ABSTRACT

Title of Document: RNAI AND MORPHOLINO AS TOOLS TO STUDY SIGNALING IN *LIMULUS* VENTRAL PHOTORECEPTORS.

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*Limulus* ventral photoreceptors have been studied using electrophysiological, immunocytochemical, and biochemical techniques. However, genetic manipulation has eluded *Limulus* research because the animal takes about 10 years to mature. As an alternative, we decided to explore the possibility of using RNA interference (RNAi), and morpholino to reduce the levels of a target protein. The purpose of this study was to test whether use of these techniques would result in any physiological changes in the ventral photoreceptors.

As a target we chose arrestin, a protein that binds to and quenches metarhodopsin, the activated form of rhodopsin. Injecting arrestin RNAi, or arrestin morpholino into the cells had a profound effect. The rate of spontaneous quantal events (‘bumps’) in the injected cells had a 5-fold increase as compared to bump rates of control cells at 24 hrs after injection. Because high levels of metarhodopsin are thought to be present in ventral photoreceptors even in darkness, this result is consistent with an increase in the amount of unquenched metarhodopsin in the cells.

To show that the increase in spontaneous dark bumps was a result of unquenched metarhodospin, we treated RNAi -injected cells with hydroxylamine, a bleaching agent that destroys metarhodopsin. The bump rate after this treatment was down to pre-injection levels confirming our hypothesis that the excess bumps were generated by unquenched metarhodopsin.

Another target protein selected was opsin. Opsin bound to the chromophore forms rhodopsin, and rhodopsin photoconverts to metarhodopsin. Therefore, opsin reduction was expected to lead to a reduction in sensitivity to light. sensitivity to light decreased by about 30 fold at 96 hrs after opsin morpholino injections.

In another set of experiments, we fixed the injected cells with the arrestin/opsin antibodies. We measured the fluorescence from thee secondary antibodies targeted against the arrestin/opsin primary antibodies to get an insight into the protein levels. We observed a 2.5 fold decrease in fluorescent counts in arrestin morpholino injected cells and a 17 fold decrease in opsin injected cells.

# RNAI AND MORPHOLINO AS TOOLS TO STUDY SIGNALING IN *LIMULUS* VENTRAL PHOTORECEPTORS.

By

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Dedication

To Mom and Dad for always believing in me even at times when I did not believe in myself. Thank you.

# ACKNOWLEDGMENTS

First and foremost I must thank Richard for being my mentor and for being so patient with me. I still remember the day that I began this wonderful, though at times frustrating journey. I was filled with anxiety having come to a new country, leaving behind everything that was familiar to me. As soon as I first met him I felt at ease; I knew I had come to the right place. At times I would get carried away in my excitement or sometimes be so frustrated that I thought of quitting, but Richard was always there for me. He taught me that even the lowly graduate students are to be respected. He taught me the value of persistence. And above all he instilled in me the love for what I do.

Richard also guided me with meeting my committee members. They have been such an inspiration for me and guided me in every step of the way. Dr. Popper was an invaluable help while I was writing my thesis. He came up with valuable suggestions which greatly enhanced the quality of my research. Dr. Wolniak was very helpful in designing the experiments. Dr. Abshire helped me immensely in the actual data analysis. He was largely responsible for dispelling the fear of math that many years of high school education had created in me. And last but certainly not the least, I must thank Dr. Dooling for all the help that he provided in ensuring that proper procedures were followed and that all the criterions were being met.

I would also take this opportunity to thank my fellow lab-mate Youjun. Over the years he has become a close friend of mine. He has been a great support for me, and a great listener. I will greatly miss him. I would also like to thank Murat for especially helping with me my Matlab difficulties, Dr. Lee for our various discussions on experiments. Sandy has been an absolutely wonderful friend and ensured that also the NACS regulations were being met. I would need to write an entire thesis just thanking her! I would also like to thank Amar and Narendra for being such good friends and being there for me through it all.

When I first joined the department, I was filled with excitement about science. But I often wondered whether I would lose this thirst for knowledge as I embarked on this road towards a Ph.D. Instead as I graduate from the University of Maryland, I am filled with even more passion and respect for science than I ever felt. I honestly believe I cannot pay a greater tribute to all the wonderful people in the NACS department who have fostered such a productive and a fun environment.

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# Introduction

## 1.1 Phototransduction

Vertebrates and invertebrates share some crucial steps in phototransduction while differing in others. The primary event in both vertebrates and invertebrates is absorption of a photon of light by the photopigment rhodopsin. This event activates a heterotrimeric G-protein and ultimately generates an electrical signal. However, the intermediate steps are different in the two classes of animals. In vertebrate phototransduction, the G-protein is transducin. Transducin- GTP activates the effector enzyme, cyclic GMP (cGMP) phosphodiesterase (PDE). A transient decrease in cGMP levels leads to the activation of cyclic nucleotide gated (CNG) channels ([1]Fesenko et al., 1985; [2]Kaupp et al., 1989). This cascade results in channel closure and causes the membrane to hyperpolarize ([3]Yarfitz & Hurley, 1994).

In contrast to vertebrates, in the microvillar photoreceptors of *Drosophila*, absorption of a photon leads to the opening of two cationic channels, TRP and TRPL, and an inward current resulting in membrane depolarization ([4]Niemeyer et al., 1996). The phototransduction is mediated by a G-protein activated phospholipase C (PLC) ([5]Bloomquist et al., 1988; [6]Ranganathan et al., 1995). Activation of PLC catalyzes the breakdown of phosphatidylinositol-4, 5-bisphosphate ($PIP_2$) leading to the production of inositol 1, 4, 5 trisphosphate ($InsP_3$) and diacylglycerol (DAG) ([7]Berridge, 1993).

Exactly how these products lead to the opening of the TRP & TRPL channels is not known.

### 1.1.1 Phototransduction in *Limulus*

*Limulus* ventral photoreceptors make an excellent model system for the study of invertebrate visual transduction for several reasons. **Firstly**, they are very large in size (~ 200 x 80 µm). This allows for multiple electrode impalement and pressure injection of substances in single cell recordings. **Secondly**, once dissected, the cells show robust responses for up to 75 days and therefore can be used to study long-term effects of manipulations ([8]Bayer et al., 1978). One optic nerve contains about 25- 35 photoreceptor cells. All the above mentioned properties have enabled several researchers to use the *Limulus* ventral photoreceptors to get an insight into the invertebrate phototransduction process using electrophysiology, biochemistry, and immunocytochemistry ([9]Drolochter & Stieve, 1997).

The *Limulus* ventral photoreceptors are divided into a rhadbomeral (R) lobe and an arhabdomeral (A) lobe ([10]Stern et al., 1982; [11]Calman & Chamberlain, 1982). The R lobe has microvilli and is light sensitive whereas the A lobe is light insensitive. The axon arises from the A-lobe. The photocurrent is localized to the R-lobe ([12]Payne & Fein 1986). The phototransduction involves $InsP_3$ as a second messenger. $InsP_3$ both excites and adapts the photoreceptors via a rise in intracellular calcium ([13]Brown et al., 1984; [14]Fein et al., 1984; [15]Payne et al., 1986). Although over the years,

substantial progress has been made in understanding the phototransduction in *Limulus* ventral photoreceptors, some questions still remain unanswered.

One of the crucial steps that are not known is the identity of the membrane channel responsible for membrane depolarization in response to light. Two likely candidates for the channel are CNG, as in vertebrates, or TRP, as in *Drosophila*. Injection of $InsP_3$ into the photoreceptors activates the light-dependent conductance ([16]Fein et al., 1984; [17]Payne et al., 1986). Intracellular injection of $Ca^{2+}$ elicits a response that mimics a light-induced response, whereas buffering $Ca^{2+}$ inhibits this response ([18]Shin et al., 1993; [19]Payne et al., 1986; [20]Frank & Fein, 1991). Collectively these results show that $InsP_3$ –induced release of stored $Ca^{2+}$ might be the message responsible for activating the channels. Further, these results suggest that TRP channels might be involved in this process. However, other studies show that cGMP injections also depolarize ventral photoreceptors and in turn suggest the possible involvement of this second messenger in phototransduction ([21]Johnson et al., 1986). Further evidence in support of the cGMP pathway comes from the work that showed that the light-dependent channels were opened by cGMP and not by $Ca^{2+}$, and that inhibitors of guanylate cyclase reduced light-induced response ([22]Bacigalupo et al., 1991; [23]Garger et al., 2001). These observations favor CNG channels in mediating photocurrent in *Limulus*. In our laboratory, putative cGMP and TRP channels have been cloned and sequenced from the *Limulus* eye cDNA ([24]Chen at al., 1999; [25]Bandyopadhyay and Payne, 2004).

The next step is to perform functional studies on these channels. Traditionally, the role of specific genes is determined by selecting or creating a mutation in the gene of interest followed by detailed analysis of the phenotype. Mutation and knockout experiments have led to a wealth of information on phototransduction in mice and in *Drosophila*. It is believed that until the effects of a gene knockout or null mutation are studied, a categorical statement can not be made about the function of the gene. This is because the knockout background may reveal multiple forms of proteins, redundant pathways, or reveal a gene as a pseudogene. Therefore, knockouts have become the "gold standard" for identifying protein function. However, despite a wealth of physiological data in *Limulus* phototransduction, mutation experiments are not performed because the animal requires about 10 years to mature. One way to overcome this problem is to use functional silencing of genes. This may bring the understanding of the physiology in *Limulus* to an approximate alignment with the molecular mechanisms found in studying *Drosophila*.

We decided to use RNA interference (RNAi) and morpholinos as tools to achieve functional silencing. Our aim was to investigate if this technique would work in *Limulus* ventral photoreceptors. We chose arrestin and opsin proteins as our targets since these two proteins are better characterized, and their role in *Limulus* phototransduction is better understood than either of the putative membrane channels.

## 1.2 Post-transcriptional gene silencing techniques

RNAi and morpholino are emerging as most effective tools in bringing about functional silencing of genes. Both of these molecules are complementary oligonucleotides that interfere with mRNA translation. This reduces protein levels and functionally silences the gene.

### 1.2.1 The RNAi technique

RNA interference (RNAi), also named RNA silencing or post transcriptional gene silencing (PTGS), is a powerful technique that allows tissue-specific suppression of gene expression. As depicted in Fig.1.1, a small double-stranded RNA (dsRNA) introduced in the cell gets cut into small 21-25 nucleotide (nt) interfering RNAs (siRNAs) that induce sequence-specific degradation of homologous single-stranded mRNA ([26]Fire et al., 1998). It is hypothesized that dsRNA resembles a viral or transposon infection and the cell has developed a defense mechanism to destroy it. The cell produces (or activates) RNA endonucleases that cleave the target RNA into small pieces ([27]Hammond et al., 2000).

Double stranded RNA-mediated RNA interference is an effective method to down- regulate the levels of proteins in cells. It has become a dominant reverse genetics method that allows one to move from gene sequence to function. This technique has been shown to work in vertebrates and invertebrates, and has been extensively used in C. *elegans*, plants, D. *melanogaster*, mouse, and several cell lines

(Fire et al., 1998; [28]Chuang et al, 2000; [29]Misquitta et al., 1999; [30]Caplen et al., 2001;[31]Tuschl et al., 1999). RNAi is especially useful in organisms that were not amenable to genetic analysis. If this technique works in *Limulus*, it would be ideal for manipulation of RNA levels.

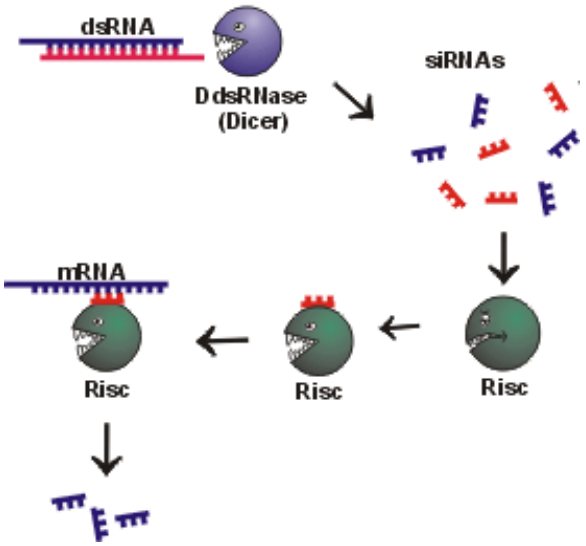



**Figure 1.1**: Mechanism of RNAi results in post-transcriptional knockdown of a gene product. dsRNA is introduced into the cell by soaking, injecting, or transfecting. The dsRNA strands are cut by Dicer (a member of the RNase III family of dsRNA-specific ribonucleases) into siRNAs. The siRNAs get attached to a nuclease, the RNA-induced silencing complex (Risc). The complex binds to the target mRNA that has a perfect complementary sequence. The targeted mRNA gets degraded by Risc activity.

Figure modified from the webpage of Wolfgang Nellen Department of Genetics at Kassel University, Germany.

http://www.uni-kassel.de/fb19/genetics/projects/prj_as.html

### 1.2.2 Use of Morpholino

Phosphorodiamidate morpholino oligomers (PMOs) are single, 25nt strands of a DNA-like molecule ([32]Summerton & Weller, 1997). They are different from DNA in two aspects. The phosphodiester linkage in DNA is replaced by a phosphorodiamidate linkage in morpholino. Secondly, the five membered deoxyribose ring in DNA is replaced by a six membered morpholine ring. These modifications make them highly resistant to degradation by enzymes while at the same time, they do not affect binding by Watson-Crick pairing ([33]Hudziak et al., 1996). They are antisense molecules that prevent ribosome binding, or interfere with pre-mRNA spicing, and subsequently block gene expression.

Morpholinos are typically designed against the first 25 bases 3' to the AUG translational start site. They presumably act by preventing ribosomal binding to the mRNA. In the absence of ribosomal binding, translation is blocked and the target gene can be silenced (Review [34]Summerton, 1999; [35]Ekker & Larson, 2001). Morpholinos show a high affinity, low toxicity and very few non-specific side effects (Summerton, 1999; Summerton & Weller 1997).

### 1.3 Selection of appropriate proteins to test the success of these techniques

We used several criteria to select proteins for testing these techniques in *Limulus* ventral photoreceptors. Firstly the proteins should to be a part of the visual transduction cascade. Secondly, they should have been sequenced (to allow designing of complementary probes). Thirdly, their turnover should be high since the above

techniques target freshly synthesized proteins and have no effect on the proteins already present in the system. The two proteins that seemed to fit these criteria were opsin and arrestin. As indicated in Fig. 1.2, both opsin and arrestin feature during photoisomerization events in invertebrates. Rhodopsin absorbs a photon and isomerizes into metarhodopsin. Both rhodopsin and metrhodopsin are made of the protein moiety opsin and a chromophore. Metarhodopsin initiates the phototransduction cascade. Quenching of metarhodopsin is necessary for terminating the light response. This process occurs by phosphorylation of metarhodopsin at multiple sites and ‘capping’ by arrestin. The quenched metarhodopsin photoconverts into an inactive rhodopsin on absorption of a photon. The inactive rhodopsin loses arrestin, gets dephosphorylated and becomes active. This rhodopsin can now initiate the cascade all over.

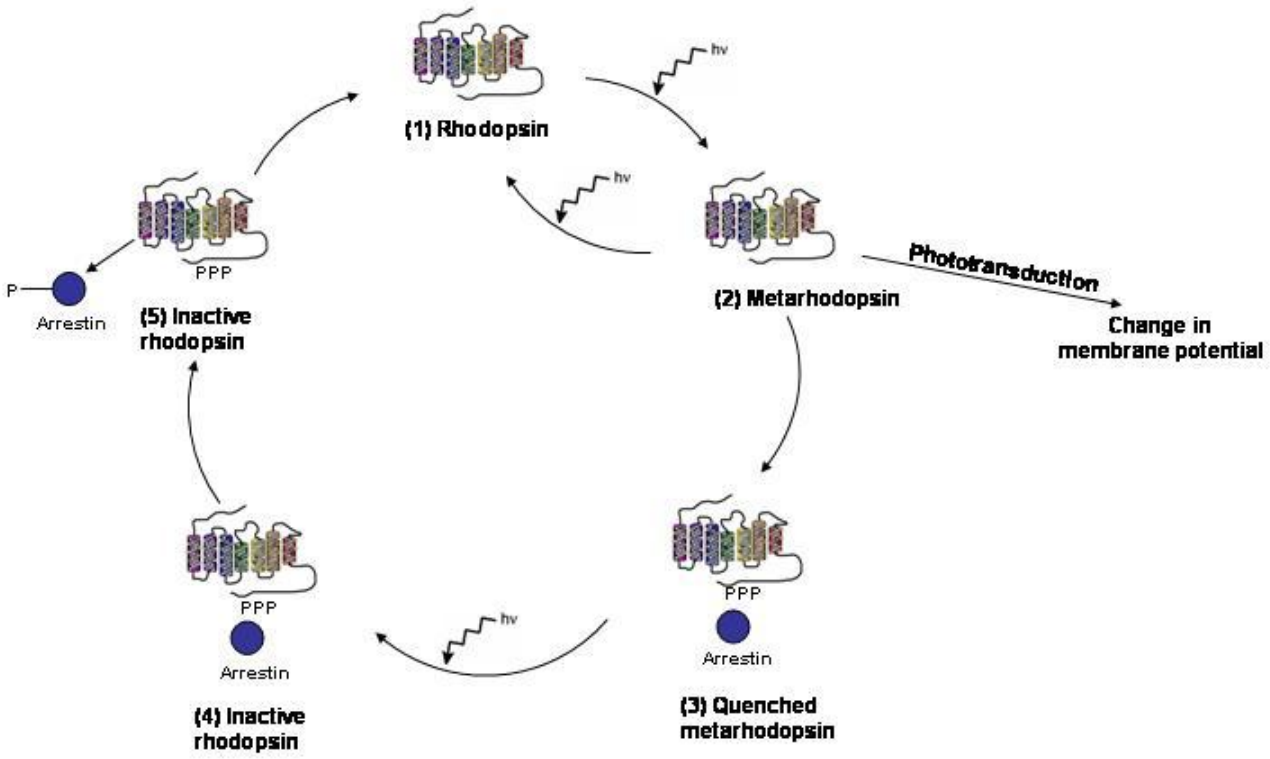



**Figure 1.2** Schematic depicting the photoisomerization events of rhodopsin in invertebrates. (1) Rhodopsin (2) Rhodopsin isomerizes to form its active form, metarhodopsin on absorption of a photon (hν). Metarhodopsin initiates the phototransduction cascade that ultimately results in a change in membrane potential. (3) Metarhodopsin is quenched after multiple phosphorylations and arrestin binding. (4) Metarhodopsin is photoconverted to the inactive form of rhodopsin that is phosphorylated and bound to arrestin. (5) Arrestin is phosphorylated and dissociates from rhodopsin. Rhodopsin is dephosphorylated and converts back to rhodopsin (1) which can then absorb a photon and start the process all over again.

## 1.4 Opsin

Visual opsin is the apoprotein moiety of the photosensitive pigment of the eye. It forms rhodopsin when it is covalently attached to the chromophore that is usually 11-cis retinal ([36]Wald, 1968). The chromophore attachment occurs at a lysine residue via a Schiff-base linkage ([37]Bownds, 1967; [38]Wang et al., 1980). Opsin is a member of a large super- family of G-protein coupled receptors (GPCRs) which have

a seven-transmembrane α-helical domain ([39]Baldwin et al., 1997). Opsin is different from the other GPCRs in that it has a lysine residue that binds retinal in the seventh helix. Many studies of vertebrate photoreceptors have increased our understanding of the role of opsins in phototransduction. Since vertebrates and invertebrates share many features of phototransduction, especially the few initial steps, these studies also give us an indication of the role of opsins in invertebrates as well.

### 1.4.1 Vertebrate opsins

Upon absorption of a photon in the photoreceptors of vertebrates, the 11-cis retinal bound to rhodopsin isomerizes to form all-trans retinal on a femtosecond time scale ([40]Wang et al., 1994). This causes conformational changes in the opsin and leads to the conversion of rhodopsin to metarhodopsin. This conversion occurs through various intermediates such as photorhodopsin, bathorhodopsin, lumirhodopsin, metarhodopsin I, metarhodopsin II, and metarhodopsin III ([41]Okada et al., 2001). These intermediates can be distinguished based on their specific absorption maxima ([42]Shichida & Imai, 1998). Metarhodopsin II initiates the phototransduction cascade by activating the heterotrimeric G-protein transducin ([43]Stryer, 1986; [44]Nathans, 1992). In a process termed bleaching, metarhodopsin eventually gets destroyed when the chromophore dissociates from it. Renewal of rhodopsin involves the binding of fresh retinal to opsin.

Point mutations in opsins lead to retinitis pigmentosa, an autosomal – dominant disease that causes blindness ([45]Dryja et al., 1990). In mice, rhodopsin mutants cause retinitis pigmentosa, retinal degeneration, and failure to develop rod outer segments ([46]Olsson et al., 1992; [47]Toda et al., 1999; [48]Humphries et al., 1997).

### 1.4.2 Invertebrate opsins

Invertebrate rhodopsin gets pohotoisomerized in the presence of blue light, and activates the $G_{q\alpha}$ subunit of the heterotrimeric G-protein, *dgq* ([49]Lee et al., 1990; [50]Scott et al., 1995). Upon activation, rhodopsin forms intermediates similar to those in vertebrates ([51]Yoshizawa & Wald, 1964). However, unlike in vertebrate photoreceptors, metarhodopsin from invertebrates is thermally stable and does not bleach ([52]Kropf et al., 1959). Upon absorption of orange light it photoconverts back to rhodopsin.

The downstream steps of this cascade differ in vertebrates in invertebrates. In vertebrates, cGMP channels close and the photoreceptors hyperpolarize ([53]Fesenko et al., 1985). Although in invertebrates the identity of membrane channel is not fully understood, the photoreceptor membrane depolarizes as a result of phototransduction ([54]Hagins et al., 1962; [55]Bortoff, 1964). Quenching of metarhodopsin is essential for ending phototransduction.

The *Drosophila* ninaE (neither inactivation nor afterpotential E) gene was isolated and identified as encoding the opsin that is expressed in R1-R6 cells of *Drosophila* photoreceptors ([56]O'Tousa et al., 1985; [57]Zuker et al., 1985). Mutations in this gene cause ultrastructural defects as well as loss of photoreceptor sensitivity ([58]O'Tousa et al., 1989). Recessive *ninaE* mutants exhibit a defective light response and photoreceptor cell death ([59]Leonard et al., 1992). Dominant *ninaE* mutants are defective in rhodopsin maturation, trafficking of rhodopsin in the endoplasmic reticulum, or cause retinal degeneration and constitutive strong desensitization in

photoreceptor cells ([60]Colley et al., 1995; [61]Kurada et al., 1998; [62]Iakhine et al., 2004).

*Limulus* has multiple opsin genes two of which (opsin1 & opsin2) are cloned and better understood than the other genes ([63]Dalal et al., 2003; [64]Smith et al., 1993).

### 1.5 Arrestin

Arrestins are a class of soluble proteins that in conjunction with receptor kinases quench the G-protein coupled receptor function ([65]Hargrave & McDowell, 1992). There are two types of visual arrestins, rod –specific (called s-antigen) and cone-specific (called x- arrestin) ([66]Review Krupnick & Benovic, 1998; [67]Sakuma et al., 1996). Several different types of visual arrestins also occur in the invertebrates. *Drosphila* has two ([68]LeVine et al., 1990; [69]Yamada et al., 1990).

