona fide oncogene, since translocations of ALK, identified in some lung cancers and lymphomas, yields the production of proteins with constitutive tyrosine kinase

activity, which can induce cell transformation *in vitro* and *in vivo* (see Chiare et al, 2008 and the references therein). MK can bind and induce phorphorylation of ALK and activate the PI3K and MAPK signal transduction pathways downstream in several cell lines that express ALK (Stoica et al., 2002). Furthermore, ALK function-blocking antibodies disrupt receptor binding of MK and MK induced anchorage-independent growth of SW-13 cells, a cell line derived from human adrenal adenocarcinomas (Stoica et al, 2002). Also, forced expression of ALK and insulin-receptor-substrate-1 (IRS-1) renders IL-3- dependent 32D murine myeloid cells independent of IL-3; growth of these transformed cells is dependent on endogenous MK production, and subsequent activation of IRS-1, PI3K, MAPK and NF-$\kappa$B (Kuo et al, 2007). Taken together these data show that ALK activation by MK results in growth and survival of transformed cells.

Integrins are a large family of transmembrane proteins constituting the principal receptors on animal cells that bind extracellular matrix proteins (ECM) and signal to the cell information on the composition of the surrounding ECM. MK was found to bind $\alpha$4$\beta$1- and $\alpha$6$\beta$1-integrins from 13 day-old mouse embryos, and antibodies against $\alpha$4 integrin inhibit migration of osteoblastic cells, whereas antibodies against $\alpha$6 integrin inhibit MK induced neurite outgrowth of embryonic neurons (Muramatsu et al, 2004). Also, MK treatment increases phorsphorylation of paxilin, a molecule associated with integrins, which suggests that integrins are part of the MK receptor-binding complex (Muramatsu et al, 2004). In addition, MK binds to a 37-kDa laminin binding protein precursor (LBP), and this interaction

leads to nuclear translocation of both LBP and MK, showing that interactions of MK to ECM linked molecules are functionally important (Salama et al., 2001).

Interesting insight comes from a recent report that shows MK can induce cross-talk between the Notch2 and Jak2/Stat3 signaling pathways and this interaction is dependent on MK binding to the Notch2 receptor (Huang et al., 2008). In this report Huang and colleagues have elegantly shown that MK induces epithelial-to-mesenchymal transition (EMT) in immortalized human keratinocytes (HaCaT) as evidenced by increased proliferation, cytoskeletal changes, disruption of the cadherin network, decrease in epithelial markers (E-cadherin, β-catenin, γ-catenin) and increase in mesenchymal markers (fibronectin, vimentin and smooth-muscle actin). Further, MK induces nuclear accumulation of Notch2, activation of Jak2/Stat3 and binding of Hes1, a signaling molecule in the Notch pathway, to activated Jak2 and Stat3. Finally, knockdown of Notch-2 inhibits MK-induced proliferation and disruption of the cadherin network (Huang et al., 2008). Given that both MK and Notch are expressed at epithelial-mesenchymal borders during development (Mitsiadis et al, 1995a and 1995b), and both play a role in cancer progression (Leong et al., 2006, Kadomatsu and Muramatsu, 2004), and tumor invasion and metastasis is characterized by epithelial-mesenchymal transformation (EMT, Baum et al, 2008), it is particularly important to understand the mechanisms by which these two signaling molecules cooperate to function at the molecular level.

The diversity of receptors that MK can bind, illustrates how versatile this molecule is and likely explains the pleiotropic nature of this growth/differentiation

factor. Biological activities described for Midkine, such as neurogenic, neurotrophic, mitogenic, transforming, anti-apoptotic, angiogenic, chemotactic and fibrinolytic, are fundamentally dependent on the specific characteristics of the biological system where these activities occur. One can easily recognize that circumstances like developmental time, the nature of the specific tissue and presence of tissue-interactions, specific compositions of the extracellular matrix, the presence and state of cell surface receptors and available downstream signaling molecules and environmental factors can profoundly influence the specific action of this molecule. Needless to say, this complexity introduces challenges when trying to understand the function of this molecule in a complex, three-dimensional, homeostatic *in vivo* environment. However, describing and understanding in detail the cellular players in a particular system brings valuable information that can lead to the understanding of critical elements and to devising strategies for successful functional experimental approaches.

## Table 1.2 Midkine receptors and signaling pathways

| Receptor | Substrate | Biological activity | Signaling pathways | Citation |
|---|---|---|---|---|
| Chondroitin-sulfate proteoglycan receptor- type **PTPζ** (binding to $Arg^{78}$) | -*in vitro* coating with MK followed by ELISA<br>-embryonic neurons in culture<br>-osteoblasts<br>-oligodenrocyte precursor cells | -neuronal migration<br>-survival of embryonic neurons in culture<br>-osteoblast migration<br>-adhesion of oligodendrocyte precursor cells | PI3K<br>MAPK<br>src protein kinase<br>G-protein linked signaling | Maeda et al. 1999<br>Sakaguchi et al., 2003<br>Owada et al., 1999<br>Qi et al., 2001<br>Rambsby et al., 2001 |
| Heparan-sulfate proteoglycans **N-syndecan** | Solid phase binding assay<br><br>Binding of Syndecans from mouse fetal brains | -neurite outgrowth<br>-differentiaion<br>and morphogenesis at epithelial-mesenchymal boundaries (suggested by expression) | | Mitsiadis et al. 1995<br>Nakanishi et al. 1997 |
| **LRP** low-density lipoprotein receptor-related protein | MK affinity chromatography with membrane glycoproteins from embryonic mice (E13) | -survival of embryonic neurons<br>-nuclear targeting of MK<br>-antiapoptotic activity<br>-anchorage-independent cell growth | | Muramatsu et al. 2000<br>Shibata et al, 2002<br>Chen et al, 2007 |
| **ALK** anaplastic lymphoma kinase | -human SW-13 cells<br>-WI-38 human fibroblasts<br>-HUVEC cells<br>-neuroblastoma (SH SY-5Y)<br>-glioblastoma (U87MG) cells<br>-IL-3 dependent myeloid cells | -colony formation (tumor growth)<br>-transformation and tumor cell growth and survival<br>-anchorage independent cell growth | PI3K<br>MAPK<br>IRS-1 and NF-κB | Stoica et al. 2002<br>Bowden et al, 2002<br>Powers et al, 2002<br>Kuo et al., 2007 |
| **integrins α4β1- and α6β1** | -osteoblastic cells | MK induced migration of osteoblastic cells | Tyrosine phosphorylation of paxilin | Muramatsu et al. 2004 |
| **Neuroglycan C** | oligodendroglial precursor-like cells | Process elongation of oligodendroglial precursor-like cells | | Ichihara-Tanaka et al., 2006 |
| **Notch-2** | Immortalized human keratinocytes | MK promotes epithelial-to-mesenchmal transition | cross talk of Notch2/Jak2/Stat3 signaling | Huang et al., 2008 |
| **Unknown receptors** | -3T3-L1 preadipocytes and adipocytes<br>-G401 rhabdoid kidney tumor cells | -mitotic clonal expansion of preadypocytes<br>-autocrine mitogen of G401 cells | Jak1, Jak2<br>Stat1, Stat3 | Cernkovich et al., 2007<br>Ratovitski et al, 1998 |

### 1.3.3 MIDKINES IN CANCER

One of the more notable characteristic of Midkine is its abundant presence in a variety of cancers (Kadomatsu and Muramatsu, 2004 and the references therein), whereas normal expression in the adult is minimal (Kadomatsu et al., 1990, Mitsiadis et al, 1995c). One of the first cancers in which increased expression of MK has been described (Tsutsui et al., 1993) is the Wilms tumor, an embryonic kidney malignancy in infants. Histopathologically this nephroblastoma has three components: a blastema, that proliferates, mesenchymal cells with different degrees of differentiation, and an epithelial component. This is indicative of a developmental defect during kidney formation. WT1 is a zinc-finger transcription factor that acts as a tumor suppressor gene and when mutated is the cause of Wilms tumors in numerous patients (Haber et al., 1990). Midkine has been shown to be a target of WT1, with WT1 binding elements in its promoter (Adachi et. al., 1996). Binding of WT1 to the midkine promoter suppresses its expression (Adachi et. al., 1996) and truncated forms of MK have been described in Wilms tumor (Mitsumoto et al., 2001). Furthermore, MK induces proliferation and inhibits apoptosis of Wilms tumor cells (Qi et al., 2000, Qiu et al., 2000).

These findings, and numerous similar others, linking Midkine to a variety of cancers in which local expression and serum levels of MK are a marker of poor prognosis (Shimada et al., 2003), have led the way towards devising novel therapeutic strategies for cancer therapy. The Midkine promoter represents a

convenient shuttle to target cancer cells for adenoviral suicide gene therapy (Adachi et al., 2002, Hattori et al., 2006). Cancer cells can be targeted with chemotherapeutic agents fused to MK antibodies (Inoh et al., 2006) and MK translation can be specifically inhibited with beneficial therapeutic effects by means of non-toxic morpholino oligonucleotides (Takei et al., 2005). While these approaches are still in their infancy, any information that enhances our understanding of the biology of Midkines will bring us closer to successful stories of cure.

### 1.3.4 INJURY-INDUCED EXPRESSION OF MIDKINE: IMPLICATIONS FOR REGENERATION AND REPAIR

Injury increases expression of MK in several tissues: transient forebrain ischemia and spinal cord injury induces MK expression in reactive astrocytes (Mochizuki et. al. 1998, Sakakima et. al. 2004a), sciatic nerve injury is followed by expression of MK in motor neurons of the anterior horn (Sakakima et. al. 2004b), myocardial infarction and skeletal muscle necrosis induces expression of MK in myocytes (Horiba et.al. 2006, Obama et. al. 1998, Sakakima et. al. 2006), and bone fractures induce MK expression in spindle-shaped mesenchymal cells and chondrocytes at the fracture site (Ohta et. al 1999). Increased expression following injury is thought to indicate a role for midkine in tissue regeneration and repair. Knockout mice lacking the midkine gene, while showing no gross abnormalities, have decreased liver regeneration following partial hepatectomy, as evidenced by reduced number of proliferating cells and by the smaller weight

of regenerated livers (Ochiai et. al, 2004).

Recently, the availability of high throughput gene profiling technology and increasing knowledge of the genome, has made it possible to analyze global changes in gene expression during regenerative events in zebrafish, an animal that can naturally regenerate various tissues to full functional integration. Two such screens performed on regenerating zebrafish hearts and fins identified *mdka* as one of the secreted factors with increased expression in the regenerating tissue, for the fin, as early as 12 hours after the lesion, and for the heart for as long as 14 days post lesion (Schebesta et al., 2006, Lien et al., 2006). This shows that, similar to mammals where Midkine is induced in a variety of tissues, fish respond to injury similarly. We don't know if functions of the mammalian Midkine are common with the zebrafish Mdka, or Mdkb or a combination of both. In light of the fact that injury induces regeneration in fish and mostly scar formation and loss of function in mammals, uncovering the particular details of Mdka and Mdkb function and regulation would enhance our understanding of regenerative biology and may open doors towards new therapeutic approaches of degenerative diseases.

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# CHAPTER 2

## THE CELLULAR EXPRESSION OF MIDKINE-A AND MIDKINE-B DURING RETINAL DEVELOPMENT AND PHOTORECEPTOR REGENERATION

### 2.1 INTRODUCTION

Midkine is a secreted heparin binding growth factor that is highly conserved throughout the animal kingdom (Kadomatsu et al. 1988, Kadomatsu et al. 1990, Winkler et al., 2003, Kadomatsu and Muramatsu, 2004). Midkine was identified in a screen for genes in embryonic carcinoma cells that are inducible by retinoic acid (Kadomatsu et al., 1988, Kadomatsu et al., 1990). Numerous functions have been ascribed to this molecule: neurogenic, mitogenic, neurotrophic, chemotactic, angiogenic, fibrinolytic and anti-apoptotic (for review see Muramatsu 2002, Kadomatsu and Muramatsu, 2004). In mammals, Midkine is expressed in many tissues during embryonic development, most prominently in the developing neural tube and at epithelial–mesenchymal boundaries (Mitsiadis et al., 1995).

Injury increases expression of Midkine in several tissues including nervous tissue, muscle, bone, skin and liver (Obama al. 1998, Sakakima al. 2006; Ohta 1999; Iwashita 1999; Jochheim-Richter et al., 2006). Increased expression following injury indicates a role for Midkine in regeneration and repair (Ohta et al. 1999). Supporting this idea, mice that are homozygous for a midkine null

mutation show decreased regenerative capacity following partial hepatectomy (Ochiai et al. 2004). Also, intravitreal injection of recombinant Midkine promotes survival and preserves function of photoreceptor cells following light-induced injury (Unoki et al., 1994, Masuda et al. 1995).

The zebrafish genome contains two *midkine* genes, *mdka* and *mdkb*, whose encoded proteins share 68% amino acid identity (Winkler et al., 2003). In early embryos, the two *midkines* have distinct patterns of expression and appear to serve different functions. The *mdka* gene is initially expressed at the tailbud stage in the paraxial mesoderm and is later observed in somites and the central neural tube (Winkler et al., 2003, Schäfer et. et al. 2005). The *mdkb* gene, however, is expressed shortly after the onset of gastrulation in the epiblast, presumptive neural plate, neural crest, the diencephalon and dorsal regions of the neural tube (Winkler and Moon 2001, Liedtke and Winkler, 2008). Also, the two *midkines* are expressed in adjacent domains at the forebrain/midbrain and midbrain/hindbrain boundaries, which serve as important organizing centers in the developing brain (Winkler and Moon, 2001, Winkler et al., 2003). Gain and loss-of-function experiments demonstrated *mdka* specifically promotes medial floorplate formation and regulates aspects of somite formation, whereas *mdkb* promotes dorsal neural fates and is required for the formation of neural crest cells and Rohon-Beard sensory neurons (Winkler and Moon, 2001, Schäfer et al., 2005, Liedtke and Winkler, 2008). Together, these data show that the two *midkine* paralogs serve different functions, perhaps through yet unidentified

receptors and mechanisms and may act as molecular determinants in defining different cell classes during neural induction and patterning.

The zebrafish retina develops from cells of the anterior neural plate that evaginate, migrate laterally and form the optic cups (Varga et al.,1999, Schmitt and Dowling, 1994, Hitchcock and Raymond, 2004). By 24 hours post-fertilization (hpf), the eyecups are well developed and consist of proliferating neuroepithelial cells. Neuronal differentiation begins at 28-32 hpf within a precocious patch that is ventro-nasal to the optic stalk (Burrill and Easter, 1994, Hu and Easter, 1999, Schmitt and Dowling, 1994, Schmitt and Dowling, 1999). Retinal cell differentiation and lamination proceed at a fast pace in sequential waves that originate in the ventro-nasal patch and sweep dorsally and then temporally through the different layers. By 72 hpf, the retina is fully laminated and functional (Schmitt and Dowling, 1999, Easter et al. 1996, Hu and Easter, 1999).

After the initial differentiation of the retinal neuroepithelium, new neurons continue to be added to the retina throughout the life of the animal. This neurogenesis persists in two regions, specialized niches that harbor stem cells and their immediate progeny: the circumferential marginal zone (CMZ), at the border between differentiated retina and the iris, and the central retina, where resident stem cells in the inner nuclear layer (INL) give rise to a lineage of cells that generates exclusively rod photoreceptors (Raymond et al., 2006, Hitchcock et al., 2004). Within the CMZ, developmental time is spatially recapitulated, such that stem cells are located peripherally in the CMZ, adjacent to the iris, and progenitors with increasingly restricted competence are located more centrally.

This spatial pattern is evidenced by a regionalized expression of genes that sequentially specify cellular identities (Raymond et al., 2006, see also Harris and Perron, 1998).

The teleost retina can regenerate in a process that generally recapitulates cellular and molecular events occuring during late retinogenesis (Raymond et al. 2006, Otteson and Hitchcock, 2003, Hitchcock et al., 2004). Recent studies identified Müller glia as the stem cells responsible for both the persistent rod genesis and neuronal regeneration (Bernardos et al,, 2007, Yurco and Cameron, 2005, Kassen et al., 2007, Raymond et al., 2006, Fausett and Goldman, 2006). Following the death of retinal neurons, Müller glia de-differentiate, reenter the cell cycle and give rise to multipotent progenitors that proliferate, migrate and differentiate to replace the missing neurons. Genes that guide developmental neurogenesis are re-expressed in regenerative Müller glia and their progeny (Raymond et al., 2006 and references therein). Little is known, however, regarding the secreted signaling molecules regulating these regenerative events.

We identified *mdkb* in a screen for genes whose expression is regulated by the selective death and regeneration of photoreceptors. As a first step to understand the role of Midkines in the retina, we analyzed the expression of *mdkb* and its paralog, *mdka*, during both retinal development and adult photoreceptor regeneration. During development, *midkines* are expressed in patterns that change temporally and spatially with the wave of retinal differentiation. The *mdka* gene is expressed in stem cells and progenitors, transiently expressed in developing Müller glia and constitutively expressed in

horizontal cells. In contrast, the *mdkb* gene is transiently expressed by newly postmitotic cells and constitutively expressed by retinal ganglion and amacrine cells. During retinal regeneration, in addition to their constitutive expression patterns, both midkines are expressed in horizontal cells and proliferating Müller glia and their neurogenic progeny. This study describes for the first time the expression of these two secreted factors in the developing, adult and regenerating retina and establishes the foundation for future studies to investigate the function of these molecules in retinal development and regeneration.

## 2.2 MATERIALS AND METHODS

### Care of zebrafish and embryos

Wild-type zebrafish (*Danio rerio*) were purchased from a local supplier and maintained in aquaria at 28.5$^{o}$C with a 10/14-hour dark/light cycle. Embryos were generated by natural mating at light onset and reared in embryonic rearing solution (Westerfield, 2000) at 28.5$^{o}$C. Zebrafish transgenic for the glial fibrillary protein (GFAP) promoter driving GFP *Tg(gfap:GFP)$^{mi2000}$ (* from Dr. Pamela Raymond) were used to facilitate the identification of Müller glia. All animal procedures were approved by the University of Michigan Committee for the Use and Care of Animals in Research Committee.

### Light treatments

Two light-lesion paradigms were used in this study. Each of these manipulations selectively kills photoreceptors and induces proliferation, which follows a common time-course (Calinescu and Hitchcock, unpublished observations). First, for the gene chip analysis, retinal lesions were induced in *albino* zebrafish (University of Oregon, Eugene, OR) as described (Vihtelic and Hyde, 2000; Vihtelic et al., 2006*).* Second, to induce photoreceptor cell death in pigmented fish, animals were dark adapted for 8 days, followed by constant exposure to intense fluorescent light (26000-31000 lux). Light intensity was measured with a Reed LX-105 Digital Light Meter (Calright Instruments, San Diego, CA).

**Microarray analysis:**

Oligonucleotide microarrays were used to identify genes that were differentially expressed in the retinas after light-induced death of photoreceptors (see above). For these experiments, retinas were sampled at a single time point, 72hrs after light onset, which is after the death of photoreceptors and at a time when the retina is replete with injury-induced photoreceptor progenitors (Vihtelic and Hyde, 2000). Control animals were maintained in normal light conditions. After the animals were sacrificed, retinas were dissected from the surrounding ocular structures, and retinal RNA was isolated and processed in 8 separate pools (4 control, 4 experimental; 12 retinas each). From each pool of RNA, probes were synthesized and hybridized to a single chip. Briefly, total RNA was amplified to yield double-stranded antisense RNA ($\alpha$RNA), which was biotinylated using the Affymetrix GeneChip Expression IVT Labeling Kit (Affymetrix, Santa Clara, CA). Ten micrograms of labeled aRNA were fragmented and hybridized to Zebrafish Genome Arrays (Affymetrix). Chips were scanned using the GeneChip Scanner 3000 (Affymetrix). The fluorescence intensity readouts were sorted into CHP files with the Affymetrix Microarray Suite v5.0.

A false discovery rate confidence interval (FDR-CI) was used to identify statistically significant changes in fluorescence intensity as described (Benjamini and Yekutieli, 2005, Hero et al., al. 2004). This approach employs robust multiarray averaging (RMA) to normalize the data, assigns fold-intensity differences and utilizes a statistical method that provides a false discovery rate-

adjusted confidence interval (FDR-CI) for each differentially-labeled probe set (Hero et al., al. 2004; see also Yoshida et al., 2002). This analysis generated a rank-ordered list of probe sets that showed 2-fold or higher changes in fluorescence intensity on the chip images. Analysis of gene ontologies was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Dennis et al., 2003; http://david.abcc.ncifcrf.gov). MAIME compliant array data will be posted at the National Center for Biotechnology Information Gene expression omnibus (http://www.ncbi.nlm.nih.gov.proxy.lib.umich.edu/geo/).

**Tissue preparation, in situ hybridization and immunohistochemistry**

At selected developmental times, embryos and larvae were fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer (2h to overnight), cryoprotected in 20% sucrose, frozen and cryosectioned. At set times following light treatment, adult fish were anesthetized in 0.05 % 3-aminobenzoic acid-ethyl ester (Sigma), eyes were enucleated, lenses removed and eyecups were processed identically to the embryos and larvae.

Sense and antisense riboprobes for *mdka* or *mdkb* were synthesized from plasmids containing the full-length *mdka* and *mdkb* cDNAs (gift from Dr. Christoph Winkler, Winkler et al., 2003). Plasmids were linearized and digoxigenin (DIG)-labeled riboprobes were generated by *in vitro* transcription using the DIG RNA Labeling kit (Roche Diagnostics, Indianapolis, IN). *In situ* hybridization was performed as described previously (Hitchcock and Kakuk-Atkins, 2004) using DIG- labeled probes and Fast Red (Roche Diagnostics,

Indianapolis, IN) as the enzymatic substrate. The enzymatic reaction was monitored using a fluorescence microscope and stopped after 2-5 hours, when the signal was distinct and the background low. Fresh Fast Red staining solution was prepared and added to the slides each hour.

To combine *in situ* hybridization with immunohistochemistry, the Fast Red color reaction was first allowed to develop, then slides were processed immediately for immunohistochemistry using standard procedures. Primary antibodies used were: anti-Proliferating Cell Nuclear Antigen-P8825 (ascites fluid from mice immunized with recombinant rat PCNA-Protein A fusion protein, Waseem and Lane, 1990, Sigma-Aldrich, St. Louis, MO) (1:1000), anti-Green Fluorescent Protein (1:1000), ab6556 (polyclonal antibody raised in rabbit against highly purified recombinant Green Fluorescent Protein made in Escherichia Coli, Abcam Inc., Cambridge, MA), or anti- Prox-1, AB5475 (polyclonal antibody raised in rabbits against a synthetic peptide from the C-terminus of mouse Prox-1 with the following sequence: EIFKSPNCLQELLHE, Chemicon International, al. Temecula, CA) (1:2000). Prox-1 is a homeodomain protein expressed in horizontal cells (Dyer et. al., 2003, Edqvist and Hallbook, 2004). Zebrafish Prox-1 is 84% identical at the amino acid level with the mouse homologue (Glasgow and Tomarev, 1998), and this antibody has been shown to specifically label Prox-1 in zebrafish (Ober et. al., 2006). In all sections, nuclei were stained with the fluorescent DNA binding dye 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen-Molecular Probes, Eugene, OR). Prior to PCNA immunostaining, sections were processed for antigen retrieval as

previously described (Raymond et al., 2006). The secondary antibodies, were either goat, anti-rabbit or goat, anti-mouse and were used at a dilution of 1:500 (Invitrogen-Molecular Probes, Eugene, OR).

**Histological analysis and measurements**

For histological analysis, eyecups were fixed overnight at 4$^{o}$C in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M Phosphate Buffer, rinsed in Phosphate Buffered Saline (PBS), dehydrated in ethanol and embedded in JB-4 compound (Polysciences Inc., Warrington, PA). Sections (4$\mu$m thick) through the optic nerve along the dorso-ventral axis were mounted on glass slides, stained with 0.25% toluidine blue, cleared in Citrisolve (Fisher Scientific) and coverslipped. Images were taken with a Nikon DMX 1200 digital camera with the 40x objective from the dorsal quadrant of the retina, corresponding to the region with the most photoreceptor damage. For quantification, six images were taken from the dorsal quadrant of six different central sections for each eyecup, three different eyecups per time-point (except the 48h and the 28day time-point for which only two eyecups were analyzed). Twelve measurements were taken for each parameter for each eyecup using the linear measurement tool in Image Pro Plus 5.0 (Media Cybernetics). The three parameters measured were: thickness of the photoreceptor layer (PL), measured from Bruch's membrane to the outer plexiform layer (OPL), thickness of the Outer Nuclear Layer: from the sclerad limit of the cone nuclei to the outer plexiform layer (OPL), the thickness of the Rod Nuclear Layer: from the outer limiting membrane (OLM) to the OPL. These measurements were normalized to the thickness of the inner nuclear layer (INL),

measured from the OPL to the inner plexiform layer (IPL), to account for individual size differences between fish (Fig. 2.5a). The mean values obtained for each time-point were compared for statistical significance (*$p<0.05$ or **$p<0.01$) by one-way ANOVA with LSD correction for multiple comparisons using SPSS software.

