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ABSTRACT

Among the recently identified vertebrate opsins, melanopsin has emerged as a photopigment critically involved in the light-mediated regulation of circadian rhythms. Melanopsin is found in a subset of intrinsically photosensitive retinal ganglion cells (ipRGCs) that directly send photic information to various non-visual light processing brain regions. Melanopsin expression is necessary for the photosensitivity of the ipRGCs and several studies have shown that the melanopsin expressing ipRGCs provide input for photoentrainment, pupillary light reflex, and regulation of the melatonin biosynthetic pathway. Although evidence points towards melanopsin being the functional photopigment of the ipRGCs, melanopsin may simply function as a photoisomerase, facilitating an as yet unidentified opsin-based photopigment in these cells. The purpose of this research was to identify whether melanopsin is in fact a photopigment and to elucidate the details of melanopsin photoactivation and signaling. We have found that heterologous expression of melanopsin in human embryonic kidney cells (HEK293) imparts photosensitivity upon the cell line. With this system, we have found that melanopsin triggers a Gq or a related G protein, activation of phospholipase C, an increase in intracellular calcium from internal calcium stores, and depolarization that may be dependent upon the presence of the canonical transient receptor potential channel, TRPC3.

MOLECULAR BASIS OF CIRCADIAN PHOTORECEPTION

Tida Kumbalasiri

**A Dissertation presented to the Graduate Faculty
of the Uniformed Services University of the Health Sciences
for the Degree of Doctor of Philosophy**

Presented July 7, 2006

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PREFACE

Since the discovery of the photopigment melanopsin in 1998, an extensive amount of research on melanopsin has emerged. This research on melanopsin-based signaling and the physiology of the attendant photoreceptors, the intrinsically photosensitive retinal ganglion cells, was recognized by *Science* as a runner-up Breakthrough of the Year in 2003.

This dissertation represents my contribution to this nascent field. It is comprised of an Introduction that provides background and context for the chapters that follow. Large portions of the Introduction have been published as a first-author review article (Kumbalasiri and Provencio, 2005). The first chapter also has been published in its entirety (Qiu et al., 2005). I earned "co-first author" status on this publication based on my contribution of developing a heterologous melanopsin expression system in a human embryonic kidney cell line (HEK293) with an easily quantified electrophysiological output. The development of a transient expression system for mouse melanopsin, a photopigment with an invertebrate character, was a primary goal of several research teams competing to characterize melanopsin's function. This competition culminated in the near simultaneous publication of three reports in high profile journals (Melyan et al., 2005; Panda et al., 2005; Qiu et al., 2005), reflecting the interest and relative difficulty in establishing such an expression system. Functional expression systems for invertebrate opsin-based photopigments have proven difficult to develop (Knox et al., 2003). Melanopsin's similarity to invertebrate opsins predicted an equally difficult task. Our success in this endeavor has been extended by others who

have subsequently used a human embryonic kidney cell line to express nonmammalian melanopsins (Koyanagi et al., 2005). Upon developing this system, I transferred this technology to David Berson's lab at Brown University to exploit their expertise in electrophysiology. I assisted in the initial characterization of the light-induced membrane potential changes in our cell-based system, but my direct involvement in characterizing the currents and spectral profile of the expressed melanopsin was more limited.

The second chapter represents a refinement of the expression system described in the first chapter. Our interest in developing a semi-quantitative calcium imaging protocol required that we maximize our signal-to-noise ratio and minimize our transient transfection variability. To this end, we developed several lines of stably transfected HEK293 cells expressing human melanopsin. Although I received assistance on this project from other members of the lab, I was directly responsible for every aspect of these studies. This chapter has been submitted to *Photochemistry and Photobiology* for consideration for peer review and publication.

Finally, the two chapters are followed by a global discussion of the cumulative results presented in the dissertation. The purpose of this section is to provide a contextual framework to illustrate the overall contributions of my dissertation work in relation to the field at large. The two published reprints that have arisen from my dissertation work are attached as an appendix.

DEDICATION

This work is dedicated to my mother and father who have worked so hard to make sure that I had all the opportunities in the world and who taught me to dream big.

INTRODUCTION

Central Hypothesis and Specific Aims

Our daily rhythmic changes in behavior and physiology are controlled by an endogenous system that generates a circadian rhythm with a period slightly greater than 24 hours (Czeisler et al., 1999). The core of this rhythmic output is the suprachiasmatic nucleus (SCN), the “master” pacemaker, which drives multiple “slave” oscillators throughout the body (Reppert and Weaver, 2002). Light input received by photoreceptive cells in the retina is used to synchronize the endogenous rhythm generated by the SCN to fit that of the environmental twenty-four hour light-dark schedule (Klein et al., 1991). This three-part system of input, master pacemaker, and output serves to maintain our physiology and behavior in the proper temporal context to the external environment.

The appropriate phasing of the circadian system is so critical such that chronic disturbances have been shown to be detrimental to health. Disturbed sleep, decreased alertness, and gastrointestinal irritability are some of the common complaints of shift workers and jet lagged travelers as their endogenous oscillators come out of phase relative to each other, and they attempt to resynchronize with the environment (Moore-Ede and Richardson, 1985; Harrington, 1994; Haimov and Arendt, 1999; Scott, 2000). Several studies have suggested that even more profound problems are associated with those who experience chronic circadian desynchronization. Temporal lobe atrophy and spatial cognitive deficits have been reported in chronically jet-lagged air flight crews (Cho, 2001). Elevated risks of developing breast cancer have been reported in nurses who have worked several years of rotating night shifts (Schernhammer et al., 2001).