#### 1.5.1 The role of arrestin in vertebrate phototransduction

Arrestin was initially characterized as a protein that translocated to the rod outer segment (ROS) after rhodopsin activation (Kuhn et al., 1984). During quenching of the light response, metarhodopsin is deactivated by a two-step process. Initially, rhodopsin kinase phosphorylates metarhodopsin at multiple serine and threonine residues on its COOH-terminal ([70]Wilden et al., 1982). This quenches the metarhodopsin partially. Phosphorylation alone decreases metarhodopsin activity by about 50% (Xu et al, 1997). Arrestin binding occurs only after metarhodopsin is phosphorylated at least at three sites ([71]Schleicher et al., 1989; [72]Mendez et al., 2000).

Arrestin does not bind to rhodopsin, has low affinity for unphosphorylated metarhodopsin and phosphorylated rhodopsin, and a high affinity for phosphorylated

metarhodopsin ([73]Vsevolod et al., 2004). Only after arrestin binding does the metarhodopsin get completely quenched ([74]Kuhn et al., 1984; [75]Wilden et al., 1986; Xu et al., 1997). Quenched metarhodopsin cannot bind to transducin and thus is inactive. Later, in the presence of retinal dehydrogenase, the all-trans-retinal gets reduced to all- trans-retinol ([76]Palczewski et al., 1994). The retinol dissociates from opsin, leaving arrestin bound to phosphorylated opsin. Arrestin is then released, and phosphatase 2A dephosphorylates the opsin ([77]Palczewski et al., 1989). The rhodopsin is regenerated when opsin binds to a freshly synthesized 11-cis retinal.

### 1.5.2 Mutations in vertebrate arrestin

In humans, a homozygous deletion of nucleotide 1147 (1147delA) in codon 309 of the arrestin gene leads to the formation of a "functional null allele**"** because although the gene product is formed, it is not functional ([78]Fuchs et al., 1995). This mutation results in a type of autosomal recessive congenital stationary night blindness (CSNB) called **Oguchi disease** ([79]Oguchi 1907). The symptoms of this disease include abnormally slow dark adaptation and diffuse yellow or gray coloration of the fundus ([80]Maw et al., 1995). This results in inability to see in darkness. After 2 or 3 hours in total darkness, the normal color of the fundus and the ability to see returns. This is called the 'Mizuo phenomenon' ([81]Mizuo 1913). All other visual functions, including visual acuity, visual field, and color vision are usually normal ([82]Carr and Ripps 1991).

The dark adaptation in Oguchi disease patients is slow because even under very dim light, the phototransduction cascade takes a long time to terminate. This may reduce the ability of the photoreceptors to detect low levels of light and thus

reduces night vision. The visual defects of Oguchi disease appear to require severe reduction in arrestin levels. Patients with heterozygous mutations in the arrestin gene do not show any defects in dark adaptation.

Correspondingly in mice ([83]Xu et al., 1997)**,** heterozygous arrestin mutants which have 33% functional arrestin still have normal responses in light. Homozygous null mutants, however, have a normal rising phase but prolonged falling phase in response to light. A defect in the falling phase is expected since in the absence of arrestin, there would be more unquenched metarhodopsin in the photoreceptors. An increase in the unquenched metarhodopsin would affect termination of the phototransduction cascade.

### 1.5.3 Arrestin function in invertebrates

In invertebrates there is one crucial difference in the fate of metarhodopsin from that of vertebrates. In vertebrates, the metarhodopsin dissociates into chromophore and opsin and rhodopsin is resynthesized in a multi-step process. However, in invertebrates, the metarhodopsin is thermostable, does not bleach, and does not regenerate spontaneously to rhodopsin. It stays as metarhodopsin until it absorbs a photon and isomerizes back to rhodopsin. In the absence of light, the only way to deactivate the metarhodopsin is phosphorylation and arrestin binding. Because a few unquenched metarhodopsin molecules might generate continuous bumps in the dark, a failure to quench metarhodopsin will lead to an increase in dark noise. Even a relatively small number of unquenched metarhodopsin molecules might therefore substantially increase background noise and decrease the sensitivity to light. So

deactivation of metarhodopsin in invertebrates is a crucial step and needs to be strictly regulated.

The mechanism of arrestin function in invertebrates is understood mainly from work in *Drosophila*. In the presence of arrestin, metarhodopsin gets phosphorylated at multiple sites and gets quenched ([84]Bentrop et al., 1993). As in vertebrates, arrestin further quenches the metarhodopsin activity and protects it from dephosphorylation by phosphatases ([85]Byk et al., 1993). Upon absorption of another photon, the arrestin-bound phosphorylated metarhodopsin is converted into arrestin-bound phosphorylated rhodopsin. Phosphorylated rhodopsin releases arrestin and gets dephosphorylated by RDGC, a rhodopsin phosphatase ([86]Vinos et al., 1997). This dephosphorylated rhodopsin is ready to absorb another photon.

### 1.5.4 Mutations in invertebrate arrestin

*Drosophila* has two arrestin genes. Mutation in the dominant arrestin gene leads to a photoresponse that has a prolonged deactivating afterpotential (PDA) that takes ten times longer to terminate the light response ([87]Dolph et al., 1993). This observation indicates that arrestin is necessary for termination of phototransduction in vivo.

There is evidence for only one arrestin gene in *Limulus* photoreceptors ([88]Smith et al., 1995). *Limulus* arrestin appears to be concentrated at the photosensitive rhabdomeral membrane, which is consistent with its participation in phototransduction. Arrestin also occurs in extra-rhabdomeral vesicles that contain internalized rhabdomeral membrane and might therefore also play a role in rhodopsin

endocytosis. In response to a bright flash of light, CaCAM PKII phosphorylates arrestin in both ventral and lateral photoreceptors ([89]Battelle et al., 2001; [90]Smith et al., 1995; [91]Calman et al., 1996).

## 1.6 Complementary oligonucleotide techniques are especially useful for molecules with high turnover since they target mRNA.

### 1.6.1 Opsin turnover

Vertebrate opsin levels are under the control of light and circadian rhythm. In mice, opsin mRNA and protein levels peak just before daylight and are lowest in the middle of the next dark cycle at night ([92]Bowes et al., 1988; [93]McGinnis et al., 1990). In toad and fish retinas, the opsin mRNA level rises just before light onset, remains high while lights are on, and decreases four to tenfold in the dark ([94]Korenbrot et al., 1989). In *Drosophila*, rhodopsin is highly stable and undergoes low levels of turnover ([95]Schwemer, 1984).

In *Limulus*, opsin mRNA levels increase during mid to late afternoon and decrease during the night. The levels are light driven and are not affected by circadian input. The opsin 1 mRNA levels were twice the opsin 2 levels. However, there was no significant change in the relative levels throughout the 24 hr cycle ([96]Dalal et al., 2003). Although there is no direct evidence of changes in the protein levels, since vertebrate opsin levels mimic opsin mRNA pattern, probably *Limulus* opsin does so as well.

### 1.6.2 Arrestin turnover

The turnover rate of arrestin varies from one organism to another. Over a 24 hr day and night cycle, arrestin mRNA levels vary in mice, rats, fish, and toads ([97]Bowes et al., 1988; [98]Craft et al., 1990; [99]McGinnis et al., 1990). In mice, the arrestin mRNA levels are lowest just before onset of the light and peak just before darkness. Arrestin mRNA levels in mammals follow rates of transcription, the rate of synthesis, and the level of arrestin protein ([100]McGinnis et al., 1994; [101]Agarwal et al., 1994; [102]Organisciak et al., 1991).

On the other hand, arrestin mRNA levels in *Drosophila,* and *Xenopus* remain constant over a 24 hr period ([103]Hartman et al., 2001; [104]Green and Besharse, 1996). In *Drosophila* (Hartman et al., 2001), arrestin protein levels are also stable over a 24 hr period and thus reflect the mRNA pattern. In *Limulus*, arrestin mRNA levels are low in the night and high in daytime ([105]Battelle et al., 2000). These changes in arrestin mRNA levels may reflect changes in the protein levels.

## 1.7 Electrophysiology can be used to assess the success of the complementary oligonucleotide techniques.

To investigate whether the RNAi and morpholino based techniques were successful, electrophysiological response of the *Limulus* photoreceptor cells to light can be studied. These cells show small depolarizations in membrane potential (1-10mV) even in complete darkness in the form of dark noise called “bumps”. Bumps also occur in several other vertebrate and invertebrate photoreceptors such as locust,

spider, Hermissenda, and toad ([106]Scholes, 1965; [107]DeVoe, 1972; [108]Takeda, 1982; [109]Baylor et al., 1979).

The occurrence of spontaneous dark bumps is attributed to two mechanisms. One is the thermal isomerization of rhodopsin ([110]Barlow et al., 1993) and the second results from the back reactions from quenched metarhodopsin. Temperature and pH contribute to the first mechanism. These bumps are heavily dependent upon surrounding temperatures since temperature strongly influences rhodopsin thermal isomerization. Toad photoreceptors show reduced dark bumps at low temperatures ([111]Aho et al., 1988). A rhodopsin unprotonated at the Schiff base is more likely to isomerize than a protonated one ([112]Birge and Barlow 1995). Therefore lowering of pH results in a reduction of dark bumps. Unquenched metarhodopsin contributes to the second mechanism. Metarhodopsin is kept quenched by the action of phosphorylation and arrestin binding.

In the dim light, the absorption of a single photon causes a similar depolarizing event called quantum bump ([113]Yeandle & Spiegler, 1973). The light induced quantum bump is generated by metarhodopsin.

The amplitude and temporal profiles of the dark and light activated bumps are identical, because thermally isomerized rhodopsin is indistinguishable from photoisomerized rhodopsin ([114]Baylor et al., 1980). The inability to distinguish between dark and light-induced bumps becomes a key limiting factor in dim light. In dim light, the dark bumps generate false signals which can not be distinguished from those generated by single photon absorption. It is therefore important for an animal to

keep the noise level as low as possible. In *Limulus* the problem of lowering thermal isomerization of rhodopsin is handled internally by lowering the photoreceptor pH via an unknown mechanism. This protonates the Schiff base of a small population of unprotonated rhodopsins and reduces noise. The problem of spontaneous activation of metarhodopsin resulting in dark bumps is reduced by keeping a tight control of quenching by phosphorylation and arrestin binding ([115]Lisman 1985). On exposure to bright light, the cells depolarize to about 60mV. A summation of bumps leads to these larger light-induced changes in membrane potential as demonstrated using a model called the "adapting bump model" ([116]Dodge et al., 1968). The spontaneous dark bumps, light-induced bumps, and depolarization in response to bright light are three parameters that can be investigated in cells treated with the complementary oligonucleotides.

### 1.8 Target proteins and predictions

Opsin and arrestin were selected as target proteins to test the feasibility of using RNAi and morpholino in *Limulus* ventral photoreceptors. The two proteins have well defined functions, which helps us to predict what to expect in the event of reduction in their levels. A reduction in opsin concentration would lead to a subsequent reduction in rhodopsin and metarhodopsin concentration. This would affect both the dark and light-induced bumps. A reduction in arrestin is expected to increase unquenched metarhodopsin which would increase the bump rate. They form a nice pair since they have opposing effects. Neither protein was expected to affect the resting membrane potential, or the bump attributes other than bump rate (such as

peak, duration, etc.). They were also not expected to affect the peak response in the presence of bright light.

# Chapter 2

# RNAi as a tool to downregulate arrestin levels in *Limulus* ventral photoreceptors

## 2.1 Introduction

RNA interference (RNAi), also named RNA silencing or post transcriptional gene silencing (PTGS), is a powerful technique that allows tissue-specific suppression of gene expression. A small double-stranded RNA (dsRNA) introduced in the cell gets cut into small 21-25nt interfering RNAs (siRNAs) that induce sequence-specific degradation of homologous single-stranded mRNA ([117]Fire et al., 1998). dsRNA seemingly resembles a viral or transposon infection and the cell has developed a defense mechanism to destroy it. The cell produces (or activates) RNA endonucleases that cleave the target RNA into small pieces ([118]Hammond et al., 2000).

Double stranded RNA-mediated RNA interference is an effective method to downregulate the levels of proteins in cells. It has become a dominant reverse genetics method which allows one to move from gene sequence to function. This technique has been shown to work in vertebrates and invertebrates, and has been extensively used in C. *elegans*, plants, D. *melanogaster*, mouse, and several cell lines (Fire et al., 1998; [119]Chuang et al, 2000; [120]Misquitta et al., 1999; [121]Caplen et al.,

2001, and [122]Tuschl et al., 1999). RNAi is especially useful in organisms that are not amenable to genetic analysis. One such organism is *Limulus polyphemus,* the horseshoe crab.

In our laboratory, we have used *Limulus* ventral photoreceptors to investigate the phototransduction cascade. These ventral photoreceptors are very large (~ 200 x 80 μm) and can be maintained in culture for several days. These properties make them highly suitable for electrophysiology, immunocytochemistry, and microscopy. If RNAi worked in this system, it would be a powerful tool to explore additional aspects of the phototransduction mechanism.

We selected arrestin as a target protein to test whether the RNAi technique works in the *Limulus* ventral photoreceptors. Arrestin binds to phosphorylated metarhodopsin and quenches it so that the physiological activity of metarhodopsin in phototransduction is completely abolished. Metarhodopsin is the photoactivated form of rhodopsin that initiates the phototransduction cascade. Quenching of metarhodopsin involves phosphorylation at multiple sites and subsequent arrestin binding. In the absence of arrestin, phosphorylated metarhodopsin is likely to be dephosphorylated spontaneously. The dephosphorylated metarhodopsin is active and may initiate the phototransduction cascade in the dark. Thus, arrestin binding is an essential part of the phototransduction cascade and a reduction in the abundance of arrestin protein should lead to a gain-of-function response.

siRNA delivery methods vary depending on the organism under investigation. The double stranded RNA can be fed to *C.elegans*, injected in *Xenopus* oocytes, or delivered through special delivery methods such as scrape-loading ([123]Timmons & Fire; 1998; [124]Nakano et al., 2000). We decided to add arrestin siRNA into the culture medium. Although we observed some effects with this delivery method, to increase accuracy, we decided to inject the siRNA into the cells. Consistent with our predictions about the effects of arrestin silencing, we observed a 5-fold increase in bump rate at 24 hrs after injection.

Since arrestin siRNA might increase unquenched metarhodopsin concentration, we looked at tools to decrease active metarhodopsin concentration. Metarhodopsin can be bleached by hydroxylamine, a very strong reducing agent. In vertebrates, hydroxylamine competes with the opsin and attacks the chromophore at the Schiff base linkage ([125]Crescitelli 1958). After binding to the chromophore, it forms retinal oxime (C=NOH) and opsin. Hydroxylamine is more likely to attack the Schiff base bond after photoisomerization of rhodopsin because the linkage in opsin is shielded either by the phospholipid layer of the outer membrane or by being buried within the hydrophobic portion of the opsin ([126]Abrahamson 1974). In rod photoreceptors, hydroxylamine treatment destroys metarhodopsin and reduces the light-induced bumps ([127]Leibrock & Lamb, 1997). The rate of breakdown of metarhodopsin in the presence of hydroxylamine shows a half-time between 12 min to 35 hr depending on the species and type of pigment being studied.

It is reasonable to suspect that, as in the case of vertebrates, hydroxylamine acts in *Limulus* by competing with opsin for the Schiff base. Another hypothesis is that probably an unstable intermediate photopigment (between rhodopsin and metarhodopsin) is formed on photon absorption. In this intermediate, the bond between the opsin and chromophore is momentarily broken and hydroxylamine attaches to the chromophore at this instant. The result of either of these processes would be breakdown of metarhodopsin in the presence of hydroxylamine. Hydroxylamine affects two indicators of reduced metarhodopsin content. One is the quantum efficiency to flashes of light. The second is the early receptor potential (ERP). ERP occurs in the presence of intense bright light, has virtually no latency, and is hypothesized to be generated directly by charge displacements that resulted from the isomerization of rhodopsin ([128]Cone & Pak, 1971). Therefore, ERP is a direct measure of the amount of rhodopsin and its intermediates present in the photoreceptor. Hydroxylamine treatment in the ventral eye has been shown to decrease the quantum efficiency, and abolish the ERP in response to flashes of light ([129]Faddis and Brown 1992). This treatment was very effective in light but not in the dark, further supporting the hypothesis that hydroxylamine destroys metarhodopsin. Our aim was to use hydroxylamine to counteract any effects of unquenched metarhodopsin in the presence of arrestin siRNA.

## 2.2 Materials and methods

### 2.2.1 Preparation of the nerve

The ventral nerve of adult male horseshoe crabs, *Limulus polyphemus*, was dissected as described by Clark et al. The nerves were placed in artificial sea water (ASW) that contained (in mM): 435 NaCl, 10 KCl, 20 $MgCl_2$, 25 $MgSO_4$, 10 $CaCl_2$, and 10 HEPES. The nerves were desheathed, and treated with 1% pronase (Calbiochem, San Diego, CA) for 1 min.

### 2.2.2 Preparation of siRNA

siRNA was produced in the laboratory of Dr. Barbara Battelle (The Whitney Laboratory & The Department of Neuroscience, Univ. of Florida) using Ambion's siRNA cocktail kit. A 314nt long piece of cDNA which is the 3' end of *Limulus* arrestin gene from nt 926-1239 was used. dsRNA was produced by transcribing in the same reaction mix two plasmids containing the target sequence: one plasmid had the T7 promoter at the 5' end of the sequence; the other had the promoter at the 3' end. After the transcription reaction, the products were annealed, treated with RNAse and DNAse, and the double stranded product was purified. The product migrated as a single band that was slightly larger than the 300 bp marker. 50ml of this purified product contained about 1 mg/ml of RNA. About 15 mg of RNA was then cut with RNAse III, and the cut pieces were purified. The yield was about 50 ml of 470 mg/ml RNA or 52 mM siRNA.

The control siRNA was ordered from Ambion. The control siRNA was diluted the same way as the arrestin siRNA and injected into cells.

### 2.2.3 siRNA treatment

In bath:

A *Limulus* ventral nerve was placed in a 200 µl well. The well was filled with 10 µl siRNA and 190 µl culture medium. A control preparation contained a nerve in a dish with 200 µl culture medium and no siRNA. The nerves were kept in a cold room ($10^0$ C) for four days covered in aluminum foil so that they were not exposed to light.

Injections:

siRNA was diluted to 1/1000 in a carrier solution (100mM potassium aspartate, 10mM Hepes, pH 7.0). This siRNA was pulse-pressure injected into the ventral photoreceptors with a glass electrode of 20MΩ resistance. All nerves were kept in a culture medium in a cold room under 12 hrs light / 12 hrs dark conditions. Control nerves with cells injected with the carrier solution alone were also treated similarly.

### 2.2.4 Hydroxylamine treatment

Cells were chemically bleached with hydroxylamine to reduce their metarhodopsin content (Faddis & Brown 1992). The protocol used is described in

Payne & Demas (2000). Briefly, a bleaching solution was prepared containing (mM) 200 hydroxylamine chloride (Sigma-Aldrich), 235 NaCl, 10 KCl, 20 $MgCl_2$, 25 $MgSO_4$, 10 $CaCl_2$, 10 HEPES, adjusted to pH 6.5 with 10 N NaOH and cooled to 4°C. Cells were bathed in this solution for 10 min under intense white light at 4°C (for bleaching experiments) and in the dark at 4°C (for dark experiments). Nerves were then washed four times at 10-min intervals with 50 ml ASW at 4°C.

### 2.2.5 Electrophysiology

The arrangement of light source and neutral density (ND) filters was similar to that described in [130]Wang et al., (2002). The unattenuated intensity of light was 80 $mW/cm^2$. Other intensities are $log_{10}$ units of attenuation of this intensity. Attenuation by -10 $log_{10}$ units typically elicited about one single photon event per second. Axon laboratory's Axoscope 9 was used for data acquisition. A Digidata 1200 (Axon instruments) analog-to-digital board installed in a personal computer was used, with temporal filtering set to 300 Hz and digital sampling at 1 kHz.

The response of cells in the dark and to various intensities of light was recorded with glass micropipettes containing carrier solution (resistance ~ 20MΩ). The same micropipette was used to inject siRNA into the cells. The siRNA injected cells were tested at 18, 20, 23, 24, and 48 hrs after injection. The cells were dark adapted for about 30 min and their response in the dark was recorded. The dark response was represented as the number of bumps/s. The cells were then exposed to

200ms flashes of light of varying intensities ($log_{10}$ -6, -5, -4, -3, -2, -1 and 0). At these intensities, we do not observe bumps but a single depolarization with amplitudes in the range of -20 to -60mV. Every flash was followed by a period of complete darkness until the cells recovered the dark-adapted state.

For experiments involving hydroxylamine treatment, in addition to the dark and bright light responses as described above, bump rate in response to continuous dim light for two min was also recorded. The dim light is represented as $log_{10}$ -10 through -7.5 in increments of 0.5. Exposure to this intensity of light increases the bump rate with increasing intensity. Every dim light exposure was followed by a period of complete darkness till the cells recovered their dark-adapted state.

### 2.2.6 Estimation of photon counts corresponding to the different $log_{10}$ light intensities.

In order to verify the accuracy of the ND filters, a photon multiplier tube (PMT) was used to count the number of photons emitted when particular ND filters were used to attenuate the light to specific $log_{10}$ intensities. The results are fitted with a line of slope one and shown in fig.2.1. This indicates a linear relationship between the incident light and the number of photons counted by the PMT. This also indicates that the filters attenuate the light quite accurately.

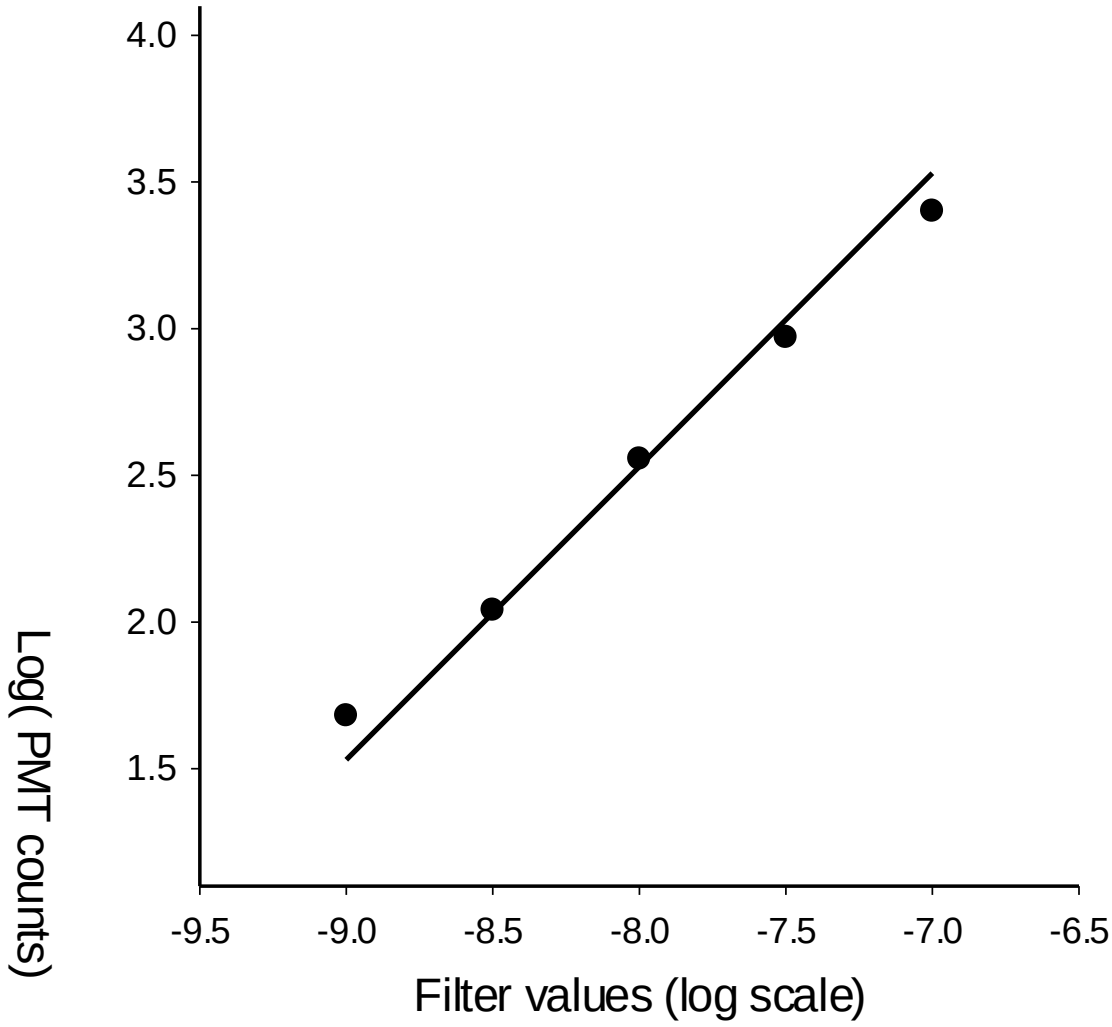



**Figure 2.1** Representative photon counts indicating the attenuation of light at various $\log_{10}$ light intensities. The attenuation decreases with increasing negative log values. The PMT saturates beyond $\log_{10}$ -6.5. The data are fitted with a line of slope 1. A close fit indicates that the filters attenuate the light quite accurately.