**Photographic images**

Images were taken with a Nikon DMX 1200 digital camera mounted on a Nikon Eclipse E800 epifluorescent compound microscope. Adobe Photoshop CS2 (Adobe Systems, San Jose, CA) was used to construct the figures. The layer tool was used to generate overlays, and the channel mixer tool was used to change the red signal to magenta. In some images, the clone stamp tool was used to remove unwanted scale bars. Images in Figure 2 and Supplemental Figure 1 were taken with an AxioCam RM digital camera and a Zeiss Axio Imager epifluorescent compound microscope (Carl Zeiss Microimaging, Thornwood, NY). Images were false colored using the Zeiss AxioVision 4.0 software and exported into Adobe Photoshop CS2 (Adobe Systems, San Jose, CA) and treated as described above.

**RNA extraction and real time reverse transcriptase polymerase chain-reaction (QRTPCR):**

Adult zebrafish (6-8 months old, 2-3 cm long) were anesthetized in 0.1% 3-Aminobenzoic Acid-Ethyl Ester (Sigma) for 10 min. Eyecups were removed and retinas dissected and carefully separated from the retinal pigment epithelium with fine forceps. Four retinas per sample from 3-4 different zebrafish were

pooled yielding 7-16 mg of tissue. Retinas were homogenized and RNA extraction was performed according to the manufacturers instruction (RNAqueous-Micro RNA isolation kit, Ambion, Austin, TX). RNA was quantified with a spectrophotometer, and RNA quality was assessed on ethydium bromide stained agarose gels. 500 ng of total RNA was used to synthesize cDNA following the manufacturers protocol (Superscript II, Invitrogen, Carlsbad, CA). The resulting first-strand reaction was diluted 1:4 and used as a template for the subsequent QRTPCR reaction (iQ™ SYBR® Green Supermix Bio-Rad, Hercules, CA) in the iCycler Real-Time PCR detection system (Bio-Rad). The following amplification and melt curve analysis protocol was used: 95$^{o}$C 3min, 40x(95$^{o}$C: 20s, 57$^{o}$C: 20s, 72$^{o}$C: 30s), 95$^{o}$C: 1min, 90X55$^{o}$C: 10s. The following primers were used: *rhodopsin* forward- agcccatacgaatacccaca; rhodopsin reverse- agcttcttgtgctcgatgg, *opsin-1* forward- aaaccacaagggaagcaatg; *opsin-1* reverse- ttgtgctggcaaacagagtc, *proliferating cellular nuclear antigen* (PCNA) forward- catccagacacttagagctgaaga; PCNA reverse- ctggtctgtgagagcttgatgtt, *connexin 52.6* forward- tggacagatggtacctttgcc; *connexin 52.6* reverse- gttgtctggaatggaccttcg (Zoidl et al. 2004). The threshold cycle (Ct) was determined by the iCycler using the maximum curvature approach and then maintained constant for subsequent runs. Relative value of gene expression was determined by the $\Delta\Delta$Ct method using connexin 52.6 as endogenous reference. This value was then divided by the relative expression in the control retina and represented graphically as fold change. Specificity of the amplification products was verified by agarose gel electrophoresis of sample wells, evidencing single

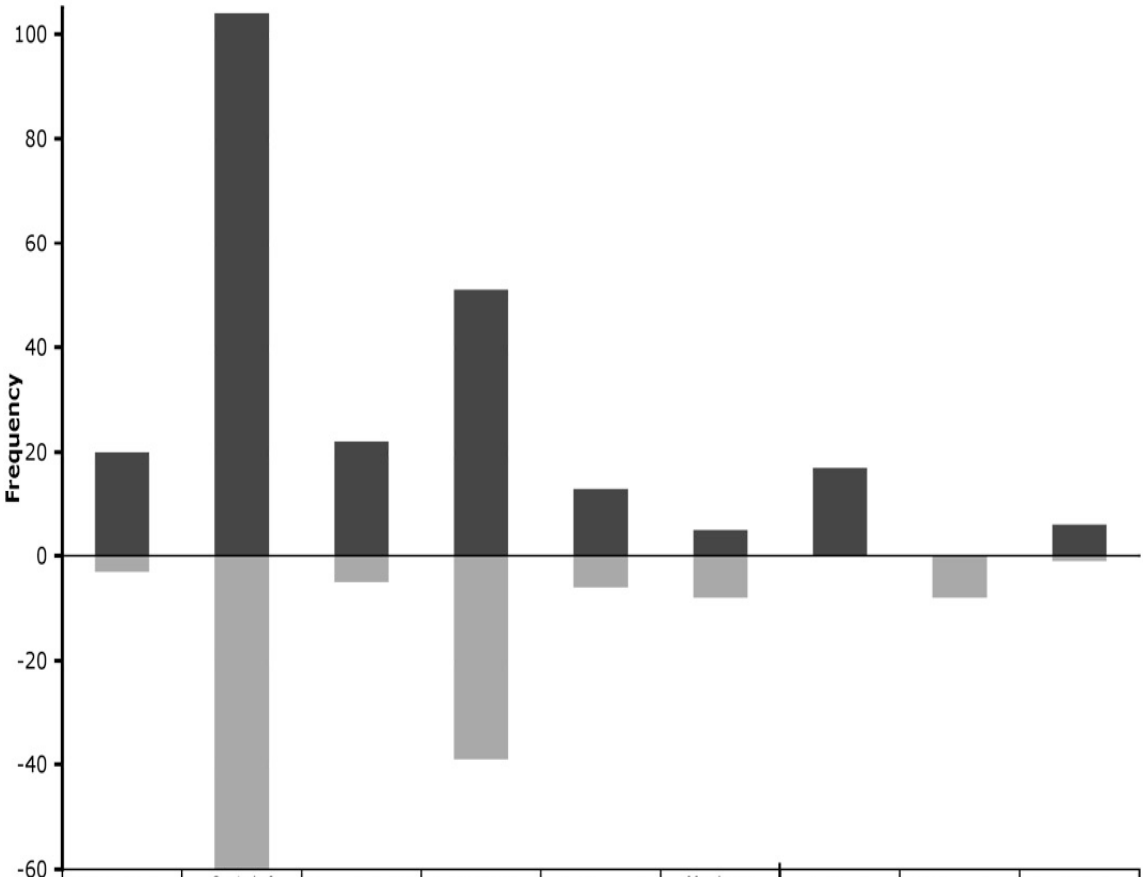
bands with the expected size. Statistical significance (*p<0.05 or **p<0.01) was calculated by one-way ANOVA with LSD correction for multiple comparisons using SPSS software.

## 2.3 RESULTS

### 2.3.1 MICROARRAY ANALYSIS

The RMA/FDR-CI identified 671 probe sets from experimental chips that showed a 2-fold or greater difference in fluorescence intensity (hereafter referred to as 'gene expression') in the microarray images when compared to controls (Table 2-2 in the Appendix). To characterize the global changes in gene expression, the differentially expressed genes were submitted to DAVID (Dennis et al., 2003) to determine the most significantly overrepresented gene ontologies. Table 2-1 lists gene ontology biological process terms that were statistically significantly overrepresented within this dataset. In addition, functional categories were identified from the gene ontology analysis, and after removing those genes lacking annotation, the direction of change for genes within each category was determined (Fig. 2.1). Finally, genes known to control the cell cycle, regulate photoreceptor physiology or encode growth factors were separately evaluated. As anticipated, the expression of each of the cell cycle control genes is increased, reflecting the accretion of mitotic photoreceptor progenitors, whereas the expression of each of the photoreceptor-specific genes is decreased, reflecting the selective death of this cell type. Genes encoding seven differentially-expressed growth factors (midkine b [see below], granulin a, granulin 1, granulin 2, dickkopf 1, galectin 1-like 2, matrixmetalloproteinase 9,

follistatin) were also identified. The direction of change in the expression for four of these genes was independently validated by *in situ* hybridization (see next section, data not shown).




| | DNA Replication | Control of Transcription | Enzymatic Activity | Cell Metabolism | Ion Transport | Membrane Proteins | Cell Cycle | Photoreceptor | Growth Factors |
|---|---|---|---|---|---|---|---|---|---|
| + | 20 | 104 | 22 | 51 | 13 | 5 | 17 | 0 | 6 |
| - | 3 | 60 | 5 | 39 | 6 | 8 | 0 | 8 | 1 |

**Figure 2-1.** Differentially-expressed genes between light-lesioned and control retinas. This graph illustrates functional gene categories and the number of genes in each category showing increased (+) or decreased (-) expression. Genes within the left-most six categories resulted from the analysis of gene ontologies, whereas right-most three categories, cell cycle, photoreceptor, growth factors, were selected by hand based on predicted experimental outcome.

**Table 2.1. Gene ontology (GO) process terms with increased frequency ($p<0.05$) in the dataset of differentially expressed genes**

| GO Biological Process Term | Rank order by p-value |
|---|---|
| intracellular part | 1.80E-06 |
| intracellular | 5.20E-05 |
| enzyme regulator activity | 1.30E-04 |
| DNA-dependent DNA replication | 3.60E-04 |
| DNA replication initiation | 6.10E-04 |
| protein polymerization | 1.30E-03 |
| cysteine-type endopeptidase activity | 2.00E-03 |
| DNA replication | 2.80E-03 |
| intracellular non-membrane-bound organelle | 6.40E-03 |
| non-membrane-bound organelle | 6.40E-03 |
| chromosomal part | 6.70E-03 |
| calcium-dependent phospholipid binding | 7.20E-03 |
| DNA metabolic process | 8.60E-03 |
| enzyme inhibitor activity | 1.00E-02 |
| intracellular organelle | 1.40E-02 |
| chromatin | 1.40E-02 |
| DNA-dependent ATPase activity | 1.60E-02 |
| nucleosome | 1.70E-02 |
| chromosome | 2.30E-02 |
| cysteine-type peptidase activity | 2.50E-02 |
| nucleoside-triphosphatase activity | 2.90E-02 |
| microtubule-based movement | 2.90E-02 |
| cytoskeleton-dependent intracellular transport | 2.90E-02 |
| translation elongation factor activity | 3.30E-02 |
| lipid binding | 3.30E-02 |
| deoxyribonucleotide metabolic process | 3.50E-02 |
| pyrophosphatase activity | 3.90E-02 |
| protease inhibitor activity | 4.10E-02 |
| GTPase activity | 4.10E-02 |
| response to stress | 4.10E-02 |
| hydrolase activity | 4.20E-02 |
| hydrolase activity, acting on acid anhydrides | 4.30E-02 |
| sensory perception of light stimulus | 4.50E-02 |
| visual perception | 4.50E-02 |
| ATPase activity | 4.50E-02 |
| biogenic amine metabolic process | 4.80E-02 |
| nucleosome assembly | 4.90E-02 |

### 2.3.2 *mdka* AND *mdkb* ARE EXPRESSED IN DISTINCT POPULATIONS OF CELLS DURING RETINAL DEVELOPMENT

To determine the cellular pattern of expression of *mdka* and *mdkb* in the developing retina, *in situ* hybridization was performed on tissue sections from embryos and larvae between 24 and 120 hpf (Fig.2.2). Neither *mdka* nor *mdkb* transcripts are detected in the eye at 24 hpf (data not shown), consistent with previous observations (Winkler and Moon, 2001, Winkler et al., 2003). However, retinal expression of *mdka* is detected at 30 hpf. At this time, a low level of expression is present throughout the retinal neuroepithelium and more intense expression is observed at the retinal margin, presaging the site of the CMZ (Fig.2.2a). At 48 hpf, *mdka* is expressed at the retinal margin and more broadly in the INL (Fig.2.2b), but *mdka* is not expressed in the ventral and central retina where differentiated cells and laminae are present. Between 48 hpf and 72 hpf, *mdka* expression becomes progressively restricted to the retinal margin. At 72 hpf, *mdka* expression appears centrally in columnar cells spanning the INL with morphology suggestive of Muller glia (Fig. 2.2c). These radial columns persist through 120 hpf (Fig.2.2c-e), but are not prominently observed in the adult retina (see below). At 120 hpf, *mdka* transcription begins in presumptive horizontal cells (Fig.2.2e see below). Interestingly, in addition to this dynamic pattern of expression in the neural retina, *mdka* is strongly expressed in cells within the nascent optic nerve (Fig.2.2d) and the lens epithelium.

The spatial pattern of expression for *mdkb* is distinctly different from *mdka,* and suggests this gene is transcribed in postmitotic cells only. *mdkb* is first

expressed at 48 hpf in the ventro-nasal patch and more broadly in the laminated central retina (Fig.2.2g, See also Appendix Fig.1). At 72 hpf, *mdkb* is expressed in differentiated ganglion and amacrine cells, which straddle the inner plexiform layer (IPL) (Fig.2.2h) and in a broad annulus of cells central to the CMZ (Fig. 2.2h). Between 96 hpf and 120 hpf, expression within the inner nuclear and ganglion cell layers persists, whereas the annulus of *mdkb* expression becomes progressively restricted to the cells just central to the CMZ (Fig. 2.2h, i, j). In contrast to *mdka*, *mdkb* is not expressed by retinal progenitors in the CMZ (Fig. 2.2h, i). These data suggest that *mdkb* is transiently expressed by cells as they differentiate and constitutively expressed by mature ganglion and amacrine cells. This pattern of expression is maintained in the adult (see below).

**Fig.2.2 Cellular expression of *mdka* and *mdkb* during retinal development.** Panels a-e are in situ hybridizations that illustrate the retinal expression of *mdka* at 30-120 hpf, respectively. The white arrowheads identify mdka-expressing cells in the circumferential marginal zone and retina. The white arrows in panels c-e identify the columnar cells within the inner nuclear layer that express *mdka*. The yellow arrowhead in panel d identifies the optic nerve. The yellow arrowheads in panel e identify presumptive horizontal cells. Panels f-j are in situ hybridizations that illustrate the expression of *mdkb* at 30-120 hpf, respectively. The arrow in panel g identifies the ventronasal patch. The arrows in panels g-j demarcate regions of transient *mdkb* expression. The arrowheads in panels h and i identify the circumferential marginal zone, which does not express *mdkb*. Note the prominent expression of *mdkb* within the gcl and inl in panels h-j. onl, outer nuclear layer; inl, inner nuclear layer; ipl, inner plexiform layer; gcl, ganglion cell layer. Scale bar equals 50μm.

30 hpf
48 hpf
72hpf
96 hpf
120 hpf
mdka
mdkb
a
b
c
d
e
f
g
h
i
j
onl
inl
ipl
gcl

Specificity of the *mdka* and *mdkb* probes used for in situ hybridization was verified by repeating the experiment for selected time-points (96hpf and adult) with sense and antisense digoxigenin riboprobes (Fig.2.2').

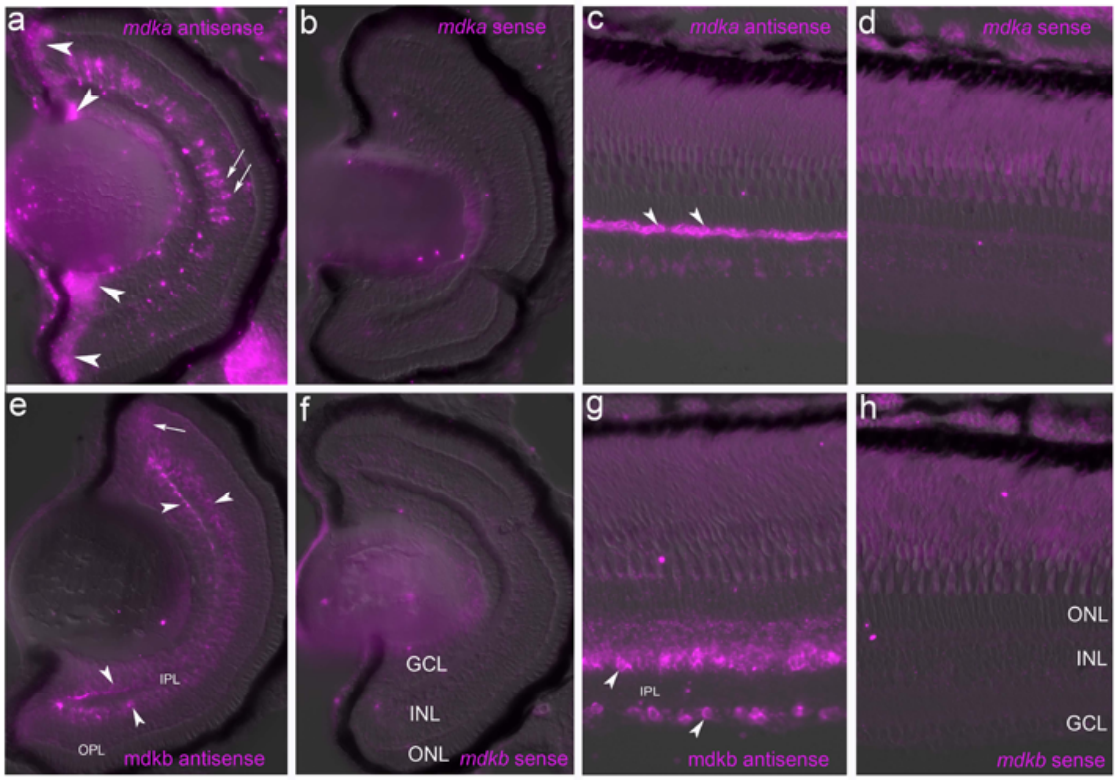



**Fig.2.2' Testing specificity of *mdka* and *mdkb* riboprobes**

Panels a and c are in situ hybridizations that illustrate the retinal expression of *mdka* at 96 hpf, and in the adult retina respectively. The white arrowheads in a identify *mdka*-expressing cells in the circumferential marginal zone. The white arrows in panel a identify the columnar cells within the inner nuclear layer that express *mdka*. White arrowheads in panel c identify presumptive horizontal cells expressing *mdka*. Panels b and d represent sections through the retina of a 96hpf larva and an adult fish, probed with a digoxigenin-labeled *mdka* sense mRNA probe. Panels e and f are in situ hybridizations that illustrate the retinal expression of *mdkb* at 96 hpf, and in the adult retina respectively. The white arrowheads in e and g identify *mdkb*-expressing cells in the ganglion and amacrine layer on both sides of the retina. Panels f and h represent sections through the retina of a 96hpf larva and an adult fish, probed with a digoxigenin-labeled *mdkb* sense mRNA probe. ONL outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

To analyze the expression of *mdka and mdkb* relative to proliferating cells, we combined *in situ* hybridization with immunohistochemistry for PCNA, a cofactor of DNA polymerases, expressed during the late G1, S and early G2 phases of the cell cycle (Kurki et al., 1986; Moldovan et al., 2007). We examined retinas at 72hpf, because at this age they contain both mature and proliferating cells. *mdka* is expressed in PCNA-positive cells within the CMZ (Fig. 2.3 a, b, c). In contrast, *mdkb* is co-expressed with PCNA only in a few cells at the interface between the CMZ and the differentiated retina (Fig.2.3 d, e, f).

Together, these data show that, during early retinal development, *mdka* is expressed in mitotic retinal progenitors, whereas *mdkb* is expressed in newly postmitotic cells. In addition, *mdka* is transiently expressed in presumptive Müller glia (see next section). Finally, both genes are constitutively expressed in subsets of neurons within the inner retinal layers.

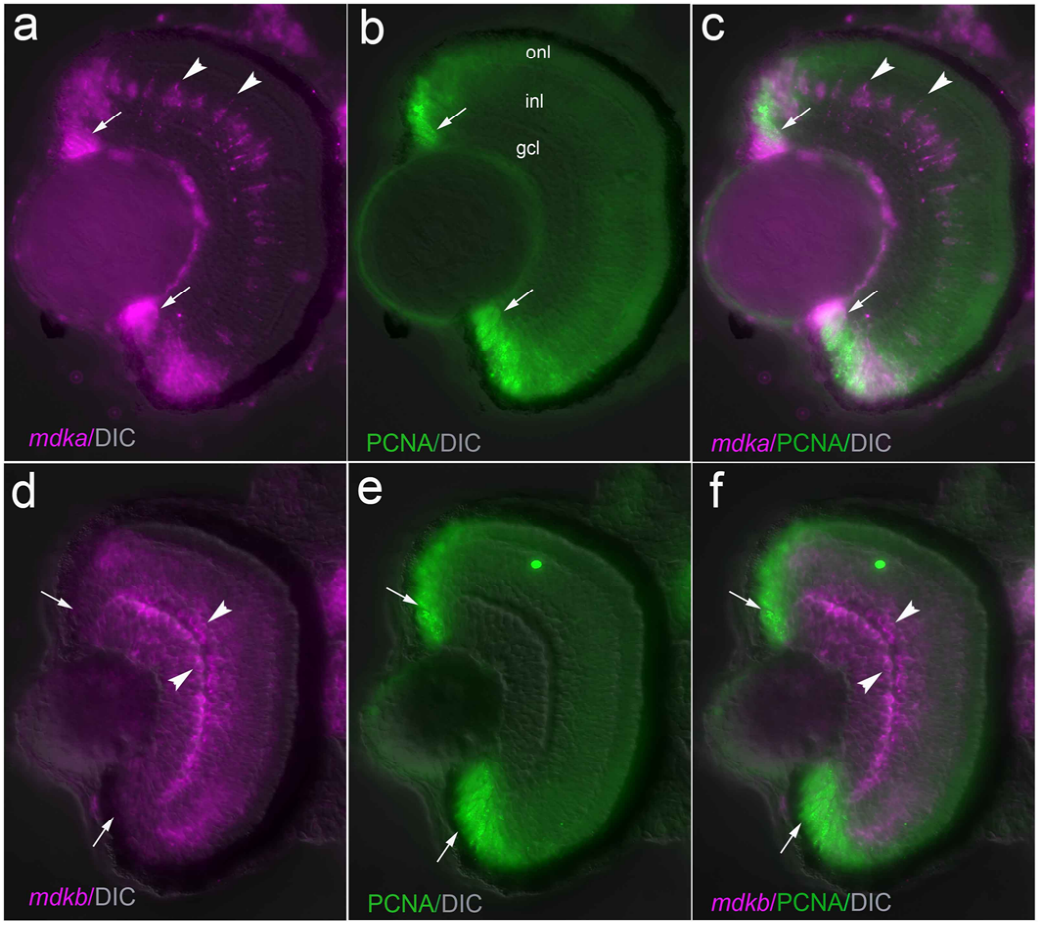



**Figure 2.3. *mdka* is expressed by retinal progenitors and *mdkb* is expressed by differentiated cells.** Panel a is an *in situ* hybridization that illustrates the expression of *mdka* at 72 hpf. Panel b is the same section as in panel a, but immunostained with antibodies against proliferating cell nuclear antigen (PCNA). Panel c is the digital overlay of panels a and b. Panel d is an *in situ* hybridization showing the expression of *mdkb* at 72 hpf. Panel e is the same section as in panel d, but immunostained with antibodies against PCNA. Panel f is the digital overlay of panels d and e. The white arrows identify the circumferential marginal zone in each panel, whereas the arrowheads identify cells expressing *mdka* (panels a and c) and *mdkb* (panels d and f) within central retina. onl, outer nuclear layer; inl, inner nuclear layer; ipl, inner plexiform layer; gcl, ganglion cell layer.

### 2.3.3 *mdka* IS TRANSIENTLY EXPRESSED IN MÜLLER GLIA AND CONSTITUTIVELY EXPRESSED IN HORIZONTAL CELLS

During larval development, *mdka*-expressing cells in the INL have centrally located large nuclei with *mdka m*RNA in processes that extend radially, from the nuclei (Fig.2.2 c, d, e; Fig.2.3 a; Fig.2.4 a). This morphology suggests *mdka* is expressed by Müller glia. To test this speculation, we combined *in situ* hybridization with immunostaining for green fluorescent protein (GFP) on retinal sections from Tg(*gfap*:GFP)$^{MI2001}$ transgenic zebrafish (Bernardos and Raymond, 2006). These fish express GFP under the control of glial fibrillary acidic protein (*gfap*) regulatory elements, which in the retina selectively marks Müller glia. The *mdka*-positive cells in the INL (Fig.2.4 a, c, d, f) overlay precisely with GFP-positive cells (Fig.2.4 b, c, e, f), confirming *mdka* transcription in Müller glia.

To test our inference that *mdka* is expressed by horizontal cells, we combined *in situ* hybridization and antibodies against Prox-1. Prox-1, a homeodomain transcription factor required for horizontal cell development (Dyer et al., 2003), is expressed in chicks and mammals by horizontal, AII amacrine and bipolar cells (Dyer et al., 2003; Belecky-Adams et al., 1997; Edqvist and Hallbook, 2004). At 72 hpf, Prox-1 expression identifies horizontal cells in the outer INL, although *mdka* expression in these cells is not detected at this time (Fig. 2.2c and 2.4g-i). At 120 hpf, however, *mdka* expression co-localizes with Prox-1 in horizontal cells (Fig. 2.4 j-l). Note that *mdka* expression is reduced or absent in the dorsal retina, which is less mature than ventral retina, showing that

*mdka* is not expressed by newly differentiated horizontal cells. In the adult retina, *mdka* is constitutively expressed in Prox-1-positive horizontal cells (Fig. 2.4 m-o).