Sleep disorders such as advanced sleep phase syndrome have also been attributed to a dysfunctional circadian system (Toh et al., 2001). Finally, aged mice forced to continously phase shift, experience dramatically higher mortality rates than non-shifting controls (Davidson et al., 2006). Only 47% of mice greater than 27 weeks of age that were exposed to weekly 6-hour phase advances in the light:dark cycle survived until the end of the seven week experiment compared to 83% of unshifted controls. These results are quite striking in light of the fact that shiftwork is so common in our industrial society.

The recognition of the importance of the circadian system has resulted in aggressive research into almost every aspect of this system. My research has been aimed at identifying and elucidating the details of light input into the system. Specifically, I have been studying the photoactivation and signaling of melanopsin, the photopigment of the intrinsically photosensitive retinal ganglion cells (ipRGCs). Photopigments are proteins that are typically coupled with at least one chromophore molecule that is photolabile. Stimulation of a photopigment results in the conversion of electromagnetic energy into a biologically relevant response. Opsins are photopigment proteins whose chromophores are derivatives of vitamin A (retinoids). In eukaryotes, opsins can be divided into three functionally distinct types. The first subclass contains those opsins which when stimulated, activate an intracellular signaling cascade. The opsin-based photopigments of vertebrate rod and cone photoreceptors are examples of such sensory opsins. The second subclass harnesses electromagnetic energy to photoisomerize the retinoid chromophore from an all-*trans* configuration to the less energetically favorable 11-*cis* configuration. Members of this subclass of opsins are

known as photoisomerases. Cephalopod retinochrome, RGR-opsin, and peropsin are representatives of this subclass. The third subclass combines the sensory and photoisomerase functions into a single photopigment whereby a photon of a given energy can photoisomerize the covalently linked 11-*cis*-retinaldehyde chromophore to the all-*trans* isomer, consequently activating a signaling pathway. Another photon, typically of lesser energy, can reisomerize the "spent" all-*trans* chromophore back to the 11-*cis* configuration, thus preparing the photopigment for another round of signaling. Cephalopod and insect visual pigments are prototypical examples of this subclass.

Since its initial identification in the light sensitive *Xenopus laevis* dermal melanophores and the subsequent identification of mammalian homologs, the role of melanopsin as a photopigment responsible for mammalian non-visual phototransduction has been debated. The **central hypothesis** of this dissertation is that melanopsin is a sensory opsin, capable of activating a second messenger signaling cascade. To test this hypothesis, two specific aims were pursued. The **first aim** was to develop a heterologous expression system to establish whether expression of rodent melanopsin can confer photosensitivity, and if so, to determine if photoactivation of melanopsin can initiate a second messenger signaling cascade. The **second aim** was to characterize this cascade and to optimize the response so a reliable measurable output could be used in future studies to address the functional significance of naturally occuring and experimentally induced single nuceotide polymorphisms in the human melanopsin gene.

Because only around 1000 retinal ganglion cells in the rodent retina express melanopsin, biochemical studies of melanopsin derived from retina are unfeasible. Accordingly, I needed to find an alternate source of melanopsin, so I generated

expression systems to address the aims outlined above. I generated multiple melanopsin expression vectors and attempted to heterologously express melanopsin in series of cell lines including a human retinal epithelial cell line (ARPE-19), and embryonic zebrafish cell line (ZEM-2S), a rodent retinal ganglion cell line (RGC-5), and the amphibian melanophore cell line fom which melanopsin was originally cloned. Only a human embryonic kidney cell line (HEK293) demonstrated proper trafficking of melanopsin to the plasma membrane, expressed high levels of melanopsin, and had measurable responses to light.

Chapter 1 of this dissertation represents the completion of the first aim and a successful test of the central hypothesis. In this chapter, I demonstrated that transient expression of melanopsin could confer photosensitivity onto previously light-insensitive HEK293 cells. I took this model system to Brown University in Providence, RI for collaborative studies with the researchers in David Berson's group. I participated in the electrophysiological measurement of light induced ionic currents in HEK293 cells transiently transfected with melanopsin. We characterized the spectral sensitivity of these resposes and discovered that this sensitivity was virtually identical to the spectral sensitivity of the intrinsic light responses of ipRGCs (Berson et al., 2002). Furthermore, the spectral sensitivity in this heterologous system closely matched the action spectrum for the light-mediated regulation of the circadian system of retinally degenerate mice (Yoshimura and Ebihara, 1996). Finally, through the use of specific pharmacologic inhibitors, we established that melanopsin activates a Gq or Gq-like G protein which likely activates phospholipase C resulting indirectly in a transient increase in intracellular calcium concentrations.

The second chapter of this dissertation represents the successful completion of the second aim. Instead of the previously mentioned transient transfections, HEK293 cells were stably transfected with human melanopsin, resulting in cell lines with dramatically improved signal-to-noise ratio. With these cells, I was able to exploit the calcium-sensitive fluorophore Rhod-2AM to observe transient light-evoked changes in intracellular calcium concentrations ten times more robust than those described in Chapter 1. With this system I also characterized the relationship between light intensity (irradiance) and the magnitude of the calcium response, laying the foundation for future functional studies on melanopsin mutants. Furthermore, I determined that the light-induced increase in intracellular calcium is largely, if not exclusively, attributable to calcium release from intracellular stores and not from the regulated influx of extracellular calcium.