### 2.2.7 Calculating volume of injections

[131]Carson and Fein (1983) used pressure injection from micropipettes into oil to get an estimate of the volume injected into ventral photoreceptor cells. They concluded that volume injected in cells was about three times the volume observed in

oil. In our experiments, we injected about 4 pl solution in oil/injection. This would mean that at we injected about 12 pl solution in the cell/ injection. Since we injected each cell 10 times, the total volume was about 120pl. This was about $1/5^{th}$ the volume of the cells. Since we injected about 55μM siRNA in a cell with an average volume of 550pl, concentration of siRNA in the cell was about 11μM.

### 2.2.8 Data analysis

The traces were analyzed for the bump number and bump amplitude using an in-house Matlab program developed by Mr. Murat Ayetikin. The program was designed to calculate threshold by two methods. The first was a manual method wherein the user manually clicked a point and all points above it were considered for bump estimation. The second method was called the "boxing" method. In this approach the user drew a box around the part of the trace where no bumps occurred. The program then calculated the mean and standard deviation (SD) of that section. A threshold for a bump was assigned as four SD above this mean. We initially used both these methods to obtain threshold for about 10 traces. Once I verified that the thresholds obtained via both the methods were similar, I used only the boxing method for subsequent analysis.

Once the threshold was selected, the program detected peaks above this level and determined their amplitude. A peak was determined as a highest value that had at least 25 consecutive points in both directions with lower values. To avoid counting multiple bumps, we also had a provision to discard a peak if it appeared within 80ms of another peak on either side (since average bump duration was about 40ms).

Amplitude was the height of the peak from the threshold value. The bumps identified in this manner were then assigned to various bins of 0.5mV amplitude. Individual differences between the bump rates were measured using a t-test. Probability (p) value of less than 0.05 was considered significant. To verify whether the bumps obtained in different categories had similar properties, we calculated the power spectra obtained from a fast Fourier transform (FFT) of the traces.

## 2.3 RESULTS

### 2.3.1 siRNA is membrane permeable in *Limulus* ventral photoreceptors:

dsRNA can be delivered to *C. elegans* by soaking the animals in a medium containing dsRNA ([132]Tabara et al., 1998). If the siRNA can be delivered to ventral photoreceptors just by adding it to the medium, it would keep the cells healthy and undamaged. We tried to deliver siRNA by adding the siRNA to the culture medium and testing the response of the cells through electrophysiological methods. Nerves kept for 96 hrs in a culture medium without siRNA added to it were used as a control. When the rate of dark bumps was compared between the two types of cells as in Fig. 2.2, those with siRNA in the medium had a rate of 3.4 $\pm$ 0.8/s as compared to a dark bump rate of 1.1 $\pm$ 0.2/s in cells that were not exposed to siRNA. The peak response of the siRNA treated cells was similar to control cells under bright light (log -3 through log 0 in increments of 1).

This led us to conclude that siRNA is membrane permeable. However, there were two problems associated with this method. Firstly, there was a lot of variability

in the bump rate of cells that had been treated with siRNA and secondly, it was not possible to determine whether the siRNA had actually penetrated the cells. So we decided to use another method, injecting the siRNA into the cells.

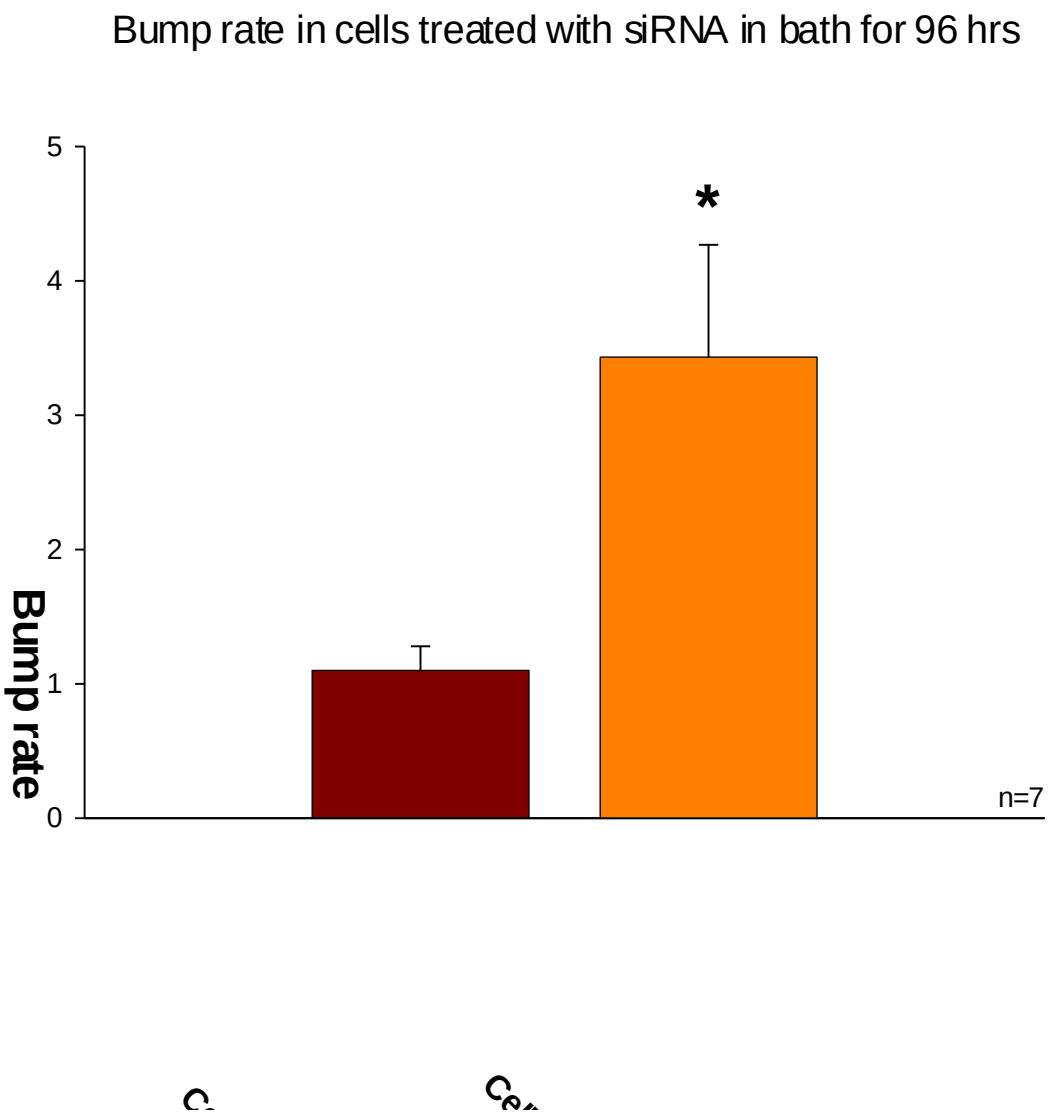



**Figure 2.2** Dark bump rate in cells treated with arrestin siRNA in bath. Cells with arrestin siRNA in bath had a bump rate of 3.4 $\pm$ 0.8 /s as compared to the 1.1 $\pm$ 0.2/s rate in control cells with no siRNA in bath. n=7. Error bars indicate SEM.

### 2.3.2 Arrestin siRNA injections lead to an increase in the dark bump rate and are most effective at 24 hrs after treatment

We injected siRNA into cells and tested them electrophysiologically at 18, 20, 23, 24, and 48 hrs after injection. Fig 2.3 indicates representative traces of cells tested at selected time points. At 24 hrs after injection, the number of bumps goes up as compared to control cells and cells tested at other time points. Uninjected cells had a dark bump rate of 1.1 $\pm$ 0.2, cells injected with the carrier solution had a bump rate of 0.9 $\pm$ 0.1, and cells injected with the control siRNA had a bump rate of 1.16$\pm$0.2. As compared to these controls, the cells injected with arrestin siRNA and tested at 24 hrs after injection had a bump rate of 4.9$\pm$ 0.3 (Fig.2.4). As compared to the control siRNA injections, the cells tested 18 hrs, and 20 hrs after injection did not show any significant difference in the dark bumps. Cells tested 24 hrs after injection showed a five-fold increase in the bump rate (4.9 $\pm$ 0.3). This effect was transient, as seen at 48 hrs after injection, when the bump rate was 3.3 $\pm$ 0.4 (Fig. 2.5).

siRNA injections had no significant effect on the kinetics of the response to bright flashes of light at all the time-points tested. A representative trace of average response to $\log_{10}$-2 of cells injected with arrestin siRNA and tested at 24 hrs after injection is indicated in Fig.2.6.

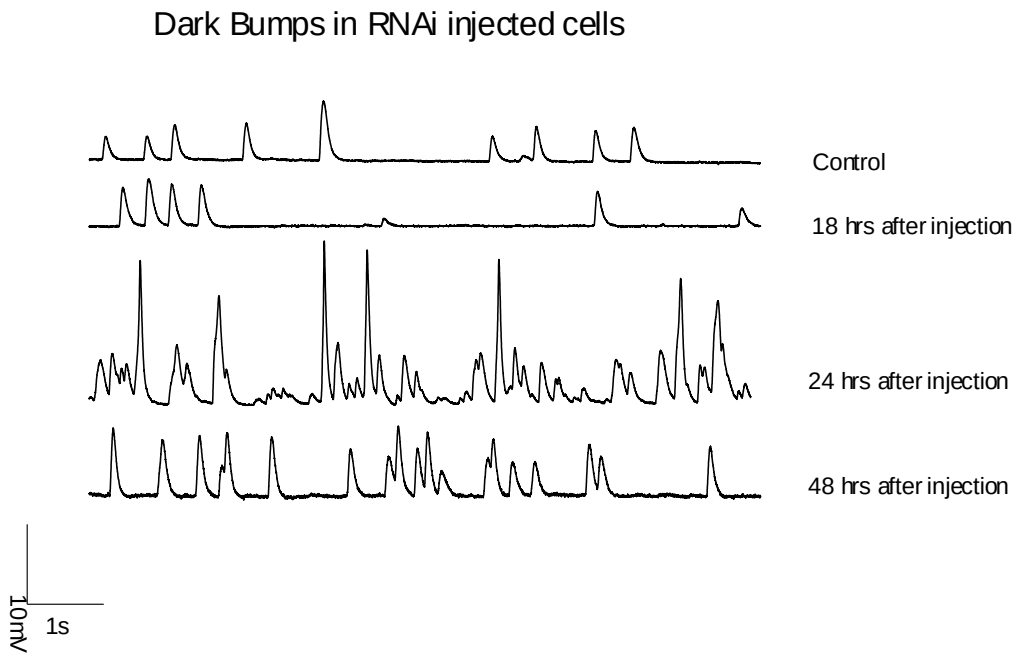



**Figure 2.3:** Representative traces of bumps observed in different categories. Cells were injected with siRNA and were tested at 18, 24, and 48 hours after injection. At 24 hrs after injection, the bump rate is almost 5 times higher than that in the control.

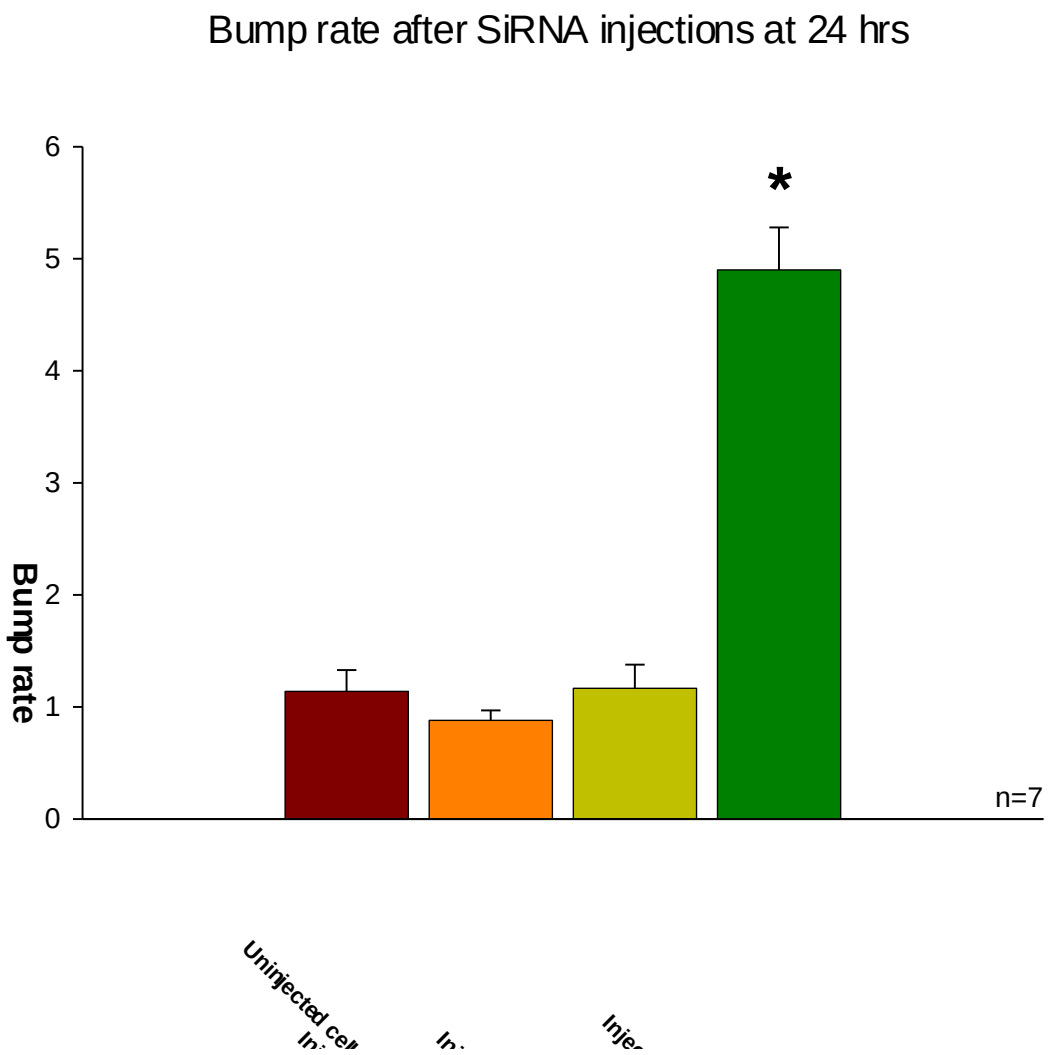



**Figure 2.4:** Cells injected with arrestin siRNA have a five-fold higher bump rate. Cells injected with siRNA had a bump rate of 5 bumps/s when tested at 24 hrs after injection. This was a five-fold increase from the bump rate in control cells. Controls consisted of three types; uninjected cells, cells injected with the carrier solution, and cells injected with a control siRNA.

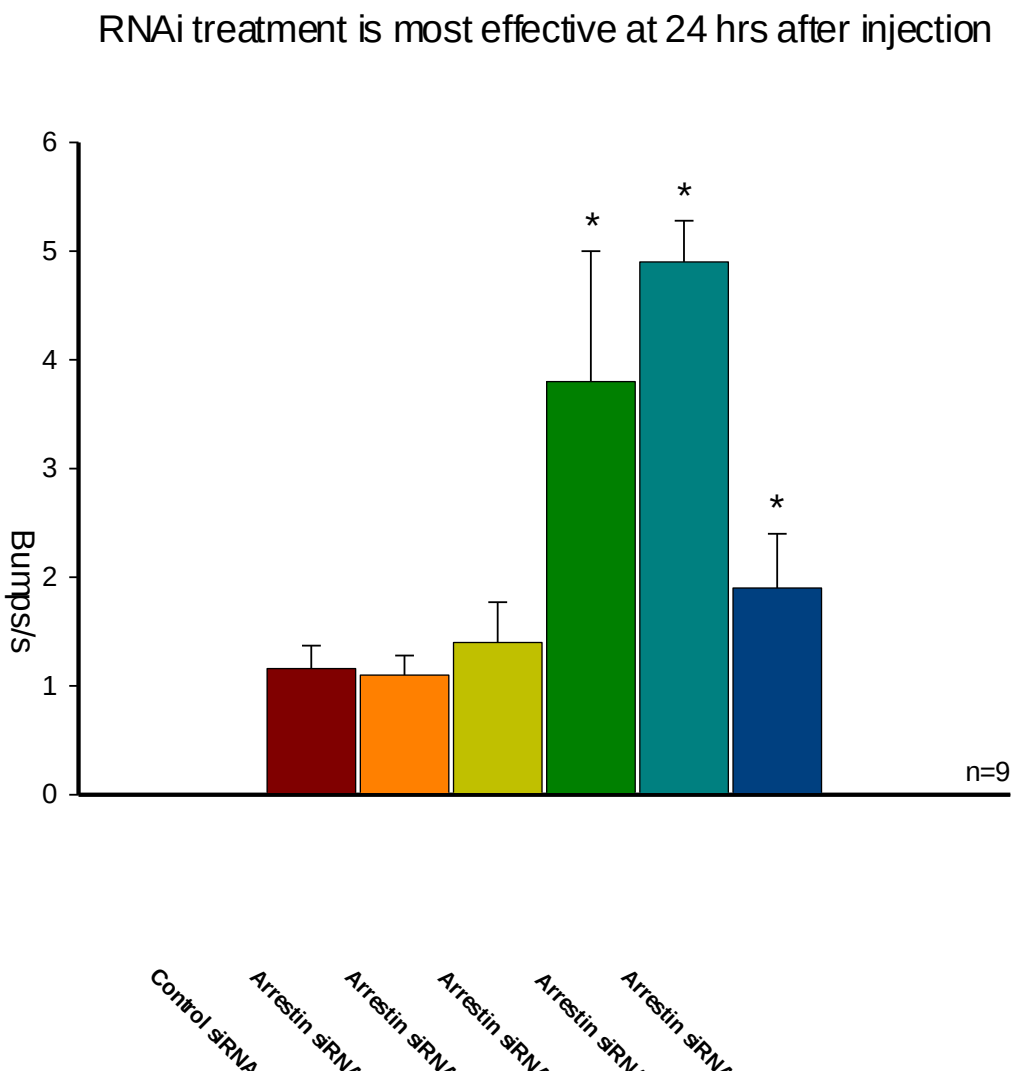



**Figure 2.5**: Spontaneous dark bump rate in cells tested at different points after arrestin siRNA injections. The dark bumps of cells injected with arrestin siRNA and tested 18, 20, 23, 24, and 48 hours after injection are compared with the dark bumps of cells injected with the control siRNA. There is no significant change in the number of dark bumps at 18, and 20 hours after injection. However, the number goes up to 4$\pm$1 at 23 hours, 4.9 $\pm$ 0.3 at 24 hours, and 3.3 $\pm$ 0.4 at 48 hours after injection. n= 9. Error bars indicate SEM.

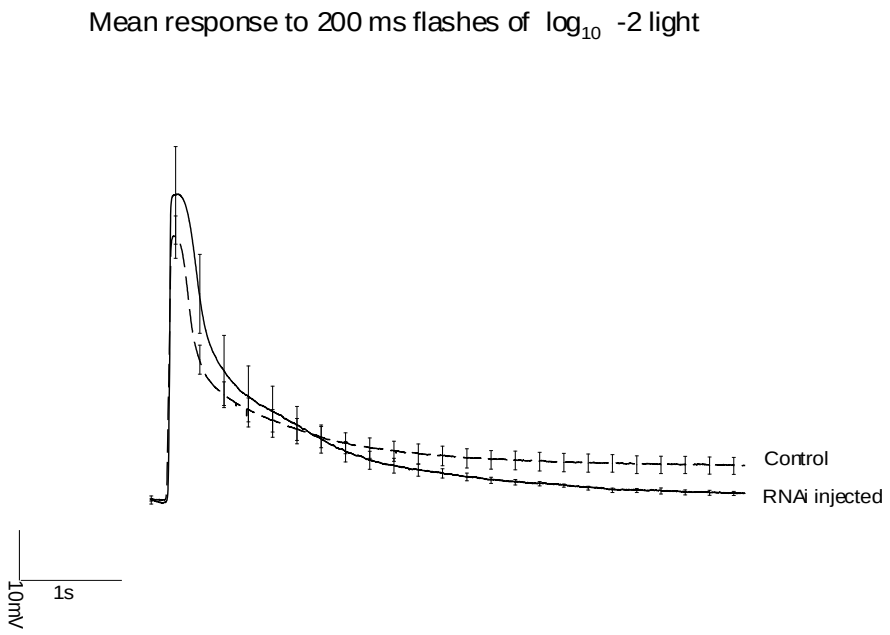



**Figure 2.6:** The response kinetics of cells tested 24 hours after injection with arrestin siRNA are compared with those of control cells. A mean trace of response to a 200ms flash of light of log-2 intensity is shown here. The dashed line represents mean and SEM of six control cells. The straight line shows a mean and SEM of six cells injected with siRNA and tested at 24 hrs after injection. The peak response of the injected cells and the decay time is not significantly different from that of the control.

### 2.3.3 The bumps generated by siRNA injections have properties similar to those generated in the presence of $\log_{10}$ -8.5 light.

To address the question whether the shape of the bumps generated by siRNA injections was different from those generated in the presence of dim light, we analyzed a power spectrum of the two categories of cells. Fourier analysis has been used to compare bumps in *Limulus* photoreceptors ([133]Ratliff et al., 1974; [134]Hardie &

Minke, 1994). Fig. 2.7 is a power spectrum plot of mean traces from two categories of cells. One set of traces is from the cells injected with siRNA and their response recorded in the dark. The second set of traces is from control cells exposed to dim light (log -8.5). This light intensity was selected because it resulted in a bump rate of about 5/s in control cells which is similar to the spontaneous dark bump rate in cells injected with siRNA. Traces from bumps generated by siRNA injections have a power spectrum similar to that of traces from bumps generated in dim light. This indicates that siRNA induced bumps and light-induced bumps may have similar properties.