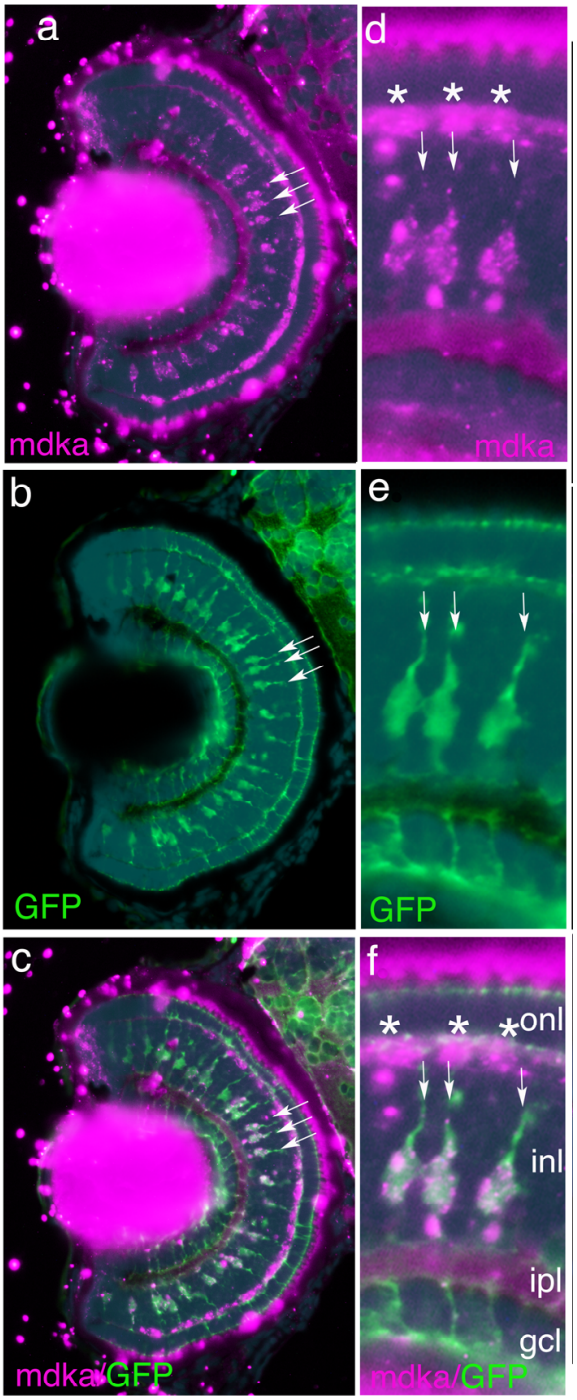



**Figure 2.4a. *mdka* is transiently expressed in Müller glia (see legend next page)**

**Figure 2.4 a and b. *mdka* is transiently expressed in Müller glia and constitutively expressed in horizontal cells**. Panels a and d illustrate *in situ* hybridizations of *mdka* expression in Tg(*gfap:*GFP)$^{Mi2001}$ fish at 120 hpf. Panels b and e illustrate Müller glia immunostained with antibodies against green fluorescent protein. Panel c is the digital overlay of panels a and b; panel f is the digital overlay of panels d and e. In each panel, the three arrows identify the same three Muller glia. In panel d, the asterisks identify *mdka* expression in presumptive horizontal cells. Panel g is an *in situ* hybridization showing the expression of *mdka* at 72 hpf. Panel h is the same section as in panel g, but immunostained with antibodies against Prox1. Panel i is the digital overlay of panels g and h. Note that at 72hpf, horizontal cells synthesize Prox1, but do not yet express *mdka.* Panel j is an *in situ* hybridization showing the expression of *mdka* at 120hpf. Panel k is the same section as in panel j, but immunostained with antibodies against Prox1. Panel l is the digital overlay of panels j and k. Note the co-localization of *mdka* mRNA and Prox1 protein. Panel m is an *in situ* hybridization showing the expression of *mdka* in the adult retina. Panel n is the same section as in panel j, but immunostained with antibodies against Prox1. Panel o is the digital overlay of panels d and e. Arrows in j-o identify horizontal cells that express *mdka* and are immunostained for Prox1. onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer; DAPI, nuclear stain 4,6-diamidino-2-phenylindole dihydrochloride. Scale bar equals 50μm; onl, outer nuclear layer; inl, inner nuclear layer; ipl, inner plexiform layer; gcl, ganglion cell layer; ipl: inner plexiform layer. Scale bar in o equals 50 μm.

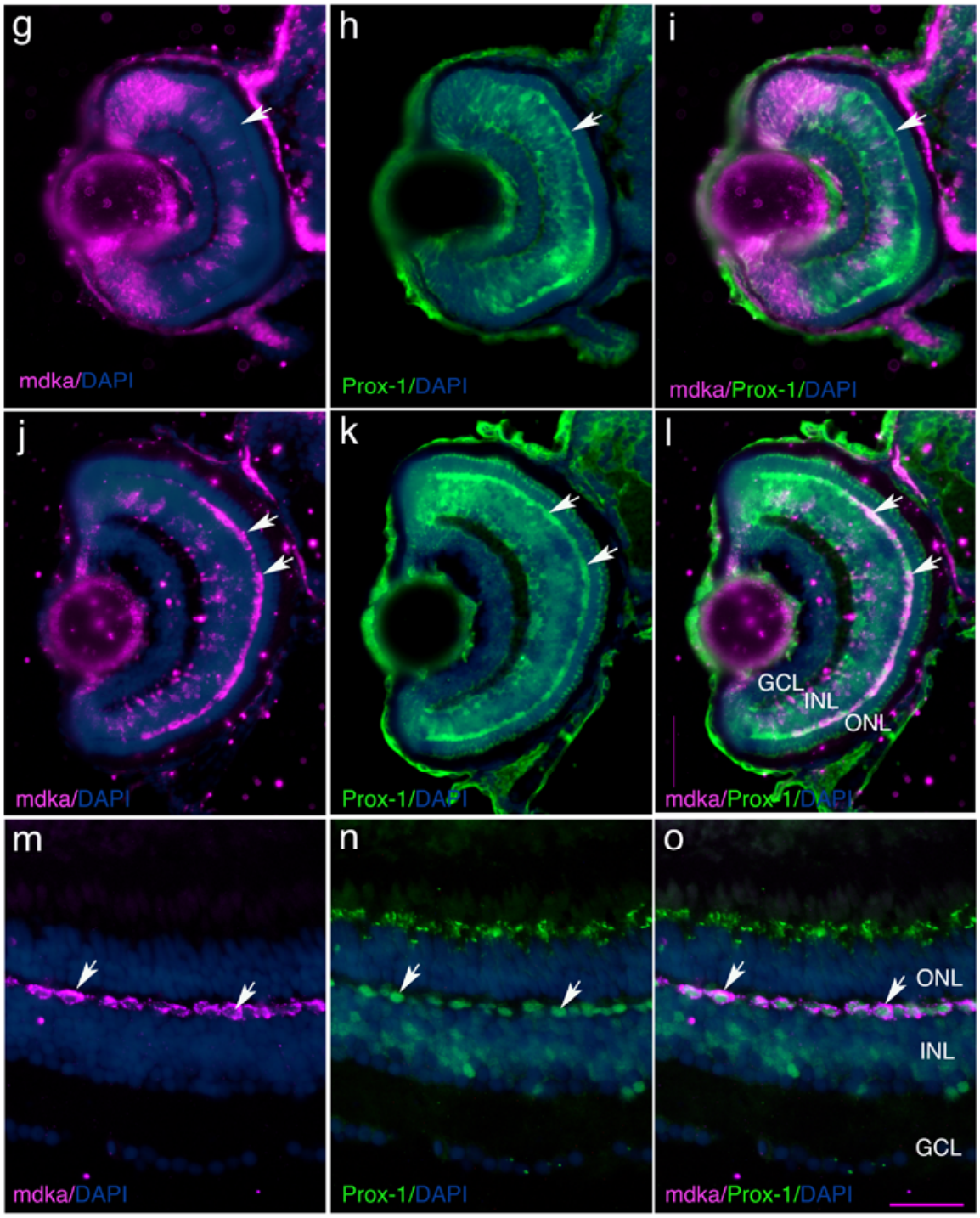


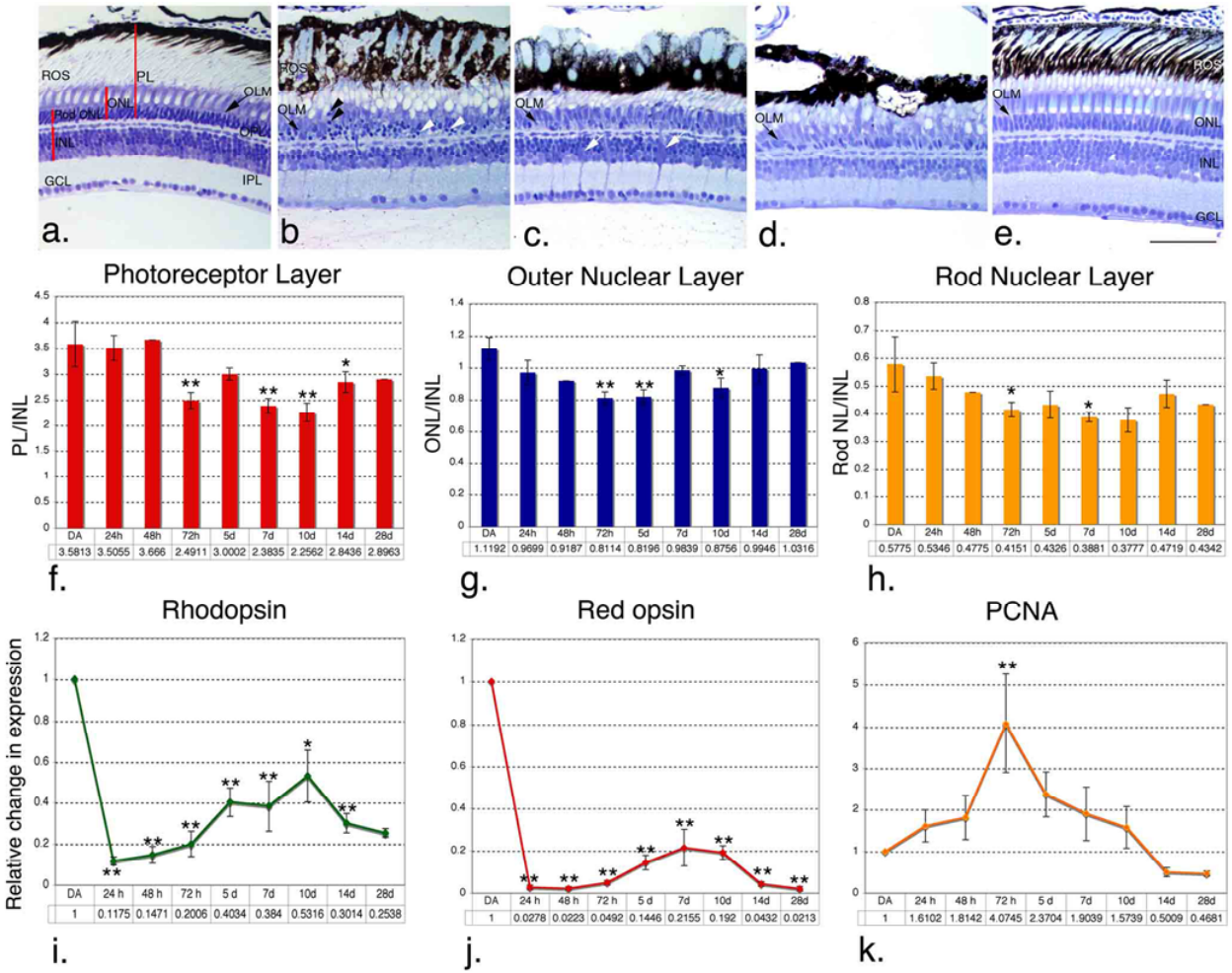

**Figure 2.4b. *mdka* is constitutively expressed in horizontal cells**

### 2.3.4 EXPOSURE TO INTENSE FLUORESCENT LIGHT INDUCES PHOTORECEPTOR LOSS, FOLLOWED BY REGENERATION IN PIGMENTED ZEBRAFISH

To analyze expression of midkines during photoreceptor regeneration we employed a similar light injury paradigm as previously described for albino zebrafish (Vihtelic and Hyde, 2000, Kassen et. al. 2006) adapted for pigmented fish. After 24h of exposure to intense fluorescent light, the rod outer segments (ROS) are swollen, irregular and damaged and numerous pyknotic nuclei are observed in the outer nuclear layer (ONL), in the region below the outer limiting membrane (OLM) that harbors the rod nuclei (Fig.2.5b and 2.5.1b' white arrowheads) as well as above the OLM, where the cone nuclei reside (Fig.2.5b and 2.5.1b' black arrowheads), indicating that rods as well as cones are dying. After 72h of light exposure, most of the outer segments have been destroyed, the ONL is visibly smaller, particularly below the OLM, Müller glia are intensely stained with Nissl stain, and their processes visible in the inner plexiform layer (IPL) (Fig.2.5c, white arrows). Measurements of the photoreceptor layer, outer nuclear layer and rod outer nuclear layer (Fig.2.5 f, g, h) indicate that there are significant decreases in the thickness of these retinal parameters, starting at 72h of light lesion; damage to the rod nuclear layer is more severe and does not fully recover by 28d (See also Table 2.1). Analysis of opsin gene expression by quantitative real time PCR (rhodopsin and red opsin, Fig.2.5 i, j) reveals marked decrease in the expression of these genes, as early as 24h after light onset. This drop in expression is not fully recovered by 28d, when the retina displays an orderly array of outer segments and a seemingly regenerated ONL (Fig. 2.5e).

PCNA expression during this light lesion time-course peaks at 72h (Fig.2.5 k) and proliferation gradually declines by 14d (See also Table 2.2). Taken together these data show that this light lesion paradigm induces photoreceptor loss (primarily rods but also cones) in pigmented zebrafish, elicits a regenerative response and the time-course of cell death and proliferation resembles other light lesion paradigms (Vihtelic and Hyde , 2000, Kassen et. al. 2006, Bernardos et. al., 2007).

**Fig.2.5 Exposure of pigmented zebrafish to intense bright fluorescent light induces photoreceptor loss followed by regeneration.** Panels a-e illustrate Nissl-stained sections from control retina (a) and retinas exposed to bright, constant light for 24hrs (b), 72hrs (c), 10 days (d) or 10 days intense bright light followed by 18 days of recovery (e). Panels f-g illustrate quantitative measurements of the photoreceptor, outer nuclear and rod nuclear layers, respectively (see panel a), normalized to the thickness of the inner nuclear layer. Note that there is a significant decrease in the thickness of the various layers starting at 72h of light exposure. Panels i-k illustrate analysis of *rhodopsin*, *red opsin* and *PCNA* expression by quantitative real-time PCR. ROS, rod outer segments; OLM, outer limiting membrane; OPL, outer plexiform layer; IPL, inner plexiform layer; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; PL, photoreceptor layer; rod ONL, rod outer nuclear layer. Scale bar equals 50$\mu$m, * $p<0.05$, ** $p<0.01$.


a.
ROS
PL
OLM
ONL
Rod ONL
OPL
INL
GCL
IPL
b
ROS
OLM
c.
OLM
d.
OLM
e.
ROS
OLM
ONL
INL
GCL
Photoreceptor Layer
PL/INL
DA 24h 48h 72h 5d 7d 10d 14d 28d
3.5813 3.5055 3.666 2.4911 3.0002 2.3835 2.2562 2.8436 2.8963
f.
Outer Nuclear Layer
ONL/INL
DA 24h 48h 72h 5d 7d 10d 14d 28d
1.1192 0.9699 0.9187 0.8114 0.8196 0.9839 0.8756 0.9946 1.0316
g.
Rod Nuclear Layer
Rod NL/INL
DA 24h 48h 72h 5d 7d 10d 14d 28d
0.5775 0.5346 0.4775 0.4151 0.4326 0.3881 0.3777 0.4719 0.4342
h.
Rhodopsin
Relative change in expression
DA 24 h 48 h 72 h 5 d 7d 10d 14d 28d
1 0.1175 0.1471 0.2006 0.4034 0.384 0.5316 0.3014 0.2538
i.
Red opsin
DA 24 h 48 h 72 h 5 d 7d 10d 14d 28d
1 0.0278 0.0223 0.0492 0.1446 0.2155 0.192 0.0432 0.0213
j.
PCNA
DA 24 h 48 h 72 h 5 d 7d 10d 14d 28d
1 1.6102 1.8142 4.0745 2.3704 1.9039 1.5739 0.5009 0.4681
k.

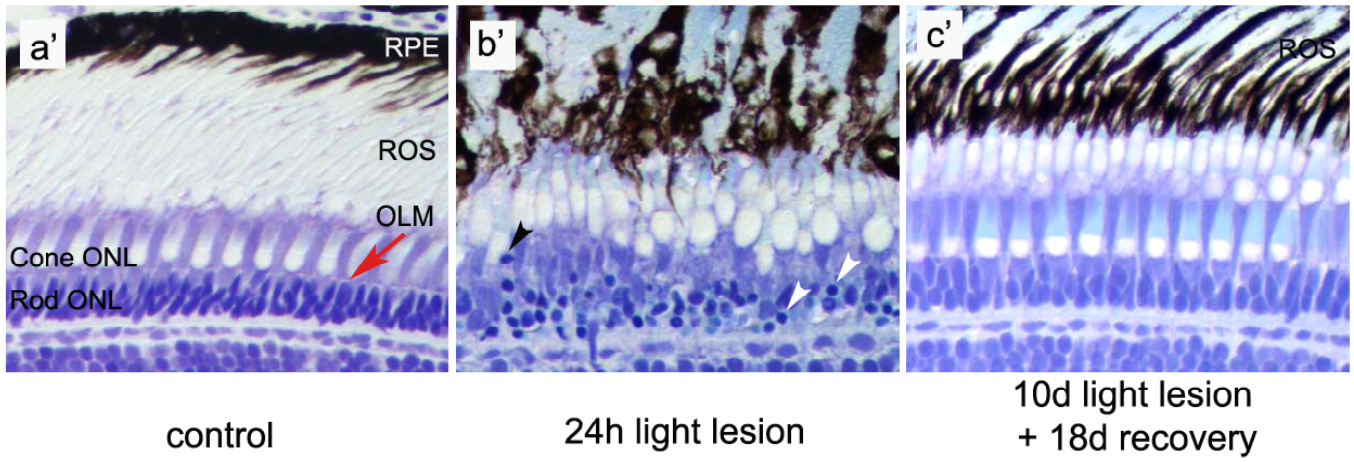





**Fig. 2.5.1 Exposure of pigmented fish to intense fluorescent light induces primarily rod, but also cone photoreceptor loss-Magnified view of the outer retina.**
Panels a'-c' represent retinal sections stained with toluidine blue at representative time-points during the light-lesion time-course. Panel a' is section through a normal, control retina, representing a magnification of panel a in Fig. 2.2. The red arrow points to the outer limiting membrane that separates the rod outer nuclear layer from the cone outer nuclear layer. Rod nuclei stain darker blue with toluidine blue than cone nuclei. Panel b', represents a magnification of panel b in Fig. 2.2, showing the histological appearance of the outer retina following 24h of exposure to intense fluorescent light. Black arrowhead points to a pyknotic nucleus in the cone nuclear layer and white arrowheads point to pyknotic nuclei in the rod nuclear layer. Note the disorganization of the rod outer segments and of the retinal pigmented epithelium. Panel c' represents a magnification of panel e in Fig. 2.2. showing the histological appearance of the outer retina recovered for 18 days in normal aquaria lighting conditions, after exposure for 10 consecutive days to intense fluorescent light. Note the orderly array of cones and rod outer segments.

Table 2.2 HISTOLOGICAL MEASUREMENTS OF CHANGES IN THE OUTER RETINA DURING THE LIGHT-LESION TIME-COURSE

| PL/INL= Photoreceptor layer (μm)/ Inner Nuclear layer (μm) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| N=3 | control | 24hLL | 48hLL | 72hLL | 5dLL | 7dLL | 10d | 14d | 21d | 28d |
| Mean | 3.58 | 3.505 | 3.665 | 2.491 | 2.999 | 2.383 | 2.259 | 2.843 | 2.896 | 2.753 |
| Std. Dev. | 0.761 | 0.421 | 0.491 | 0.268 | 0.203 | 0.245 | 0.308 | 0.346 | 0.230 | 0.030 |
| Std. Error | 0.439 | 0.243 | 0.347 | 0.154 | 0.117 | 0.141 | 0.177 | 0.199 | 0.163 | 0.021 |

| ONL/INL= Outer Nuclear Layer (μm)/ Inner Nuclear layer (μm) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| N=3 | control | 24hLL | 48hLL | 72hLL | 5dLL | 7dLL | 10d | 14d | 21d | 28d |
| Mean | 1.119 | 0.969 | 0.918 | 0.81 | 0.819 | 0.983 | 0.875 | 0.994 | 1.096 | 1.031 |
| Std. Dev. | 0.123 | 0.132 | 0.115 | 0.066 | 0.076 | 0.047 | 0.104 | 0.145 | 0.129 | 0.052 |
| Std. Error | 0.071 | 0.076 | 0.082 | 0.038 | 0.044 | 0.027 | 0.060 | 0.084 | 0.091 | 0.037 |

| (OLM-OPL)/INL= Distance from the Outer Limiting Membrane to the Outer Plexiform Layer (Rod ONL[μm])/ Inner Nuclear layer (μm) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| N=3 | control | 24hLL | 48hLL | 72hLL | 5dLL | 7dLL | 10d | 14d | 21d | 28d |
| Mean | 0.577 | 0.534 | 0.477 | 0.415 | 0.432 | 0.388 | 0.377 | 0.471 | 0.399 | 0.434 |
| Std. Dev. | 0.17 | 0.08 | 0.038 | 0.046 | 0.084 | 0.03 | 0.076 | 0.083 | 0.007 | 0.492 |
| Std. Error | 0.098 | 0.046 | 0.027 | 0.026 | 0.048 | 0.017 | 0.044 | 0.048 | 0.005 | 0.034 |

TABLE 2.3 CHANGES IN RODOPSIN, RED OPSIN AND PROLIFERATING CELL NUCLEAR ANTIGEN GENE EXPRESSION DURING THE LIGHT LESION TIME-COURSE. DATA OBTAINED WITH QUANTITATIVE REAL-TIME REVERSE TRANSCRPTASE POLYMERASE-CHAIN-REACTION (QRTPCR). RELATIVE VALUES COMPARED TO CONTROL

| Light-lesion time-course of *rodopsin* gene expression | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| N=3 | Control N=6 | 24hLL N=6 | 48hLL N=6 | 72hLL N=6 | 5dLL N=4 | 7dLL N=4 | 10d N=4 | 14d N=4 | 28d N=4 |
| Mean | 1 | 0.107 | 0.1693 | 0.1748 | 0.4020 | 0.384 | 0.515 | 0.301 | 0.253 |
| Std. Dev. | 0 | 0.047 | 0.097 | 0.137 | 0.138 | 0.243 | 0.255 | 0.093 | 0.041 |
| Std. Error | 0 | 0.019 | 0.039 | 0.056 | 0.069 | 0.121 | 0.127 | 0.046 | 0.02 |

| Light-lesion time-course of *red opsin* gene expression | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| N=3 | Control N=4 | 24hLL N=4 | 48hLL N=4 | 72hLL N=4 | 5dLL N=4 | 7dLL N=4 | 10d N=4 | 14d N=4 | 28d N=4 |
| Mean | 1 | 0.027 | 0.084 | 0.049 | 0.144 | 0.215 | 0.192 | 0.043 | 0.021 |
| Std. Dev. | 0 | 0.023 | 0.13 | 0.023 | 0.068 | 0.172 | 0.064 | 0.02 | 0.021 |
| Std. Error | 1 | 0.011 | 0.065 | 0.011 | 0.034 | 0.086 | 0.032 | 0.01 | 0.01 |

| Light-lesion time-course of *pcna gene* expression | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| N=3 | Control N=5 | 24hLL N=5 | 48hLL N=5 | 72hLL N=5 | 5dLL N=4 | 7dLL N=4 | 10d N=4 | 14d N=4 | 28d N=4 |
| Mean | 1 | 1.609 | 1.8128 | 4.0744 | 2.3699 | 1.903 | 1.572 | 0.498 | 0.467 |
| Std. Dev. | 0 | 0.849 | 1.182 | 2.626 | 1.084 | 1.285 | 0.995 | 0.208 | 0.118 |
| Std. Error | 0 | 0.379 | 0.528 | 1.174 | 0.542 | 0.642 | 0.497 | 0.104 | 0.059 |

### 2.3.5 DURING PHOTORECEPTOR REGENERATION *mdka* and *mdkb* ARE EXPRESSED BY PROLIFERATING MÜLLER GLIA AND PHOTORECEPTOR PROGENITORS

To investigate the expression of the *midkine* genes during retinal regeneration, we compared their expression patterns in normal adult retinas to adult retinas following the selective death of photoreceptors. The patterns of *midkine* expression in the adult retina are similar to 120 hpf larvae: *mdka* is selectively expressed in horizontal cells (Fig. 2.6a), while *mdkb* is transcribed by cells in the vitreal aspect of the INL and the GCL (Fig. 2.6e).

Light-induced photoreceptor death is clearly evident following 24 hrs of intense light exposure, and the subsequent cell proliferation nears its maximum at 72 hrs after initiating light treatment (Fig. 2.5). During the proliferative phase of photoreceptor regeneration, the expression of *mdka* in horizontal cells persists. In addition, *mdka* is expressed by the PCNA-positive photoreceptor progenitors in the inner and outer nuclear layers (Fig.2.6b, c, d). In comparison, *mdkb* transcription is also actively upregulated following photoreceptor death, validating the change in expression first detected with the gene arrays. The expression of *mdkb* expands throughout the INL, including the horizontal cells. In addition, *mdkb* transcripts are detected in the PCNA-positive photoreceptor progenitors (Fig.2.6f, g, h). Intense light treatment of the Tg(gfap:GFP)$^{MI2001}$ zebrafish confirmed the proliferating cells expressing *mdka* and *mdkb* are Müller cells and their progeny (see Appendix Fig. A3). These data show that in contrast to the expression of *mdka* and *mdkb* in separate cell populations in the developing and

adult retina, both genes are induced in a common set of cells in the regenerating retina:  horizontal cells and injury-induced photoreceptor progenitors.