Together these studies have contributed to the groundwork of understanding the non-visual phototransduction system. While there still remains a great deal to understand in this system, I hope that a greater understanding of the melanopsin-based photoreceptive system will help identify specific pharmacological targets for attenuating or even eliminating circadian desynchrony.

Background

In mammals, light input to the SCN is essential to entraining circadian rhythms to the day: night cycle (Van Gelder, 2004). Circadian entrainment is an adaptation that synchronizes endogenously generated circadian rhythms to the environment's light: dark cycle. This allows organisms to prepare their physiology and behavior for the

drastic changes associated with the dawn and dusk transitions. For example, proper circadian entrainment permits animals to inhabit precise “temporal niches” that minimize exposure to predation (Davidson and Menaker, 2003), thereby increasing fitness. Within the past decade, a much clearer understanding of the molecular components of the circadian clock has emerged (Reppert and Weaver, 2002), but the mechanism by which the components of the clock are reset by light remains to be elucidated.

Non-visual photoreception in the eye

Only recently have the photoreceptors involved in mammalian photoentrainment been identified (Berson et al., 2002). Prior to their identification, it was known that the eyes are necessary for the light-mediated regulation of the circadian axis because bilateral removal of the eyes abolished photoentrainment (Nelson and Zucker, 1981). However, in an apparent paradox, the rods and cones are not necessary for circadian photoregulation because genetic ablation of these photoreceptors has no effect on the photic circadian phase shifting response (Nelson and Zucker, 1981; Foster et al., 1991; Freedman et al., 1999). In fact, several mouse models of rod and cone degeneration have been tested and none of these have demonstrated any decline of circadian photoresponses. Similarly in humans, some individuals remain capable of photic resetting of their circadian rhythms despite being cognitively blind (klerman et al., 2002). Taken together, these data indicate the existence of a non-rod, non-cone, ocular photoreceptor involved in photoentrainment.

The retina is one of the most extensively studied tissues. The prospect of an unidentified photoreceptor lurking within such familiar anatomical territory was met with appropriate skepticism. Action spectrum studies for light-induced circadian phase

shifting were undertaken to establish the relative roles of the visual photoreceptors in entrainment or to provide insight into the spectral sensitivity of any unidentified circadian photoreceptors. Such a study in the golden hamster (*Mesocricetus auratus*) indicated a spectral sensitivity peaking around a blue-green wavelength of 500 nm (Takahashi et al., 1984). This was coincident with the spectral maximum of rods, thus implicating the rods in photoentrainment. A later action spectrum study in retinally degenerate mice alluded to a photoreceptor with a sensitivity peaking in the blue wavelengths (480 nm) (Yoshimura and Ebihara, 1996). This sensitivity did not correspond to any of the known visual photoreceptors, again raising the specter of an as yet unidentified photoreceptor. Action spectra studies using other endpoints believed to be mediated at least in part by non-visual photoreceptors, such as the pupillary light response (Lucas et al., 2001) and the acute photosuppression of serum melatonin (Brainard et al., 2001; Thapan et al., 2001), also pointed to a blue-sensitive photoreceptor distinct from the visual photoreceptors. As several new putative opsin-based photopigments have been identified, it has been proposed that the cells containing these opsins may function in circadian photoentrainment or other forms of non-visual photophysiology (Provencio et al., 2000).

Melanopsin

Of the cohort of non-canonical opsins identified in the past few years, melanopsin is the most extensively studied. Melanopsin (gene symbol *Opn4*) was initially cloned from the intrinsically photosensitive dermal melanophores of *Xenopus laevis* (Provencio et al., 1998b). In addition to its dermal expression, melanopsin is also expressed in the amphibian brain and eye. The ocular expression pattern was of particular interest

because melanopsin mRNA is localized in non-rod, non-cone cells. This localization inspired the search for mammalian homologs of melanopsin, because as previously mentioned, the rods and cones are not required for circadian photoentrainment. Localization of a mammalian melanopsin homolog potentially could point to a candidate photoreceptor involved in the photic regulation of circadian rhythms.

Using probes derived from the amphibian melanopsin sequence, human and mouse homologs of melanopsin were identified (Provencio et al., 2000). Indeed, melanopsin expression in the retina is absent from rods and cones, but restricted to a small subset of RGCs (approximately 2% of RGCs in the rodent retina) (Hattar et al., 2002; Sollars et al., 2003). The low number of cells contained within this subset coupled with their broad, sparse distribution along the retinal surface parallels the number and distribution of RGCs known to project to the SCN in rodents (Pickard, 1980, 1982; Murakami et al., 1989; Balkema and Drager, 1990; Moore et al., 1995; Provencio et al., 1998a). This finding led to the proposition that melanopsin-containing RGCs may serve as photoreceptors that can provide the SCN with photic information independent of the rod-cone system (Provencio et al., 2000). Gooley and colleagues combined melanopsin *in situ* hybridization with retrograde tract tracing from the SCN to show that at least 74% of the RGCs that project to the SCN express melanopsin transcripts (Gooley et al., 2001). A subsequent study combining viral tract tracing and melanopsin immunohistochemical techniques suggested that as many as 80-90% of SCN-projecting RGCs contain melanopsin (Sollars et al., 2003). The generation of melanopsin knock-in mice, where the *tau-LacZ* reporter gene was knocked into the melanopsin locus, was useful in identifying other central sites that receive input from the melanopsin-containing RGCs

(Hattar et al., 2002). Among these sites are the intergeniculate leaflet and the olivary pretectal nuclei, two sites previously shown to be involved in the assessment of ambient illuminance levels. The ventral subparaventricular zone and the ventrolateral preoptic area, structures involved in the acute regulation of behavior and sleep, also receive input from melanopsin cells indicating a broad role for melanopsin in non-visual photoreception (Gooley et al., 2003; Hattar et al., 2006). Also, melanopsin-containing RGCs provide inputs to the peri-supraoptic nucleus, which may influence the photic modulation of neuroendocrine output (Hattar et al., 2006). With such few cells projecting to so many sites, it is not surprising that many of these sites receive collateral projections from the same melanopsin RGCs (Morin et al., 2003).