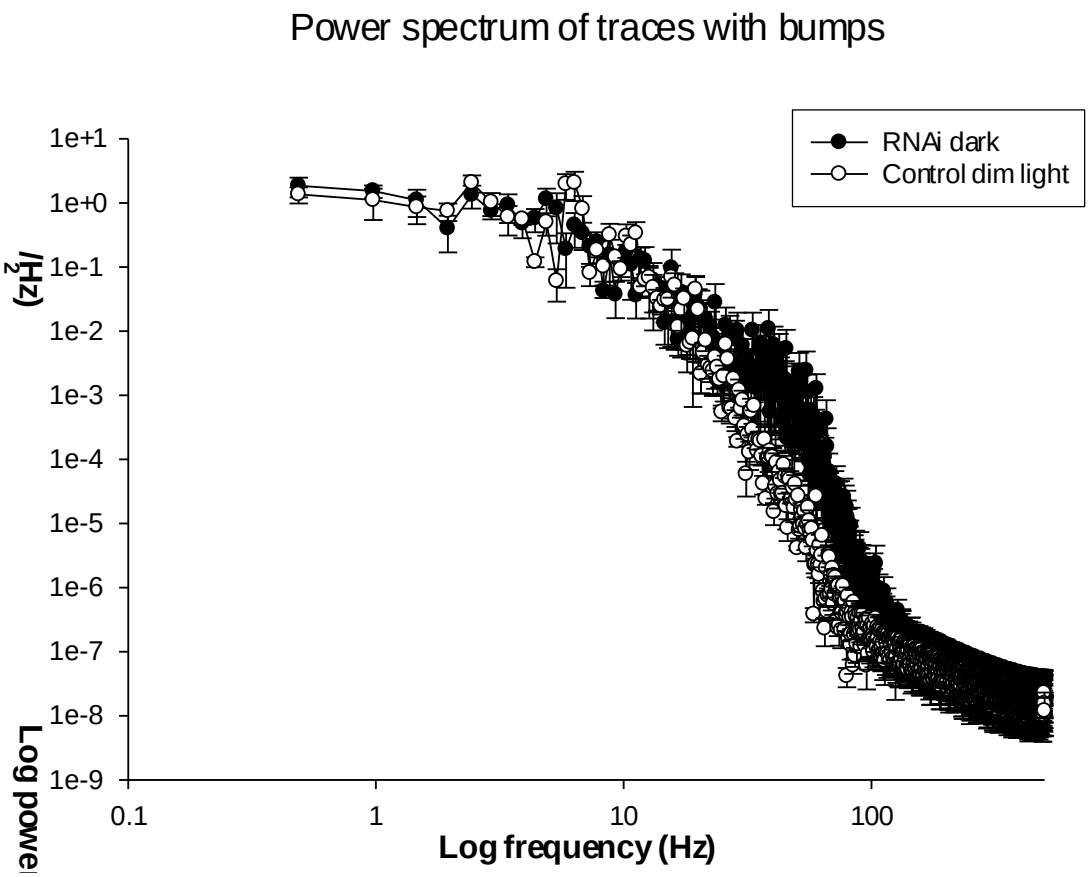



**Figure 2.7**: Power spectrum determined from traces containing bumps. siRNA dark = mean of traces from cells injected with siRNA and their response recorded in the dark. Control dim light = mean of traces from control cells exposed to dim light (log -8.5). There is no significant difference between the two spectra. Error bars indicate SEM. n=6

### 2.3.4 Bleach with hydroxylamine reduces dark noise

The effects of hydroxylamine treatment on *Limulus* photoreceptors in dim light have been investigated previously by Faddis and Brown (1992). We conducted

similar experiments to verify that hydroxylamine does not reduce bump rate when treated in darkness but leads to a reduction in bump rate when treated under intense white light (fig. 2.8). The dark bump rate in control cells and cells treated with hydroxylamine in the dark is not statistically different. However, the dark bump rate in cells treated with hydroxylamine under intense bright light is significantly lower than that of control cells. This indicates that bleaching with hydroxylamine reduces spontaneous dark noise in uninjected control cells. Dark noise is generated by rhodopsin present in the cell. Bleaching with hydroxylamine reduces metarhodopsin content. However, in *Limulus* since rhodopsin-metarhodopsin ratio is always maintained at 50:50 on illumination, reduction in metarhodopsin results in some rhodopsin photoconverting to metarhodopsin. This process continues for the duration of bleaching treatment (10 min) by the end of which both the rhodopsin and metarhodopsin content should have decreased equally. Once the cell has been dark adapted, this reduced rhodopsin content likely results in the observed reduction in dark noise.

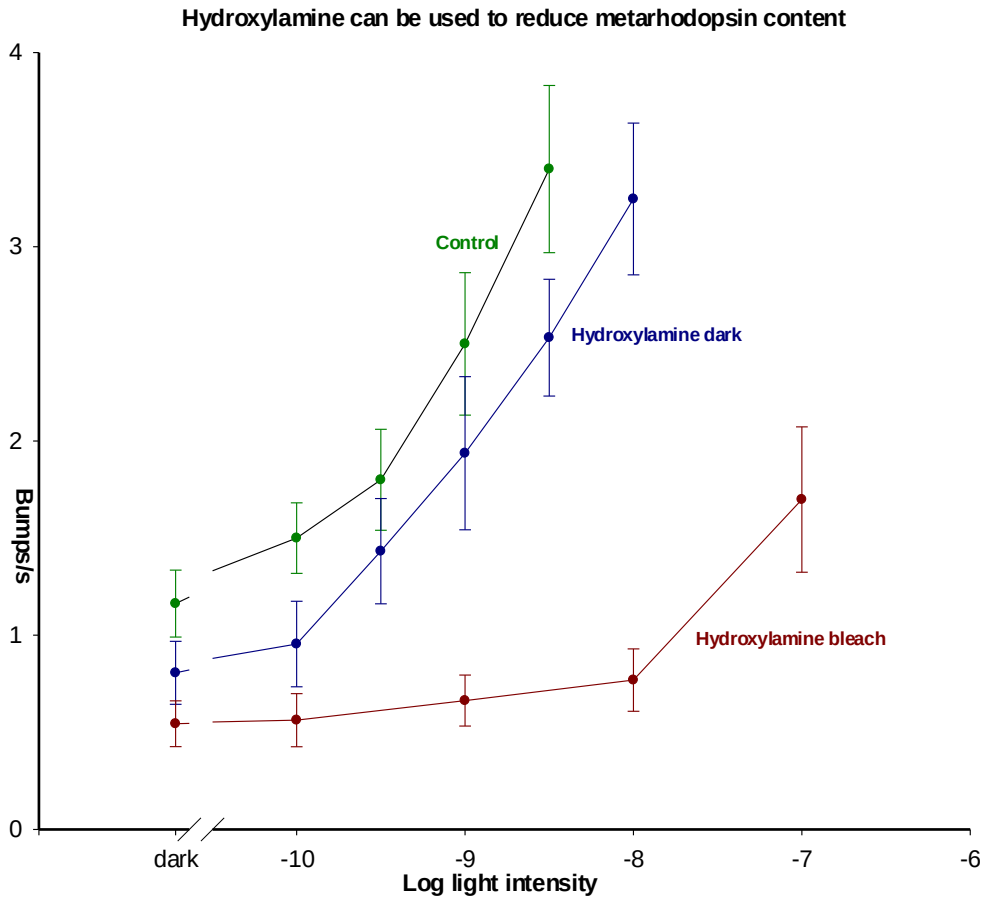



**Figure 2.8:** Effect of hydroxylamine treatment on control cells. Uninjected control cells were treated with hydroxylamine in the dark and in the light. The bump rate was tested at dim light intensities. Hydroxylamine treatment in the dark had no significant effect on the bump rate of cells. However, bleach with hydroxylamine in intense bright light led to a reduction in sensitivity to light.

### 2.3.5 The effects of hydroxylamine are consistent with an attack on metarhodopsin but not rhodopsin.

Hydroxylamine treatment in the dark has been shown to have no effect on the light-induced bump rate of control cells (Faddis and Brown, 1992). We observed that

after treatment with hydroxylamine in the dark, there is no reduction in the dark bumps generated by the uninjected control cells. Since hydroxylamine attacks metarhodopsin and does not affect rhodopsin, this result confirms that dark bumps in control cells do not originate from metarhodopsin. We also tested the effect of treating cells with hydroxylamine in the dark on the bump rate in cells injected with the arrestin siRNA. Representative traces from control cells, cells injected wit arrestin siRNA, and injected cells treated with hydroxylamine are shown in Fig. 2.9. We measured the dark bump rate in control cells before and after hydroxylamine treatment, and in injected cells before and after hydroxylamine treatment. The bump rate in injected cells treated with hydroxylamine ($1.1 \pm 0.2$) was educed significantly from that at 24hr after injection ($5 \pm 0.4$). The bump rate of control cells before and after treatment was $1.4 \pm 0.1$ and $1.2 \pm 0.1$ respectively (Fig. 2.10).

Since hydroxylamine reduces the concentration of metarhodopsin, the corresponding reduction in bump rate in hydroxylamine – treated cells is indicative of metarhodopsin being the source of these bumps. The bump rate after treatment is almost indistinguishable from that of pre-treatment in uninjected control cells. This indicates that the hydroxylamine treatment probably led to the dissociation of the excess metarhodopsin that was generated as a result of the RNAi treatment.

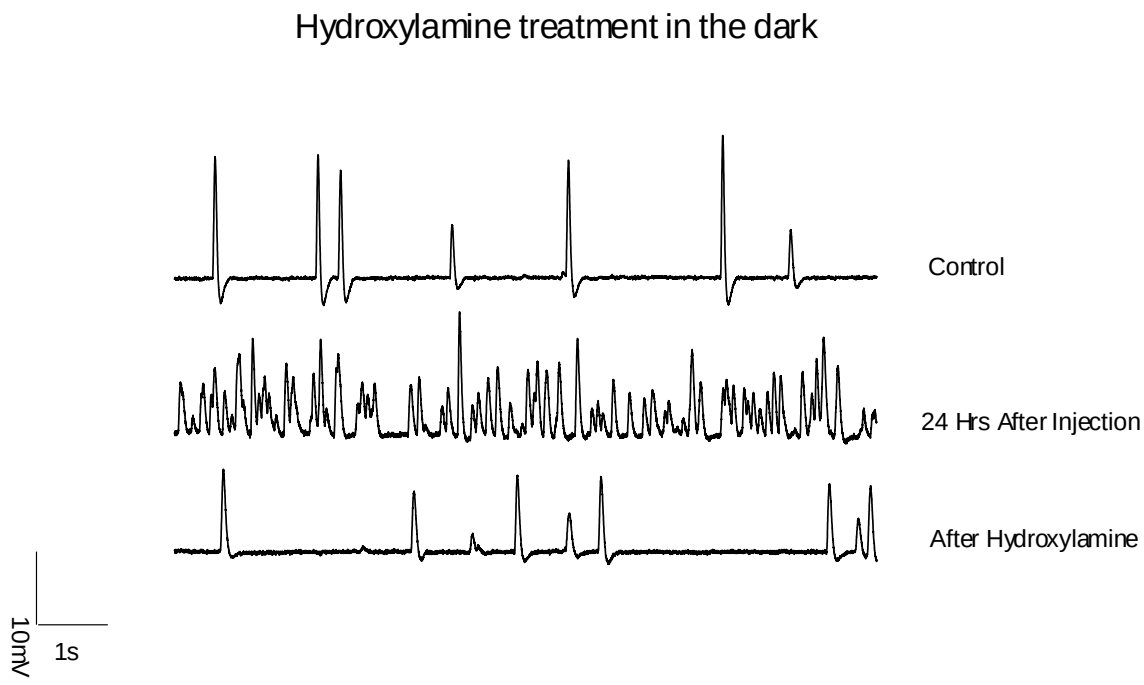



**Figure 2.9:** Representative traces depicting the bumps in cells treated with hydroxylamine in the dark. Cells were injected with arrestin siRNA and tested at 24 hrs to verify the increase in bump rate. The cells were then treated with hydroxylamine in complete darkness and the bump rate was checked again. After hydroxylamine treatment, the bump rate reduced to pre-injection control level.

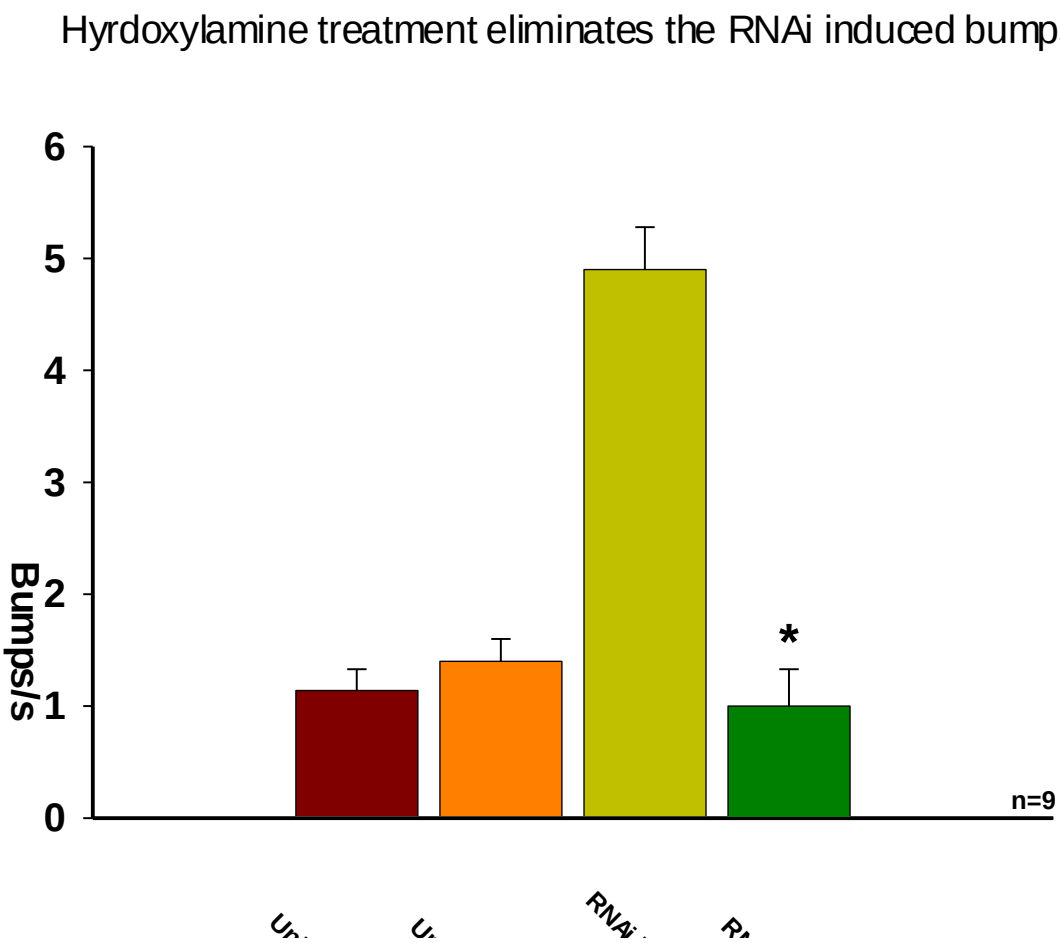



**Figure 2.10**: Dark bump rate after hydroxylamine treatment in the dark. In the cells injected with arrestin siRNA and then treated with hydroxylamine in the dark, the bump rate goes down from 4.9 $\pm$ 0.38 to 1 $\pm$ 0.33. Bump rate in uninjected cells is 1.3 $\pm$ 0.19 and that in uninjected cells treated with hydroxylamine is 1.1 $\pm$ 0.2. n= 7. Error bars indicate SEM.

### 2.3.6 siRNA injected cells bleached with hydroxylamine show a marked decrease in dark bumps.

We studied the effect of bleach with hydroxylamine on the cells injected with arrestin siRNA. The dark noise in uninjected cells was recorded. These cells were then injected with siRNA, and after 24hrs, the dark noise was recorded again to verify the increase in bumps. Then these cells were bleached with hydroxylamine in the presence of intense bright light for 10 min. After several washes, the dark noise of the cells was recorded again. Representative traces are depicted in Fig. 2.11. As indicated in Fig. 2.12, the bump rate in bleached cells ($0.4 \pm 0.1$) was reduced significantly from that at 24hr after injection ($4.8 \pm 0.3$). In uninjected control cells, the bump rate after bleach ($0.8 \pm 0.2$) was also lower than that of pre-bleach ($1.3 \pm 0.2$).

During 10 min of bleaching, the hydroxylamine is expected to inactivate metarhodopsin by binding to all-trans retinal. This will reduce the metarhodopsin concentration in the cell. In bright light, some rhodopsin is expected to convert to metarhodopsin. This metarhodopsin is again attacked by the hydroxylamine. Since rhodopsin-metarhodopsin is at equal ratio in vivo, we may see a decrease in rhodopsin content as well. As this process continues, the content of both rhodopsin and metarhodopsin is decreased. The reduction in bump rate occurs due to elimination of bumps generated by unquenched metarhodopsin, and the bumps generated by some rhodopsin.

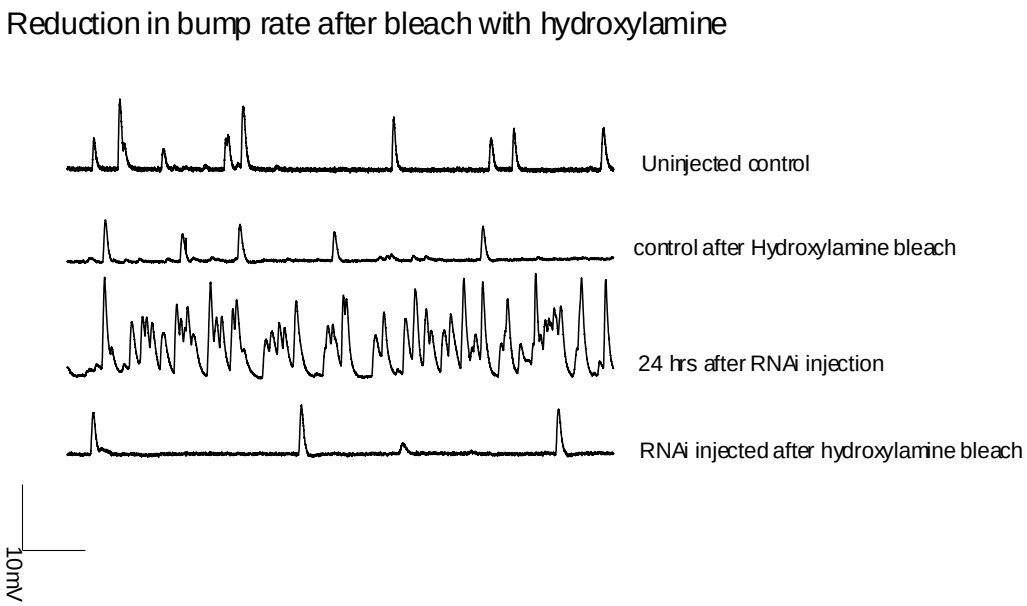



**Figure 2.11:** Traces indicating the bump rate in cells injected with arrestin and bleached with hydroxylamine. Cells were injected with arrestin SiRNA and the increase in bump rate was verified at 24 hrs. These cells were then bleached with hydroxylamine under intense bright light. The bump rate in the bleached cells was significantly lower than the uninjected cells.

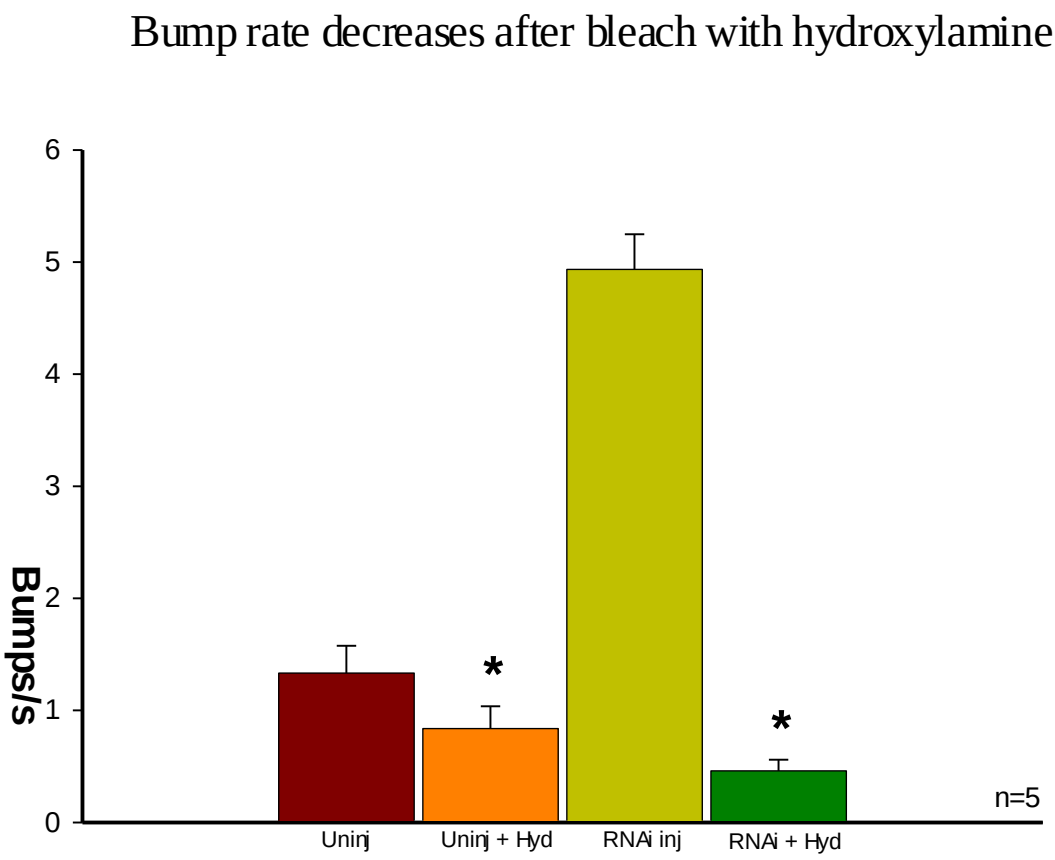



**Figure 2.12:** The dark bumps are compared for a cell before injection, 24hr after injection and 30 min after bleaching with hydroxylamine. The bump number significantly goes down after bleaching with hydroxylamine and subsequent four washes. n=5. Error bars indicate SEM.

**2.4 Discussion**

The major findings of this study are that siRNA when delivered through the bath or via injections leads to an increase in *Limulus* ventral photoreceptor dark bump rate, and that these bumps are abolished in the presence of hydroxylamine.

### 2.4.1 Injecting siRNA is the optimum delivery method.

Delivery methods vary widely. siRNA can be fed, injected, or scrape loaded into cells. Although in *Limulus* ventral photoreceptors arrestin siRNA in bath seems to have the expected effect on the dark noise, we observed considerable levels of variability in the response. This could result probably because of the variation in positioning of cells on the nerve. Some cells are more accessible than others. The nerve is treated with pronase before the treatment. The pronase cleaves the peptide bonds in the glia that surround the photoreceptor cells on the optic nerve. However, this action of pronase might not be uniform and could affect accessibility to the photoreceptor cells. These factors might lead to varying amounts of siRNA entering into the cells and might account for the observed variability of response. Also, the only verification of the success of the technique was the electrophysiological data. There was no independent confirmation of the success of the RNAi technique since we could not mark the cells that had taken up the siRNA. We therefore decided to inject cells with the arrestin siRNA. This allowed us to estimate the amount of siRNA injected into the cells (~11nM) and later also allowed us to mark the cells that had been injected. With injections, we also had an internal control of uninjected cells from the same nerve.

### 2.4.2 Increased bump rate after siRNA injections gives an estimate of arrestin turnover rate.

We expected the arrestin RNAi injections to be effective if the turnover rate of arrestin was high since the RNAi affects the levels of freshly made protein and has no

effect on the pre-existing protein in the system. The turnover rate of arrestin protein varies from one organism to another. Over a 24 hr (12h D/12h N) cycle, diurnal rhythms of arrestin mRNAs have been observed in mice, rats, fish, and toads ([135]Bowes et al., 1988; [136]Craft et al., 1990; [137]McGinnis et al., 1990; [138]Korenbrot et al., 1989). On the other hand, there is no change in arrestin mRNA levels over a 24hr period in *Drosophila,* and *Xenopus* ([139]Hartman et al., 2001; [140]Green and Besharse, 1996). In *Limulus*, arrestin protein turnover has not been determined. Light causes a reversible movement of the arrestin protein to the photosensitive membrane. Light-driven shedding of the rhabdomeral membrane probably involves arrestin ([141]Sacunas et al., 2002). Arrestin mRNA levels are regulated mostly by clock input. Levels of arrestin mRNA are higher during the day in the light than during the night in the dark ([142]Battelle et al., 2000). In mice, levels of arrestin biosynthesis mimic the mRNA levels ([143]Agarwal et al., 1994). In *Limulus* too, changes in arrestin mRNA levels may reflect changes in the protein levels. In the present study, we observed a maximum effect of arrestin siRNA injections at 24 hrs.