**Figure 2.6.** In the light-lesioned retina, *mdka* and *mdkb* are expressed by horizontal cells and injury-induced photoreceptor progenitors. Panel a is an *in situ* hybridization showing the expression of *mdka* in a control retina. The white arrow identifies *mdka*-expressing horizontal cells. Panel b is an *in* situ hybridization showing *mdka* expression in a retina following 72 hrs of light exposure. Panel c is the same section as in panel b, immunostained with antibodies against PCNA. Panel d is the digital overlay of panels b and c. Arrowheads and arrows in panels b-d identify double-labeled cells in the ONL and INL, respectively. Panel e illustrates an *in situ* hybridization showing the expression of *mdkb* in a control retina. Panel f is an *in* situ hybridization showing *mdkb* expression in a retina following 72 hrs of light exposure. The arrowhead and arrow identify *mdkb*-expressing cells in the inner and outer nuclear layers, respectively. Panel g is the same section as in panel f, immunostained with antibodies against PCNA. Panel h illustrates the digital overlay of panels f and g. In panels b-d and f-h, arrowheads and arrows identify double-labeled cells in the ONL and INL, respectively. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; PCNA, Proliferating Cellular Nuclear Antigen; DAPI: nuclear stain 4,6-diamidino-2-phenylindole, dihydrochloride. Scale bar equals 50μm.

a
ONL
INL
GCL
mdka/DAPI/PCNA
b
mdka/DAPI
c
PCNA
d
mdka/DAPI/PCNA
e
ONL
INL
GCL
mdkb/DAPI/PCNA
f
mdkb/DAPI
g
PCNA
h
mdkb/DAPI/PCNA

## DISCUSSION

We used oligonucleotide microarrays in an initial experiment to identify genes that are differentially expressed in the retina of the zebrafish after a light lesion. The light-lesion model was selected, because, 1) it selectively kills photoreceptors; 2) we infer that degeneration of only two cell types (rods and cones) will result in less complex changes in the retinal transcriptome, thereby simplifying the array analysis; and 3) the selective death of photoreceptors, while sparing the rest of the retina, closely resembles photoreceptor degeneration in mouse genetic models of human disease. The specific goal of the array experiment was to identify novel growth factors that we hypothesize function to regulate aspects of retinal and/or photoreceptor regeneration.

Following the microarray screen, we characterized the expression of the two midkine genes, *mdka* and *mdkb,* during two neurogenic events: retinal development and photoreceptor regeneration. This analysis revealed that during retinal development the expression of *mdka* and *mdkb* is differentially regulated, and each gene has a pattern of cellular expression with distinct temporal and spatial features. *mdka* is expressed in the CMZ, transiently expressed in Müller glia, and constitutively expressed in horizontal cells. In contrast, *mdkb* is transiently expressed by newly postmitotic cells in the INL and constitutively expressed in differentiated ganglion and amacrine cells. During photoreceptor regeneration, the expression of both midkines is markedly upregulated in proliferating Müller glia and their progeny. Additionally, *mdka* expression persists in horizontal cells while *mdkb* expression expands to include all INL neurons,

including horizontal cells. These results suggest that both Midkines function in signaling pathways to direct stem cells and progenitors to regenerate retinal neurons and photoreceptors (Fischer and Reh, 2003, Fischer *et al*, 2004, Ooto *et al*., 2004, Osakada *et al*., 2007). Further, these results add Midkines to the molecular signature of Müller glia when they exhibit features of neural stem cells (Raymond et al., 2006).

During retinal development, two cell populations transiently express the *midkines* in non-overlapping domains, which are progressively restricted to adjacent annuli at the retinal margin. Double labeling with antibodies against PCNA indicate that *mdka* is expressed by retinal progenitors, whereas *mdkb* is expressed in newly postmitotic cells. The adjacent expression of these paralogous genes is similar to the developing neural tube at the forebrain/midbrain and midbrain/hindbrain boundaries, where *mdka* and *mdkb* are also expressed in contiguous, non-overlapping domains and function to pattern the early neural tube (Winkler et al., 2003). Our data suggest that the two Midkines may function to pattern the developing retina by acting on signaling pathways with separate domains of gene expression among progenitors and newly post-mitotic neurons (see Harris and Perron, 1998, Raymond et. al., 2006).

The initial and persistent expression of *mdka* in the peripheral-most portion of the CMZ suggests that Mdka is a molecular component of the retinal stem cell niche (Raymond et al., 2006) and may serve to regulate proliferation of retinal progenitors. In adult zebrafish brain, *mdka* transcripts are detected in the subventricular zone of the optic tectum (Winkler et.et al, al. 2003), a site of

persistent neurogenesis (Marcus et al., 1999). During tissue repair following chemical lesion of fetal rat brains, mitotic neuroepithelial cells at the ventricular surface of the cerebral cortex express Midkine (Kikuchi-Horie et al., al. 2004), and it was speculated that Midkine served to maintain proliferation. Indeed, neurospheres generated from Midkine deficient mice remain small, and derivatives of the neural precursors proliferate less than wild-type cells (Zou et. al. 2006). This function is consistent with the expression of *mdka* in retinal progenitors within the CMZ and regenerative Müller glia. These data indicate that expression of Midkine is associated with neural progenitors, in both the normal and injured brain, and may promote proliferation of neural stem and/or progenitor cells.

The strong and distinct expression of *mdka* in horizontal cells in the adult retina is intriguing. The presence of this secreted molecule, which acts in a paracrine manner (Schäfer et. al. 2005), in close proximity to the proliferating rod progenitors in the ONL, suggests that Mdka may regulate the persistent rod genesis in the zebrafish retina. Dynamic change in expression that leads to the presence of both *mdka* and *mdkb* in horizontal cells during retinal regeneration indicates that horizontal cells may also play a role, as yet unknown, in orchestrating the regenerative response. Their laminar position is ideal for influencing proliferation, migration and differentiation of photoreceptor precursors through signaling molecules, which may be distributed over large areas through their complex network of intercellular junctions. It has been proposed that horizontal cells play a pioneering role in the postnatal development of

photoreceptors (Messersmith and Redburn, 1990, Hagedorn et. al, 1998). It is possible that Midkines, secreted by horizontal cells, regulate aspects of persistent (Mdka) and regenerative neurogenesis (Mdka and Mdkb).

*mdkb* expression begins in the precocious ventro-nasal patch and follows the wave of retinal differentiation in the INL. The tight coupling of *mdkb* expression and the location of new neurons suggests it may regulate aspects of neuronal differentiation. In the zebrafish retina, *sonic hedgehog* (*shh*) initiates and sustains the circumferential waves of neuronal differentiation that pass through the inner layers (Neumann and Nuesslein-Volhard, 2000, Shkumatava et al., 2004). The expression of *mdkb* appears to follow the wave of *shh* and, thereby, may serve as an additional molecular component of signaling cascades that regulate early differentiation. Alternatively, *mdkb* could regulate events associated with neuronal maturation. Studies of mammalian neurons *in vitro* show that Midkine promotes neurite outgrowth (Muramatsu and Muramatsu, 1991). Similarly, we found the expression of *mdkb* is coincident with neurons that are elaborating axons and dendrites into the nascent plexiform layers, which suggests Mdkb may promote process formation and elongation from newly differentiated retinal neurons.

The constitutive expression of *mdkb* in ganglion and amacrine cells is maintained throughout adulthood. In rat retina, midkine expression is detected in the ganglion cell layer, in the inner INL and in the retinal pigmented epithelium (RPE) (Miyashiro et. al., 1998). The zebrafish *mdkb* expression pattern is similar to the mammalian homologue, although zebrafish lack RPE expression. The two

zebrafish *midkine* paralogs map to different linkage groups, although both chromosomal regions display extensive synteny to human chromosome 11, the location of the unique human midkine ortholog (Winkler et al., 2003). This suggests *mdkb* is more likely to serve similar functions as the mammalian midkine compared to *mdka*, which is expressed in different cell populations.

During photoreceptor regeneration both *midkines* are expressed in horizontal cells, and mitotic Müller glia and their progeny. Expression of *mdka* and *mdkb* in the same cells is not observed during retinal development suggesting the two growth factors act together to regulate regenerative events. The mammalian Midkine forms dimers *in vitro* and its biological activities are dependent on the crosslinking status of ligand molecules (Kojima et al., 1997, Iwasaki et al., 1997, Qiu et al., 2000). When produced by the same cells, the two zebrafish Midkines may form heterodimers which may bind different receptors to activate regeneration-specific signaling pathways. Alternatively, the functions of each Midkine could be preserved to differentially regulate proliferation and early photoreceptor differentiation during regeneration.

In summary, the expression of the *mdka* and *mdkb*, which encode secreted heparin-binding neurogenic factors, is dynamically regulated during retinal development and injury-induced retinal regeneration. The two *midkine* genes are expressed in distinct populations of stem cells, retinal progenitors and mature neurons, suggesting these secreted molecules subserve different functions. Following retinal injury, *mdka* and *mkdb* transcription is upregulated in Müller glia and their neurogenic offspring to regenerate the lost photoreceptors.

In vertebrates, midkines are upregulated following injury in numerous tissues (see Introduction and Results), and in humans midkine is integral to the growth of transformed cells. An emerging concept in biology is that the same regulatory proteins control common cellular events during development, tissue repair and carcinogenesis (Beachy et al., 2004, Gardiner, 2005). Our studies reinforce this concept and add Midkine-a and Midkine-b to the family of signaling molecules involved in both development and regeneration of the vertebrate retina.

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# CHAPTER 3

# IN HORIZONTAL CELLS OF THE ZEBRAFISH RETINA EXPRESSION OF MIDKINES IS MODULATED BY THE CIRCADIAN CLOCK

## 3.1 INTRODUCTION

The circadian clock maintains intrinsic rhythmical changes in biochemical and physiological processes, which provide optimal adaptation to environmental changes, such as light, temperature and access to food. The retina is the primary tissue that entrains the circadian clock to changes in the dark/light cycle in animals with eyes (Nelson and Zucker, 1981). This occurs through direct projections to the suprachiasmatic nucleus, the master circadian pacemaker, indirect projections to the pineal gland (Moore et al., 1995, Reppert and Weaver, 2002), as well as through synthesis of endocrine/paracrine factors, such as melatonin and dopamine (Wiechmann and Summers, 2008, Ribelayga et al., 2004). Numerous processes that occur in the retina are dependent on circadian rhythms, such as retinomotor movements (Pierce and Besharse 1985), disc-shedding of photoreceptor outer segments (Matthew LaVail, 1980), visual sensitivity (Li and Dowling, 1998), dopamine release (Ribelayga et al., 2004), expression of the interphotoreceptor retinoid binding protein (Rajendran et al, 1996) and photoreceptor input to cone horizontal cells (Wang and Mangel, 1996, Ribelayga et al., 2002).

Midkine (MK) is a member of the family of secreted heparin-binding growth/differentiation factors that also includes pleiotrophin (Obama et al, 1994,

Kadomatsu and Muramatsu, 2004). Identified in a screen of retinoic acid inducible genes in embryonic carcinoma cells (Kadomatsu et al.,1988, Kadomatsu et. al. 1990), Midkine is highly conserved throughout the animal kingdom, and has numerous functions: neurogenic, transforming, neurotrophic, chemotactic, mitogenic, and anti-apoptotic (Muramatsu 2002, Kadomatsu and Muramatsu 2004, Winkler et. al, 2003). In mammals, MK is expressed in numerous tissues during embryonic development, most prominently in the developing neural tube and at epithelial–mesenchymal boundaries (Mitsiadis et al., 1995). The zebrafish genome encodes two distinct midkine genes, *midkine-a* (*mdka*) and *midkine-b* (*mdkb*), which, during early zebrafish development have distinct patterns of expression and have different biological functions (Winkler and Moon 2001, Winkler et al., 2003, Schäfer et al., 2005, this study).

In zebrafish, cells of the retinal stem cell compartments (retinal stem and progenitor cells in the CMZ and injury-activated Müller glia) express midkines during developmental and regenerative neurogenesis (Chapter 2). In the developing retina, *mdka* and *mdkb* are expressed in distinct, adjacent domains at the boundary between the CMZ and the newly differentiated retina, as well as in distinct subtypes of retinal neurons in the adult retina: *mdka* is constitutively expressed by horizontal cells and *mdkb* is constitutively expressed by ganglion cells and cells in the amacrine layer of the INL. In addition, during the proliferative phase of photoreceptor regeneration both *mdka* and *mdkb* are expressed by proliferating Müller glia, their neurogenic progeny in the INL and photoreceptor precursors in the ONL (Chapter 2).

During the light-lesion experiments described in Chapter 2, I consistently observed a decrease in the expression of *mdka* in horizontal cells at 12 hours following light onset. I followed up on this observation, and here I report that expression of Midkine-a in retinal horizontal cells, is regulated by the circadian clock, resulting in robust changes in expression during a 24h period at both mRNA and protein levels. The expression of *mdka* increases during subjective day and decreases during subjective night. Similarly, the cellular expression of *mdkb* appears also to be regulated by the circadian clock, with expansion of the expression domain during the second half of the subjective night, to include horizontal cells. The functional significance of the circadian expression of *mdka* and *mdkb* in horizontal cells is yet to be determined.

## 3.2 MATERIALS AND METHODS

### Animals

Wild-type zebrafish (*Danio rerio*), mixed strains and strain AB, 4.5 to 7 months old, were purchased from Aquatica Tropicalis (Plant City, Florida) and acclimated for at least 2 weeks in aquaria at 28.5$^{o}$C with a 14/10 hour light/dark cycle, lights on at 8am (or 9am during daylight savings time) and lights off at 10pm (or 11pm during daylight savings time). For circadian experiments, fish were maintained in complete darkness for 24hrs and sacrificed at 4-hour intervals (six time-points), starting at either 12am or 4am. All animal procedures were approved by the University of Michigan Committee on the Use and Care of Animals.

### Light treatments

Wild-type zebrafish were housed in complete darkness for 7 days and then exposed to intense fluorescent light (30,000 lux) for 12 hours as described (Chapter 2).

### Tissue preparation, in situ hybridization and immunohistochemistry

At selected times, adult fish were anesthetized in 0.05% 3-aminobenzoic acid-ethyl ester (Sigma-Aldrich, St. Louis, MO), eyes were enucleated, lenses removed and eyecups fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer with 5% sucrose. Eyecups were cryoprotected in 20% sucrose, embedded in 2 parts 20% sucrose 1 part Tissue-Tek® O.C.T. Compound (Electron Microscopy Sciences, Hatfield, PA), frozen in Tissue-Tek® OCT and stored at -80$^{o}$C. Ten micron cryosections through the dorso-ventral axis of the

eyecups were mounted on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and processed for *in situ* hybridization with riboprobes encoding *mdka* and *mdkb* labeled with digoxigenin (DIG)-labeled riboprobes, as described (Chapter 2). Nitro-blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) or Fast Red (Roche Diagnostics, Indianapolis, IN) were used as enzymatic substrate. For comparison of *mdka* and *mdkb* expression between circadian samples, four or five cryosections from eyes harvested at each of the six time points were mounted on the same slide, for each of the two midkine probes in three independent experiments. This ensured that sections from all time points were identically processed and allowed comparison of expression levels based on the intensity of the color reaction. Proliferating cells were identified on retinal sections using antibodies that recognize Proliferating-Cell Nuclear Antigen (p-8825, Sigma-Aldrich, St. Louis, MO) used at a dilution of 1:1000 following antigen retrieval (Raymond et. al., 2006). Goat anti-mouse Alexa-Fluor 555, used at a dilution of 1:500 (Invitrogen-Molecular Probes, Eugene, OR).

**Photographic images**

Images were taken with a Nikon DMX 1200 digital camera mounted on a Nikon Eclipse E800 epifluorescence microscope equipped with a differential interference contrast filter. Adobe Photoshop CS2 (Adobe Systems, San Jose, CA) was used to construct the figures. The layer tool was used to generate overlays. For any given composit figure, brightness and contrast were adjusted, if needed, identically for all panels of each figure. Images presented in Fig.3.6 were

taken with an AxioCam RM digital camera and a Zeiss Axio Imager epifluorescent compound microscope (Carl Zeiss Microimaging, Thornwood, NY). Images were false colored using the Zeiss AxioVision 4.0 software and exported into Adobe Photoshop CS2 (Adobe Systems, San Jose, CA) and treated as described above.

**Western Blot Analysis**

Affinity purified polyclonal antibodies against C terminal peptides of zebrafish Mdka and Mdkb were generated in rabbits (Invitrogen, Carlsbad, California). Peptides were selected with a protein toolbox plot (Invitrogen, Camarillo, CA), from the amino acid sequences of the proteins available in the NCBI database (accession numbers NP_571145 for Mdka and NP_571791 for Mdkb) based on antigenicity, hydrophilicity, flexibility and surface probability. C-terminal peptides composed of 16 amino acid residues were chosen as immunogens (for Mdka amino acids 131-145: KVKNKPKGKKGKGKGC, and for Mdkb amino acids 132-147: CKPKGGEKKKGKGKEN).

Specificity of the antibodies was tested using recombinant Mdka-MYC and Mdkb-MYC proteins. To generate Mdka-MYC and Mdkb-MYC proteins, 293T human embryonic kidney cells were plated on 100mmx20mm tissue culture dishes, transfected at 70% confluency with 2-4μg of plasmid: *pCS2mdka-myc* and *pCS2mdkb-myc* (gift from Dr. Christoph Winkler) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Three days after transfection, supernatants were collected and proteins purified with aMYC-coated agarose beads, following the manufacturer's recommendations (Medical and Biological Laboratories

International, Woburn, MA). 2μl of purified protein, 10μl of supernatant and 125 ng of human recombinant Midkine (R&D Systems, Minneapolis, MN) were separated by electrophoresis on a 12% Sodium-Dodecyl-Sulphate Polyacrylamide Gel (SDS-PAGE) and transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Membranes were incubated at 4$^{o}$C for at least 4 hours in Blocking Buffer (Phosphate-Buffered-Saline with 0.5% Tween [PBST] and 5% Non-Fat Dry Milk) followed by overnight incubation in primary antibodies, αMdka or aMdkb, diluted 1:500 in Blocking Buffer. Membranes were washed for one hour in PBST with 4-5 changes of the washing buffer, then incubated for one hour in secondary antibodies (goat anti-rabbit IgG conjugated to horse-radish peroxidase [Amersham Biosciences, Arlington Heights, IL]). Following 4-5 vigorous washes in PBST, proteins were visualized with the enhanced chemiluminescence (ECL) detection system (ECL- Amersham Biosciences, Arlington Heights, IL) and radiographic film.

To test the specificity of the antibodies, 293T cells were transfected with *pCS2mka-myc* or *pCS2mdkb-myc* (as described above), supernatants were collected and cell lysates were prepared in L-RIPA lysis buffer (150mM NaCl, 50mM Tris, 0.1% TritonX, 2mM EGTA, pH 7.5) supplemented with protease inhibitors (Complete-mini EDTA free, Roche, Indianapolis, IN). Supernatants (10μl/ from a total of 7ml) and cell lysates (20μl of 150 μl) from *pCS2mdka-myc* and *pCS2mdkb-myc* transfected 293T cells were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with affinity purified Mdka and Mdkb antibodies diluted 1:500 in blocking buffer or anti-c-myc

antibodies (9E10, Santa-Cruz Biotechnology, Santa-Cruz, CA), diluted 1:1000 in blocking buffer, washed, incubated in secondary antibodies (anti-mouse or anti-rabbit IgG-HRP) and specific bands were detected by ECL.

To assay expression of endogenous Mdka and Mdkb in the zebrafish retina, 5 retinas were dissected from 5 different fish for each selected time-point of the circadian cycle. The retinal pigment epithelium was carefully removed using Dumont #55 fine forceps (Fine Science Tools, Foster City, CA), and retinas homogenized with a Kontes pellet pestle (Fisher Scientific, Pittsburgh, PA) in 75ml of lysis buffer (Phosphate Buffered Saline with 1% Triton X and protease inhibitors (Complete-mini EDTA free, Roche, Indianapolis, IN). Lysates were centrifuged for 5 minutes at 5000 rpm at 4$^{o}$C to pellet nuclei, and transferred to fresh tubes. The amount of retinal protein was determined with a BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were loaded onto a 12% SDS-PAGE and processed for immunoblotting, as described above. Equal protein loading was verified by reprobing membranes with a monoclonal antibody recognizing $\alpha$, $\beta$ and $\gamma$-actin in a broad range of species, used at a dilution of 1:3000 (JLA20, Calbiochem, San Diego, CA), followed by incubation with the secondary antibody conjugated to HRP conjugate, at a dilution of 1:7000 and ECL detection of specific bands as described above.

To quantify proteins detected on the radiographic film, the film was scanned using a flatbed scanner (Epson expression 1600) to obtaine a digital image that had both the Mdka and the actin band for each circadian time point. Adobe Photoshop CS2 (Adobe Systems, San Jose, CA) was used to invert the

image, the magic wand or lasso tool to select the specific bands, and the expanded histogram tool to measure the number and intensity of the pixels within each band. The product of these numbers, for the Mdka band was divided by the number obtained for the actin band at each time point. The resulting value was compared to the value obtained for the 8pm time-point, in each circadian experiment; values were averaged between experiments and plotted on a graph as fold-change. The 8pm time-point was chosen as reference, in order to compare relative expression values between experiments.

## RNA extraction and Quantitative Reverse Trascriptase Real-Time PCR (QRTPCR)

To obtain retinal RNA, at selected times during the circadian cycle, adult zebrafish were anesthetized in 0.1% 3-Aminobenzoic Acid-Ethyl Ester (Sigma-Aldrich, St. Louis, MO). Eyecups were removed and retinas dissected and carefully separated from the retinal pigment epithelium with fine forceps. Three to four retinas per time-point (7-16 mg) were pooled and homogenized with a sterile pestle (Kontes Pellet Pestle, Fisher Scientific) in 200 ml lysis buffer from the Ambion RNAqueous-Micro RNA isolation kit (Ambion, Austin, TX). RNA extraction was performed according to the manufacturer's instruction. RNA was quantified with a spectrophotometer and RNA quality was assessed on ethydium bromide stained formalin-agarose gels. 0.5 or 1 mg of total RNA was used to synthesize cDNA using the Superscript II First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) following the manufacturers protocol. The

resulting first-strand reaction, diluted 1:4 was used as a template for the subsequent QRTPCR reaction performed with the iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA) in the iCycler Real-Time PCR detection system (Bio-Rad). The following amplification and melt curve analysis protocol was used: 95$^{o}$C 3min, 40 cycles (95$^{o}$C: 20s, 57$^{o}$C: 20s, 72$^{o}$C: 30s), 95$^{o}$C: 1min, 90X55$^{o}$C: 10s. Gene specific primers (0.4mM) were as follows: for *mdka* (NM_131070) forward: tgaagttttgttactgagctttgtg, and reverse: agccagtgtacataagtgtgtgtgt; for *mdkb* (NM_131716) forward: gctgttgtaatttgtagcaggtttt, and reverse: cattcaatctcgttgtcatttacag; for *connexin 52.6* (NM_212819) forward: tggacagatggtacctttgcc and reverse: gttgtctggaatggaccttcg. (Connexin 52.6 (Cx52.6) is a gap junction protein expressed exclusively in horizontal cells [Zoidl et. al. 2004]). Serial dilutions of the first strand reaction were run for efficiency calculations of each primer using the Pfaffl method (Pfaffl, MW, 2001). The threshold cycle (Ct) was determined by the iCycler using the maximum curvature approach and then maintained constant for subsequent runs. For each time-point, relative gene expression values were determined using the calculated primer efficiencies and threshold cycle with the formula: $E^{-Ct}$ (Pfaffl, MW, 2001) divided by the expression at 8pm and represented graphically as fold-change. Specificity of the amplification products was verified by agarose gel electrophoresis of sample wells showing single bands with the expected size. Values obtained were averaged for 3-6 independent experiments and statistical significance (*$p<0.05$ or **$p<0.01$) was calculated by one-way ANOVA with Bonferroni correction for multiple comparisons using SPSS software. Samples

within one experiment (6 time-points collected at 4 hour intervals within one 24h period) were generated identically, enabling comparison within one experiment. Normalizing results to one timepoint (8pm) allowed comparison between experiments. Independently, to verify that equal amounts of RNA were used for the circadian samples within one experiment, QRTPCR was performed with specific primers for ribosomal protein L-19 (rpl19, accession number: NM_213208, primers forward: gagtatgctcagacttcagaagagg and reverse: atcaaaccatccttcaccaacttac), showing no difference in expression between the different time-points.