Berson *et al.* recognized that a class of SCN-projecting RGCs is intrinsically photosensitive with a peak spectral sensitivity in the blue wavelengths (λ_{max} = 484 nm) (Berson et al., 2002). SCN-projecting RGCs were identified by retrograde transport of rhodamine-labled beads injected into the SCN. Labeled RGCs were tested electrophysiologically for light responses under pharmacologic conditions that blocked synaptic transmission. These conditions ensured that any observed photoresponses were indeed intrinsic and not a consequence of classical retinal signaling via rod and cone pathways. To independently validate these remarkable findings, these investigators accomplished the herculean task of physically isolating a labeled cell and recording responses to light. The remarkable finding of intrinsic photosensitivity in RGCs has been independently verified by other investigators (Warren et al., 2003; Dacey et al., 2005).

The multitude of central projections arising from the ipRGCs predicts that they will have numerous functions. To assess their function, several labs created melanopsin-null mice (Hattar et al., 2002; Panda et al., 2002; Ruby et al., 2002; Hattar et al., 2003; Lucas et al., 2003; Mrosovsky and Hattar, 2003; Panda et al., 2003). These studies show that melanopsin-containing ipRGCs play a role in circadian photoentrainment, adjustment of circadian phase in response to light pulses, regulation of circadian period in response to constant light, the pupillary light reflex, acute photoinhibition of nocturnal activity, and the photic regulation of the melatonin biosynthetic pathway. Many of the deficits in these responses were subtle or not apparent in melanopsin-null mice (Panda et al., 2002; Ruby et al., 2002; Lucas et al., 2003; Mrosovsky and Hattar, 2003). Only after these mice were crossed with mice lacking functional rods and cones were extreme phenotypes observed (Hattar et al., 2003; Panda et al., 2003). For example, transgenic mice lacking functional photoreceptors (Freedman et al., 1999; Barnard et al., 2004) or mice homozygous for a naturally occurring retinal degeneration allele (*rd*) (Foster et al., 1991) remain capable of regulating circadian locomotor activity by light in a manner indistinguishable from sighted controls. This ability to shift the phase of circadian activity rhythms in response to light pulses is attenuated in melanopsin-null mice (Panda et al., 2002; Ruby et al., 2002). Interestingly, a residual capacity for light-induced phase shifting remains. Mice lacking functional rods and cones and null for melanopsin are completely incapable light-induced phase shifts (Hattar et al., 2003; Panda et al., 2003). Similarly, *rd/rd* mice show a 1.5 log unit loss in the sensitivity of the consensual pupillary light response, although the maximal response can be achieved at very high irradiances ($\geq 10^{14}$ photons$\bullet$sec$^{-1}\bullet$cm^{-2}). By contrast, melanopsin-null mice

show no decreased sensitivity although they exhibit about a 10% decrease in the response amplitude at the highest irradiances tested ($\geq 10^{14}$ photons•sec^{-1}•cm^{-2}) (Panda et al., 2003). Again, mice lacking functional rods and cones and null for melanopsin fail to show any pupillary response at any irradiance. Taken together, these data suggest that the visual photoreceptors complement the melanopsin system's role in the regulation of non-visual photophysiology.

It remains to be seen whether the melanopsin system plays a role in vision. Ultrastructural evidence suggests that dendrites of ipRGCs receive input from amacrine and bipolar cells, thereby providing an anatomical underpinning by which ipRGCs may regulate visual pathway (Belenky et al., 2003). Furthermore, melanopsin has been implicated in the regulation of the human cone visual pathway in response to long-term light exposure (Hankins and Lucas, 2002). Recently, melanopsin-containing "giant" RGCs, the likely paralogs of rodent ipRGCs, have been shown to combine with rod-based scotopic and cone-based photopic mechanisms to encode irradiance over the entire dynamic range of the primate visual system (Dacey et al., 2005). Although the process of vision has been extensively studied, these finding indicate that future models of vision may have to account for the contributions of the melanopsin-based photoreceptive system.

Possession of melanopsin confers photosensitivity upon ipRGCs (Lucas et al., 2003). The most parsimonious explanation of melanopsin's role in these cells is that it is functioning as the photopigment initiating the observed photoresponses. However, the possibility exists that melanopsin is functioning exclusively as a photoisomerase. Photoisomerases are opsin-based photopigments that do not directly activate signaling

pathways but rather use light to isomerize all-*trans*-retinaldedhyde to11-*cis*-retinaldehyde, the light-absorbing isomer used by signaling opsins to initiate phototransduction (Pepe and Cugnoli, 1992). In essence, photoisomerases play an auxiliary role by providing true signaling opsins with an appropriate retinoid isomer required to activate their respective transduction cascade.