At 48 hrs after injections, we observed a decline in the bump rate from that at 24 hrs after injections. siRNA gets amplified once it is inside cells. The decrease in bump rate could be indicative of deleterious effects of high siRNA concentration. It could also be due to the degradation of siRNA inside the cells. There is no direct evidence to indicate either of the two possibilities.

### 2.4.3 Excess bumps are probably caused by unquenched metarhodopsin.

The source of these bumps was of interest to us. The increase in bump rate was probably caused due to the presence of unquenched metarhodopsin in the photoreceptors. Hydroxylamine is often used to reduce metarhodopsin concentration pharmacologically since it competes for the chromophore, retinal. We treated siRNA-injected cells with hydroxylamine and observed that the bump rate went down to about 1/s which is similar to control levels. In the presence of siRNA, there was a high concentration of unquenched metarhodopsin present in the cell and this presumably led to an increase in bump rate. After hydroxylamine treatment, the metarhodopsin concentration was decreased and the bump rate also was reduced. Since the RNAi-induced bumps in toads are generated by metarhodopsin, a decrease in the bump rate in the ventral photoreceptors could be indicative of a decrease in metarhodopsin concentration ([144]Leibrock and Lamb, 1997).

### 2.4.4 Equal rhodopsin and metarhodopsin ratio in *Limulus* results in a decrease in spontaneous dark bumps after bleach with hydroxylamine.

When hydroxylamine was administered in the presence of intense bright light, there was a decrease in the spontaneous dark bump rate in cells injected with arrestin siRNA. This decrease was significantly lower than the spontaneous bump rate in the control cells. This can be explained if we consider that in *Limulus* photoreceptors the ratio of rhodopsin and metarhodopsin is always maintained at 50:50 in the light. In

this set of experiments, we expect arrestin levels to go down due to siRNA injections. Pharmacologically the injections cause an increase in unquenched metarhodopsin. The unquenched metarhodopsin could be destroyed by hydroxylamine treatment. This could skew the ratio towards rhodopsin. The intense bright light could aid in converting large amounts of rhodopsin into metarhodopsin and the ratio could be maintained. In this process, however, the total amount of both rhodopsin and metarhodopsin would be reduced than in the dark state. Since the dark bumps are generated in part by rhodopsin in toads, the spontaneous dark bumps after bleach with hydroxylamine in *Limulus* will probably be lower than those in the dark-adapted state ([145]Baylor et al., 1980).

**2.4.5 siRNA injections do not abolish bump rates completely.**

In the above experiments, I did not observe a difference in the peak amplitude or the kinetics of falling phase in response to flashes of bright light. This could be due to two reasons. Firstly, metarhodopsin may be partially deactivated when it is phosphorylated. So even in the absence of arrestin, the phosphorylated metarhodopsin is quenched to a large extent and the falling phase of the light is not significantly affected. Secondly, the reduction in arrestin content might not be sufficient to leave a large number of metarhodopsin molecules unquenched. A similar phenomenon has been observed in arrestin mutant mice ([146]Xu et al., 1997). The heterozygous mutants with 33% arrestin protein showed no change in response to light. On the other hand, the null homozygous mutants showed an abnormal falling phase in response to light while the rising phase of light response was unaffected. In *Drosophila* arrestin mutants, there was no change in response to bright light when the arrestin1 expression

was reduced to 10% and 80% respectively ([147]Dolph et al., 1993). However, when the expression of the dominant arrestin, arrestin2 was reduced to 10%, a prolonged depolarized afterpotential (PDA) was observed. This confirms the idea that only a drastic change in arrestin concentration will sufficiently change the concentration of unquenched metarhodopsin that would affect the falling phase of the response.

### 2.4.6 siRNA technique can be a very powerful tool to study *Limulus* phototransduction.

Our experiments indicate that using siRNA might be a feasible option for manipulating the concentration of the proteins involved in *Limulus* phototransduction. This method can be used to explore the function of *trp* and *cGMP* genes that have been sequenced in our lab. siRNA has opened the doors for exploration of a large number of processes in vertebrates and invertebrates alike. The feasibility of siRNA as a potential therapeutic drug is being explored. RNAi in *Limulus* has the potential to be a very powerful tool that can be used to understand the transduction mechanisms better.

# Chapter 3

# Inhibiting *Limulus* arrestin and opsin gene expression using Morpholino Antisense Oligonucleotide

## 3.1 Introduction

The ventral photoreceptor cells of the horseshoe crab *Limulus polyphemus* are highly suitable for studying phototransduction. Their large size renders them ideal for single-unit recordings. They are also amenable to other techniques such as immunocytochemistry and microscopy. However, genetic studies have not been possible so far because the animal takes about 10 years to mature.

We decided to use morpholino oligonucleotides to silence target genes functionally to overcome the problem of genetic manipulation. Phosphorodiamidate morpholino oligomers (PMOs) are potent antisense molecules used to inhibit gene expression by preventing translation or by inhibiting pre-mRNA splicing ([148]Summerton & Weller, 1997;[149] Summerton, 1999). PMOs differ from DNA in that they have a nonionic phosphorodiamidate intersubunit linkage instead of the negatively charged phosphorodiester linkage, and have a six-membered morpholine moiety (morpholine = $C_4H_9NO$) instead of a five-membered deoxyribose ring. The neutral nature of non-ionic PMOs overcomes the limitations of traditional nucleotides in that they are not toxic at high concentrations, do not have non-specific interactions with cellular proteins, and are more amenable to delivery in conjugation with cationic

peptides ([150]Iversen, 2001;[151] Summerton et al., 1997). The morpholine ring renders them resistant to enzymes and degradation ([152]Hudziak et al., 1996). In spite of these differences, they bind to complementary sequences by Watson-Crick pairing. These and other properties have made morpholinos very popular in the past decade, especially in the field of vertebrate developmental biology ([153]Heasman, 2002). They have been used in *Xenopus laevis,* Zebrafish, Sea urchin, and snails ([154]Haesman, 2000; [155]Nasevicius & Ekker, 2000; [156]Howard et al., 2001; [157]Bogulavsky et al., 2003). We decided to use this technique on two target proteins; arrestin and opsin.

Arrestin is a protein involved in quenching of metarhodopsin. Metarhodopsin is formed by photoconversion of rhodopsin in which the chromophore 11-cis-retinal isomerizes to form all-trans-retinal. Metarhodopsin initiates excitation and the ensuing cascade of events results in a change in membrane potential. It was first shown in vertebrates that quenching of metarhodopsin is a two-step process. Firstly, metarhodopsin gets phosphorylated by rhodopsin kinase and subsequently it binds to arrestin for complete silencing of activity ([158]Kuhn et al., 1984; [159]Palczewski & Benovic, 1991). In *Drosophila*, genes encoding two types of visual arrestins have been isolated, arrestin 1 (*arr1*) and arrestin 2 (*arr2*) ([160]Hyde et al., 1990; [161]Smith et al., 1990; [162]Levine et al., 1990). Arrestin in *Drosophila* mimics its role in vertebrates. Severe arrestin mutants cause a prolonged depolarizing afterpotential (PDA) indicating their role in metarhodopsin inactivation ([163]Dolph et al., 1993).

Dark noise in *Limulus* is seen in the form of events called "bumps". The bumps appear as small (1-10mV) depolarizing events. To reduce noise, metarhodopsin needs to be kept quenched. Since arrestin is involved in inactivating metarhodopsin, arrestin plays an important role in reducing noise. The question addressed here is whether injecting arrestin morpholino into the *Limulus* ventral photoreceptor would reduce the arrestin mRNA. If it does, the arrestin protein concentration would go down and we would observe an increase in noise levels. To complement these studies, we decided to use opsin. A reduction in opsin protein content would presumably lead to a reduction in sensitivity to light, and decrease dark noise.

There is one known arrestin gene in *Limulus* and two opsin genes (opsin1 & opsin2) ([164]Smith et al., 1995; [165]Smith et al., 1993). The morpholinos were designed against the region near the start codon. The sequence of this region is identical in the two *Limulus* opsin genes so we expect the morpholino sequence to target both these genes. We injected known concentrations of the morpholinos into the ventral photoreceptors and used predicted changes in the dark noise and sensitivity to light as a measure of success. We observed an increase in dark noise in the presence of arrestin morpholino and a decrease in sensitivity to light in the presence of opsin morpholino.

## 3.2 MATERIALS and METHODS

### 3.2.1 Preparation of the nerve

Adult male horseshoe crabs, *Limulus polyphemus*, measuring about 5-7 inches across the carapace were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts. The crabs were maintained on a 12 hr light/dark cycle. The ventral nerve was dissected as described by [166]Clark et al. (1969). The nerves were kept in artificial sea water (ASW) that contained (in mM): 435 NaCl, 10 KCl, 20 $MgCl_2$, 25 $MgSO_4$, 10 $CaCl_2$, and 10 HEPES. The nerves were desheathed, and treated with 1% pronase (Calbiochem, San Diego, CA) for 1 min.

### 3.2.2 Morpholino injections

Three different morpholinos were used in this study. One was directed against the 16-40 nt of *Limulus* arrestin and had the sequence 5'-AATCATTGGGCTGCCGTTTTACTTT -3'. A second morpholino was directed against 16-40 nt of *Limulus* opsin and had a sequence of 5'-GGCATTACCATTAGTTGACTCGAT-3'. The third morpholino was an unrelated standard control 5'-CCTCTTACCTCAGTTACAATTTATA-3'. All morpholinos were designed and prepared by Gene Tools (Philomath, OR, USA).

Three concentrations of all of these PMOs were injected into cells, 0.1 µM, 1 µM, and 10 µM, all diluted in a carrier solution (100mM potassium aspartate, 10 mM

Hepes, pH 7.0). PMO was delivered into the cells by pulse-pressure injections with a glass micropipette of 20MΩ resistance. The nerves were kept in a moist chamber covered with aluminum foil at $10^0$C. Control nerves with cells injected with the carrier solution were also treated similarly.

Ten injections were made in each cell with a total volume of injected solution being ~ 120pl. This resulted in a final concentration of ~17pl inside the cell. The reliability of the injections was monitored by observing a disturbance in the cell's cytoplasm with each pressure pulse.

### 3.2.3 Electrophysiology

A glass micropipette containing PMO (resistance ~ 20MΩ) was used to test the bump rate before injections. The cells were kept in the dark for about 30 min so that their bump rate was at the dark adapted level (1/s). Arrangement of light source and neutral density (ND) filters was similar to that described in [167]Wang et al., (2002). Attenuation by $\log_{10}$ -10 typically elicited about one single photon event per second. Before exposing the cells to the next flash of light, the cells were kept in dark till the spontaneous bump rate recovered to that of dark-adapted state.

The spontaneous dark bump rate, bump rate in response to dim light, and membrane depolarization in response to bright light were recorded for uninjected cells. The same micropipette was used to inject morpholino into cells. The cells were kept in a chamber covered with aluminum foil at $10^o$C. At 24, 48, 72, 96, 108, 120,

and 144 hrs after injections (n = at least 6), the injected and uninjected cells were tested for the spontaneous dark bump rate, bump rate in response to dim light, and membrane depolarization in response to bright light

### 3.2.4 Immunocytochemistry

The anti-arrestin and anti-opsin antibodies were a generous gift from Dr. Barbara Battelle (The Whitney Laboratory & The Department of Neuroscience, Univ. of Florida). The antibody treatment protocol as described in Battelle at al., (2001) was used. Briefly, nerves left in the modified organ culture medium (MOCM) overnight in the dark were treated with 4% paraformaldehyde and 0.1M PBS for 2 hrs. They were then washed with PBS, permeabilized by incubation for 1 hour with PBST (PBS plus 0.1% Triton X-100), blocked for 1 hour with PBST containing 2% BSA and 2% normal goat serum, and rinsed with 2% BSA in PBST (PBST/BSA).The nerves were then incubated for 1 hour at room temperature with 1:200 dilutions of the primary antibodies in PBST/BSA, and rinsed three times with PBST/BSA. The secondary antibody conjugated to Molecular Probes' Alexa Fluor 488 fluorescent dye was diluted 1:200 in PBST/BSA. The nerves were incubated with this antibody for 1 hr at room temperature. Finally, the nerves were rinsed three times with PBS. Immunofluorescence staining was detected using a Zeiss LSM 510 laser-scanning confocal microscope.

### 3.2.5 Confocal microscopy

The immunostained nerves were imaged on a Zeiss LSM 510 laser-scanning confocal microscope (Zeiss, Germany) using a 40X oil lens. The secondary antibodies for arrestin and opsin were excited using a 488nm line of the Argon ion laser and detected after passing through a BP505-550 emission filter. Alexa fluor 568 10,000 MW dextran conjugate (Molecular Probes, OR) was used as a marker for morpholino injections. It was injected with morpholino at a final concentration of 200 µM. Dextrans are water soluble inert polysaccharides that have low toxicity. They have been used as markers for microinjection ([168]Pepperkok et al., 1988). The dextran dye was excited by a 543nm light from the green HeNe laser and imaged through the LP 560 emission filter. The sections were scanned in a multi-track mode. One track was used for dextran fluorescence (presence of fluorescence indicated that the cell had been injected) and a second track was used for the antibody fluorescence. Zeiss LSM image browser was used to acquire images. The images were exported to the NIH image J software (http://rsb.info.nih.gov/ij/). The histogram tool of image-J was used to quantify the fluorescence counts.

## 3.3 RESULTS

### 3.3.1 Arrestin morpholino leads to a 5-fold increase in bump rate 24 hrs after injection.

In previous studies with arrestin RNAi injections, we showed that arrestin suppression is most effective at 24 hrs after injection (section 2.3.2). To investigate the effects of arrestin morpholino, we decided to use this time-point and a later time point (48 hrs), which would give us an insight into the longevity of the morpholino-induced effect. We compared uninjected cells, cells injected with a control morpholino, and cells injected with 1 μM arrestin morpholino. We tested them at 24 hrs and 48 hrs after injection. The cells were "mapped" while injecting the morpholino by drawing a diagram of the nerve with cells and marking the injected and uninjected cells on this diagram.

Representative traces from cells in these three categories (uninjected, injected with control morpholino, and injected with arrestin morpholino) are shown in Fig.3.1. The traces of uninjected control, and cells injected with the control morpholino look identical. However, in the cell injected with arrestin morpholino there appear to be many more bumps. Quantification of the data is indicated in Fig. 3.2A. The dark bump rate in cells injected with arrestin morpholino and tested 24 hrs after injection was $5.1 \pm 0.4$. This was about a five-fold increase over that of the bump rate in uninjected cells ($1 \pm 0.1$) ($p<0.05$), and in cells injected with control morpholino (1.2

± 0.1). This mimicked a previous result obtained with arrestin RNAi injections. At 48 hrs after injection, there was no (p=0.55) further increase in the average bump rate beyond the 24 hr time-point.

The responses of cells to 200ms flashes of bright light ($log_{10}$-3 to $log_{10}$0 in increments of 1) were also tested. The parameters recorded were the peak amplitude and the width at half the peak amplitude. The response to bright light in all categories of cells was same as that in the control cells.

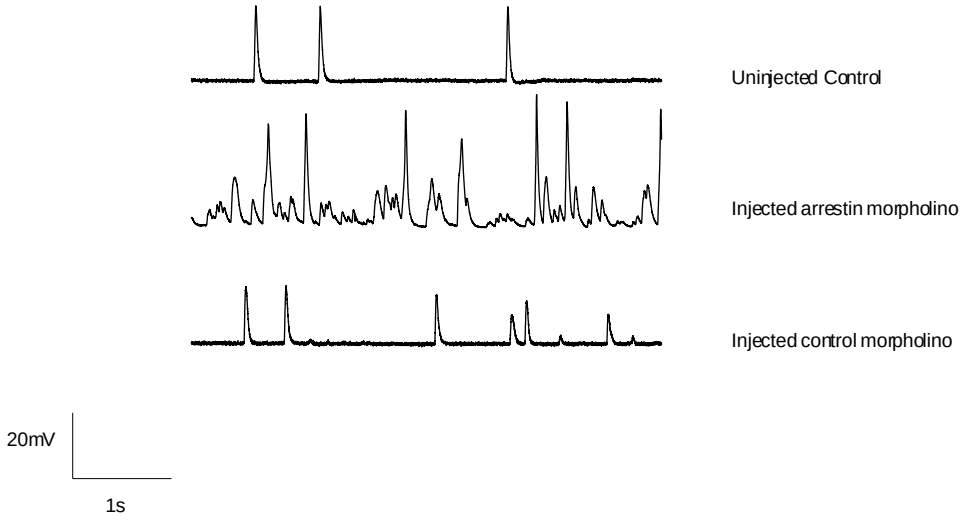



**Figure 3.1:** 1 µM arrestin morpholino injections lead to an increase in dark bumps. Shown above are representative traces from cells under three conditions. The trace on top is from the uninjected control and shows the normal bump rate. The middle trace shows the increase in bumps when cells are injected with arrestin morpholino and tested 24 hrs later. The trace at the bottom is from a cell that had been injected with a

control morpholino and tested at 24 hrs. This trace indicates that the process of injecting 25nt long morpholino in itself does not have any effect on the bump rate.

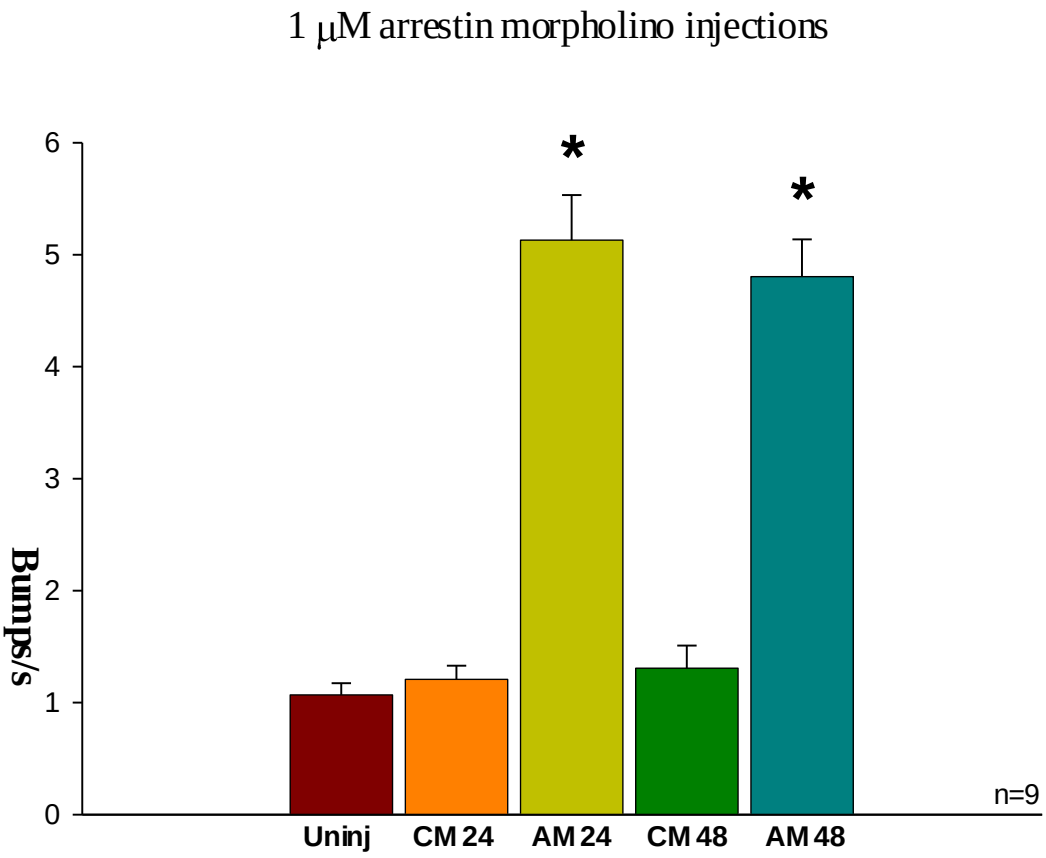



**Figure 3.2:** Dark bumps in cells injected with arrestin morpholino. Cells were injected with 1 μM arrestin morpholino and checked at 24 and 48 hrs after injections for their bump rate. Uninj= uninjected control cells CM= cells injected with the control morpholino AM= cells injected with the arrestin morpholino. The bumps in cells tested at 24 hrs after injection (AM 24) (5.1 $\pm$ 0.4) and 48 hrs after injection (AM 48) (4.8 $\pm$ 0.3) show about a 5-fold increase as compared to cells injected with control morpholino and tested at similar times (1.2 $\pm$ 0.1 and 1.3 $\pm$ 0.2 respectively). Control morpholino did not induce any increase in bump rate when compared to the uninjected cells (1 $\pm$ 0.1). n=9. Error bars indicate SEM.

### 3.3.2 Dose-dependence in arrestin morpholino injected cells

Typically in oligonucleotide studies, multiple sequences and/or multiple concentrations are used to verify that the effect seen is not spurious. We used two additional concentrations of arrestin morpholino (0.1 μM, and 10 μM) with corresponding concentrations of control morpholinos. We tested the effect of these concentrations at 24 hrs after injection (Fig. 3.3). Cells injected with 0.1 μM arrestin morpholino had a bump rate of $3 \pm 0.3$ which is lower than that observed with 1μM arrestin morpholino injections ($4.8 \pm 0.33$). However, with 10 μM morpholino injections, we did not observe any further ($p=0.71$) increase in bump rate as compared to 1 μM morpholino injections. This indicates that morpholino injections at 1μM may have a saturating effect.