## 3.3 RESULTS

### 3.3.1 EXPRESSION OF *mdka* IN HORIZONTAL CELLS DECREASES AT THE END OF THE DIURNAL LIGHT CYCLE

I have previously shown that the secreted growth factors, Midkine-a and Midkine-b are present in the zebrafish retina and that their cellular expression is actively modulated in the retinal stem cell niche during two neurogenic events: retinal development and photoreceptor regeneration following photoreceptor death induced by exposure to intense fluorescent light (Chapter 2). Intriguingly, time-course analysis of *mdka* expression in the light-lesioned retina consistently showed a marked decrease at twelve hours after light onset. This time corresponded in each experiment with approximately 9pm in the evening. To pursue this observation and determine if this decrease is a consequence of light-induced injury or a diurnal phenomenon that normally occurs in the retina, I analyzed *mdka* expression in parallel, in control retinas collected in the morning, 15min after light onset, retinas from animals exposed for 12h to intense fluorescent light, and retinas from animals kept in normal aquaria lighting conditions, 12 hours after light onset, at the end of the day. This revealed that shortly after light onset in the morning, *mdka* is strongly expressed in horizontal cells, as well as in a few columnar cells in the INL, cells with morphology resembling Müller glia (Fig. 3.1 panel a). Following 12 hours of exposure to intense fluorescent light, *mdka* expression in the retina decreases markedly, to levels undetectable by in situ hybridization (Fig.3.1, panel b). Similarly, decreased expression of *mdka* is observed at the end the day, 12 hours after

light onset, in retinas of animals kept under normal lighting aquaria conditions (Fig. 3.1, panel c).

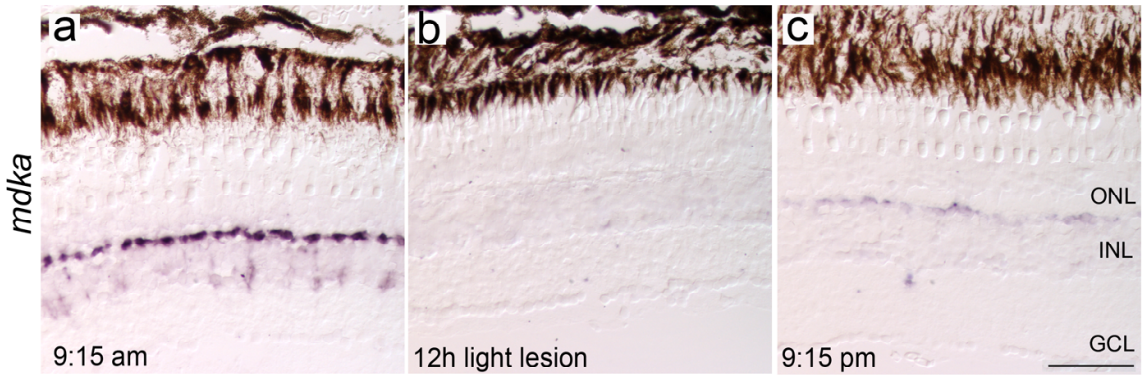


**Fig. 3.1 Expression of *mdka* in horizontal cells decreases at the end of the diurnal light cycle.**
In situ hybridizations that illustrate *mdka* expression in the adult zebrafish retina at the beginning of the diurnal light cycle (panel a), following 12 hours of treatment with intense fluorescent light (panel b) or at the end of the diurnal light cycle in normal aquaria conditions (panel c). Sections for all conditions were processed on the same slide. ONL; outer nuclear layer, INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar in panel c equals 50 μm.

### 3.3.2 THE CIRCADIAN CLOCK REGULATES EXPRESION OF *mdka* mRNA AND PROTEIN IN HORIZONTAL CELLS

To more fully characterize *mdka* expression and its putative regulation by the circadian clock, I maintained zebrafish in total darkness for a period of 24 hours and collected retinas at four-hour intervals. Retinas were divided into three groups, and *mdka* expression was evaluated by *in situ* hybridization, quantitative real-time PCR (QRTPCR) and Western blot analysis. In situ hybridization revealed that expression of *mdka* in horizontal cells is dynamically modulated in the absence of light during the circadian cycle. Maximum expression is observed at 8am (Fig.3.2, panel c), one hour prior to the normal time of light onset (9am). Expression is high during the first half of the subjective day (Fig.3.2 panels d, e) and gradually decreases during the second half of the subjective day (Fig.3.2 panel f) reaching a minimum at 12am, the beginning of the subjective night, one hour after the usual end of the light cycle in our zebrafish facility (Fig.3.2 panel a).

To confirm this circadian rhythm of expression using a rigorous quantitative method I performed quantitative real-time reverse-trascriptase polymerase chain reaction (QRTPCR) with *mdka* specific primers. I collected RNA from zebrafish retinas, at selected time-points during the subjective day and night, as described above, in three independent experiments. These quantitative data parallel the in situ hybridization data and confirm the circadian rhythm of *mdka* expression, showing maximum expression at 8am, one hour prior to the usual onset of the light period and minimum expression at 12 am, one hour after

the usual onset of the dark period (Fig. 3.2 a). Analysis of variance with Bonferroni correction for multiple comparisons shows that the changes in *mdka* expression levels are strongly statistically significant (Table 3.1).

**Fig. 3.2 The circadian clock regulates expression of *mdka* and *mdkb* in the zebrafish retina.**
Panels a-f are in situ hybridization that illustrate retinal epression of *mdka* during subjective day (panels a-c) and subjective night (panels d-f). Panels g-n are in situ hybridizations that illustrate retinal expression of *mdkb* during subjective day (panels g-i) and subjective night (panels l-n). Arrows in panels a-n point to the location of horizontal cells. Arrowheads in panels g-n point to the location of amacrine and ganglion cells respectively. Sections for all time-points for each probe were processed on the same slide. ONL; outer nuclear layer, INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar in f equals 50 μm.

mdka
mdkb
a
b
c
d
e
f
g
h
i
l
m
n
ONL
INL
GCL
12am
4am
8am
12pm
4pm
8pm
Subjective night
Subjective day

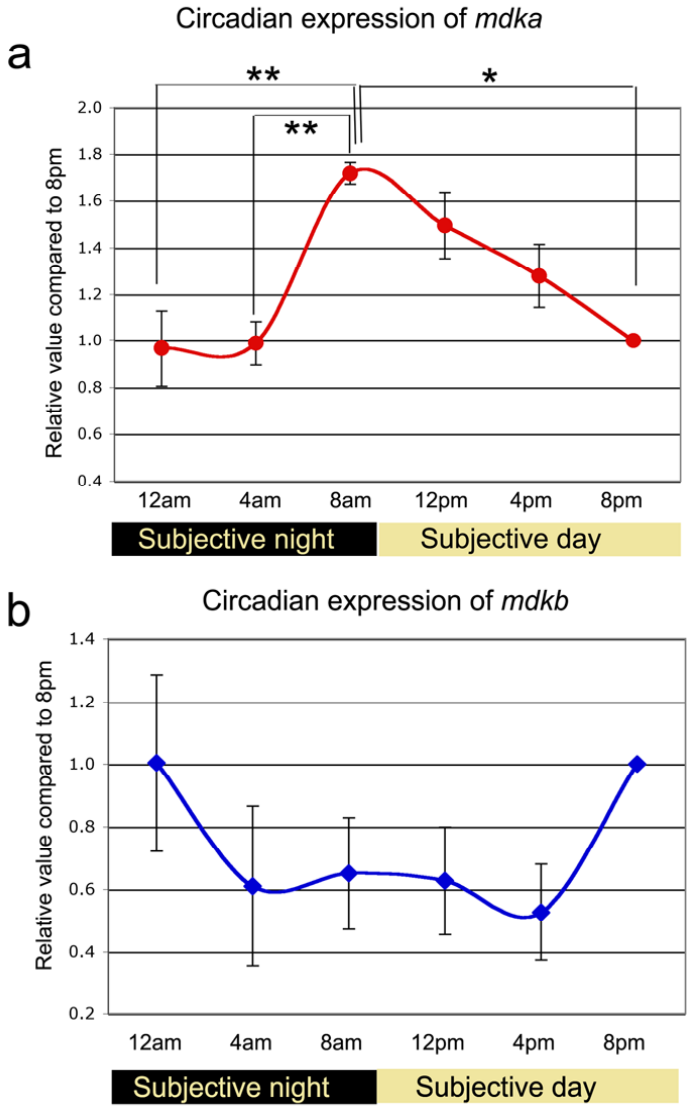


**Fig. 3.3 Quantitative analysis of circadian variations of *mdka* and *mdkb*** Graphical representations of QRTPCR analysis of circadian expression of *mdka* (panel a), *mdkb* (panel b). Gene expression is represented as fold change compared to expression values at the end of the subjective day (8pm). Data represent average values from 3 experiments with standard errors represented for each time-point. Statistical significance was determined through one-way ANOVA with Bonferroni correction for multiple comparisons using SPSS (** $p<0.01$, * $p<0.05$). Descriptive statistics are presented separately in tables 3.1 and 3.2.

I next asked whether this rhythm in *mdka* mRNA expression results in dynamic changes in levels of Mdka protein. To answer this question, antibodies that recognize Mdka protein were generated. To determine the specificity of the antibodies I transfected 293T cells with plasmids encoding mdka-MYC and *mdkb-MYC* (gift from Dr. Christoph Winkler), I collected lysates and supernatants from cell cultures and purified Mdka-MYC and Mdkb-MYC proteins from their respective supernatants. On immunoblots the Mdka antibody recognized the Mdka-MYC proteins in the cell lysate, supernatant and the purified protein as well as the human recombinant MK (hrMK), but not the purified Mdkb-MYC protein (Fig.3.4-1 panel a), showing that, the antibody is specific for Mdka.

Western blot analysis of retinal lysates, from fish sacrificed at the same selected circadian time-points during a 24h period revealed that the circadian rhythm in Mdka protein expression mirrors the mRNA rhythm. Maximum expression is found at 8am and minimum expression at 12am (Fig. 3.4-2 a). This rhythm was consistent over two independent experiments. Taken together, these data show that the expression of Mdka in horizontal cells of the zebrafish retina is regulated by the circadian clock and both the mRNA and the protein follow the same dynamic changes with maximum expression prior to the onset of the subjective day and minimum expression at the beginning of the subjective night.

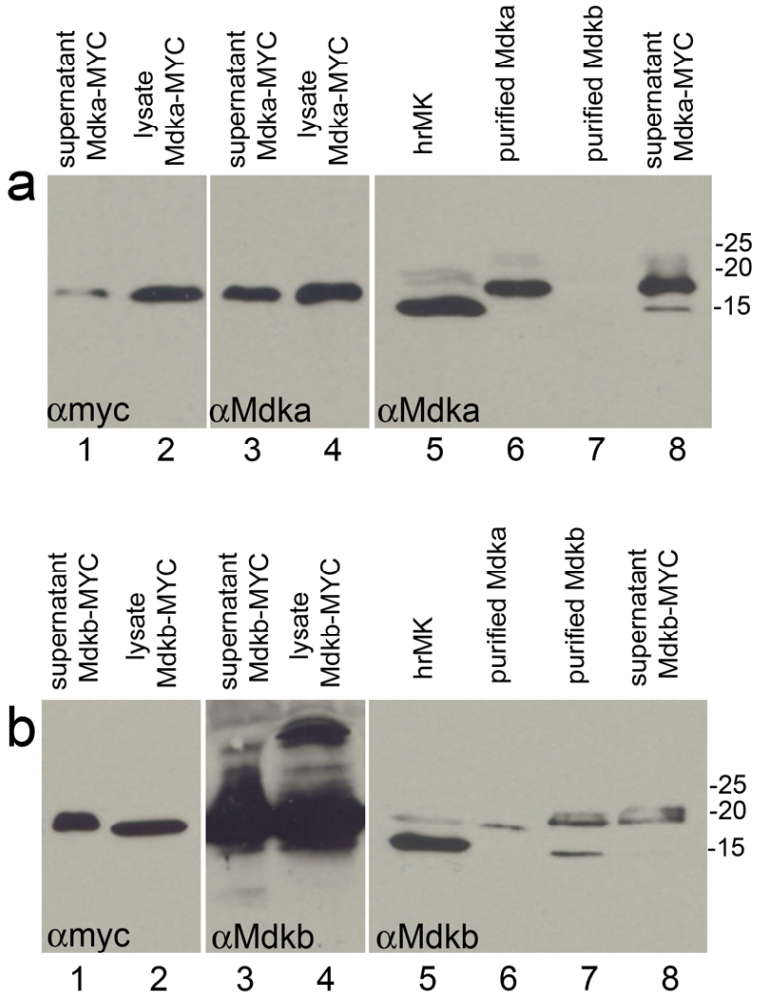


**Fig. 3.4-1 Specificity of zebrafish αMdka and αMdkb antibodies**
**Panel a.** Lanes 1-4 represent immunodetection with αc-myc antibodies (lanes 1 and 2) or αMdka antibodies (lanes 3 and 4) of Mdka-MYC protein separated by SDS-PAGE from the same supernatants (lanes 1 and 3) and cellular lysates (lanes 2 and 4) of 293T cells transfected with *pCS2mdka-myc*. Lanes 5-8 represent immunodetection of Mdka with αMdka antibodies of human recombinant midkine (hrMK, lane 5), purified Mdka (lane 6) and supernatant from 293T cells transfected with *pCS2mdka-myc* (lane 8). Note that the antibody does not detect the purified Mdkb (lane 7).
**Panel b.** Lanes 1-4 represent immunodetection with αc-myc antibodies (lanes 1 and 2) or αMdkb antibodies (lanes 3 and 4) of Mdkb-MYC protein separated by SDS-PAGE from the same supernatants (lanes 1 and 3) and cellular lysates (lanes 2 and 4) of 293T cells transfected with *pCS2mdkb-myc*. Lanes 5-8 represent immunodetection of Mdkb and Mdka with αMdkb antibodies of human recombinant Midkine (hrMK, lane 5), purified Mdka (lane 6), purified Mdkb (lane7) and supernatant from 293T cells transfected with *pCS2mdkb-myc*. Note that the antibody detects the purified Mdka (lane 6).

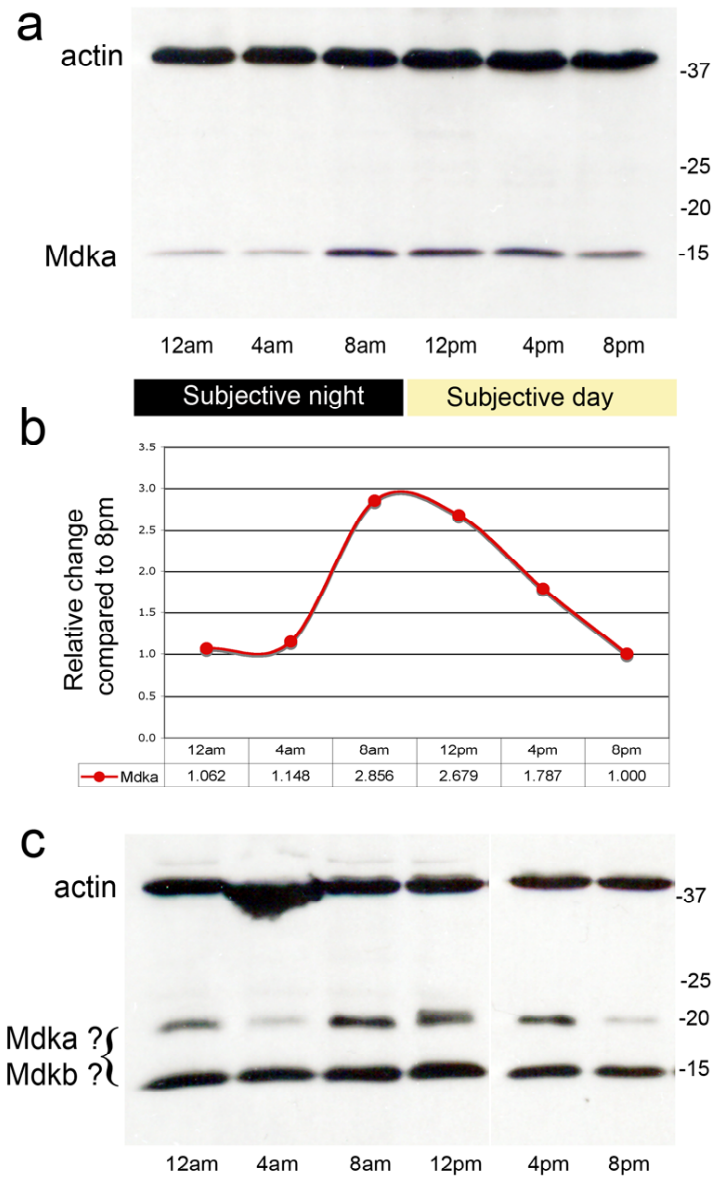


**Fig. 3.4-2 The circadian clock regulates the expression of Mdka protein**
**Panel a** shows an immunoblot with antibodies against Mdka and actin of retinal lysates obtained from zebrafish at specified times during the circadian cycle and separated by SDS-PAGE. Lower bands represent the Mdka protein and upper bands represent actin, used as loading control.
**Panel b** illustrates the quantification of circadian changes in retinal Mdka protein expression, normalized to expression of actin, average of 2 experiments. Results are shown as mean fold change compared to the expression at 8pm.
**Panel c** shows an immunoblot with antibodies against Mdkb and actin of retinal lysates obtained from zebrafish at specified times during the circadian cycle and separated by SDS-PAGE. Lower bands (approximately 15 and 20 kDa) likely represent Mdka and Mdkb proteins and upper bands (approximately 40kDa) represent actin, used as loading control.

### 3.3.3 THE CIRCADIAN CLOCK MAY MODULATE CELLULAR EXPRESSION OF *mdkb* IN HORIZONTAL CELLS

To test if expression of *mdkb* is modulated by the circadian clock, we performed in situ hybridization with specific probe against *mdkb*, on retinas collected during the subjective day and night, as described above. This analysis reveled that during the day, expression of *mdkb* is confined to the inner (vitreal) retina, where *mdkb* is expressed in the ganglion cell layer (GCL) and the amacrine region of the inner nuclear layer (INL) (Fig.3.2, panels l and m). At the end of the subjective day and the beginning of the subjective night, expression of *mdkb* in the INL increases (Fig.3.2 panels n and g). Midway through the subjective night (4am and 8am time-points) *mdkb* is distinctly expressed in horizontal cells in addition to the GCL and cells of the vitreal INL (Fig. 3.2, panels h and i).

To test if there is a quantitative change in the circadian expression of *mdkb*, similar to *mdka*, we performed QRTPCR analysis with primers specific for *mdkb* on retinal RNA samples collected at circadian time-points, as described above. Three independent experiments revealed a trend for increased expression of *mdkb* at the end of the subjective day (8pm) and beginning of the subjective night (12am), and decreased expression during the day (12pm and 4 pm, Table 3.2), these quantitative changes in expression are more variable, and do not reach statistical significance (see Table 3.2).

To analyze expression of Mdkb protein in the retina we generated antibodies against a C-terminal peptide of Mdkb. This antibody appears to recognize both Mdka and Mdkb, since it detects bands not only in the lanes with lysates, supernatant from *mdkb-myc* transfected cells or purified Mdkb-MYC protein, but also in the lane with Mdka-MYC purified protein (Fig. 3.4-1b). On immunoblots with retinal lysates collected at the specified circadian times, the Mdkb antibody reveals two distinct bands, at approximately 15kDa and 20kDa. The higher molecular weight band appears to follow the same circadian rhythm as Mdka (Fig. 3.4-2c). According to their amino-acid composition (146 amino-acids for Mdka and 147 amino-acids for Mdkb), both Midkines are 13 kDa proteins. In our gel conditions, Mdka runs approximately with the 15kDa marker (Fig.3.4-2a), and Mdkb should run very similarly. We cannot determine whether the lower molecular weight bands in the circadian lysates immunoblotted with Mdkb antibodies represent Mdkb or Mdka or both, or the identity of the protein approximately 20kDa in size. Thus we cannot conclude whether Mdkb protein is modulated during the circadian cycle.

### 3.3.4 *mdka* IS EXPRESSED IN PRESUMPTIVE MÜLLER GLIA AT THE RETINAL MARGIN

The rod lineage in zebrafish retina has been described to have at the origin Müller cells in the INL which divide asymmetrically, give rise to rod progenitors that migrate in the ONL, where they divide a few times more to generate rod precursors that differentiate into rods (Johns, 1982, Bernardos et al. 2007, Otteson and Hitchcock, 2003). Young Müller cells at the periphery of the zebrafish retina have been shown to express numerous molecules characteristic of neural stem cells, such as Brain Lipid Binding Protein and apo-lipoprotein E (Raymond et al., 2006). We observed that at the time when *mdka* expression in horizontal cells is lowest (12am, Fig. 3.5a), cells with Müller cell morphology at the retinal margin express *mdka* (Fig.3.5b). In addition, at both times, *mdka* is expressed in a few cells at the periphery of the retina, the location of pluripotent undifferentiated retinal stem cells (Raymond et. al., 2006).

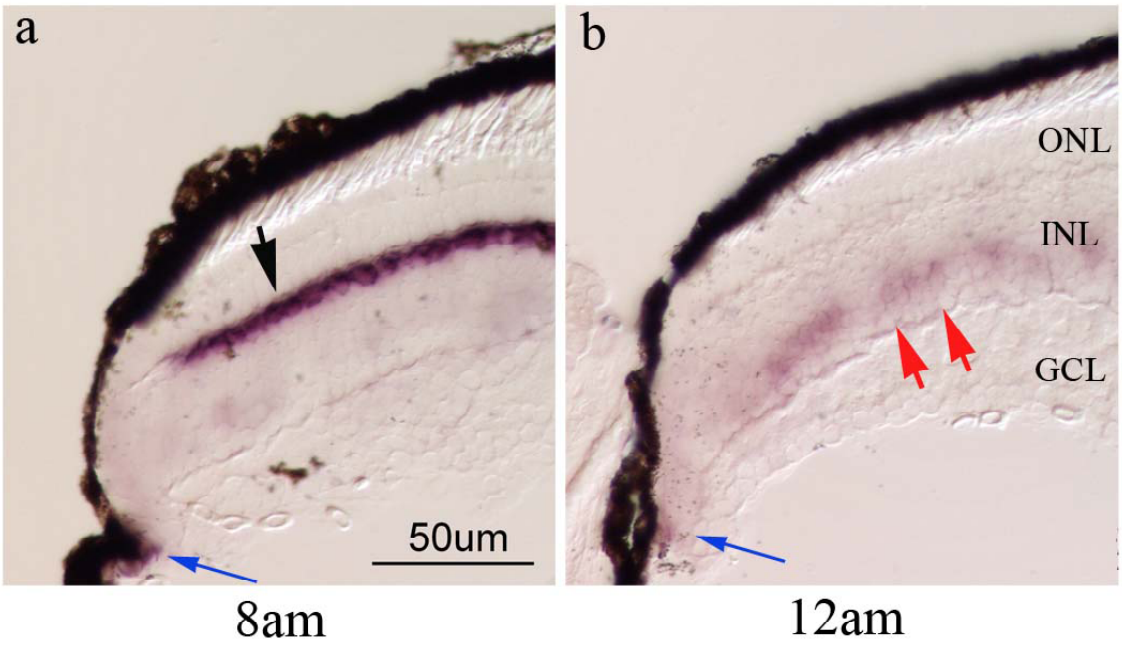


**Fig.3.5 At the retinal margin, *mdka* is expressed in the CMZ and presumptive Müller glia**
Panels a and b are in situ hybridization that illustrate epression of *mdka* at the retinal margin at the circadian time of maximum *mdka* expression (8am, panel a) and minimum *mdka* expression (12am, panel b). Black arrow in panel a points to horizontal cells that strongly express *mdka* at this time. Blue arrows in panels a and b point to the location of peripheral-most cells in the CMZ that express *mdka*. Red arrows in panel b point to the presumptive Müller glia that express *mdka* at the time when expression of *mdka* in horizontal cells is minimal. Sections were processed on the same slide. ONL, outer nuclear layer, INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar in f equals 50 μm

### 3.3.4 ROD PRECURSORS IN THE OUTER NUCLEAR LAYER DO NOT EXPRESS *mdka* or *mdkb*

In the course of the experiments described in Chapter 2 we observed that photoreceptor precursors in the ONL of light-lesioned retinas, express both *mdka* and *mdkb*. To test if expression of *mdka* and *mdkb* in photoreceptor progenitors

is a regeneration specific event or if it is characteristic of growth-associated neurogenesis I performed in situ hybridization in combination with proliferating cell nuclear antigen (PCNA) immunohistochemistry, on retinas from zebrafish collected at 12pm, a time during the circadian cycle when *mdka* shows high expression. This analysis revealed that neither *mdka* nor *mdkb* are expressed in rod precursors, identified as such, by their laminar location in the ONL, and expression of PCNA (Fig.3.6).