Significance to the Military

Many military situations require around the clock activity and are frequently unpredictable. Situations such as night operations and/or rapid transmeridian deployment cause an undesirable discord between the circadian rhythms and environment of these individuals. This desynchrony can result in sleep loss, fatigue, decreased alertness, stress, and performance degradation (Goh et al., 2000). Reentrainment of phases may take several days during which time the soldier may suffer a decline in cognitive and physiological function. So much is at stake in the high-stress environment of combat such as the health of the involved individuals, the millions of dollars worth of equipment they are operating, and the welfare of their fellow pilots, soldiers, or sailors. Therefore, concerns about the performance of military personnel in situations of circadian desynchrony has resulted in a great deal of research (Ferrer et al., 1995; Comperatore et al., 1996; Goh et al., 2000; Caldwell and Gilreath, 2001). Each study has recognized the importance of maintaining alertness and optimal physical performance in these tactical situations. As such, they each have investigated countermeasures against the abnormal phase relationships experienced while in the state of circadian desynchrony. Sleep therapy, light therapy, exogenous melatonin, exercise, and the combination of hypnotics and stimulants have all been used as

countermeasures. Some have shown greater effectiveness among the different studies, but none have really proven to be effective enough to utilize in all cases of desynchronization. Therefore, elucidation of the phototransduction for non-visual light perception is necessary in order to identify a countermeasure with specific and predictable efficacy.

CHAPTER 1:
INDUCTION OF PHOTOSENSITIVITY BY HETEROLOGOUS EXPRESSION OF MELANOPSIN

THIS CHAPER HAS BEEN PUBLISHED IN ITS ENTIRETY:

Qui, X., Kumbalasiri, T., Carlson, S.M., Wong, K.Y., Krishna, V., Provencio, I., and Berson, D.M. (2005) Induction of photosensitivity by heterologous expression of melanopsin. *Nature*, 433, 745-749.

ABSTRACT

Melanopsin (Provencio et al., 1998b; Provencio et al., 2000; Bellingham et al., 2002; Hannibal and Fahrenkrug, 2002; Hannibal et al., 2002; Hattar et al., 2002; Provencio et al., 2002; Berson, 2003) has been proposed to be the photopigment of the intrinsically photosensitive retinal ganglion cells (ipRGCs)(Gooley et al., 2001; Berson et al., 2002; Panda et al., 2002; Provencio et al., 2002; Ruby et al., 2002; Berson, 2003; Lucas et al., 2003; Panda et al., 2003; Warren et al., 2003); these photoreceptors of the mammalian eye drive circadian and pupillary adjustments through direct projections to the brain (Gooley et al., 2001; Lucas et al., 2001; Berson et al., 2002; Hannibal et al., 2002; Hattar et al., 2002; Panda et al., 2002; Ruby et al., 2002; Berson, 2003; Gooley et al., 2003; Hattar et al., 2003; Lucas et al., 2003; Panda et al., 2003). Their action spectrum ($\lambda_{max} \approx 480$ nm) implicates an opsin (Berson et al., 2002) and melanopsin is the only opsin known to exist in these cells. Melanopsin is required for ipRGC photosensitivity (Lucas et al., 2003) and for behavioral photoresponses that survive disrupted rod and cone function (Hattar et al., 2003; Panda et al., 2003). Heterologously expressed melanopsin apparently binds retinaldehyde and mediates photic activation of G proteins (Newman et al., 2003). However, its amino-acid sequence differs from vertebrate photosensory opsins (Provencio et al., 1998b; Bellingham and Foster, 2002) and some have suggested that melanopsin may be a photoisomerase, providing retinoid chromophore to an unidentified opsin (Bellingham and Foster, 2002; Bellingham et al., 2002). To determine whether melanopsin is a functional sensory photopigment, here we transiently expressed it in HEK293 cells that stably expressed TRPC3 channels. Light triggered a membrane depolarization in these cells and increased intracellular calcium.

The light response resembled that of ipRGCs, with almost identical spectral sensitivity ($\lambda_{max} \approx 479$ nm). The phototransduction pathway included Gq or a related G protein, phospholipase C and TRPC3 channels. We conclude that mammalian melanopsin is a functional sensory photopigment, that it is the photopigment of ganglion-cell photoreceptors, and that these photoreceptors may use an invertebrate-like phototransduction cascade.

Melanopsin Expression Induces Photosensitivity

To test melanopsin's capacity to form a functional sensory photopigment, we heterologously expressed mouse melanopsin (Opn4) complementary DNA in human embryonic kidney cells (HEK293) stably expressing TRPC3 receptor-operated non-specific cation channels (Hurst et al., 1998). The mouse melanopsin open reading frame was cloned into a bicistronic expression vector (pIRES2–EGFP). HEK293-TRPC3 cells transfected with this vector faithfully coexpressed the enhanced green fluorescent protein (EGFP) reporter protein (Fig. 1a, c) and mouse melanopsin (Fig. 1b, c). Melanopsin was localized predominantly in the cell membrane (Fig. 1b), as it is in ipRGCs (Belenky et al., 2003).

Many of these cells were photosensitive. They exhibited large light-evoked changes in transmembrane voltage that were sluggish and sustained (Figs 1d, e). Light responses were never observed among neighboring untransfected, EGFP-negative cells (n = 11), nor in cells transiently transfected with empty pIRES2–EGFP, which expressed EGFP but not melanopsin (n = 10; Fig. 1f). Increasing stimulus irradiance augmented response amplitude (Fig. 1e; see also Fig. 4a) and decreased onset latency from >10 s to a minimum of ~500 ms. Maximal responses were typically >15mV. Thresholds were approximately (5.6 ± 1.7) x 10^{12} photons s^{-1} cm^{-2} at 480 nm (mean ± standard error of the mean, s.e.m.; n = 12), about one log unit higher than for ipRGCs10. Although there was no retinaldehyde in the bath, responsiveness persisted for hours.