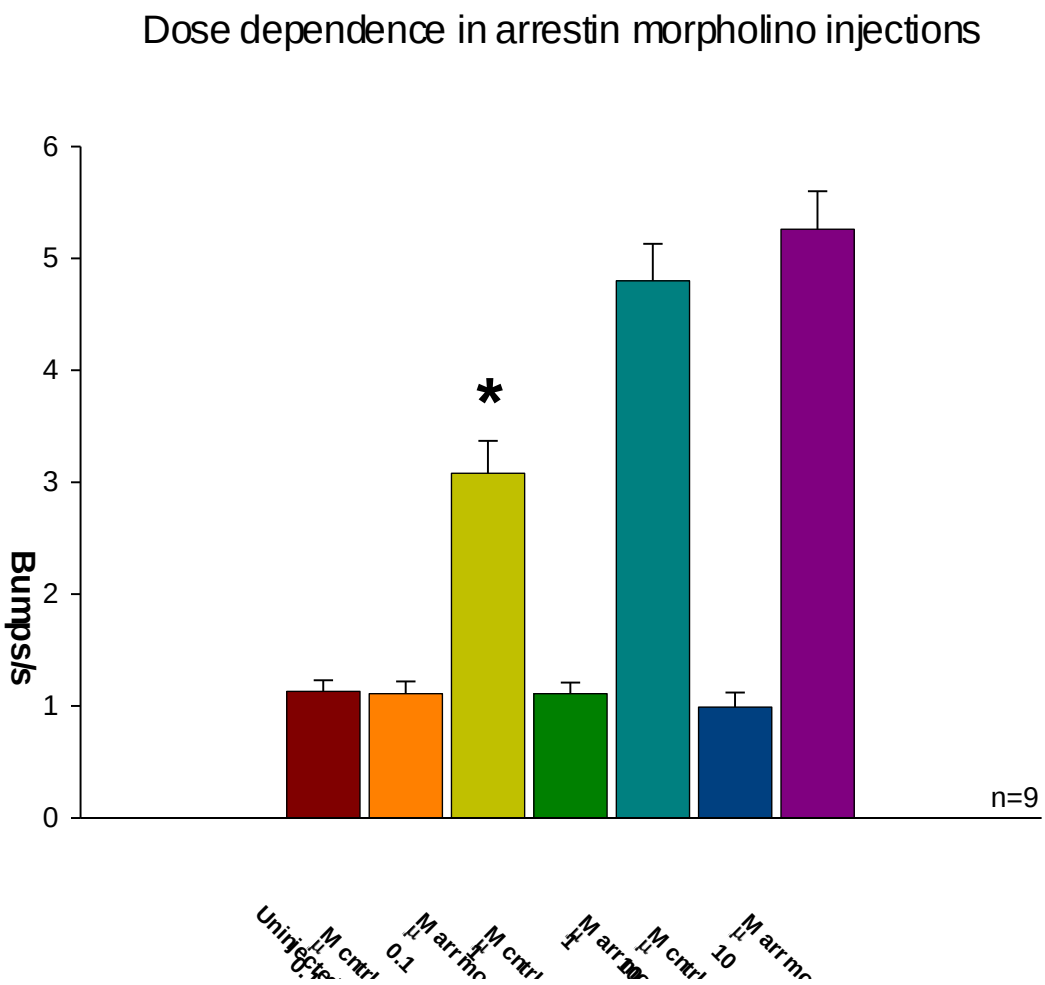



**Figure 3.3:** Arrestin morpholino injections show a dose-dependent effect. Bump rates in cells injected with 0.1 µM, 1 µM, and 10 µM arrestin morpholino were compared. Control morpholinos at corresoponding concentrations were used as controls. At 0.1 µM injections, cells have a bump rate of 3 $\pm$ 0.3, at 1 µM they have a bump rate of 4.8 $\pm$ 0.33, and at 10 µM they have a rate of 5.3 $\pm$ 0.35. n=9. Error bars indicate SEM.

### 3.4 Opsin morpholino Injections

#### 3.4.1 Opsin morpholino injections reduce dark noise

To verify that the morpholino technique works in *Limulus* ventral photoreceptors, we decided to check its effect on a different protein. Since the previous experiments led to a gain-of-function response, we decided to use a protein that would lead to a loss-of-function response. Opsin seemed to be an ideal candidate for this purpose.

One μM opsin morpholino was injected into cells. Time-points selected were 24, 48, 72, 96, 108, 120, and 144 hrs after injections. It was possible to extend the time point until six days since the morpholino does not get degraded and also because the *Limulus* nerve preparations stay responsive when kept in the culture medium ([169]Hudziak et al., 1996; [170]Bayer et al., 1978). As shown in Fig. 3.4, at 96 hrs after injections, opsin morpholino reduced the dark bump rate from 1/s to 0.4/s ($p<0.05$). This is consistent with the role of opsins (rhodopsin and/or metarhodopsin) in generating the spontaneous dark bumps.

Even at 144 hrs after injections, the spontaneous dark bump rate is $0.36\pm0.07$. The bump rate does not go down to zero. This occurs probably not because of reduced concentration of morpholino over time since as mentioned above; morpholino does not get degraded in the cells. One possibility is that the concentration of opsin morpholino in the cell body may decrease over time if the morpholino moves down the axons. This reduced concentration might be ineffective

in interrupting translation and could therefore result in the residual bumps observed at 144 hrs after injections. Another possibility is that the opsin turnover rate is low in dissected cells and the levels of opsin protein do not decrease further in spite of waiting 6 days after injections. A third possibility if that the residual bumps are formed by sources other than rhodopsin and/or metarhodopsin such as the G-protein.

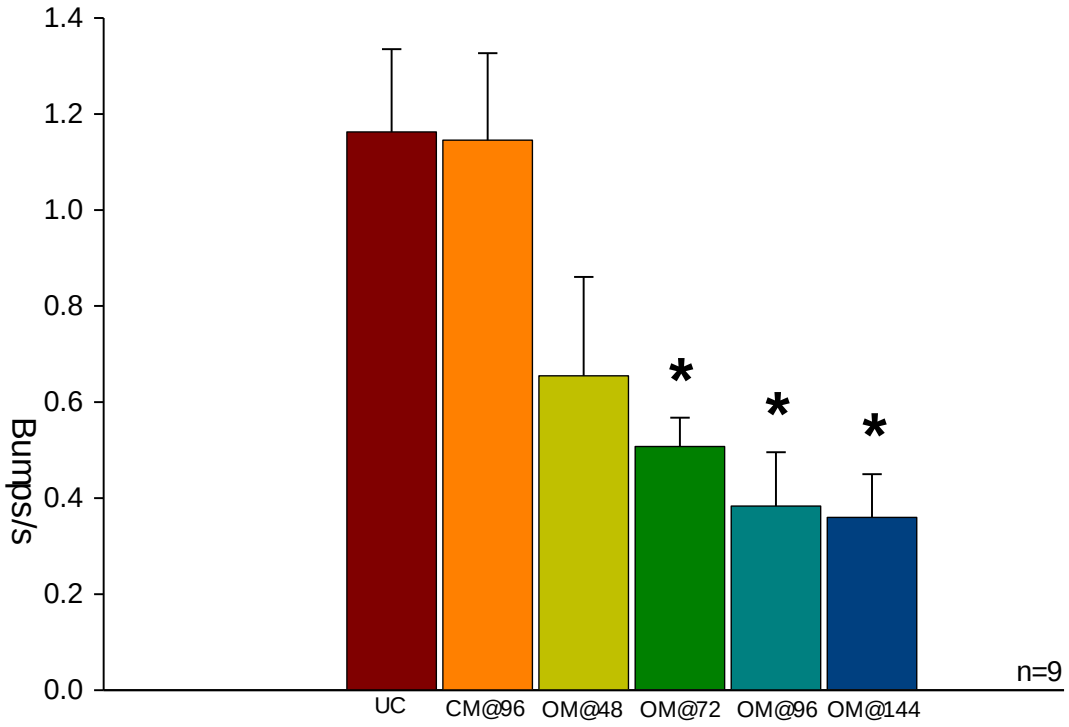



**Figure 3.4:** Dark bump rate in cells injected with 1 uM opsin morpholino and checked at various time points. UC = uninjected control CM = control mprpholino OM = opsin morpholino. 48, 72, 96, and 144 = hrs after injection. The bump rate in uninjected control cells and cells injected with the control morpholino is the same. However, on opsin morpholino injections the bump rate starts declining. The bump

rate is lowest at 96 hrs after injections (0.4/s). There is no further significant decrease beyond 96 hrs. n=8. Error bars indicate SEM.

### 3.4.2 Opsin morpholino injections lead to a 1.5 $\log_{10}$ reduction in sensitivity at 96 hrs after injection.

We decided to explore the effect of 1 µM opsin morpholino injections by checking the cells for their light sensitivity. Cells were tested 24, 48, 72, 96, 108, 120, and 144 hrs after injections. We observed their response to dim light of intensities ranging from $\log_{10}$ -10 to $\log_{10}$ - 7 in increments of 0.5. In all cases, the bump rate was lower in cells injected with opsin morpholino as compared to cells injected with control morpholino, or those not injected at all (Fig. 3.5). However, the largest difference, reduction in light sensitivity by 1.5 $\log_{10}$, was observed at 96 hrs after injection.

These results are consistent with opsin morpholino reducing the rhodopsin content.

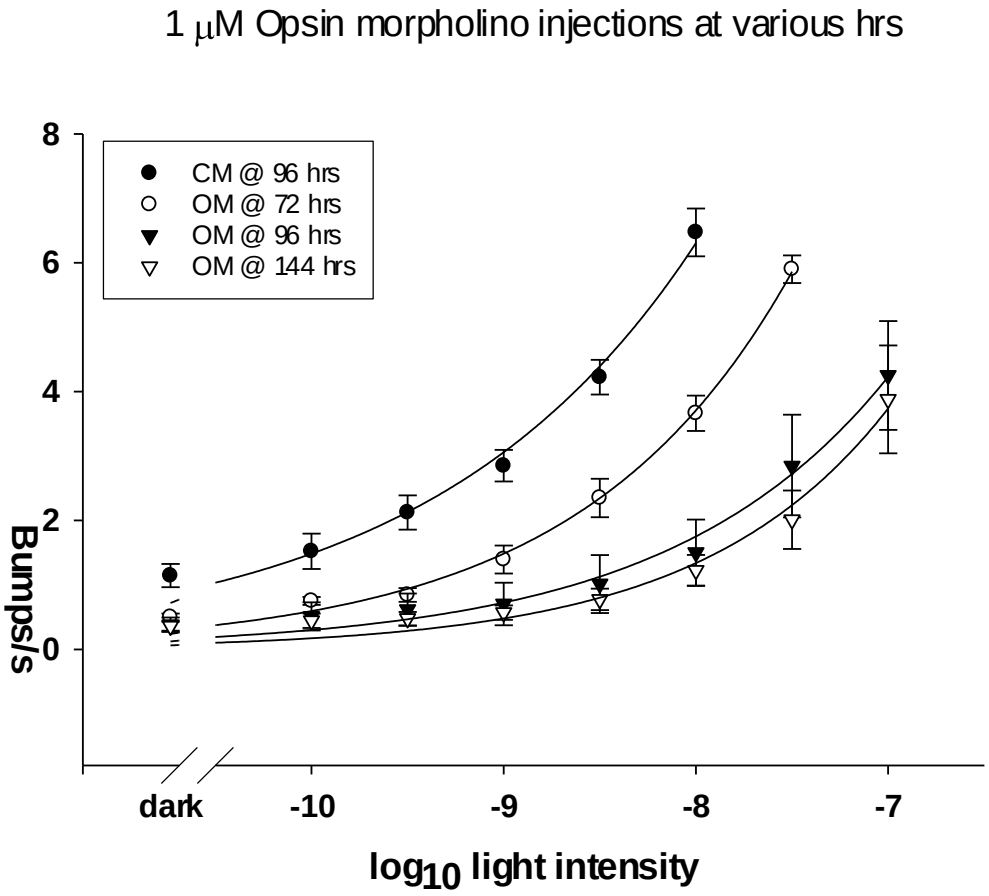



**Figure 3.5:** Effect of opsin morpholino injections on the bump rate. CM= control morpholino OM= opsin morpholino. Cells were injected with 1 μM opsin morpholino. This figure indicates representative time-points at which the response of cells to the dark and dim light was measured. With increasing time after injections, the bump rate gets lower at all intensities including darkness. At 96 hrs after injection, the sensitivity of cells to light is decreased by 1.5 $\log_{10}$ units. n= 9. Error bars indicate SEM.

### 3.4.3 Bump rates in response to opsin morpholino injections show a dose-dependent effect

To investigate dose-dependence of opsin morpholino injections, we injected 0.1 µM, 1 µM, and 10 µM into cells and tested the bump rate at 96 hrs after injection. As indicated in Fig. 3.6, after 0.1 µM opsin morpholino injections, the bump rate is lower than after control morpholino injections at all time points tested. There is a further decrease in bump rate after 1 µM opsin morpholino injections. However, no dose dependence was observed in cells when they were tested with 10 µM opsin morpholino injections. This could probably be explained if morpholino at 1µM has a saturating effect and 10 µM injections have no additional effect on the sensitivity of cells.

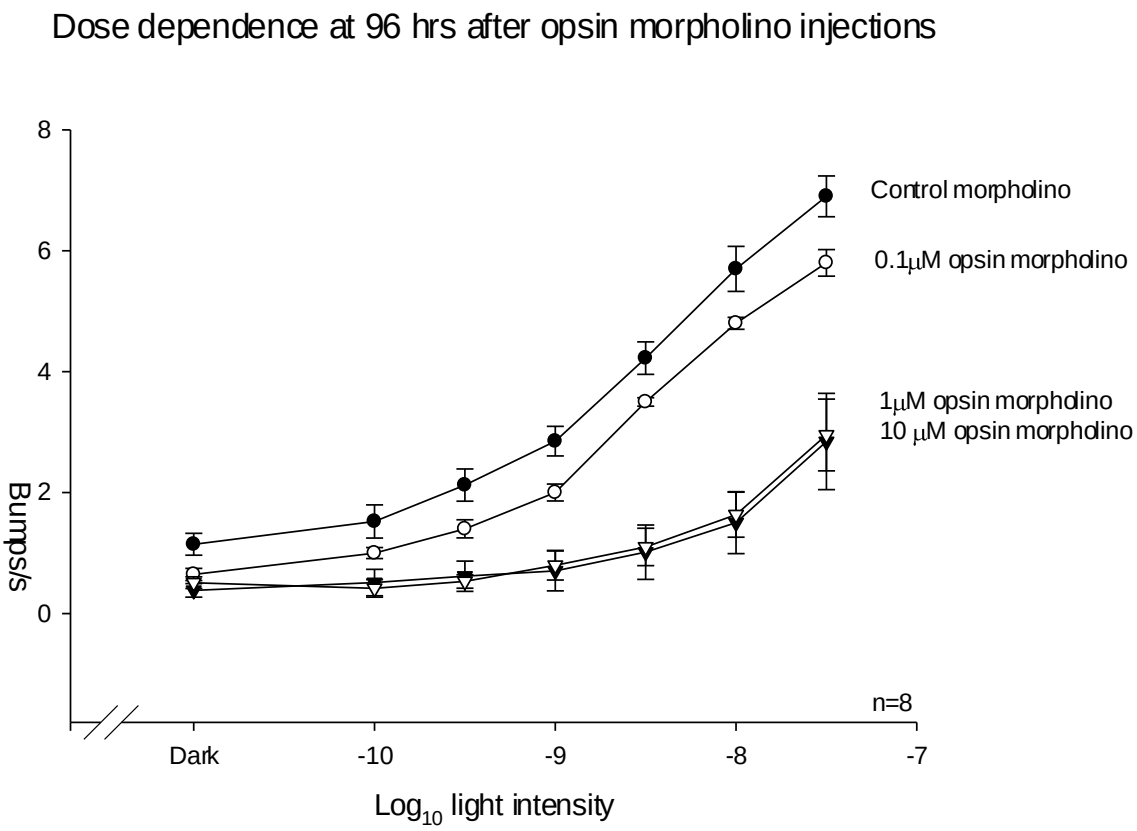



**Figure 3.6:** Varying concentrations of opsin morpholino injections. Cells were injected with 0.1 μM, 1 μM, and 10 μM opsin morpholino. The bump rate in 0.1 μM injected cells is lower than control morpholino injected cells at all time points tested. There is a further decrease in bump rate in cells injected with 1 μM opsin morpholino. After 10 μM opsin injections, there is no further decrease in bump rate. n=8 Error bars indicate SEM.

### 3.4.4 Light sensitivity decreases in opsin morpholino injected cells.

The reduction in sensitivity occurred gradually over time. Shown in fig. 3.7 are two representative traces, one in the dark and one in response to log-8 light

intensity. Both traces show a decrease in bump rate over time. As shown in fig. 3.7A the largest drop in sensitivity occurs between 48-96 hrs after injections. Beyond 96 hrs, there is no significant decrease in sensitivity. The decrease in the spontaneous dark bump rate occurs earlier, by 48 hrs (Fig. 3.7b). The biggest drop observed is not immediate. This indicates that the drop in bump rate is not due to damage during electrode withdrawal or reimpalement. Both the reduction in the dark, and light-induced bumps, are consistent with a reduction in opsin concentration as a result of the morpholino injections.

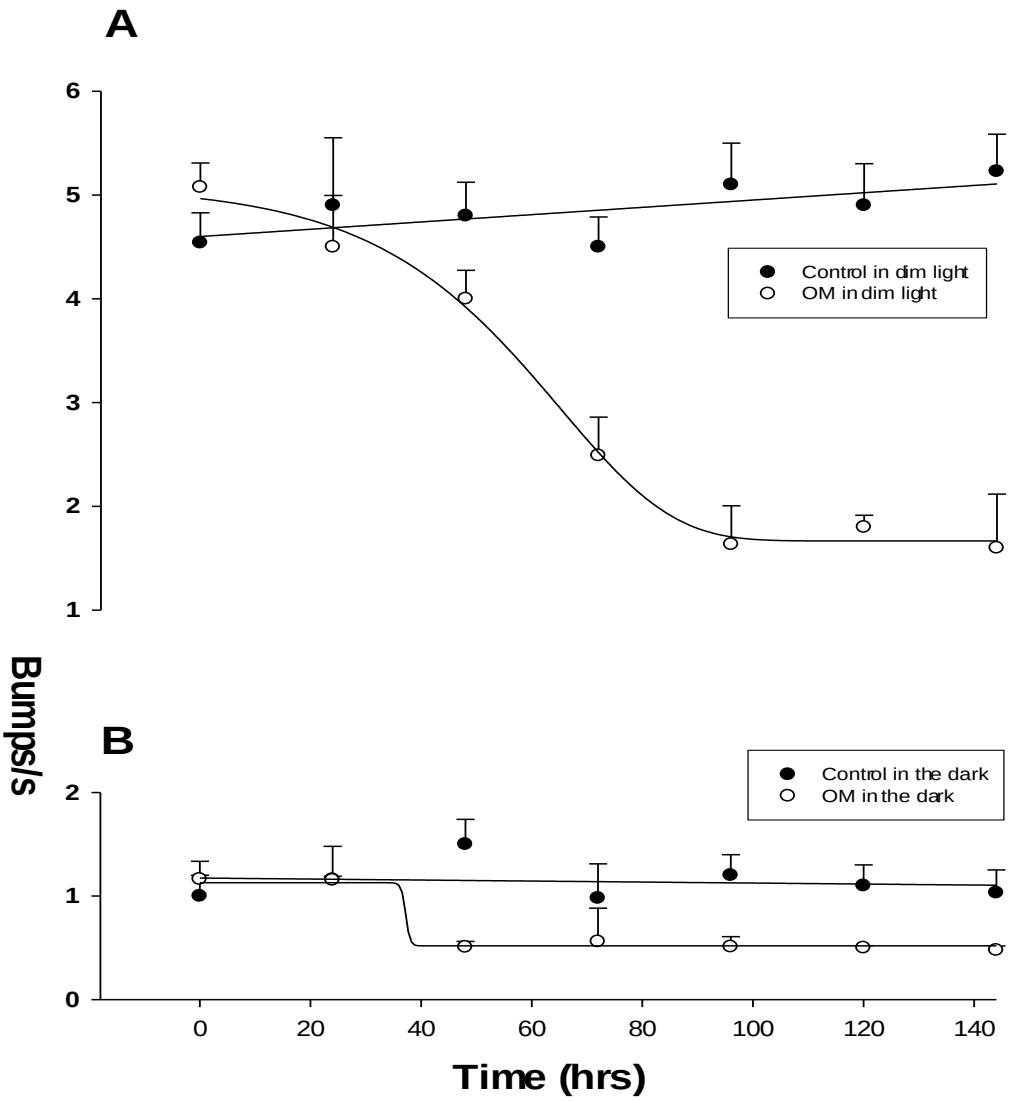



**Figure 3.7:** Sensitivity to light decreases with time. OM = cells injected with opsin morpholino A) Response to $\log_{10}$ -8 light is plotted over time in cells injected with 1 μM opsin morpholino. The bump rate decreases with time. In control cells, the bump rate remains constant. B) Bump rate in the dark is plotted in cells injected with 1 μM opsin morpholino. With time, there is a decrease in the bump rate in injected cells while the bump rate in control cells remains constant. The bump rate in injected cells is fitted with a sigmoid curve while that in control cells is fitted with a straight line. n=8. Error bars indicate SEM.

### 3.4.5 Bump amplitude is not reduced in injected cells

We addressed the issue of whether the light induced bumps generated in the presence of opsin morpholino had similar characteristics to the light induced bumps in control cells. For this, we selected traces from injected and uninjected cells that had similar bump rate. We then analyzed the average bump amplitude in these traces. As shown in table 3.1, this process confirmed the decreased sensitivity shown in fig. 3.7 since the bump rate generated by $\log_{10}$-9 light in control cells was achieved by shining $\log_{10}$ -7.5 light on morpholino injected cells. The natural variation in the bump amplitude of uninjected cells is pretty large ($4.2\pm0.9$). This has been attributed to the multi-step deactivation of metarhodopsin ([171]Kirkwood & Lisman, 1994). The average bump amplitude in these two categories of cells was not significantly different ($p=0.22$). This indicates that although there may be reduction in opsin, bumps generated by the remaining opsin have the same amplification characteristics. We also tested these cells for their dark bumps and their amplitudes were also statistically similar ($p>0.05$).

| | Bump rate | Light intensity | Light bump amplitude | Dark bump amplitude |
|---|---|---|---|---|
| Control cells | 2.4±0.1 | 9 ± 0.2 | 4.2±0.9 | 1.36±0.2 |
| Cells injected with OM | 3.1±0.7 | 7.5 ±0.1 | 2.8±0.5 | 1.26 ± 0.2 |

**Table 3.1:** Properties of bumps in the two categories of cells. OM = opsin morpholino. Light intensities were selected such that the bump rate would be between 2-3/s. At these light intensities, the amplitude of bumps was compared. The bump amplitude in morpholino injected cells was not statistically different from that in control cells (P=0.22). The cells had similar dark bump amplitude (P=0.74) indicating that the cells were healthy and bumping normally.

### 3.4.6 Peak amplitude is reduced in response to a saturating flash of light

As indicated in Fig. 3.8, the peak amplitude in response to a 200ms flash of saturating ($\log_{10}0$) light is lower in cells injected with opsin morpholino. This is consistent with the decrease in quantal sensitivity observed in dim light. However, this is not an accurate measure of sensitivity since the peak amplitude is a function of sensitivity and driving force. This is the force driving the ion flux changes and is dependent on the membrane potential. Besides a decrease in quantal sensitivity, the decrease in the amplitude of the saturating response could also be contributed in part by a decrease in the driving force mediated by a drift in the membrane potential.

Therefore, the response to a non-saturating light might give a better estimate of sensitivity.