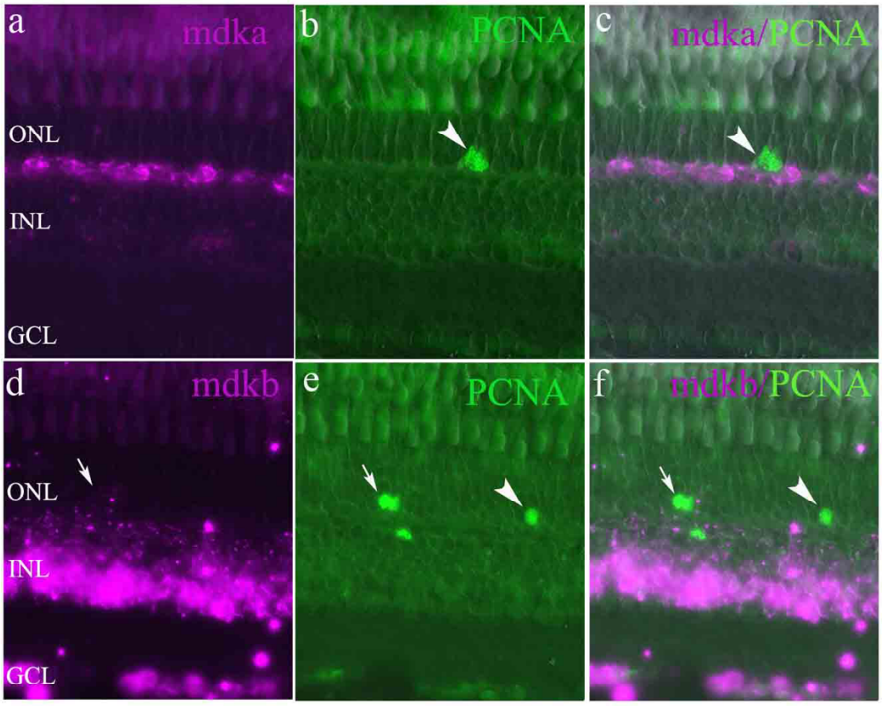


**Fig.3.6 Rod precursors in the ONL do not express *mdka* or *mdkb***
Panels a and d are *in situ* hybridization that illustrate expression of *mdka* and *mdkb* respectively in the retina of a zebrafish collected at 12pm. Panels b and e are the same sections as in a and d, immunostained with antibodies against PCNA. Panels c and f are are digital overlays of panels a and b, and d and e respectively. Arrowheads identify rod precursors that express PCNA but not *mdka* or *mdkb*. PCNA, Proliferating Cell Nuclear Antigen.

Table 3.1 CIRCADIAN EXPRESSION OF *mdka* IN THE ZEBRAFISH RETINA

Data obtained with Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction (QRTPCR)
Relative values compared to 8pm.

| Experiment | 12 am | 4 am | 8 am | 12 pm | 4 pm | 8 pm | Min | Max |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.6599 | 0.8043 | 1.6980 | 1.2189 | 1.1373 | 1 | 0.6599 (12am) | 1.6980 (8am) |
| 2 | 1.0282 | 1.0572 | 1.6504 | 1.6875 | 1.1493 | 1 | 1.0282 (12 am) | 1.6875 (12pm) |
| 3 | 1.2099 | 1.1042 | 1.8055 | 1.5799 | 1.5501 | 1 | 1.1042 (4am) | 1.8055 (8am) |
| Mean | 0.9660 | 0.9886 | 1.7180 | 1.4955 | 1.2789 | 1 | | |
| Std. Dev. | 0.2802 | 0.1613 | 0.0794 | 0.2455 | 0.235 | 0 | | |
| Std. Error | 0.1618 | 0.0931 | 0.0458 | 0.1417 | 0.1356 | 0 | | |
| 95% CI lower bound | 0.2698 | 0.5879 | 1.5207 | 0.8856 | 0.6952 | 1 | | |
| 95% CI upper bound | 1.6623 | 1.3893 | 1.9153 | 2.1054 | 1.8627 | 1 | | |

| One way ANOVA with Bonferroni correction for multiple comparisons | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Pairwise compari-son | Mean differenc e | p value | Pairwise compari-son | Mean differenc e | p value | Pairwise compari-son | Mean difference | p value |
| 12am vs 4am | -0.02253 | 1.000 | 8am vs 12am | 0.75197 | 0.007** | 4pm vs 12am | 0.31290 | 1.000 |
| 12am vs 8am | -0.75197 | 0.007** | 8am vs 4am | 0.72943 | 0.009** | 4pm vs 4am | 0.29037 | 1.000 |
| 12am vs 12pm | -0.52947 | 0.089 | 8am vs 12pm | 0.2225 | 1.000 | 4pm vs 8am | -0.43907 | 0.255 |
| 12am vs 4pm | -0.3129 | 1.000 | 8am vs 4pm | 0.43907 | 0.255 | 4pm vs 12pm | -0.21657 | 1.000 |
| 12am vs 8pm | -0.03397 | 1.000 | 8am vs 8pm | 0.71800 | 0.01* | 4pm vs 8pm | 0.27893 | 1.000 |
| 4am vs 12am | 0.02253 | 1.000 | 12pm vs 12am | 0.52947 | 0.089 | 8pm vs 12am | 0.03397 | 1.000 |
| 4am vs 8am | -0.72943 | 0.009** | 12pm vs 4am | 0.50693 | 0.115 | 8pm vs 4am | 0.1143 | 1.000 |
| 4am vs 12pm | -0.50693 | 0.115 | 12pm vs 8am | -0.22250 | 1.000 | 8pm vs 8am | -0.718 | 0.01* |
| 4am vs 4pm | -0.29037 | 1.000 | 12pm vs 4pm | 0.21657 | 1.000 | 8pm vs 12pm | -0.4955 | 0.132 |
| 4am vs 8pm | -0.01143 | 1.000 | 12pm vs 8pm | 0.49550 | 0.132 | 8pm vs 4pm | -0.27893 | 1.000 |

Table 3.2 CIRCADIAN EXPRESSION OF *mdkb* IN THE ZEBRAFISH RETINA

Data obtained with Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction (QRTPCR)
Relative values compared to 8pm.

| Experiment | 12 am | 4 am | 8 am | 12 pm | 4 pm | 8 pm | Min | Max |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.6127 | 0.1035 | 0.2938 | 0.4176 | 0.2846 | 1 | 0.1035 (4 am) | 1 (8pm) |
| 2 | 1.5518 | 0.9176 | 0.834 | 0.9625 | 0.8120 | 1 | 0.8120 (4pm) | 1.55 (12 am) |
| 3 | 0.8471 | 0.8083 | 0.8227 | 0.5001 | 0.4856 | 1 | 0.4846 (4pm) | 1 (8pm) |
| Mean | 1.0039 | 0.6098 | 0.6502 | 0.6242 | 0.5274 | 1 | | |
| Std. Dev. | 048878 | 0.44186 | 0.30867 | 0.29642 | 0.26617 | 0 | | |
| Std. Error | 0.2822 | 0.25511 | 0.17821 | 0.17114 | 0.15368 | 0 | | |
| 95% CI lower bound | -0.2103 | -0.4878 | -0.1166 | -0.1121 | -0.1338 | 1 | | |
| 95% CI upper bound | 2.2181 | 1.7074 | 1.4170 | 1.3605 | 1.1886 | 1 | | |

One way ANOVA with Bonferroni post-hoc correction for multiple comparisons

| Pairwise comparison | Mean difference | p value | Pairwise comparison | Mean difference | p value | Pairwise comparison | Mean difference | p value |
|---|---|---|---|---|---|---|---|---|
| 12am vs 4am | 0.39407 | 1.000 | 8am vs 12am | -0.35370 | 1.000 | 4pm vs 12am | -0.47647 | 1.000 |
| 12am vs 8am | 0.35370 | 1.000 | 8am vs 4am | 0.04037 | 1.000 | 4pm vs 4am | -0.08240 | 1.000 |
| 12am vs 12pm | 0.37967 | 1.000 | 8am vs 12pm | 0.02597 | 1.000 | 4pm vs 8am | -0.12277 | 1.000 |
| 12am vs 4pm | 0.47647 | 1.000 | 8am vs 4pm | 0.12277 | 1.000 | 4pm vs 12pm | -0.968 | 1.000 |
| 12am vs 8pm | 0.00387 | 1.000 | 8am vs 8pm | -0.34983 | 1.000 | 4pm vs 8pm | -0.4726 | 1.000 |
| 4am vs 12am | -0.39407 | 1.000 | 12pm vs 12am | -0.37967 | 1.000 | 8pm vs 12am | -0.00387 | 1.000 |
| 4am vs 8am | -0.4037 | 1.000 | 12pm vs 4am | 0.0144 | 1.000 | 8pm vs 4am | 0.39020 | 1.000 |
| 4am vs 12pm | -0.1440 | 1.000 | 12pm vs 8am | -0.2597 | 1.000 | 8pm vs 8am | 0.34983 | 1.000 |
| 4am vs 4pm | 0.08240 | 1.000 | 12pm vs 4pm | 0.0968 | 1.000 | 8pm vs 12pm | 0.37580 | 1.000 |
| 4am vs 8pm | -0.39020 | 1.000 | 12pm vs 8pm | -0.37580 | 1.000 | 8pm vs 4pm | 0.47260 | 1.000 |

## DISCUSSION

The two zebrafish midkine paralogs, evolved following a genome-wide duplication in fish (Winkler et al 2003), are encoded by genes located on different chromosomes, have different expression patterns in the developing zebrafish, and appear to subserve different functions (Winkler and Moon, 2001, Winkler et al, 2003, Schäfer et al., 2005, Liedtke and Winkler, 2008). In the developing and adult retina, *mdka* and *mdkb* are expressed in different types of retinal cells (Chapter 2), and likely have distinct biological functions. Here we show that the circadian clock regulates expression of Mdka in horizontal cells, which results in changes in expression that follow the same time-course at both mRNA and protein levels, expression being higher during the subjective day than during the subjective night. In horizontal cells, expression of *mdkb* appears also to be modulated by the circadian clock, since in situ hybridization consistently shows expression of *mdkb* in these cells only during the second half of the subjective night. Since *mdkb* is expressed by other retinal cells (ganglion and amacrine cells), and we observe circadian changes in horizontal cells, quantitative analysis of *mdkb* expression in the whole retina didn't reveal a statistically significant change in the circadian expression of mdkb. This analysis showed only the existence of a trend for lower expression during the subjective day compared to the subjective night.

The dynamic and selective changes in expression of *mdka* and *mdkb* in the zebrafish retina during the circadian cycle indicate that expression of these

genes is regulated by different mechanisms. From these observations I infer that the two proteins primarily exert their biological actions at different times during the circadian cycle. This reinforces the suggestion that *mdka* and *mdkb* have developed independently, under purifying selection (Winkler and Moon, 2001), because they have different patterns of expression and have so far been described to subserve different functions during early embryogenesis (Winkler and Moon, 2001, Winkler et al., 2003, Schäfer et al, 2007, Liedtke and Winkler, 2008).

In the adult retina, midkines are expressed in very distinct populations of cells: *mdka* in horizontal cells and *mdkb* in ganglion cells and in the amacrine layer of the INL. There is, however, a period during the diurnal cycle, the second half of the subjective night, when expression of both midkines coincides within horizontal cells (Fig. 3.2 panels b,c,h,i). This is similar to the expression of both midkines in horizontal cells in the regenerating retina, following photoreceptor apoptosis induced by exposure to intense light (Chapter 2). Thus, circadian events that occur in the retina during this period of the circadian cycle, may be similar to events in the regenerating retina, and these events may be linked to growth-associated neurogenesis in the retina.

No information exists to date as to receptors through which Mdka and Mdkb exert their function in zebrafish. Also unknown are the signaling pathways and downstream targets of Mdka and Mdkb. In mammals, Midkine (MK) can form dimers and its biological activities are dependent on the crosslinking status of ligand molecules (Kojima et al., 1997, Iwasaki et al., 1997, Qiu et al., 2000). It is

possible that zebrafish Midkines can dimerize as well, since the two glutamine residues responsible for ligand dimerization by tissue-type 2 trans-glutaminase (Kojima et al., 1997) are conserved in both *mdka* and *mdkb* (Winkler et al., 2003). It may also be possible that *mdka* and *mdkb*, when expressed in the same cells, heterodimerize to activate different downstream targets, than when they are expressed in different cells. Alternatively, the functions of each Midkine in horizontal cells could be distinct, and they may independently regulate different cellular events.

Since I have previously shown that Midkines in the zebrafish retina are actively modulated during retinogenesis and photoreceptor regeneration, I wanted to know if there is any evidence for involvement of Midkines in growth-associated neurogenesis. In this chapter I showed expression of *mdka* in presumptive Müller glia at the retinal margin, cells that we know are at the origin of the rod photoreceptor lineage (Bernardos et al., 2007). I also showed that, unlike the regenerating retina, when both *mdka* and *mdkb* are expressed in proliferating photoreceptor precursors in the outer nuclear layer, in the intact retina, rod progenitors do not express *mdka* or *mdkb*. This suggests that expression of Midkines in photoreceptor precursors is a regeneration-specific event, possibly restricted to cone photoreceptors precursors, which are not present in the central retina of growing fish.

The consistent and robust circadian rhythm of *mdka* expression in horizontal cells is very intriguing but its function is unknown. In zebrafish, horizontal cells produce GABA (Sandell et al, 1994, Connaughton et al, 1999)

and expression of GABA is influenced by the light/dark cycle, with more GABA produced in the light adapted retina compared with the dark adapted retina (Dominic Lam, 1972, Connaughton et al., 2001). Aside from its roles as an inhibitory synaptic neurotransmitter, non-synaptic, non-vesicular release of GABA has been shown to inhibit proliferation of GFAP-positive progenitors in the sub-ventricular zone (Liu et al., 2005). In some teleost fish a diurnal pattern of rod genesis has been described, with increased numbers of proliferating cells in the ONL during the night than during the day (Chiu et al, 1995, Julian et al., 1998). I found a similar trend in the retina of zebrafish, with increased expression of *pcna* during the subjective night compared to the subjective day, but variability is high and this trend does not reach statistical significance (see Appendix, section A4). It is possible that diurnal proliferation of rod precursors in the ONL of the fish retina is inhibited through a similar mechanism as in the subventricular zone of mammals, by means of GABA secreted by horizontal cells or maybe Mdka. These inferences are purely speculative, based on the presence of GABA in horizontal cells in increased amounts during the light phase of the diurnal cycle, similar to Mdka, and a trend for decreased proliferation in the zebrafish retina during the subjective day.

An alternate possibility for a functional significance of the increased levels of Mdka during the subjective day is that Mdka has a neuroprotective effect on photoreceptor cells during the time when they are exposed to increased amounts of light. In mammals Midkine has been shown to have antiapoptotic effects on a

variety of transformed cells, and this effect is exerted by activation of the PI3K signaling pathway or reduction of active caspase-3 (Owada et al, 1999, Tong et al., 2007). In albino rats, intravitreal injection of Midkine has been shown to promote survival and preserve function of photoreceptor cells following light-induced injury (Unoki et al., 1994, Masuda et al., 1995). These studies were done with no evidence that Midkine is normally expressed in the retina. In zebrafish, Mdka in horizontal cells may protect photoreceptors from deleterious effects of light during the light phase of the circadian cycle.

In conclusion, our data show, for the first time, that the circadian clock regulates quantitative expression of Mdka and cellular localization of *mdkb* in horizontal cells the zebrafish retina, with increased expression of Mdka in horizontal cells during the subjective day and increased expression of *mdkb* in horizontal cells during the subjective night. The two midkines therefore show asynchronous circadian regulation in horizontal cells, suggesting different biological activities for these molecules, at distinct circadian times. Expression of *mdkb* in horizontal cells during the subjective night, similar to the regenerating retina, suggests a role in persistent neurogenesis. The robust circadian rhythm of Mdka expression in horizontal cells is intriguing, and we have yet to determine its functional importance.

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# CHAPTER 4
# CONCLUSIONS AND PERSPECTIVES

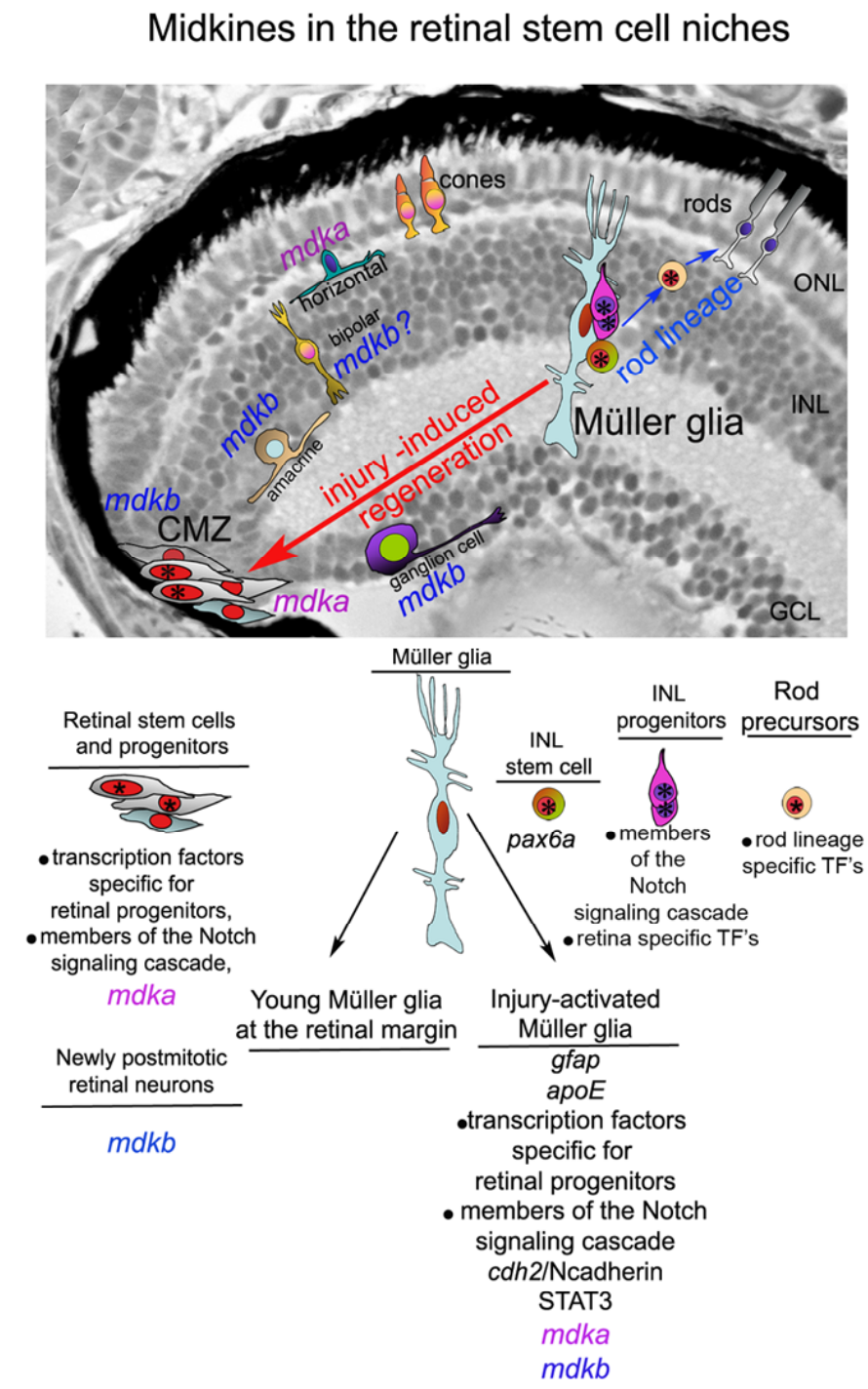
## 4.1 MIDKINES JOIN THE FAMILY OF MOLECULAR COMPONENTS IN THE NEURAL STEM CELL NICHE OF THE ZEBRAFISH RETINA

This project was initiated from an unbiased screen to identify secreted molecules in the regenerating retina, with increased expression following photoreceptor cell death, which we hypothesized trigger, sustain or regulate important regenerative events. Several candidate molecules were identified from a gene array experiment analyzing differential gene expression in lesioned and control retinas, and my work has focused on *mdkb* and its paralog *mdka*. Given that no information about these signaling molecules in the zebrafish retina existed prior to this work, I first set out to describe in detail their cellular expression, during both retinal development and regeneration. *mdkb* is expressed in postmitotic neurons in the developing retina, ganglion and amacrine cells in the intact retina, and Müller cells, photoreceptor precursors and horizontal cells in the regenerating retina. Unlike two other gene-chip array screens that analyzed gene expression changes during heart and fin regeneration in zebrafish (Schebesta et al., 2006, Lien et al., 2006), which identified *mdka*, our analysis of regenerating retina identified *mdkb.* This suggests that *mdkb* is a molecule more involved in retina and, by extension, CNS regeneration. Its expression in early postmitotic neurons suggests it may be important in neuronal differentiation, possibly in the earliest steps of differentiation or neuronal process elongation. Independently, I identified *mdka* as a molecular component of the retinal stem

cell niches in both the developing and regenerating retina and this, together with the evidence that *mdka* is induced in the regenerating heart and fin, suggests that *mdka* is a component of more general regenerative events, and that its actions are more general and not limited to specific tissues. Expression of *mdka* in the peripheral-most region of the CMZ, the location of pluripotent retinal stem cells (Raymond et al., 2006), further underscores that *mdka* exerts its biological activities during early phases of retinogenesis and/or injury-induced regeneration and that *mdka* specific actions may be common in different tissues.

The transient expression of *mdka* in Müller cells during retinal development as well as in young Müller cells at the retinal margin and also in proliferating Müller glia during photoreceptor regeneration shows that Mdka is a consistent and important component of the molecular signature of Müller cells when they exhibit features of neural stem cells. Young Müller cells at the retinal margin express the radial glia/astrocyte marker brain lipid binding protein (BLBP [Raymond et. al, 2006]), which is associated with neurogenic stem cell niches and immature astrocytes (Raymond et al., 2006, Campbel and Gotz, 2002). BLBP has been shown to be a target of Notch signaling (Anthony et al., 2005) and Midkine has been reported to bind the Notch2 receptor and activate target molecules downstream of Notch (Huang et al., 2008). Similar to BLBP, *mdka* is expressed in young Müller cells at the retinal margin and induced upon light-lesion distinctly in proliferating Müller glia at the site of the lesion (Raymond et al., 2006, this report Chapter 2 and Appendix section A3). Numerous members of the Notch signaling cascade are expressed in the circumferential marginal zone

of the zebrafish. Notch signaling is important for maintenance of the neural stem cell pool and differentiation of Müller cells (Gaiano and Fishell, 2002, Bernardos et al, 2005). It is possible that Mdka expressed in the CMZ and in young Müller glia activates members of the Notch signaling cascade contributing to the aforementioned functions.




adapted from Raymond et al., 2006, BMC Dev Biol.6:36

**Fig. 4.1 Midkines in the retinal stem cell niches**

Analyzing *mdka* and *mdkb* expression patterns shows that these molecules are complementary in the timing and location of their expression: *mdka* is expressed in the CMZ, *mdkb* is expressed in postmitotic neurons; *mdka* is expressed at the outer rim of the INL, *mdkb* is expressed in the inner retina; *mdka* expression in horizontal cells is increased during the day, *mdkb* expression is increased during the night in horizontal cells. It is tempting to speculate that the two Midkines exert their actions through different receptors at different times of the day. If their actions are different, which I believe they are, the presence of just one Midkine paralog in the extracellular milieu would increase its specificity and prevent unwanted crosstalk.

Mammalian Midkine can form dimers and multimers, *in vitro* and *in vivo* through the crosslinking activity of tissue type-2 transglutaminase on glutamines ($Gln^{42}$, $Gln^{44}$ and $Gln^{95}$ [Kojima et al., 1995, Kojima et al., 1997, Iwasaki et al., 1997, Qiu et al., 2000]). Dimer and multimer formation alters the biological activities of MK (plasminogen activator activity and neurite outgrowth promoting activity [Kojima et al., 1995 and 1997]). Two of the glutamines responsible for dimerization of the Midkine ligand ($Gln^{43}$ and $Gln^{95}$) are highly conserved across species, and are present in both zebrafish *mdka* and *mdkb* (Winkler et al., 2003). Whereas in the normal adult retina *mdka* and *mdkb* are largely expressed in different cells at different times of the day, there are instances when they are co-expressed in the same cells at the same time: during retinal regeneration *mdka* and *mdkb* are present in proliferating Müller glia, their progeny and in horizontal

cells, and during the normal circadian cycle, at the end of the subjective day, both midkines are expressed by horizontal cells. This suggests that co-expression of Midkines in horizontal cells may regulate aspects of neurogenesis during retinal regeneration and that similar events may occur during the latter half of the subjective night. I hypothesize these events are linked to growth-associated neurogenesis. It is possible that during the times when the two Midkines are expressed by the same cells, they heterodimerize to activate neurogenic-specific signaling pathways.

Alternatively, in the normal retina, during the circadian cycle, *mdka* and *mdkb* may regulate each other, and increased expression of *mdka* during subjective day, could be initiated by the transient expression of *mdkb* in horizontal cells prior to the onset of the subjective day, or increased expression of *mdka* may down-regulate *mdkb*. Given that during retinal development *mdka* and *mdkb* are expressed sequentially in subsets of cells with different degrees of differentiation, it is easy to envision a mechanism by which turning one midkine on results in turning off the other.

To test this prediction one could use an in vivo or an in vitro approach. This would require generating reporter constructs that drive, for example, GFP under the control of *mdka* or *mdkb promoter elements,* and expressing these constructs in a cell line that responds to Midkine or in transient transgenic zebrafish. The 5' promoter sequence used for generating the reporter constructs could be determined by analyzing 1000-2000 base-pairs upstream of the translation initiation site, for both genes, and identifying important putative regulatory

elements. We know that *mdkb* can be modulated by retinoic acid in a dose dependent manner (Winkler and Moon, 2001). This information could be useful to test promoters in vitro, to see if treatment with retinoic acid (RA) can modulate the expression of the reporter. It can be assumed that if the promoter directs (in transient transgenics) expression of the reporter (for example GFP) to the appropriate cellular location in the retina, then it likely harbors many, if not all, necessary regulatory elements required for proper function. Several constructs could be generated encoding various segments from the 5' upstream genomic sequence, and tested in vitro for modulation of the reporter signal upon RA administration in a cell line that is likely to respond to MK or in vivo, in transient transgenic zebrafish, for appropriate cellular localization.