With voltage clamped at negative potentials, light triggered an inward current (Fig. 1g) exhibiting strong outward rectification and reversing near 0mV (4.0 ± 3.8mV,

mean ± s.e.m., n = 9; Fig. 1h). Maximal white-light stimuli evoked peak inward currents averaging 30 pA (±13 pA standard deviation, s.d.; n = 5; V_{hold} ≈ 244mV). Light-evoked inward currents were never observed in untransfected cells (Fig. 1h, bottom) or in cells expressing EGFP, but not melanopsin.

Calcium Imaging of Light Response

Light also triggered increases in intracellular free calcium in these cells. Only transfected cells exhibited light-evoked increases in fluorescence of the calcium indicator Rhod-2-AM (Fig. 2). Calcium responses were also detectable in melanopsin-transfected cells lacking the EGFP reporter (Supplementary Fig. 1).

Phototransduction Cascade

Melanopsin is presumably the photopigment in these cells because it was required for the observed photosensitivity. To test the hypothesis that melanopsin, like other opsin photopigments, triggers a G-protein signaling cascade, we infused guanosine 50-O-(2-thiodiphosphate) (GDPβS). Light-evoked depolarizations were reduced by more than half their initial amplitude within 30 min (Fig. 3a; in 3 of 4 cells tested). This was a specific effect, not an artifact of cell dialysis, because cells recorded with the control internal solution maintained stable response amplitudes for ≥70 min (data not shown). Gq or a related protein seems most likely to be the cognate G protein for melanopsin. The light response was effectively abolished within 20 min by internal application of GPant-2a, a competitive inhibitor of Gq, and was suppressed 75–100% by bath-application of U73122, an antagonist of phospholipase C (PLC; n = 3; Fig. 3c), the effector enzyme for Gq-like G proteins (Fields and Casey, 1997). An inactive analogue of the PLC antagonist (U73343; n = 3; Supplementary Fig. 2a) and pertussis toxin (a Gi

inactivator; 250 ng ml^{-1}, 2 h) were ineffective. TRPC3 channels apparently carry the light-activated current in these cells (see Supplementary Data and Supplementary Figs 2b and c).

Spectral Sensitivity

Relative sensitivity at different wavelengths was assessed for individual cells as shown in Fig. 4a. The optimal wavelength (λ_{max}) for each cell was estimated from the best-fitting retinaldehyde template function (Lamb, 1995) (see Methods). These values, plotted in the histogram of Fig. 4b, had a mean value of 479.2nm (±1.5nm s.e.m.; n = 12), very close to the optimal wavelength for isolated rat ipRGCs10 (484 nm). Mean estimates of relative sensitivity at each tested wavelength (Fig. 4b, points) adhered closely to the best-fitting retinaldehyde template function (Fig. 4b, curve), supporting the assumption that the action spectrum in this system adheres to the standard form for opsin-mediated responses.

Melanopsin as a Functional Photopigment

This study provides the first direct physiological evidence that melanopsin is a functional sensory photopigment and that it can activate a signaling cascade that gates an ionic conductance. Melanopsin is an opsin, with the canonical retinaldehyde binding site (Provencio et al., 1998b; Bellingham and Foster, 2002), and most cells expressing it are known or presumed to be directly photosensitive (Provencio et al., 1998b; Provencio et al., 2000; Berson et al., 2002; Hattar et al., 2002; Panda et al., 2002; Provencio et al., 2002; Ruby et al., 2002; Berson, 2003; Lucas et al., 2003; Panda et al., 2003). Its overexpression increases photosensitivity in dermal melanophores (Rollag et al., 2000), and its deletion abolishes the light response in ganglion-cell photoreceptors (Lucas et

al., 2003). Heterologously expressed melanopsin reportedly mediates photic activation of a G protein (Newman et al., 2003), again supporting a photosensory role.

Our findings contradict the idea that melanopsin could be exclusively a photoisomerase instead of a photosensory pigment (Bellingham and Foster, 2002; Bellingham et al., 2002). Introducing melanopsin into HEK293-TRPC3 cells transformed them into photoreceptors. The action spectrum of the induced photoresponse peaked at 479 nm, closely matching the spectral tuning of isolated rat ipRGCs10 ($\lambda_{max} \approx 484$ nm) and of melanopsin-dependent behavioral light responses of rodless/coneless rodents (pupillary light reflex: $\lambda_{max} \approx 479$ nm, (Lucas et al., 2003); circadian phase shifting: $\lambda_{max} \approx 480$–481 nm, (Yoshimura and Ebihara, 1996; Hattar et al., 2003)). Such correspondence was lacking in an earlier study of heterologously expressed melanopsin, which reported maximal absorption at 421nm (Bellingham and Foster, 2002). The discrepancy with our spectral results could stem from the earlier study's use of a different expression system, protein modifications for epitope tagging, effects of opsin solubilization and immunopurification, or the use of chemical rather than photic bleaching to generate the difference spectrum.