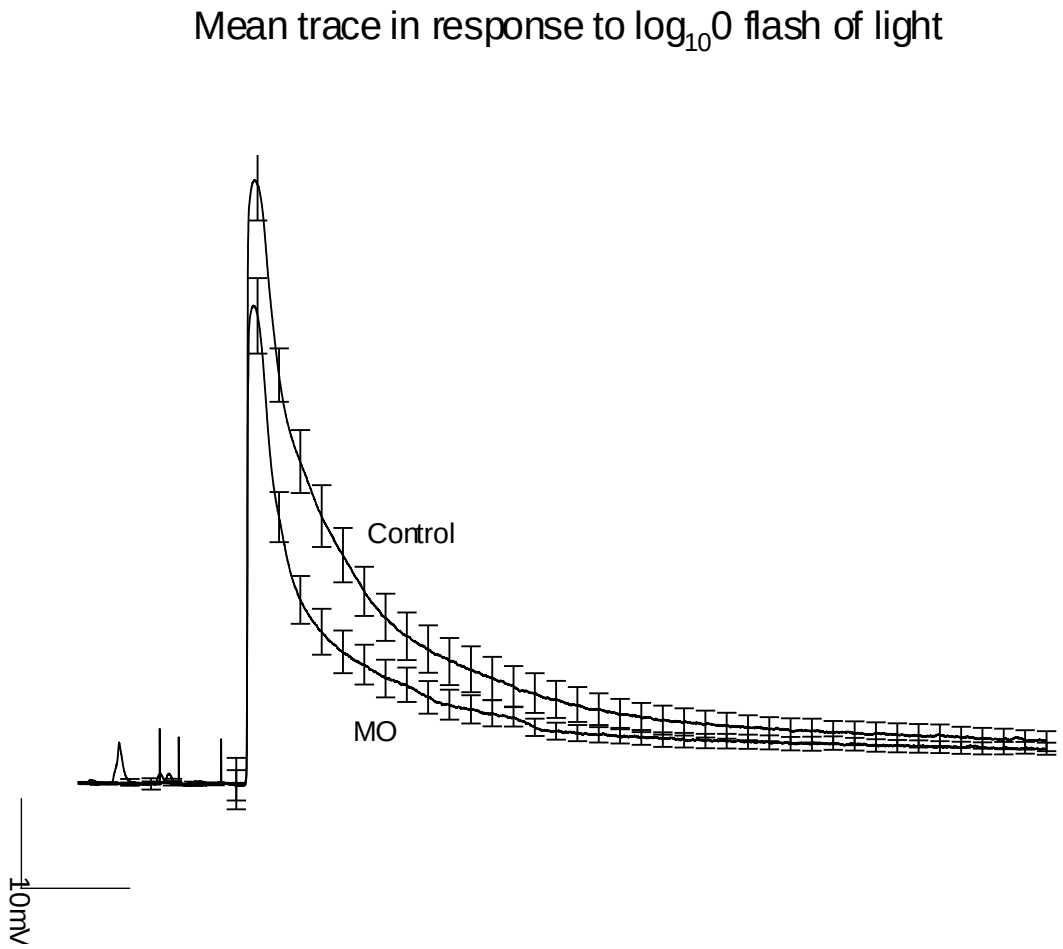



**Figure 3.8:** Response to a 200ms saturating flash ($log_{10}0$) is compared between cells injected with opsin morpholino (MO) and control cells. The reduction in peak amplitude and faster recovery kinetics are consistent with a reduction in opsin. n= 8. Error bars indicate SEM.

### 3.4.7 Response kinetics affected in the presence of a non-saturating flash of light

Cells were exposed to a 200ms flash of $\log_{10}$-4 light. At this intensity, the response of the cell is not saturated. As compared to control cells, opsin morpholino injected cells had smaller peak amplitude and the response terminated earlier (Fig. 3.9). This is consistent with the decrease in sensitivity of cells to light as observed in the analysis of bump responses.

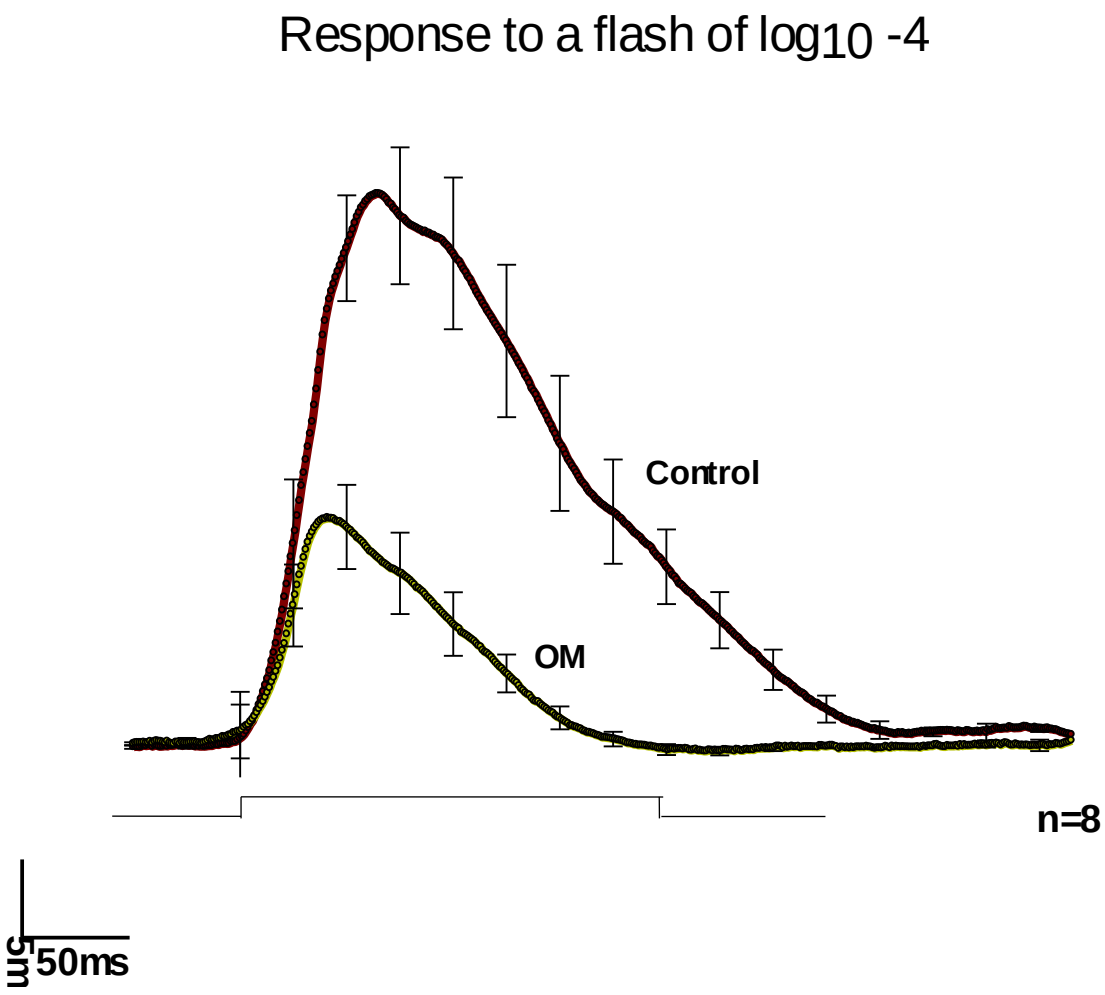



**Figure 3.9:** Response to a 200ms non-saturating flash ($\log_{10}$-4) is compared between opsin morpholino injected (MO) and control cells. Reduction in peak amplitude is

consistent with a reduction in opsin levels and mimics the decreased bump rate observed in response to dim light. n=8. Error bars indicate SEM.

### 3.5 Reduction in protein levels after morpholino injections

As described in methods, cells injected with either arrestin or opsin morpholino were co-injected with dextran. Alexa fluor 568 10,000 MW dextran conjugate is a fluorescent dye that is water soluble and has low toxicity. This dye was used to mark the cells that had been injected with opsin morpholino. The nerves were fixed and probed with antibodies against opsin and arrestin. The fixed nerves were imaged on an LSM 510 confocal microscope. For analysis of fluorescence, cells were imaged in optical sections via a focus series (z-series).

#### 3.5.1 Dextran injections do not damage the cell

We first assessed the dextran fluorescence pattern by injecting cells with dextran alone (Fig. 3.10). This also gave us an opportunity to observe that dextran injections did not cause any bloating or shrinking in cells. We also checked the arrestin/opsin fluorescence in the presence of dextran injections. The secondary antibodies for arrestin and opsin were excited using a 488nm laser and detected after passing through a BP 505-550 emission filter. The dextran dye was excited by a 543nm laser and imaged through the LP 560 emission filter. Once we verified that dextran injections do not interfere with arrestin/opsin fluorescence, we then co-

injected the arrestin/opsin morpholino with dextran. Cells with dextran fluorescence were marked as being injected. Fluorescent counts of for stacks of these cells were compared with similar counts in uninjected cells.

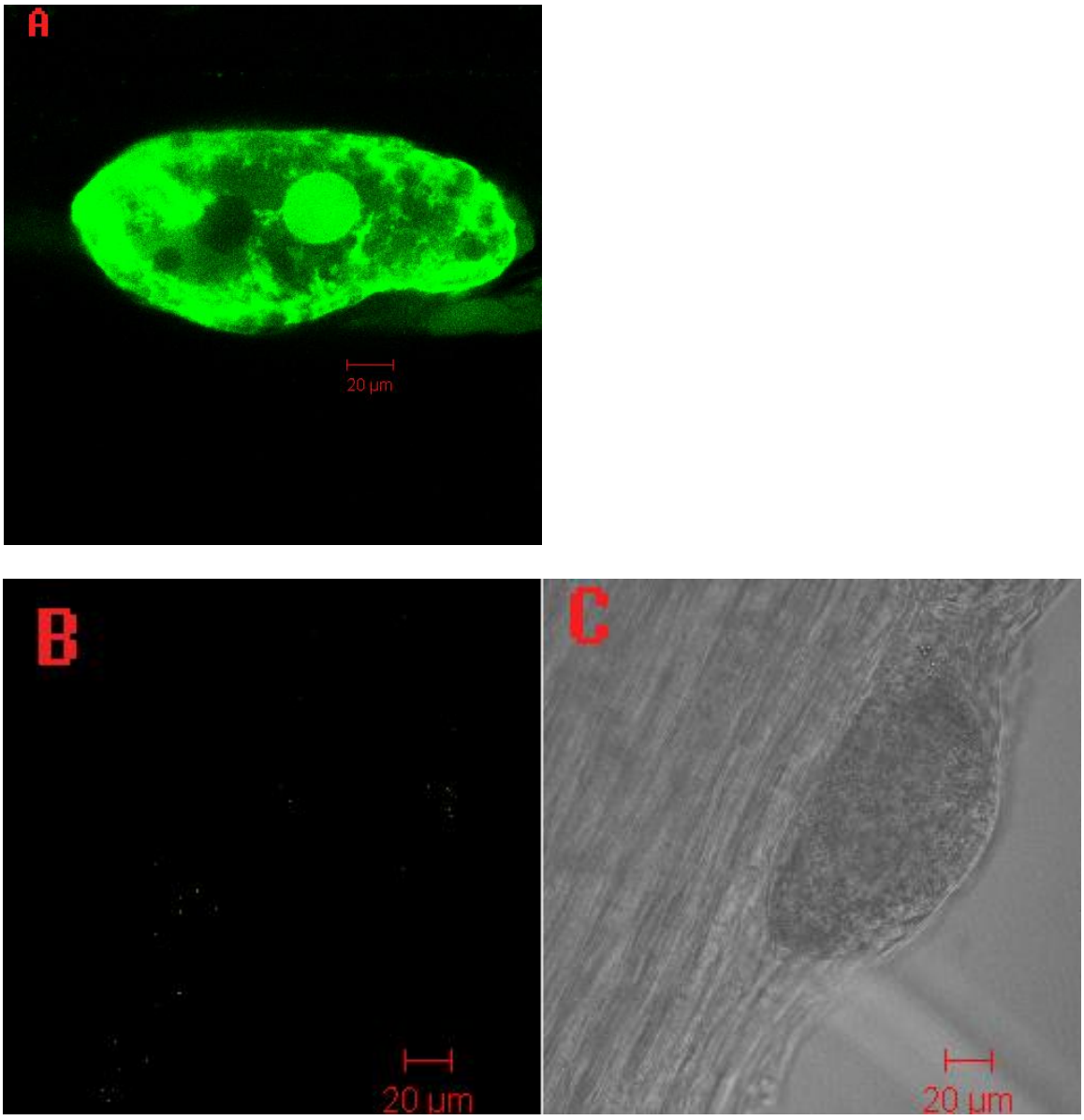



**Figure 3.10:** Immunofluorescece micrograph of dextran staining. A) Cell injected with dextran. The cell does not look damaged, bloated, or shrunken. B) Image of a cell not injected with dextran. No immunofluorescence is observed. C) Phase-contrast image of the cell in B.

### 3.5.2 Arrestin morpholino injections lead to a 2.5 fold reduction in fluorescent counts

Cells were co- injected with arrestin morpholino and dextran, and fixed at 24 hrs after injection. The fixed cells were imaged at the confocal microscope. Control cells were uninjected cells from the same nerve. As shown in Fig. 3.11, cells injected with arrestin morpholino were less bright and had a diffused pattern of fluorescence. Confocal z-section analysis of the stacks of images obtained from the cells was used to calculate the fluorescent counts from the images. As shown in Fig. 3.12, there is a 2.5 fold reduction in fluorescent counts in injected cells as compared to the counts in uninjected cells.

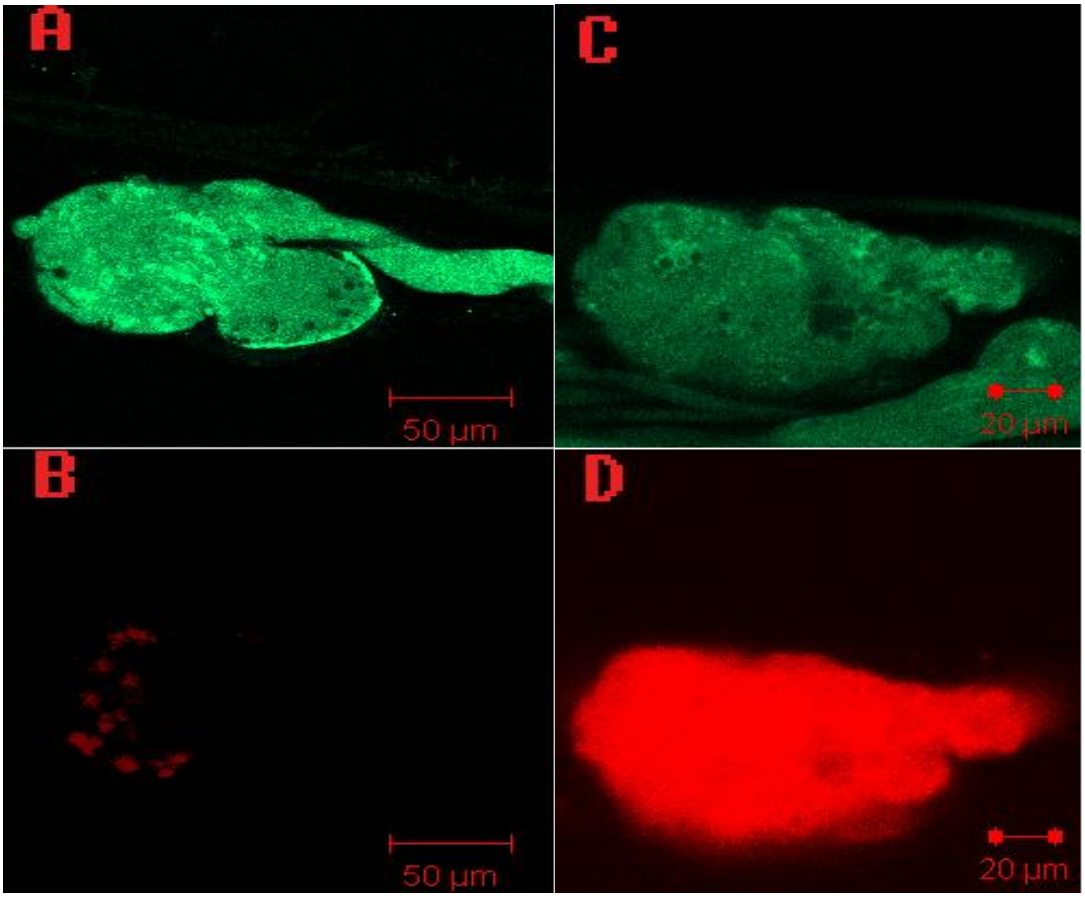



**Figure 3.11:** Fluorescent counts are lower in the images of cells injected with arrestin morpholino. Immunofluorescence micrograph recorded from cells that have been probed with anti-arrestin antibody. The green indicates arrestin and red indicates dextran fluorescence. A and B are images from a cell that has not been injected with arrestin morpholino and dextran. C and D are images from a cell that has been injected. A- Cell with a characteristic arrestin staining pattern. B- Absence of dextran fluorescence indicates that the cell was not injected C- Arrestin staining is diffused and less bright. D- Cell injected with morpholino as indicated by the dextran staining.

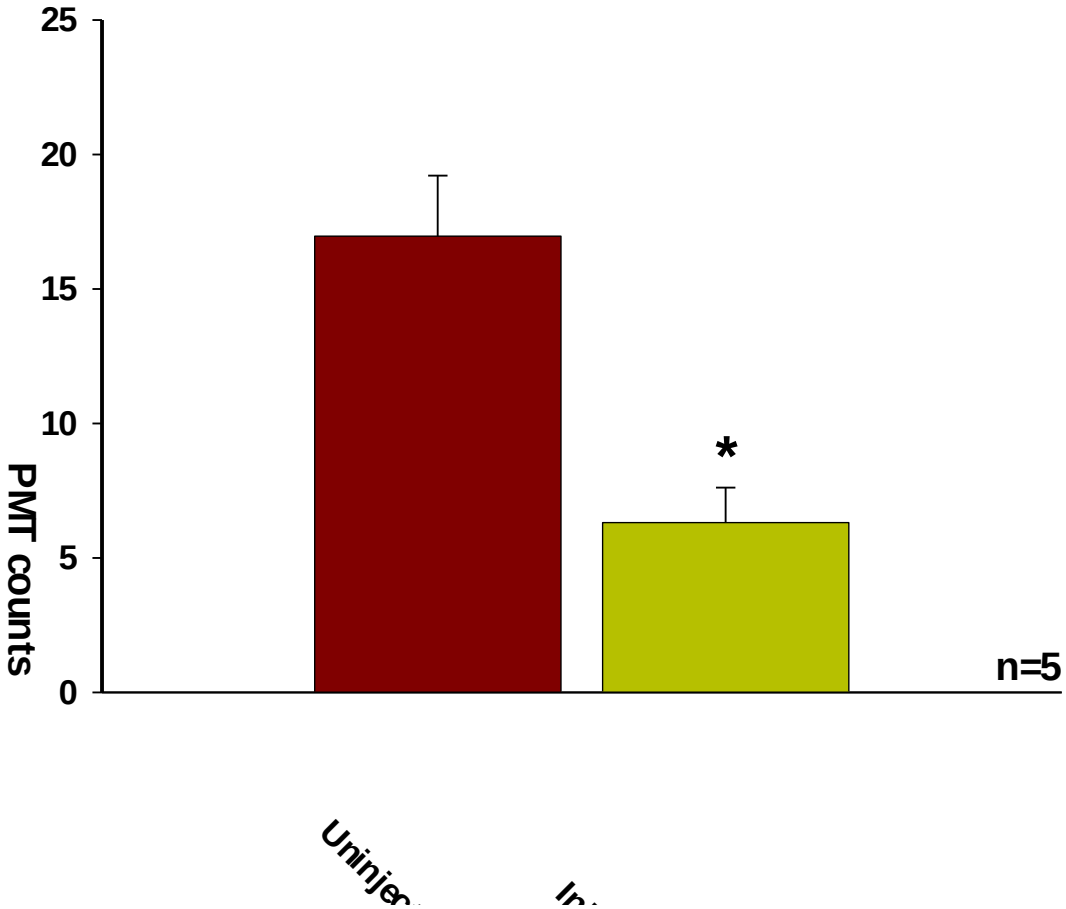
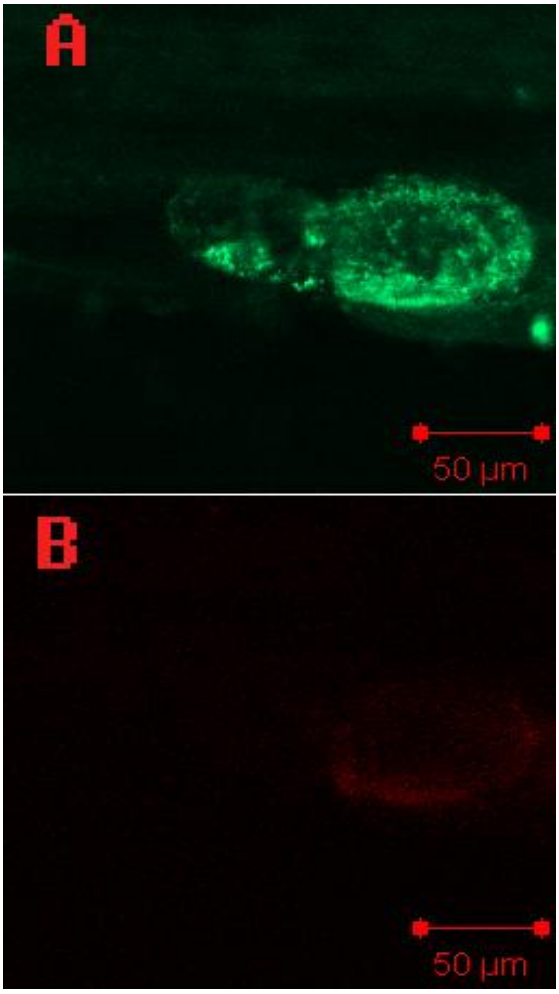



**Figure 3.12:** Fluorescent counts from z-section images of cells. AM= arrestin morpholino. Images of cells injected with arrestin had an average of 6.3 $\pm$ 1.3 fluorescent counts as compared with the uninjected control cells that had an average count of 16.9 $\pm$ 2.2. This indicates about 2.5 fold decrease in fluorescence in injected cells. n=3. Error bars indicate SEM.

### 3.5.3 Opsin morpholino injections lead to a 17 fold reduction in fluorescent counts

The opsin morpholino injected cells were also less bright (Fig. 3.13) and had a diffused pattern of fluorescence. Quantitative analysis of the fluorescent counts from the confocal images indicated a 17 fold reduction in fluorescent counts in injected cells (Fig. 3.14).

Although we can not quantify actual protein levels by this method, the decrease in fluorescent counts arising from the antibody staining is consistent with a decrease in protein levels in the injected cells.


A
50 µm
B
50 µm

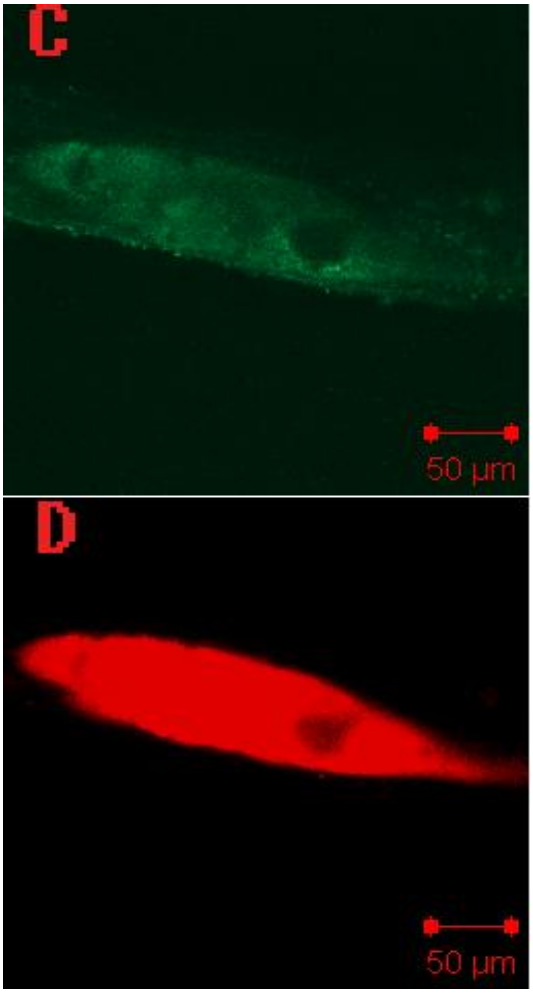



**Figure 3.13:** Confocal fluorescent images of ventral photoreceptors probed with the opsin antibody. The green indicates opsin and red indicates dextran fluorescence. A and B are images from a cell that has not been injected with opsin morpholino and dextran. C and D are images from a cell that has been injected. A) Cell has a characteristic opsin pattern B) Cell not injected with opsin morpholino as indicated by the absence of dextran fluorescence. C) Cell looks less bright and has a diffused pattern. D) Cell injected with opsin morpholino as confirmed by the dextran fluorescence.