Müller cells may harbor receptors and intracellular signaling molecules needed for the biological actions of both Midkines, since they express *mdka* and *mdkb* during retinal regeneration. Zebrafish Müller cells could be isolated by flow-cytometry from the gfap:GFP$^{MI2001}$ transgenic zebrafish line generated by Rebecca Bernardos (Bernardos and Raymond, 2006), cultured in vitro, transfected with the reporter constructs and reporter activity measured following addition of RA, Mdka or Mdkb. Also, since Müller glia are able to synthesize both Mdka and Mdkb, endogenous production of Mdka and Mdkb can be tested by Western blot analysis of supernatants in cells treated with RA, Mdka or Mdkb. This system could be useful in uncovering other factors that regulate expression of Mdka and Mdkb.

A caveat of this experiment would be that dissociating the retina may alter the composition of the receptors presented on cell membranes. If this should be the case, a similar experiments could be performed in organotypic retinal cultures, which could be electroporated with the reporter constructs (Kusterman et al., 2008), or endogenous production of Mdka or Mdkb could be assayed following administration of Mdka-MYC or Mdkb-MYC. In vivo, this question could be addressed using transgenic fish that conditionally express a dominant negative form of Mdka or Mdkb (see section 4.4).

## 4.2 HORIZONTAL CELLS

One of the important findings in my work is that it revealed a potential and unexpected role of horizontal cells in retinal regeneration and possibly in growth associated rod photoreceptor production. Their laminar position is ideal for influencing the proliferation, migration and differentiation of photoreceptor precursors. Horizontal cells can form large networks by means of their complex intercellular junctions, and thus may distribute cytoplasmic molecules over large areas and potentially alter the makeup of the extracellular milieu, to make it permissive for the proliferation, migration and functional integration of newly formed neurons. In mammals, horizontal cells are among the first retinal neurons to differentiate. It has been proposed that they play a pioneering role in the postnatal development of the outer plexiform layer (OPL) (Messersmith and Redburn, 1993) and in the development of rod photoreceptors (Hagedorn et. al, 1998). Selective destruction of type A horizontal cells results in a reversal of the

normal complement of photoreceptor cells with an abnormally high rod/cone ratio and abnormal synaptic connections in the OPL (Messersmith and Redburn, 1990). Zebrafish horizontal cells have been less studied compared to other retinal cell types (Song et al., 2008), but their function is largely similar to the mammalian retina. It is possible that Mdka and Mdkb in horizontal cells regulate the cone/rod ratio and/or synaptogenesis of the newly generated neurons during photoreceptor regeneration.

The strong circadian regulation of Mdka in horizontal cells is a very intriguing finding, the functional importance of which is not known. I identified *mdka* as a component of the neural stem cell niche during neurogenic events, and in other teleosts neurogenesis follows a diurnal rhythm, so I hypothesized that the circadian expression of *mdka* may be linked to circadian neurogenesis in the zebrafish retina. I therefore analyzed proliferation in retinas from fish kept in constant darkness, over a period of 24 hours. This revealed a trend for proliferation to be higher at the beginning of the subjective night than at the end of the subjective day, similar to other teleosts, yet with high variability (Appendix section A4). I also hypothesized that the decrease in expression of *mdka* at the end of the subjective day is linked to the circadian rhythm of proliferation and that, if the rhythm of *mdka* expression is perturbed by injecting exogenous MK at the time of its minimum endogenous expression, the rhythm of proliferation in the retina would be altered. Initial experiments have shown, by QRTPCR, that following exogenous intra-peritoneal administration of human recombinant MK *pcna* expression increases and the circadian rhythm in *pcna* expression is

altered. However, attempts to replicate these results have produced inconsistent results. Moreover, analyzing retinal sections by PCNA immunohistochemistry did not reveal an increase in PCNA+ cells in the ONL of hrMK injected fish. Finally, intravitreal injection of hrMK showed no difference in proliferation between MK and PBS injected fish. In both of these groups expression of *pcna* was increased over the uninjected fish, suggesting the effect of the injection itself was to induce proliferation.

Similar experiments, with intravitreal injections of recombinant Mdka-MYC produced in 293T cells as described in Chapter 2, showed no difference in proliferation between MK and PBS injected animals, over a time-course of three days. These experiments indicate that Mdka is likely not a direct mitogen for retinal stem cells or rod precursors cells of the zebrafish retina, although we cannot exclude alternate explanations for these negative data. Mdka may induce limited proliferation, lower than the injury-induced response generated by the injection itself. To address this possibility I injected hrMK intraperitoneally, but found no consistent changes in proliferation between the control and MK injected group. Since I have no independent evidence that systemic administration of MK makes the protein available in the retina, it is difficult to interpret these data.

These experiments have led me to believe that the circadian expression of *mdka* in horizontal cells is not directly linked to growth-associated rod genesis. Also, during the light-injury induced retinal regeneration I have observed expression of *mdka* and *mdkb* in proliferative photoreceptor precursors in the ONL (Fig. 2.6). In the uninjured retina, I have not observed *mdka* expression in

PCNA+ rod precursors in the ONL. (Fig. 3.6). I cannot exclude that due to the small number of rod precursors in the uninjured retina, the likelihood of finding co-expression of *mdka* and PCNA is lower, and therefore, such examples may escape observation. Nevertheless, expression of *mdka* in rod precursors in the ONL may be an event exclusively related to regeneration. However, *mdka* is expressed in presumptive Müller glia at the retinal margin (Fig.3.5) and we know that these cells are at the origin of the rod photoreceptor lineage (Bernardos et al., 2007). Therefore, while *mdka* in horizontal cells may not be linked to the rod genesis, *mdka* in Müller cells may be. Further, expression of *mdka* in horizontal cells does not decrease with increase in age, though this question has not been systematically addressed. However, labeling of presumptive Müller glia with *mdka* is increased in younger animals compared with older ones. In the zebrafish retina, neurogenesis decreases with age and adult fish grow at a very slow rate. If *mdka* in horizontal cells is linked to rod genesis, expression therefore would be expected to decrease with age, which was not observed.

The question remains, what does Mdka do in horizontal cells? There is little information to build upon, since expression of Midkines in the zebrafish retina is novel and so is its circadian regulation. Although mice engineered to lack functional MK protein are grossly normal (Nakamura et al, 1998), a striking finding from the analysis of transcriptional profiles in aortae from Mk -/- and wildtype mice is the robust elevation of key enzymes of the catecholamine biosynthesis pathway, specifically of tyrosine hydroxylase, DOPA decarboxylase and dopamine β-hydroxylase, suggesting that Midkine negatively regulates the

norepinephrine synthesis pathway in the aorta of mice (Ezquerra et al., 2006). In the retina of goldfish, dopamine release is regulated by the circadian clock, increasing during the subjective day and decreasing during the subjective night (Ribelayga et al., 2004). Horizontal cell responses are also under the control of the circadian clock and this is dependent on dopamine release (Ribelayga et al., 2002). Both Mdka and dopamine are increased in the retina during the light phase of the dark/light cycle, and if Mdka negatively regulates dopamine in the retina, we would expect the opposite relationship. Alternatively, this function could be a property of *mdkb*, which shows a mild trend of increased expression during the subjective night compared to the subjective day.

In the teleost retina, cone retinomotor movements, the adaptive positioning of outer segments within the optimal focal point of the eye, are controlled by endogenous clocks and mediated by dopamine (McCormack and Burnside, 1992). MK has been shown to promote migration of osteoblastic cells in culture by binding to integrins (Muramatsu et al., 2004). It is possible that in the zebrafish retina Mdka facilitates motility of photoreceptor outer segments, possibly by regulating extracellular matrix components. This hypothesis could be tested by analyzing retinomotor movements in transgenic zebrafish in which the function of Mdka is disrupted by means of a dominant negative form (see section 4.4).

Interesting to note is that dopamine in the retina is made by interplexiform cells, with cell bodies in the amacrine section of the INL, where Mdkb is synthesized, and cell processes in the outer plexiform layer and surrounding

horizontal cells, the location of Mdka expression. It would be interesting to investigate if there is a functional connection between Midkines, dopamine and melatonin, given their robust circadian rhythms and their location in the retina.

## 4.3 MIDKINES IN THE RETINA AND DOWNSTREAM TARGETS

As reviewed in the introduction to this dissertation, MK exerts its biological functions through many receptors or receptor complexes and activates several signaling cascades. Given that none of the receptors for zebrafish Midkines have been yet identified, it will be challenging to parse out the molecular players regulated by Mdka and Mdkb, and to decipher the specificities for the two paralogs. One possible place to start would be analyzing the effect of Midkines on identified molecular components in the retinal stem cell niches that are induced in the regenerative retina.

For example, light-injury induces increased expression and activation of the Signal transducer and activator of transcription-3 (Stat3) in proliferating Müller glia (Kassen et al, 2007). In mammalian pre-adipocytes in culture, MK has been shown to be an autocrine activator of Stat3 and this MK-dependent activation of Stat3 is required for the mitotic clonal expansion of preadipocytes and their subsequent differentiation into adipocytes (Cernkovich et al, 2007). I attempted to show that Mdka can induce expression of Stat3 in the zebrafish retina, by injecting either Mdka-MYC (produced as described in Chapter 3) or PBS into the vitreous of zebrafish and analyzing expression of Stat3 by

immunohistochemistry with Stat3 antibodies (gift from Tom Vihtelic). While I was able to reproduce the results of Kassen and colleagues, showing that in the light-lesioned retina there is increased expression of Stat3 in cells with Müller cell morphology, both control injected and Mdka-Myc injected retinas showed increased Stat3 expression as compared with uninjected, indicating that the injection itself activates the Stat3 signaling pathway. An alternate approach to test the hypothesis that Mdka or Mdkb can activate Stat3 in Müller cells, would be to use an in vitro organotypic culture system of zebrafish retinas, as recently described (Kusterman et al, 2008). This system allows for culturing zebrafish retinas up to 7 days in vitro, after which the histology of the retina becomes compromised. To analyze the effect of Mdka and Mdkb on Stat3 in the retina, only a short period of culture would be required, since MK can directly activate Stat3 in cultured cells within minutes (Cernkovich et al, 2007). Using this system, I anticipate that depriving the culture medium of serum for up to one day, will render the retinas quiescent and make the specific actions of MK more discernable.

In the same system, other potential targets of Midkines, selected from the molecules described in the retinal stem cell niches (Raymond et al, 2006) could be tested. In human keratinocytes in culture, MK has been shown to induce epithelial-to-mesenchymal transition and this transition occurs via binding the Notch2 receptor, activating the Jak2/Stat3 pathway and binding Hes1 (Huang et al., 2008). These molecular players are present in injury-activated Müller cells, though the Notch receptors are different (Notch1a, 1b, 3). Mdka or Mdkb or both

may be regulating their activity. During development, MK is expressed at sites where epithelial-to-mesenchymal transitions (EMT) occur (Mitsiadis et al., 1995) and also at sites of mesenchymal to epithelial transition during secondary neurulation (Griffith and Zile, 2000). EMT is a complex biological process during which epithelial cells loose their polarity, their adhesions to neighboring cells and begin to migrate and divide. It is a developmental process involved in generation of numerous tissues and also characteristic of metastatic invasions (Baum et al, 2008). The process of Müller glia de-differentiation and generation of retinal progenitors that proliferate and migrate to the ONL can be regarded as EMT. A hallmark of EMT is loss of adherens junctions, and in the regenerating zebrafish retina, proliferating Müller cells show a redistribution of N-cadherin, from the localized adherens junctions in the outer limiting membrane and plexiform layers to a diffuse distribution in the entire plasma membrane (Raymond et al., 2006). Mdka or Mdkb or both could be responsible for regulating aspects of EMT, possibly in a sequential manner.

To test this hypothesis, the same in vitro system described above (Kusterman et al, 2008) could be used in combination with immunohistochemistry and biochemical techniques to assay known molecular players of EMT following addition of Mdka-MYC or Mdkb-MYC. It is expected that, similar to the study on keratinocytes, addition of Mdka and/or Mdkb will lead to disruption of adherens junctions, decrease in epithelial markers, increase in mesodermal markers and cytoskeletal rearrangements. Addition of specific Mdka or Mdkb antibodies may block these effects and confirm specificity of the changes observed.

## 4.4 TRANSGENIC ZEBRAFISH TO ASSAY MIDKINE FUNCTIONS

While in vitro assays are extremely useful and offer a high level of control over experimental parameters, in vivo analysis is always more gratifying when proposed mechanisms are confirmed in live animals. Winkler and colleagues have shown that a C-terminal truncation of Mdkb functions as a dominant negative molecule that can rescue the effects induced by over-expression of *mdkb* RNA. I used truncation mutants (gift from Dr. Christoph Winkler [Winkler and Moon, 2001]) to generate plasmids that encode a Mdkb (full length or truncated form) heatshock-inducible full length or truncated Mdkb fused to GFP, driven by the heat-shock promoter Hsp70 (gift from Dr. John Kuwada). I expressed this construct in P19 cells and transiently in zebrafish. Similarly, dominant negative constructs with a C-terminus truncation of Mdka can be generated. I hypothesize that Midkines, when expressed in different cell types exert different functions. To investigate these functions, cell type specific promoters are available. The *connexin 52.6* promoter (Shields et al., 2007) drives specific expression of the GFP reporter in horizontal cells. The *gfap* promoter (Bernardos and Raymond, 2006) enables specific expression in Müller cells. The mifepristone-inducible LexPR system (Emelyanov and Parinov, 2008) could be used to generate transgenic zebrafish that can be induced to express the full-length or truncated forms of Mdka or Mdkb in horizontal cells or Müller cells. With this conditional expression system, effector gene activation can be induced at any time in zebrafish embryo, larvae or adults. This timed expression can be

combined with light lesion to investigate the role of Mdka and Mdkb in injury induced photoreceptor regeneration. These fish would be instrumental in answering numerous questions. Being able to turn Mdka or Mdkb on or off in Müller cells or horizontal cells, at specific times prior or during retinal regenerative events following light lesion, would allow one to monitor closely gain- and loss-of-function effects of Mdka or Mdkb produced in horizontal or Müller cells on photoreceptor survival, Müller cell dedifferentiation, proliferation and migration of newly generated retinal progenitor cells. In addition, these fish could be used to analyze the function of Mdka and Mdkb during retinogenesis. Given that zebrafish larvae are transparent and amenable to live imaging of developmental events, these fish could be used to elegantly analyze the specific functions of Mdka and Mdkb in Müller and horizontal cells during late retinogenesis.

My work presented in this dissertation describes for the first time the dynamic expression of Mdka and Mdkb in the zebrafish retina during development, photoreceptor regeneration and the circadian cycle. Numerous fascinating questions arise from this thorough analysis. Why are these two paralogs so different in their cellular expression in the developing and the adult retina? Why are they so similar during photoreceptor regeneration when expressed in stem cells, their progeny and in horizontal cells? What role do they play during regenerative events? Why is Mdka so robustly controlled by the circadian clock and what is its role in horizontal cells? Are horizontal cells critical players during photoreceptor regeneration? What is the function of Mdka in the

circumferential marginal zone? Are Midkines important regulators of growth-associated neurogenesis? Approaches to analyze the function of these molecules will not be trivial, particularly *in vivo*, however, my work has established a reliable foundation of knowledge, which enables one to formulate clear hypotheses.

Midkine is a highly conserved pleiotropic molecule with increased expression during midgestation, tumorigenesis and following injury in a variety of tissues in many animal models. It is therefore, similar to the Wnt, BMP, Notch and Hedgehog families of signaling molecules which function during development, cancer and injury-induced regeneration to regulate fundamental processes such as cell division, migration, differentiation and tissue integration (Bailey et. al., 2007, Beachy et al., 2004). Understanding how Mdka and Mdkb are regulated in the zebrafish retina, identifying their specific molecular targets and the particular cellular processes they modulate, will bring important contributions to the fields of developmental, regenerative and cancer biology and may open doors towards novel therapeutic approaches.

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# APPENDIX

## A1 LIGHT-INJURY INCREASES EXPRESSION OF *mdkb* IN THE RETINA OF ALBINO FISH.

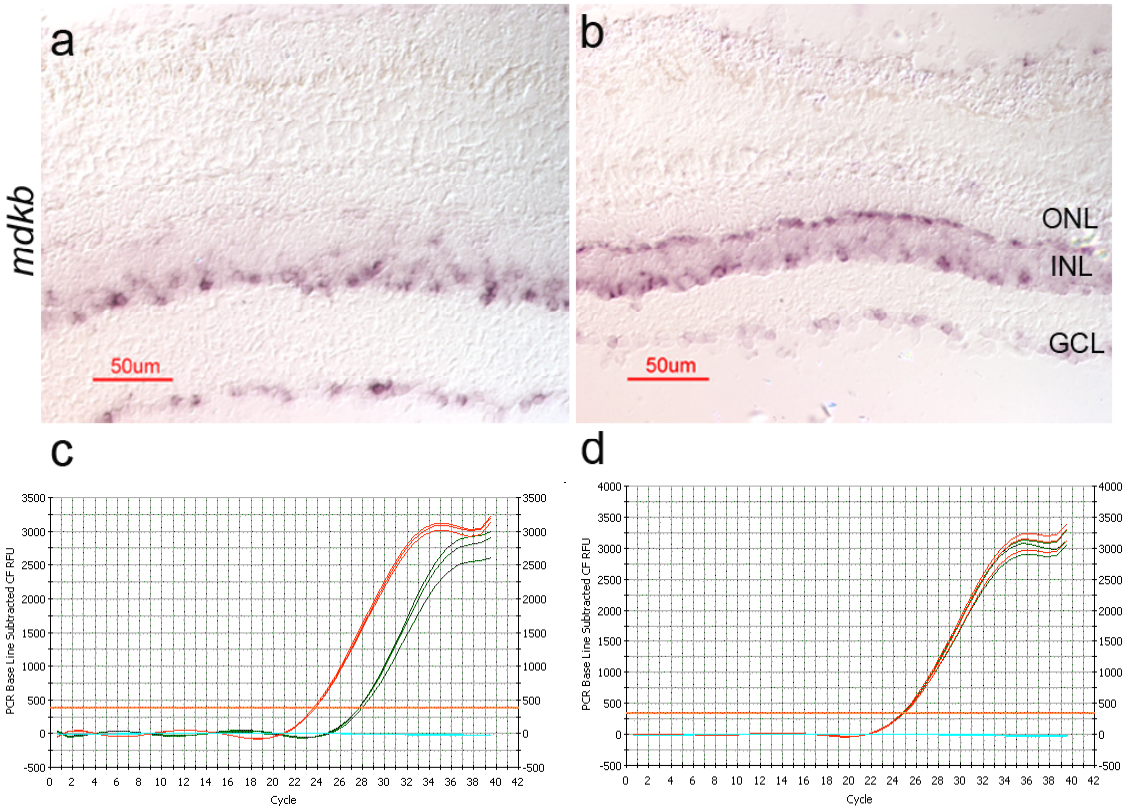


**Fig.A1. Light injury increases expression of *mdkb* in the retina of albino zebrafish.**
Albino zebrafish were dark-adapted for 7 days and then exposed for 72h to intense fluorescent light (8000lux). Panel a is an *in situ* hybridization showing *mdkb* expression in a control retina from a fish maintained in normal aquaria conditions. Note expression of *mdkb* in the ganglion cell layer (GCL) and the basal inner nuclear layer (INL). Panel b is an *in situ* hybridization showing *mdkb* expression in a retina from an albino zebrafish exposed to intense fluorescent light for 72h. Note increased expression of mdkb throughout the INL and expression of *mdkb* in horizontal cells, at the outer rim of the INL. Panel c illustrates a quantitative real-time RTPCR (QRTPCR) reaction with specific primers for *mdkb*, presenting triplicate samples from control fish (green curves) and from light-lesioned fish (red curves). The threshold cycle difference is 4.1, indicating an increase in expression of *mdkb* of approximately 16 fold in the light-lesioned retina compared to control. Panel d illustrates a QRTPCR reaction with specific primers for connexin 52.6 (*cx52.6*) presenting triplicate samples from control fish (green curves) and from light-lesioned fish (red curves). Note that there is no difference in the threshold cycle between the two groups of curves, indicating that expression of connexin 52.6 does not change following light lesion.

## A2 IN THE DEVELOPING ZEBRAFISH RETINA EXPRESSION OF *mdka* BEGINS AT THE SITE OF THE FUTURE CIRCUMFERENTIAL MARGINAL ZONE (CMZ) AND EXPRESSION OF *mdkb* BEGINS IN THE PRECOCIOUS VENTRO-NASAL PATCH

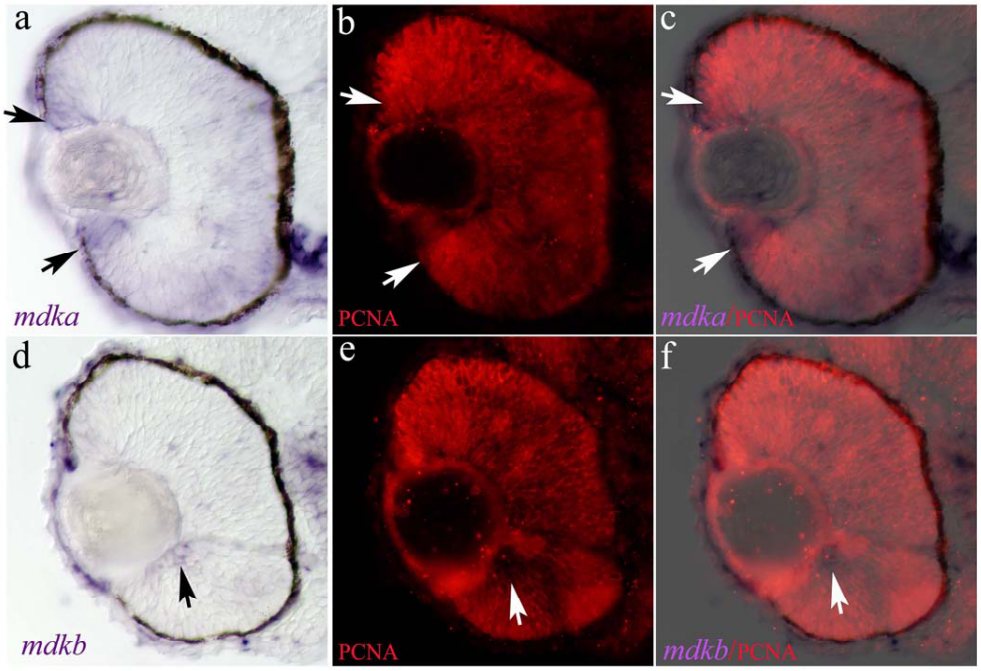


**Fig. A2 Expression of *mdka* begins in the future CMZ and expression of *mdkb* begins in the precocious ventro-nasal patch.** Panel a is an in situ hybridization showing expression of *mdka* in the retina of a 48hpf zebrafish embryo. Panel b is the same section as in panel a, but immunostained with antibodies against PCNA. Panel c is a digital overlay of panels a and b. Arrows point to the future site of the CMZ where *mdka* is first expressed. Panel d is an in situ hybridization showing expression of *mdkb* in the retina of a 48hpf zebrafish embryo. Panel e is the same section as in panel a, but immunostained with antibodies against PCNA. Panel f is a digital overlay of panels d and e. Arrows point to the precocious ventro-nasal patch where *mdkb* is first expressed, and where PCNA is not expressed. PCNA, proliferating cell nuclear antigen.

## A3 IN THE LIGHT-LESIONED RETINA *mdka* IS EXPRESSED IN PROLIFERATING MÜLLER GLIA AT THE SITE OF THE LESION.

Transgenic zebrafish expressing GFP under the control of glial fibrillary acid protein (GFAP) regulatory elements Tg(gfap:GFP)$^{MI2001}$ were exposed to ultra-high intensity light (as described, Bernardos et al., 2007) for 30min. Three days after the light-lesion zebrafish were sacrificed, enucleated and retinas were processed for immunohistochemistry and *in situ* hybridization, as described in Chapter 2. Panel a. represents a low magnification view of a section through the light-lesioned retina processed for *in situ* hybridization with *mdka* specific probe. Arrows point to the region where *mdka* is expressed in columnar cells in the INL in addition to the expression in horizontal cells. Panel b. is the same section as in a, but immunostained with antibodies against PCNA. Panel c. is the digital overlay of panels a. and b. Panel d is a higher magnification view at the site of the lesion processed for *in situ* hybridization with *mdka* specific probe. Panel b. is the same section as in a, but immunostained with antibodies against GFP. In these transgenic fish, in the retina, GFP is specifically expressed in Müller glia. Panel f. is the digital overlay of panels d. and e. Arrows point to Müller cells expressing *mdka.* Panel g is a high magnification view at the site of the lesion processed for *in situ* hybridization with *mdka* specific probe. Panel h. is the same section as in g, but immunostained with antibodies against PCNA. Panel i. is the digital overlay of panels g. and h. Arrows point to proliferating cells expressing *mdka.* Arrowheads point to photoreceptor precursors in the outer nuclear layer. PCNA, proliferating cell nuclear antigen, GFP, green fluorescent protein. Scalebar in c equals 200μm. Scalebar in i equals 50μm.