Though melanopsin is now firmly established as a photosensory pigment, it may also possess photoisomerase activity. In bistable invertebrate photopigments, the chromophore–opsin linkage is non-dissociating and light can trigger photoreversal of metarhodopsin to rhodopsin. Support for possible melanopsin bistability comes from its invertebrate-like aromatic amino acid at the 'counter-ion' position (Provencio et al., 1998b), a key determinant of chromophore binding, and, as shown here, the persistence for hours of melanopsin-based light responses without supplementary

retinaldehyde despite repeated exposure to bright illumination. Still, there is no direct evidence that melanopsin is bistable. In photoreceptors using bistable pigments, appropriate narrow-band stimuli can terminate persistent poststimulus responses and augment sensitivity to subsequent stimuli by converting metarhodopsin to rhodopsin. We have not detected such behavior in transfected HEK293-TRPC3 cells (see Supplementary Data). If melanopsin instead dissociates from its chromophore like other vertebrate photopigments, its regeneration in HEK293 cells may be attributable to the intrinsic retinoid processing capacity of these cells (Brueggemann and Sullivan, 2002).

Phototransduction Mechanism in ipRGCs

By demonstrating that melanopsin is activated maximally near 480 nm and can indirectly gate cation channels in the plasma membrane, our findings provide nearly all of the missing links in the chain of evidence for melanopsin as the photopigment of ipRGCs (Bellingham and Foster, 2002; Berson et al., 2002; Berson, 2003). There are striking similarities between the photoresponses of melanopsin-expressing HEK293 cells and those of ipRGCs including response polarity, low sensitivity, sluggish onset, tonic response to continuous illumination, and slow poststimulus recovery. It seems probable that some of these properties, like the spectral tuning, derive from features of melanopsin itself, while others are shaped by the downstream signaling cascade.

In HEK293-TRPC3 cells, this cascade involves Gq or a related pertussis toxin-insensitive G protein. Some G-protein-coupled receptors (GPCRs) couple promiscuously to multiple G-protein families (Wong, 2003) and melanopsin itself reportedly interacts with highly concentrated rod transducin (Gt) in a biochemical assay (Newman et al., 2003). Under physiological conditions, however, most GPCRs couple

mainly to only one of the four G-protein families (Wong, 2003), so the cognate G protein for melanopsin in ganglion cells is probably a member of the Gq family (Gq, G11, G14, G15 and/or G16). In HEK293-TRPC3 cells, the G protein activated by melanopsin stimulates PLC to open TRPC3 channels and depolarize the plasma membrane (Fields and Casey, 1997; Hurst et al., 1998; Oh et al., 2003). Drosophila photoreceptors apparently use a similar phototransduction cascade, but it is not yet known whether ipRGCs use (or even express) these specific downstream components.

Methods

Cell culture and melanopsin expression

Cells were cultured conventionally. The mouse melanopsin (Opn4) open reading frame (GenBank accession number NM_013887) was amplified by polymerase chain reaction (PCR) from a vector containing the open reading frame. PCR primers were generated from the melanopsin sequence with restriction enzyme sequences appended to the 5' end to facilitate directional cloning. Two different PCR products were generated for two expression vectors: the plasmid pcDNA 3.1(+) (Invitrogen) and the bicistronic pIRES2–EGFP (BD Biosciences). PCR products were digested with restriction enzymes and ligated into the multiple cloning site of the vectors, also digested with restriction enzymes. Expression vector sequences were confirmed by dideoxy terminator sequencing. Cells were transfected with the calcium phosphate method and seeded on coverslips. From transfection until recording, cells were provided with retinaldehyde (0.5 μM 11-*cis* retinaldehyde for spectral studies, 2 μM all-*trans*-retinaldehyde for all other experiments) and were kept in darkness, except for brief exposures to laboratory lighting. Omitting these retinoids did not preclude photosensitivity (see Supplementary

Data).

Melanopsin immunohistochemistry

Cells were fixed overnight (4% paraformaldehyde in phosphate-buffered saline, PBS), incubated in polyclonal rabbit anti-melanopsin antiserum (UF006, (Provencio et al., 2002), 1:2,500; 24 h, 4°C), then in Cy-3-conjugated anti-rabbit IgG (1:500; Jackson ImmunoResearch; 1 h; 21°C). Photomicrographs were obtained on a Zeiss Pascal confocal microscope using a 40 X objective.

Electrophysiology

Cells transfected 1–2 days earlier were mounted in a chamber perfused with Tyrodes solution containing no retinaldehyde (1–2 ml min^{-1}, 21°C). Although incubated in darkness, cells were exposed to normal laboratory lighting during preparation for recording except in spectral studies, which were conducted in darkness. Cells were visualized with a 40 X water-immersion objective on an upright epifluorescence microscope equipped with a cooled charge-coupled device (CCD) camera, integrating frame grabbers and video monitor. Except for brief blue epifluorescence to identify EGFPpositive cells (Chroma no. 41001), only infrared light was used to view cells.

Whole-cell patch recordings were made conventionally. Micropipettes (3–5MΩ) contained 120mM K-gluconate, 5mM NaCl, 4mM KCl, 0.5–2mM EGTA, 10mM HEPES, 0.5–4mM ATP-Mg, 7mM phosphocreatine, 0.05–0.3mM GTP-Tris; (280–300 mOsm, pH 7.3). Liquid junction potentials (-14mV) were corrected. In darkness, most cells had series resistance <10MΩ, Vm ≈ -35 to -45mVand input resistance of 0.5–1.5GΩ. Evoked currents or voltages are baseline-subtracted.

Photic stimulation and spectral analysis

Light stimuli from a 100-W tungsten source were filtered (neutral density and narrowband interference filters;10 nm width; Oriel), gated by a shutter (Uniblitz VS35;Vincent Associates) and calibrated by a radiometer (S370, UDT Instruments). The irradiance of the unfiltered ('white') stimulus was (in photons s^{-1} cm^{-2}): 4 X 10^{12} at 400 nm, 6 X 10^{13} at 500 nm and 1 X 10^{14} at 600 nm.