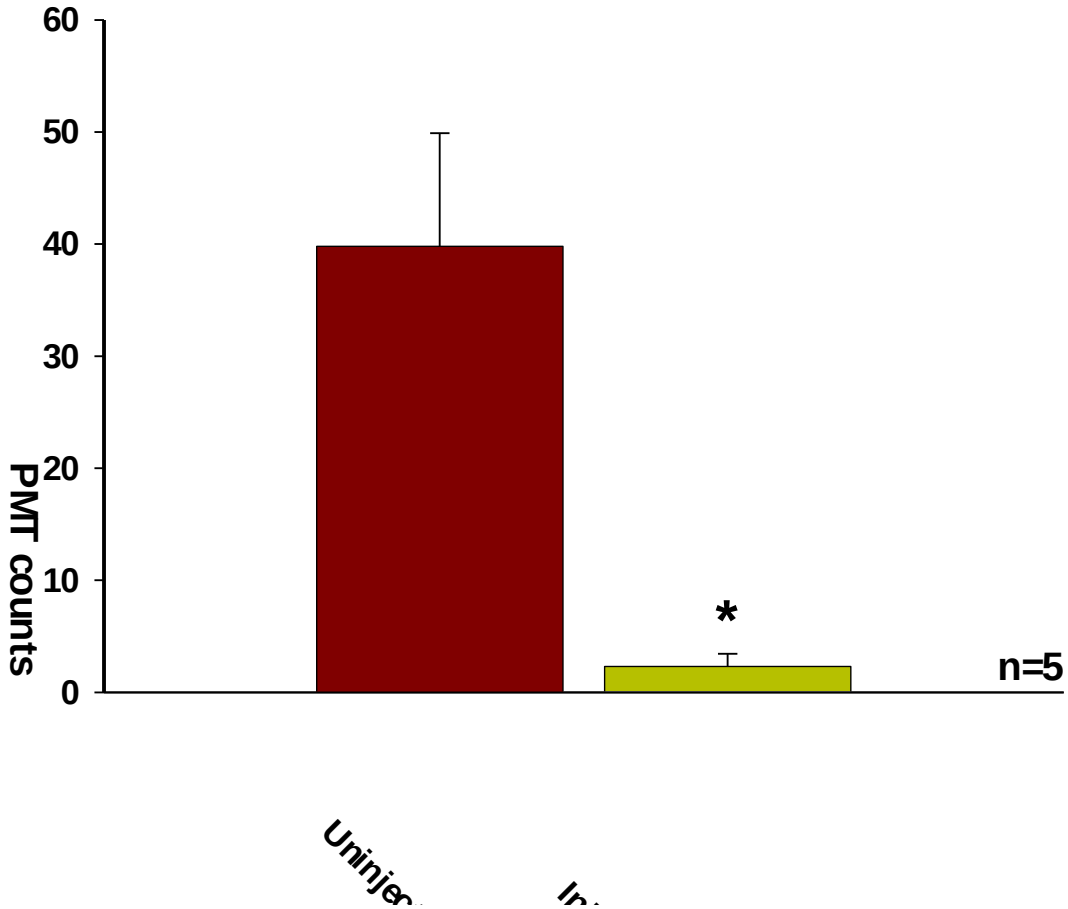



**Figure 3.14:** Fluorescent counts from z-section images of cells. OM= opsin morpholino. Images of cells injected with opsin had an average of 2.3 ± 1.1 fluorescent counts as compared with the uninjected control cells that had an average count of 39.8 ± 10. This indicates about 17 fold decrease in fluorescence in injected cells. n=3. Error bars indicate SEM.

### 3.6 DISCUSSION

Functional silencing of genes is a very powerful technique for model systems in which mutagenesis studies can not be carried out. *Limulus* is therefore an ideal

candidate to make the most use of this technique. We have shown here that morpholino can be injected directly into individual ventral photoreceptor cells and its long term effects can be investigated. It is possible to study the effect of morpholino up to 6-7 days after injections because morpholino is very stable and the cells themselves can be maintained in culture for a long time.

The results with the arrestin morpholino support the data obtained from arrestin RNAi studies since both these procedures lead to a 5-fold increase in bump rate 24 hrs after injections. It is interesting to note that although RNAi and morpholino work through different mechanisms, their functional output in our studies was very similar. At the same time there were some differences too. The bump rate at 48 hrs after arrestin RNAi injections decreased significantly from that at 24 hrs after injections. However, morpholino injections at 48 hrs had no significant difference in bump rate from those at 24 hrs. This could be due to low toxicity of morpholinos observed both in vitro and in vivo ([172]Iversen et al., 2003).

Opsin morpholino injections lead to a loss of sensitivity to light. The degree of the loss increases with longer time points after injections. However, after about 96 hrs, there is no further significant reduction observed in the bump rate. Although the decrease in sensitivity is maintained at later hours, there is an increase in cell death and difficulty in obtaining good recordings from cells. Thus we decided that 96 hrs after injections was the optimum time to readily test the effectiveness of opsin morpholino. We hypothesize that failure of injections to further reduce bump rate

could be due to morpholino diffusing out of the cell body into the axon. Another possibility is that the morpholino significantly reduces the content of the freshly synthesized opsin but the residual bumps at 96 hrs are produced due to the opsin that was already present in the system before the injections.

The reduction in bump rate at 96 hrs after morpholino treatment is indicative of a slow turnover of opsin in *Limulus* ventral photoreceptors. Turnover of opsin in vertebrates is rapid. In the presence of light, the discs in the outer segment of rods get degraded and new discs are continuously assembled. Each photoisomerization event leads to the formation of the active photoproduct, metarhodopsin. To replenish the rhodopsin concentration, fresh opsin needs to bind the chromophore in the pigment epithelium. This mechanism requires rapid turnover of opsin. In invertebrates, however, rhodopsin regeneration is not long and involved but occurs when metarhodopsin absorbs a photon. Thus the rate of synthesis of opsin is much slower. In *Drosophila*, there is no change in opsin mRNA or opsin protein levels over a 24 hr D/L cycle ([173]Hartman et al., 2001). In *Limulus* lateral photoreceptors, opsin mRNA levels spike at 9-12 hrs after sunrise but stay constant for the rest of the day and night ([174]Dalal et al., 2003). However, no information is available on the opsin protein levels. Morpholino injections reduce the production of fresh opsin protein but have no effect on the opsin that was already present in the system before the injections. Since it takes about 96 hrs to maximally reduce the bump rate, this might indicate a slow turnover of opsin in *Limulus*.

### 3.6.1 Consequence of Rhodopsin and metarhodopsin with similar spectral properties

Rhodopsin and metarhodopsin in most species absorb light maximally at different wavelengths. In *Drosophila*, a 480nm (blue) light can convert about 80% of rhodopsin to metarhodopsin and a 580nm (orange) light can convert > 80% of metarhodopsin back to rhodopsin ([175]Matsumoto et al., 1982). However, in *Limulus*, both forms of rhodopsin have the same absorption maxima. This means that light that photo-converts rhodopsin to metarhodopsin also photo-converts metarhodopsin to rhodopsin. This process quickly reaches equilibrium and there is a 1:1 ratio of rhodopsin and metarhodopsin present in the ventral photoreceptors. As a consequence of this, we observed some unique results in the opsin experiments.

In cells injected with opsin morpholino, the dark bump rate is significantly lower than those of control cells (Fig. 3.3). This is presumably because the opsin morpholino injections reduce the opsin content in the cell. This results in reduction in both rhodopsin and metarhodopsin. So the dark bump rate goes down.

The confocal experiments were designed to get an idea about the protein levels in the injected cells. Analysis of fluorescent counts indicates that in the presence of arrestin morpholino and opsin morpholino, there might be a reduction in protein levels. This is in agreement with the electrophysiology data and is indicative of the success of the morpholino technique in *Limulus* ventral photoreceptors.

We have demonstrated here that morpholino technique can be used to study the different proteins involved in *Limulus* phototransduction. It is a powerful technique that can be used to overcome the inability to do genetic studies in this system.

# Chapter 4

# Discussion

## 4.1 Controls used in the experiments.

For the arrestin RNAi experiment, we tried two delivery methods. First was adding RNAi to the bath in the culture medium. The controls for this experiment were nerves placed in a separate dish that had no RNAi added to the culture medium. Although this experiment resulted in an increased dark bump rate in the RNAi treated cells, there was a lot of variability from cell to cell. So we decided to inject RNAi into the cells instead. This also gave us the opportunity to use an internal control. Experimental cells were the ones on a nerve that were injected while control cells were the ones on the same nerve that were not injected. This meant that both categories of cells were exposed to the same conditions of light, temperature, pH, and osmolarity. Another control group was cells injected with the carrier solution. This served to test the effect of injecting a specific volume of solution into the cells and testing them at various time points after that.

For the morpholino experiment, there were two types of control. On a nerve, some cells were injected with opsin morpholino and formed the experimental set. The cells from the same nerve that were not injected formed the uninjected control set. In some cells, an unrelated standard control morpholino was injected and these formed another set of control. These controls addressed the issues of injecting large

molecules into cell, keeping the cells in the culture medium for up to 6 days, and of specificity. For some experiments, the cells injected with opsin morpholino, cells injected with control morpholino, and uninjected control cells were all from the same nerve. These provided us with an excellent internal control.

### 4.2 RNAi vs. Morpholino; Morpholino is recommended.

What I have shown here is that the complementary oligonucleotide technique seems to work for *Limulus* ventral photoreceptors. The arrestin data indicates that although RNAi and morpholino work through different mechanisms, their effect on the electrophysiology of cells is similar; that of a five-fold increase in bump rate. Either of these techniques can be further used to understand the role of membrane channels and other proteins in the photoreceptors. However, I would recommend using morpholino as opposed to RNAi. This recommendation is based on the production of these oligonucleotides and their mechanism of action. RNAi is targeted against a long length of the gene (in case of arrestin RNAi it is about 300 base pairs). Once the RNAi is delivered into the cells it breaks down into 25 bp –long strands and gets amplified. This introduces a lot of uncertainties. We can neither identify specific sequences that might have had the silencing effect nor can we estimate the concentration of RNAi that was reached inside the cells. Once inside the cell, the RNAi gets amplified and the concentration inside the cell can not be determined.

Morpholino on the other hand is manufactured as a 25bp long sequence against an intentionally chosen site on the gene. Once delivered to the cell it does not get amplified but functions for a long time because of its resistance to degradation by endonucleases. Therefore it introduces a lot of specificity in our system. We can control the sequence and the concentration of morpholino and study its effects on the ventral photoreceptors. Although RNAi has been used as a preferred method in several systems, in our system morpholino seems to be a better choice.

**4.3 Arrestin reduces dark noise.**

Visual arrestins forms a part of a large family of arrestins found in both vertebrates and invertebrates ([176]review Palczewski 1994). In mammals, four members of the arrestin gene family have been identified. Two of these are visual arrestins. One is a form present in the rods and is called the s-antigent or arrestin ([177]Review Krupnick & Benovic, 1998). The second occurs in the cones and in pineal glands and is called x-arrestin, arrestin 4, or cone arrestin (CAR) ([178]Sakuma et al., 1996). The other two types of arrestins are expressed ubiquitously and are called β-arrestin1, and arrestin2. The first invertebrate arrestin homologue was characterized in *Drosophila* ([179]Yamada et al., 1990; [180]Smith et al., 1990).

Functionally, visual arrestin has been implicated in termination of light response and retinal degeneration. To terminate the light response, it binds to phosphorylated metarhodopsin ([181]Kuhn et al., 1984). Arrestin knock-out mice exhibit

prolonged photoresponses ([182]Xu et al., 1997). In *Drosophila* arrestin mutants there is an increase in the prolonged depolarizing afterpotential (PDA) ([183]Dolph et al., 1993). The observations above are indicative of a role of arrestin in terminating the light response. In humans, a frameshift mutation in the arrestin gene causes slowed dark adaptation and reduced sensitivity, a condition called Oguchi's disease ([184]Fuchus et al., 1995). This suggests that arrestin is involved in dark adaptation. Dark adaptation is the process that occurs when photoreceptors have been exposed to intense bright light and are then exposed to darkness. Immediately after the onset of darkness, the photoreceptors can not return to pre-light exposure sensitivity because their pigment is "bleached". The process in which this pigment recovers its sensitivity may take tens of minutes in vertebrates and is called dark adaptation.

Arrestin plays a crucial role in retinal degeneration, a process in which it forms stable arrestin-rhodopsin complexes that lead to apoptosis ([185]Alloway et al., 2000). Higher arrestin levels have been observed in retinal degeneration slow (*rds*) mutant mice. In these mice the inner segments are normal but the outer segments fail to form. Accumulation of high concentration of arrestin is implicated in this degeneration ([186]Agarwal et al., 1994). In arrestin KO mice, light driven degeneration is observed in the photoreceptors ([187]Chen et al., 1999). In *Drosophila*, failure to dissociate arrestin from the arrestin-rhodopsin complexes eventually leads to light-dependent apoptosis of retinal photoreceptors ([188]Alloway et al., 2000).

Arrestin in *Limulus* has been implicated in internalization of rhodopsin in response to light ([189]Sacunas et al., 2002). However, the function of arrestin in the quenching of the light response has not been investigated. In our experiments we show that a reduction in arrestin leads to an increase in dark noise. This was demonstrated by the use of two different methods; RNAi, and morpholino injections. Both of these techniques work through different mechanisms but have the same end effect on the photoreceptor cells; that of increasing the spontaneous dark noise 5-fold. This supports a role of arrestin in keeping the spontaneous dark noise level down.

**4.4 Opsin confirmed as the photopigment in *Limulus.***

Visual pigments across different species are all made of a 7-transmembrane protein (opsin) covalently linked to a chromophore. Most species have multiple opsin genes. In vertebrates several 'visual' and 'non-visual' opsins have been identified ([190]review Kumbalasiri & Provencio 2005). In humans, there are four visual opsins. The one expressed in rods is the rhodopsin and the ones expressed in cones are S, M, and L cone opsins. Invertebrates also have multiple opsin genes. In *Drosophila*, there are 6 opsin genes, Rh1 to Rh-6, that are expressed in different cell types and no cell expresses more than one type of opsin.

In *Limulus*, about four opsin genes have been sequenced ([191]Dalal et al., 2003). Of those, two proteins generated from genes opsin1, and opsin 2, have been identified. Opsin 1 and opsin 2 express in both the lateral and ventral eyes and opsin 1

is the more abundant transcript in the ventral photoreceptors. Information about the expression of the other two possible opsin genes is not available.

Although these studies strongly indicate that opsins form the photopigment in *Limulus*, there have been no functional studies to confirm that. Our immunocytological studies show that there is a reduction in opsin content in the presence of opsin morpholino. Our electrophysiological studies indicate that the functional consequence of reducing opsin content through opsin morpholino is a 30-fold decrease in sensitivity to light. This is consistent with the gene product of opsin 1 and opsin 2 forming the photopigment in *Limulus* ventral photoreceptors.

**4.5 Consequence of opsins with similar spectral properties not resolved.**

Multiple opsins occurring within a single eye have different spectral properties. In humans, rhodopsin absorbs maximally at 500nm whereas the three cone opsins S, M, and L absorb maximally at <500nm, ~530nm, and ~560nm respectively. In some vertebrates there is a fifth opsin gene that senses ultraviolet light. Mice, rabbits, guinea pigs, and probably marsupials appear to coexpress two opsins per cell ([192]Rohlich et al., 1994; [193]Applebury et al., 2000[194]Hemmi & Grunert, 1999). Fish, eels, tiger salamander, and gecko express two or three opsins in the same cell ([195]Archer & Lythgoe, 1990; [196]Hope et al., 1998; [197]Makino & Dodd, 1996; [198]Loew et al., 1996).Each of the six opsins expressed in *Drosophila* has different absorption maxima ([199]Review Montell 1999). Butterfly photoreceptors express two visual

pigments per cell ([200]Kitamoto et al., 1998). The distinct absorption maxima of the opsins work towards avoiding sensory overlap and broadening spectral sensitivity of eyes.

Interestingly, the two opsins expressed in the ventral photoreceptors presumably have the same spectral sensitivity since the domain thought to influence this property is the same in both genes ([201]Briscoe, 2000; Dalal et al., 2003). A similar phenomenon has been observed in the crab Hemigrapsus sanguineus where the two opsins presumably have similar spectral properties ([202]Sakamoto et al., 1996). However, the physiological significance of having multiple opsins with similar spectral properties is not known.

In our experiments, the opsin morpholino was targeted against the 5' region of the gene. In the two opsin genes that express in the ventral photoreceptors have identical sequence in this region. So presumably the morpholino was targeted against both the gene products. The antibody used was made against the C-terminus of the opsin DNA. This antibody also presumably recognizes both the opsin products. We observed a decrease in opsin levels in morpholino injected cells as indicated by a decrease in fluorescence counts. Although this experiment indicates the success of the morpholino technique, it still does not give any indication as to the significance of multiple opsins with similar spectral properties.

**4.6 Regulation of metarhodopsin deactivation is extremely critical in *Limulus.***

In *Limulus*, both rhodopsin and metarhodopsin share the same absorption maxima ($\lambda_{max}$ =500nm). This phenomenon is also observed in the squid photoreceptors but is unlike other animals in which the absorption maxima for rhodopsin and metarhodopsin are different. For example, in *Drosophila*, rhodopsin $\lambda_{max}$ =480nm (blue) and metarhodopsin $\lambda_{max}$ = 580nm (orange). Blue light converts rhodopsin to metarhodopsin and orange light converts metarhodopsin to rhodopsin. However, in *Limulus*, a photon that can activate rhodopsin to form metarhodopsin is equally likely to change a metarhodopsin back into rhodopsin. As a result of this, unlike vertebrates that have no metarhodopsin in the dark, and invertebrates such as *Drosophila*, that has about 20% metarhodopsin in the dark, *Limulus* photoreceptor cells have equal number of rhodopsin and metarhodopsin molecules. Since the total opsin is estimated to be $10^9$ molecules, it means that there are about $5* 10^8$ metarhodopsin molecules present at any time ([203]Lisman et al., 1977).

The metarhodopsin stays inactive due to phosphorylation and arrestin binding. However, sometimes it can spontaneously lose arrestin and become dephosphorylated. This reaction, even though rare, may produce spontaneous bumps in the dark. The rate of dark bump production in dissected *Limulus* photoreceptors from about $5* 10^8$ metarhodopsin molecules is about 1/s. In the event of reduction in quenching by arrestin even by a fraction, the dark noise would go up several folds. This shows that it is very crucial to tightly regulate metarhodopsin activation and deactivation and arrestin forms an important part of this cycle.

This is consistent with our observation in arrestin RNAi and arrestin morpholino experiments wherein a reduction in arrestin levels led to a five-fold increase in the spontaneous dark bump rate.

**4.7 Membrane channels involved in phototransduction.**

Two types of photoreceptors have been identified in vertebrates and invertebrates, ciliary, and rhabdomeric. The ciliary photoreceptors occur primarily in vertebrates but are also seen in invertebrates. They respond to light either by hyperpolarization or depolarization. However, they all use the cGMP pathway for transduction. The membrane channels in these photoreceptors, the CNG channels, either close or open during phototransduction. CNG channels were first implicated in rod photoreceptors ([204]Fesenko et al., 1985). The same year these channels were found to be involved in cone phototransduction ([205]Haynes & Yau, 1985). The channel was cloned and expressed a few years later ([206]Kaupp et al., 1989). Involvement of cGMP as a second messenger in the ciliary photoreceptors of scallop was shown by [207]Gomez & Nasi (1995). Scallop photoreceptors hyperpolarize in response to light ([208]McReynolds, 1976).

The rhabdomeric photoreceptors, on the other hand, occur only in invertebrates and absorption of photons always results in depolarization. Phototransduction involves activation of the phorphoinositide second messenger pathway and the membrane channels in *Drosophila* have been reported to be

TRP/TRPL channels that are part of the transient receptor potential (TRP) family ([209]Hardie & Minke, 1992). However, the second messenger involved in activating these channels is not known. Heterologous studies indicated a role of $InsP_3$ in activating these channels since addition of thapsigargin reduced $Ca^{2+}$ levels and activated these channels ([210]Vaca et al., 1994; [211]Xu et al., 1997). However, this result has not been duplicated *in vivo*. When $Ca^{2+}$ was depleted using thapsigargin *in vivo*, phototransduction was not affected ([212]Ranganathan et al., 1994). Caged $InsP_3$ did not activate any light sensitive channels ([213]Hardie and Raghu, 1998). $InsP_3R$ null mutant did not have any defect in phototransduction ([214]Acharya et al., 1997). All of these studies indicate that the TRP/TRPL channels are probably not activated *via* the $InsP_3$-mediated pathway. There is some indication that the DAG branch of the phototransduction might be involved in the activation process. These channels were shown to be activated in the presence of polyunsaturated fatty acids (PUFAs) which are presumably produced after DAG hydrolysis ([215]Chyb et al., 1999). TRP channel was shown to be constitutively active in *Drosophila* retinal degeneration A (*rdgA*) mutants ([216]Raghu et al., 2000). The *rdgA* are diacylglycerol kinase mutants. Diacylglycerol kinase converts DAG to phosphotidic acid and so the DAG concentration in these mutants is expected to be high. These experiments support the idea of DAG pathway to be involved in channel activation.

The success of the complementary oligonucleotide technique studied with respect to arrestin and opsin provides us with a valuable tool that can be used to manipulate the expression of CNG and/or TRP channels and study their effect on

phototransduction. As mentioned in the introduction section, the identity of the membrane channel leading to membrane depolarization in *Limulus* is not known. Injection of $InsP_3$ and $Ca^{2+}$ in the photoreceptors activates light-dependent conductance ([217]Fein et al., 1984; [218]Payne et al., 1986). This supports the role of $Ca^{2+}$ in activating the channels. However, cGMP injections also activate light-dependent conductance and indicate involvement of this second messenger ([219]Johnson et al., 1986). Further evidence in support of this pathway comes from the work that shows that the light-dependent channels open by cGMP and not by $Ca^{2+}$ ([220]Bacigalupo et al., 1991). In our laboratory, the cGMP and TRP channels have been cloned and sequenced from the *Limulus* eye cDNA ([221]Chen at al., 1999; [222]Bandyopadhyay and Payne, 2004). We can now use this technique to study the functional consequence of lowering the concentration of cGMP and trp.

**4.8 Future directions.**

The success of the RNAi and morpholino injection techniques opens new doors to study phototransduction in *Limulus* ventral photoreceptors. These techniques give us a handle on manipulating protein levels in the cells and thus overcoming the shortcomings imposed by inability to perform mutagenesis.

An ERP experiment can be performed in the immediate future to show the changes in pigment concentration in the presence of morpholinos. ERP occurs in the presence of very bright light, has virtually no latency, and is hypothesized to be

generated directly by charge displacements produced by the isomerization of rhodopsin ([223]Cone & Pak, 1971). Therefore, ERP is a direct measure of the amount of rhodopsin and its intermediates present in the photoreceptor. The hydroxylamine experiments indicate that the increased bumps are generated due to unquenched metarhodopsin. The results from the hydroxylamine experiments are consistent with the idea that bumps in the dark are generated by rhodopsin while bumps in the light are generated by metarhodopsin. ERP experiments will be another way to prove this point.

As mentioned before, a big question in *Limulus* phototransduction concerns the identity of the membrane channel. There is indirect evidence supporting the finvolvement of both cGMP and TRP channels. Since the sequences for both *Limulus* TRP and cCMP are available, morpholinos against them can me made. It may now be possible to use cGMP morpholino and *trp* morpholino to manipulate the levels of these proteins and study the electrophysiological consequences. Defects in electrophysiological responses in the presence of the cGMP morpholino or *trp* morpholino would strongly indicate the involvement of that channel in *Limulus* phototransduction. This technique can be extended to the study of any protein in the ventral photoreceptors. Currently sequences are available for Gq and myosin. Morpholinos can be made against these. As more and more sequences become available, this technique can be extended to those proteins and play a valuable role in understanding their function.

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