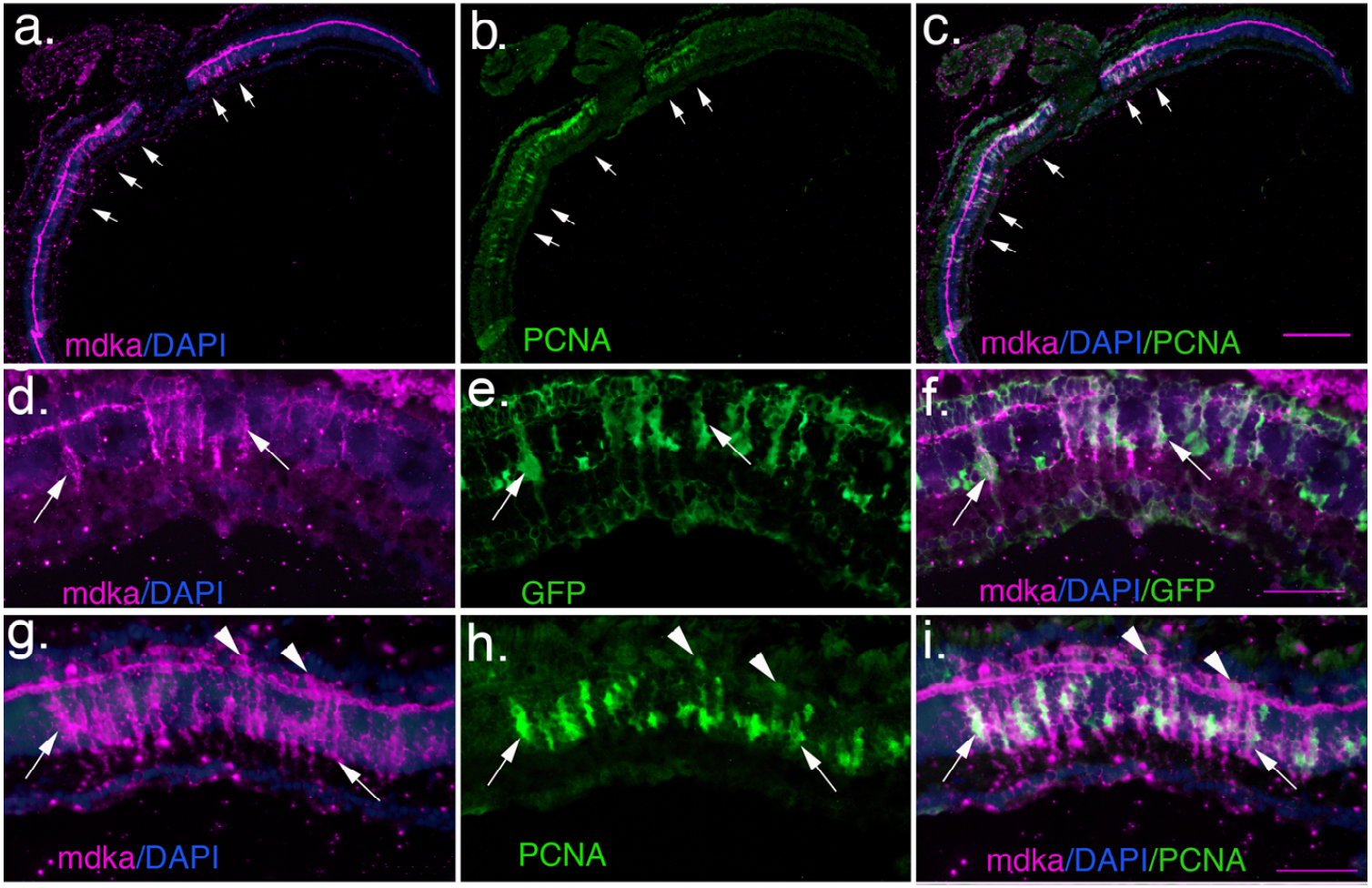


**Fig. A3 In the light-lesioned retina *mdka* is expressed in proliferating Müller glia restricted to the site of the lesion**

## A4 IS GROWTH-ASSOCIATED NEUROGENESIS IN THE ZEBRAFISH RETINA REGULATED BY THE CIRCADIAN CLOCK?

### A4.1 INTRODUCTION

A fundamental biological process regulated by the circadian clock is the cell cycle (see Levi et al., 2007 and the references within). In zebrafish larvae it has been shown that light regulates cell cycle in several tissues, such that increased number of cells pass through S phase in a narrow time-window at the end of the light cycle/beginning of the dark cycle, and this rhythm is maintained in constant darkness (Dekens et al., 2003). In other species, as diverse as algae and humans, DNA replication has been shown to occur preferentially during the dark phase (Levi et al., 2007) and this is thought to prevent DNA damage from the harmful effect of ultraviolet light (Vallone et al., 2005, Tamai et al., 2004).

In adult fish, new neurons are continuously added to the retina and brain as the fish grow. In the retina, cells divide within two neurogenic zones, the circumferential marginal zone (CMZ) that generates all retinal cell types, except rod photoreceptors, and within the central retina, where stem cells located in the inner nuclear layer (INL) divide giving rise to a lineage of cells that proliferate, migrate to the outer nuclear layer (ONL) and differentiate exclusively into rod photoreceptors (Johns, 1982, Otteson and Hitchock, 2003, Hitchcock et al, 2004, Bernardos et al., 2007). In some teleost fish, rainbow trout (*Onchoryncus mykiss*) and cichlids (*Haplochromis burtoni*), a diurnal pattern in rod genesis has been described, with higher numbers of proliferating cells present in the ONL during the night than during the day (Julian et al., 1998, Chiu and Fernald, 1995). No

data as to the presence of a rhythm of rod precursor proliferation exists to date for zebrafish.

## A 4.2 MATERIALS AND METHODS

### Animals

Wild-type zebrafish (Danio rerio), mixed strains and strain AB, 4.5 to 7 months old, were handled as described in Chapter 3.

### Tissue preparation, in situ hybridization and immunohistochemistry.

These procedures were performed as described in Chapter 2.. Proliferating cells were identified on retinal sections using anti-Proliferating-Cell Nuclear Antigen antibodies (p-8825, Sigma-Aldrich, St. Louis, MO) at a dilution of 1:1000 following antigen retrieval, as previously described (Raymond et. al., 2006). As secondary antibodies goat anti-mouse Alexa-Fluor 555 were used at a dilution of 1:500 (Invitrogen-Molecular Probes, Eugene, OR). Nuclei were stained with 4,6-diamidino-2- phenylindole, dihydrochloride (DAPI, Invitrogen-Molecular Probes, Eugene, OR).

## A 4.3 RESULTS AND DISCUSSION

To examine circadian rhythm of proliferation in the zebrafish retina I analyzed expression of *proliferating cell nuclear antigen (pcna)*, a co-factor of DNA polymerase, expressed during the late G1, S and early G2 phases of the cell cycle (Kurki et al, 1986, Moldovan et al., 2007). QRTPCR revealed that *pcna* expression is highest at the beginning of the subjective night (12am) and lowest

at the end of the subjective day (Fig. A4a). However, there is a high variability between animals and this trend does not reach statistical significance (Table A4). Increasing the sample size may render this trend statistically significant. Also analysis of younger fish, which have robust proliferation in the retina, may show different results. Analysis of proliferating cells by immunohistochemistry with antibodies against proliferating cell nuclear antigen (PCNA) shows an increased number of PCNA positive cells in the outer nuclear layer (ONL) of retinas at 12am compared to 8pm (Fig.A4.1 panels b and c), however there is high degree of variability between different animals. This variability is probably a result of zebrafish growing at a much slower rate than the other teleosts studied (cichlids, and rainbow trout). This results in decreased numbers of proliferating cells in the retina at any one time, and increased variability due to other factors such as intake of food and other growth-associated endocrine factors. Also in cichlids the amplitude of the daily rhythm of proliferation in the ONL decreases when the fish are maintained in constant darkness (Chiu and Fernald, 1994).

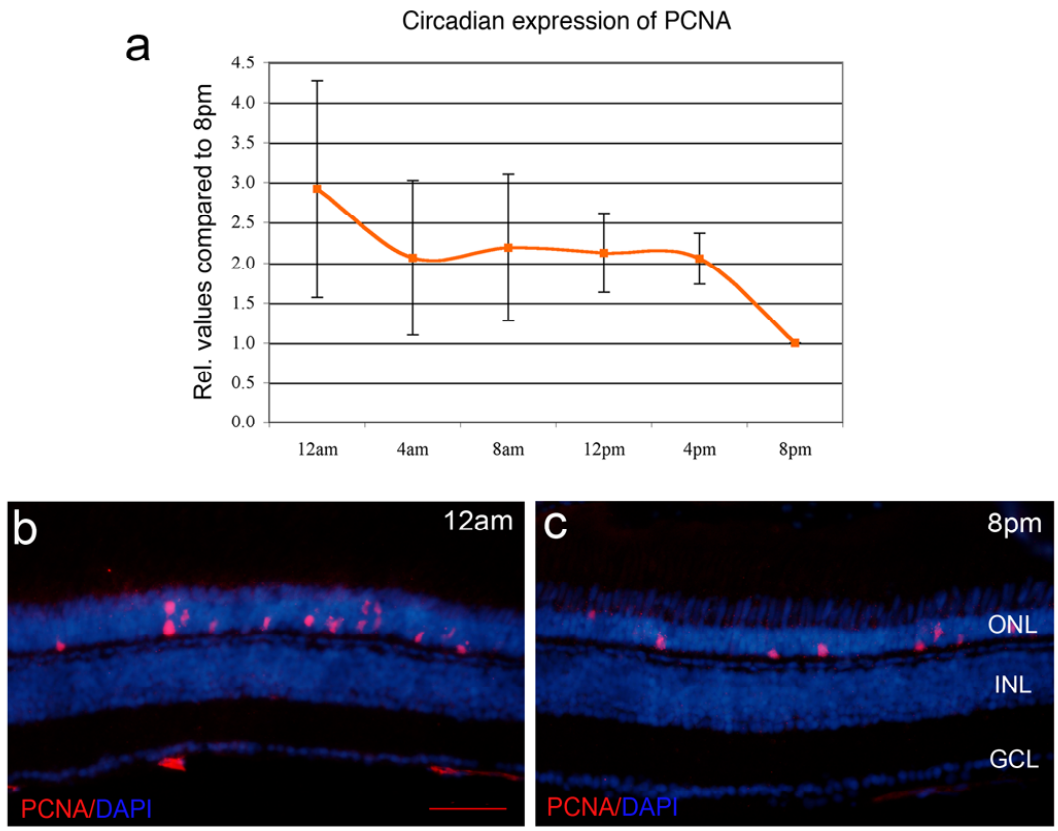


**Fig. A4 Proliferation in the zebrafish retina follows a circadian trend, being increased during the subjective night and decreased during the subjective day.**

**Panel a** represents a graphical representations of QRTPCR analysis of circadian expression of *pcna.* Gene expression is represented as fold change compared to expression values at the end of the subjective day (8pm). Proliferating cell nuclear antigen (NM_131404) specific primers used were: forward: catccagacacttagagctgaaga and reverse: ctggtctgtgagagcttgatgtt. Reactions and analysis was performed identical to Chapter 3. Data represent mean values from 3 experiments with standard errors represented for each time-point. Descriptive statistics are presented separately in table A4.

**Panels b and c** represent immuno-histochemical detection of proliferating cells with antibodies against proliferating cell nuclear antigen (PCNA) in retinas collected at the beginning of the subjective night (panel a) and the end of the subjective day respectively (panel b). Sections were counterstained with the nuclear dye 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) to evidence the retinal layers. ONL; outer nuclear layer, INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar in b equals 50 μm.

**Table A4 Circadian expression of *pcna* in the zebrafish retina.**

Data obtained with Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction (QRTPCR)
Relative values compared to 8pm.

| Experiment | 12 am | 4 am | 8 am | 12 pm | 4 pm | 8 pm | Min | Max |
|---|---|---|---|---|---|---|---|---|
| 1 | 5.6225 | 3.9662 | 4.0142 | 3.0806 | 2.6029 | 1 | 1 (8pm) | 5.6225 (12 am) |
| 2 | 1.4513 | 0.8416 | 1.1639 | 1.4714 | 1.5126 | 1 | 0.8416 (4am) | 1.5126 (4pm) |
| 3 | 1.6864 | 1.3753 | 1.3929 | 1.8204 | 2.0417 | 1 | 1 (8pm) | 2.0417 2(4pm) |
| Mean | 2.92 | 2.0610 | 2.1903 | 2.1241 | 2.0524 | 1 | | |
| Std. Dev. | 2.34334 | 1.67136 | 1.58369 | 0.84646 | 0.54523 | 0 | | |
| Std. Error | 1.35293 | 0.96496 | 0.91435 | 0.48871 | 0.31479 | 0 | | |
| 95% CI lower bound | -2.9012 | -2.0909 | -1.7438 | 0.0214 | 0.6980 | 1 | | |
| 95% CI upper bound | 8.7412 | 6.2129 | 6.1244 | 4.2269 | 3.4068 | 1 | | |

One way ANOVA with Bonferroni post-hoc correction for multiple comparisons

| Pairwise comparison | Mean difference | p value | Pairwise comparison | Mean difference | p value | Pairwise comparison | Mean difference | p value |
|---|---|---|---|---|---|---|---|---|
| 12am vs 4am | 0.859 | 1.000 | 8am vs 12am | -0.72971 | 1.000 | 4pm vs 12am | -0.86762 | 1.000 |
| 12am vs 8am | 0.72971 | 1.000 | 8am vs 4am | 0.12929 | 1.000 | 4pm vs 4am | -0.00862 | 1.000 |
| 12am vs 12pm | 0.79589 | 1.000 | 8am vs 12pm | 0.06618 | 1.000 | 4pm vs 8am | -0.13791 | 1.000 |
| 12am vs 4pm | 0.86762 | 1.000 | 8am vs 4pm | 0.13791 | 1.000 | 4pm vs 12pm | -0.07173 | 1.000 |
| 12am vs 8pm | 1.92 | 1.000 | 8am vs 8pm | 1.19031 | 1.000 | 4pm vs 8pm | 1.05240 | 1.000 |
| 4am vs 12am | -0.859 | 1.000 | 12pm vs 12am | -0.79589 | 1.000 | 8pm vs 12am | -1.92 | 1.000 |
| 4am vs 8am | -0.12929 | 1.000 | 12pm vs 4am | 0.06311 | 1.000 | 8pm vs 4am | -1.061 | 1.000 |
| 4am vs 12pm | -0.6311 | 1.000 | 12pm vs 8am | -0.6618 | 1.000 | 8pm vs 8am | -1.19031 | 1.000 |
| 4am vs 4pm | 0.00862 | 1.000 | 12pm vs 4pm | 0.07173 | 1.000 | 8pm vs 12pm | -1.1243 | 1.000 |
| 4am vs 8pm | 1.06102 | 1.000 | 12pm vs 8pm | 1.12413 | 1.000 | 8pm vs 4pm | -1.05240 | 1.000 |

It is possible that zebrafish have a diurnal rhythm of rod precursor proliferation, which becomes more variable when fish are kept in total darkness. This could be easily tested by QRTPCR with *pcna* specific primers on retina RNA samples from fish at different times during the diurnal cycle, under normal lighting

conditions. Similar to *pcna*, expression of *mdkb* is also higher during the subjective night than during the subjective day, and more variable than *mdka*. It would be useful to know if the same trend is found in the retina of fish maintained under normal lighting conditions, during a diurnal cycle. In the retinas from fish maintained in constant darkness maximum expression of *mdkb* precedes and overlaps the maximum expression of *pcna*, at the end of the subjective day and beginning of the subjective night (Chapter 3). I do not yet have evidence for a functional significance of this temporal correlation. Since I have shown that *mdkb* is expressed in photoreceptor precursors in the ONL during retinal regeneration (Chapter 2) and *mdkb* is expressed during the subjective night in horizontal cells (Chapter 3), similar to the expression during photoreceptor regeneration (Chapter 2), I can speculate that quantitative changes of *mdkb* in the INL may influence aspects of growth-associated rod genesis.

## A5 CONNEXIN 52.6 IS REGULATED BY THE CIRCADIAN CLOCK

In the course of the experiments described in Chapter 3, serendipitously, I consistently observed that expression of *connexin 52.6,* (*cx52.6*) also follows a circadian rhythm, with statistically significant maximum expression in the middle of the subjective day and minimum expression in the middle of the subjective night (Fig. A5 and Table A5). Cx 52.6 is a gap-junction protein, specifically expressed in the zebrafish retina by horizontal cells (Zoidl et al., 2004, Shields et al, 2007). Previously, during light-lesion experiments, I have observed that, unlike other common ubiquitous genes used as a reference to normalize amount of RNA in QRTPCR assays (*actin, gapdh, hgprt, rpl19*), expression of *cx52.6* did not vary with the intense light treatment (Chapter 2 and Appendix section A1).

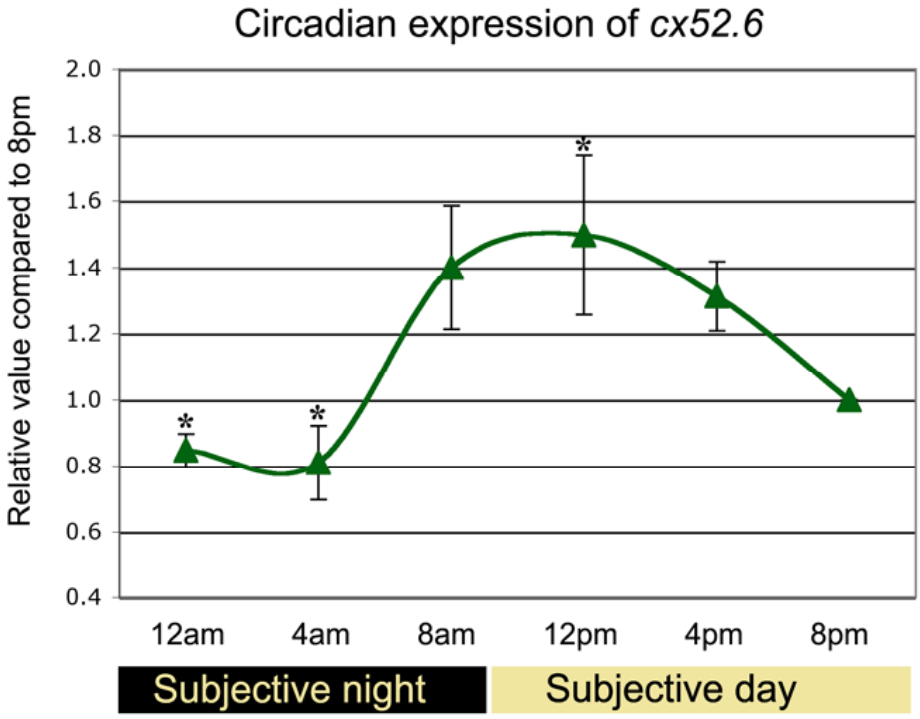


**Fig. A5 Quantitative analysis of circadian variations of *cx52.6***
Graphical representations of QRTPCR analysis of circadian expression of *cx52.6* Gene expression is represented as fold change compared to expression values at the end of the subjective day (8pm). Primers used for *connexin 52.6* (NM_212819) were forward: tggacagatggtacctttgcc and reverse: gttgtctggaatggaccttcg. QRTPCR reaction was performed as described in Chapter 3. Data represent average values from 6 independent experiments with standard errors represented for each time-point. Statistical significance was determined through Analysis of Variance with Bonferroni post-hoc correction for multiple comparisons using SPSS (* $p<0.05$). Descriptive statistics are presented separately in Table A5.

**Table A5. Circadian expression of *cx52.6* in the zebrafish retina.**

Data obtained with Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction (QRTPCR)
Relative values compared to 8pm.

| Experiment | 12 am | 4 am | 8 am | 12 pm | 4 pm | 8 pm | Min | Max |
|---|---|---|---|---|---|---|---|---|
| 1 | 1.0428 | 1.3594 | 2.3104 | 2.5833 | 1.7477 | 1 | 1.0428 (12am) | 2.5833 (12pm) |
| 2 | 0.8468 | 0.7034 | 1.2751 | 1.6260 | 1.3594 | 1 | 0.7034 (4am) | 1.6260 (12pm) |
| 3 | 0.6581 | 0.7566 | 1.3217 | 1.3796 | 1.3945 | 1 | 0.6581 (12am) | 1.3945 (4pm) |
| 4 | 0.8286 | 0.7143 | 1.2310 | 1.0301 | 1.0301 | 1 | 0.7566 (4am) | 1.2310 (8am) |
| 5 | 0.8605 | 0.7120 | 1.2165 | 1.4231 | 1.1102 | 1 | 0.7120 (4am) | 1.4231 (12pm) |
| 6 | 0.8449 | 0.6118 | 1.0430 | 0.9454 | 1.2344 | 1 | 0.6118 (4am) | 1.2344 (4pm) |
| Mean | 0.8469 | 0.8096 | 1.3996 | 1.4979 | 1.3127 | 1 | | |
| Std. Dev. | 0.12207 | 0.27353 | 0.45612 | 0.5896 | 0.25495 | 0 | | |
| Std. Error | 0.04984 | 0.11167 | 0.18621 | 0.2407 | 0.10408 | 0 | | |
| 95% CI lower bound | 0.7188 | 0.5225 | 0.9209 | 0.8791 | 1.0451 | 1 | | |
| 95% CI upper bound | 0.9750 | 1.0966 | 1.8783 | 2.1167 | 1.5802 | 1 | | |

| One way ANOVA with Bonferroni post-hoc correction for multiple comparisons | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Pairwise comparison | Mean difference | p value | Pairwise comparison | Mean difference | p value | Pairwise comparison | Mean difference | p value |
| 12am vs 4am | .03733 | 1.000 | 8am vs 12am | 0.55270 | 0.139 | 4pm vs 12am | 0.46577 | 0.388 |
| 12am vs 8am | -0.55270 | 0.139 | 8am vs 4am | 0.59003 | 0.087 | 4pm vs 4am | 0.5031 | 0.252 |
| 12am vs 12pm | -0.65098 | 0.04 | 8am vs 12pm | -0.09828 | 1.000 | 4pm vs 8am | -0.08693 | 1.000 |
| 12am vs 4pm | -0.46577 | 0.388 | 8am vs 4pm | 0.08693 | 1.000 | 4pm vs 12pm | -0.18522 | 1.000 |
| 12am vs 8pm | -0.15308 | 1.000 | 8am vs 8pm | 0.39962 | 0.800 | 4pm vs 8pm | 0.31268 | 1.000 |
| 4am vs 12am | -0.3733 | 1.000 | 12pm vs 12am | 0.65098 | 0.040* | 8pm vs 12am | 0.15308 | 1.000 |
| 4am vs 8am | -0.59003 | 0.087 | 12pm vs 4am | 0.68832 | 0.024* | 8pm vs 4am | 0.19042 | 1.000 |
| 4am vs 12pm | -0.68832 | 0.024* | 12pm vs 8am | 0.09828 | 1.000 | 8pm vs 8am | -0.39962 | 0.800 |
| 4am vs 4pm | -0.50310 | 0.252 | 12pm vs 4pm | 0.18522 | 1.000 | 8pm vs 12pm | -0.49790 | 0.268 |
| 4am vs 8pm | -0.19042 | 1.000 | 12pm vs 8pm | 0.49790 | 0.268 | 8pm vs 4pm | -0.31268 | 1.000 |

Expression of Cx52.6 in the zebrafish retina is restricted to horizontal cells and has been shown to localize to the gap junctions between them (Zoidl et al, 2004, Shields et al, 2007). Gap junctions in horizontal cells are highly permeable to small molecules and ions, enable chemical and electrical coupling of horizontal cells and allow the formation of receptive fields across the retina that can spread beyond the reach of their immediate dendritic fields (Bloomfield et al., 1995). Gap junction coupling between horizontal cells, and thus the extent of the resulting receptive fields can be drastically reduced by light and the presence of extracellular neuromodulators such as dopamine, retinoic acid and nitric oxide (Mangel and Dowling, 1985, John Dowling, 1991, Pottek et al, 1997, Weiler et al., 1998). The responses of cone horizontal cells to light are influenced by the circadian clock (Wang and Mangel, 1996, Ribelayga et al, 2002), but gap-junction coupling has been shown to be independent of the circadian clock (Ribleayga et al, 2004, Ribelayga and Mangel, 2007). We find that the expression of *cx52.6* mRNA is regulated by the circadian clock, and peaks during subjective midday. This finding is intriguing for two reasons. First, since horizontal cell coupling is independent of the circadian clock (Ribleayga et al, 2004, Ribelayga and Mangel, 2007), we would expect not to see a circadian variation in the synthesis of a protein expressed specifically in gap junctions between horizontal cells (Shields et al., 2007). Second, since gap junctions are used primarily during the dark phases of the day/light cycle, we would expect to see more mRNA synthesized during the subjective night. I did not examine whether the rhythm of *cx52.6*

mRNA production is paralleled by the same dynamic changes at the protein level. If it does, then perhaps increased levels of Cx52.6 inhibit the formation of hemichannels between cones and horizontal cells, since immuno-electron microscopy shows the presence of unpaired connexin channels composed of Cx52.6 on horizontal cell lateral dendrites and spinules in the proximity of cone synaptic terminals (Shields et al., 2007). During the day, increased ambient light allows for a heightened spatial sensitivity through narrow receptive fields, and light responses to cone horizontal cells are modulated by the circadian clock (Wang and Mangel, 1996). Circadian changes of Cx52.6, with increased levels during the day, may be required for modulating connections between cones and horizontal cells.

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