For spectral analysis, the culture medium contained 11-cis-retinaldehyde. Current injection held cells near -44mV. Stable sensitivity, confirmed by retesting with a standard stimulus, required long interstimulus intervals (7 min). Stimuli of 480nm were interleaved with those of two to three other wavelengths (420, 440, 540, 570 or 600 nm). Each response to one of these other wavelengths yielded an estimate of relative sensitivity normalized to that at 480 nm (see Fig. 4a), and these were averaged for each wavelength. For each cell, we determined the retinaldehyde template function (Lamb, 1995) that best fitted these data (least-squares method). The two free parameters for the fit were λ_{max} and the vertical offset. Relative sensitivities were then re-normalized to that at the theoretical optimum (λ_{max}).

Calcium imaging

Cells loaded with the long-wavelength Ca^{2+} indicator Rhod-2-AM (4–6 μM in Tyrodes, 30 min, 37 ^{o}C) were imaged at 30 Hz using green excitation (525–550 nm; emission 580–650 nm; Chroma 31002a) attenuated 128- to 512-fold by neutral density filters. Integration time was fixed within trials (typically 32 frames) to optimize sensitivity and avoid saturation. Responses, analysed by ImageJ software (W. Rasband; http://rsb.info.nih.gov/ij/; 2004), were expressed as post-stimulus change in

fluorescence intensity above baseline divided by the baseline intensity (DF/F). Further methodological details are provided in the Supplementary Methods.

Figures

Figure 1

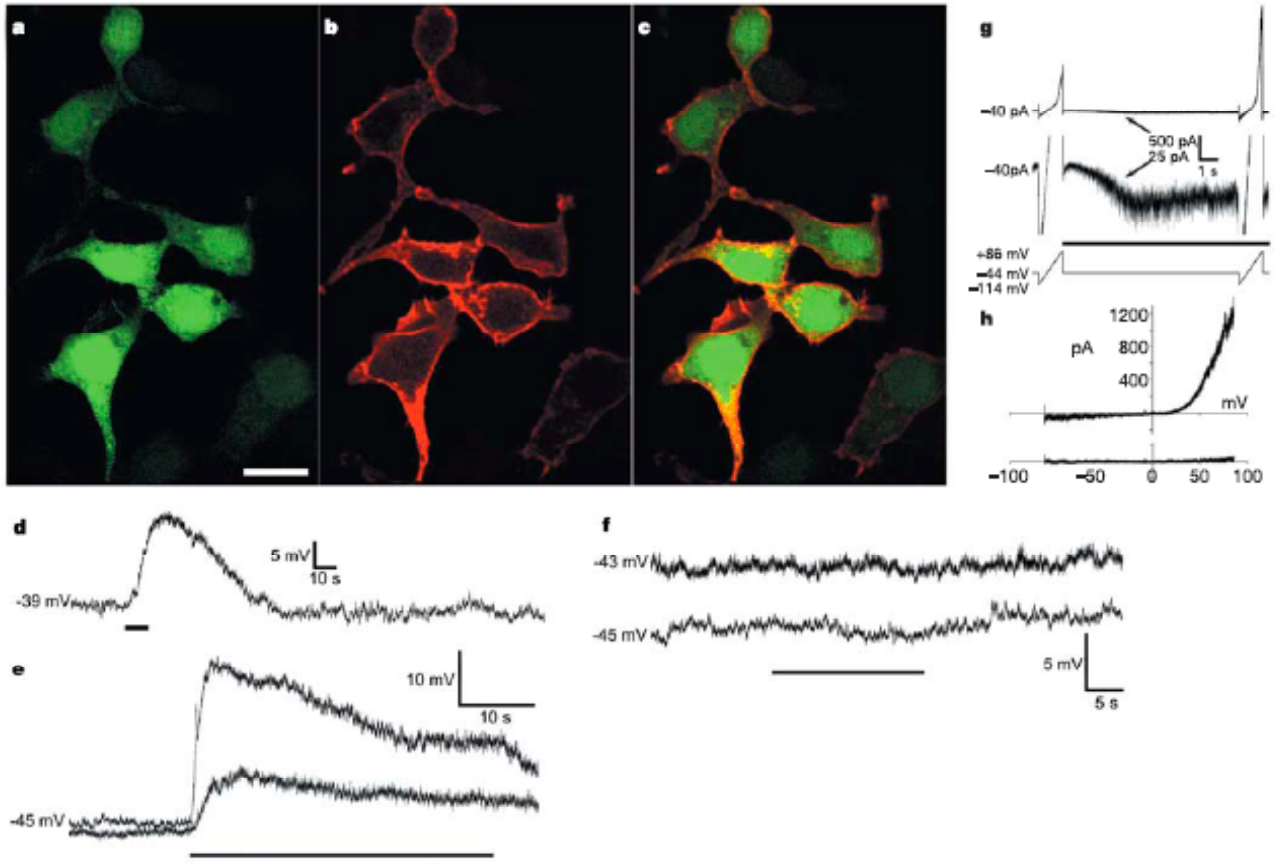


Figure 1 Light-evoked responses of melanopsin-expressing HEK293-TRPC3 cells. a–c, Melanopsin expression. Confocal photomicrographs showing EGFP (a, green), melanopsin immunofluorescence (b, red) and merge (c). Scale bar, 20 μm. d, e, Current clamp recordings of light-evoked depolarization in transfected cells. d, Sluggish response to 10-s flash (bar marks stimulus; 480 nm, 9 X 10^{14} photons s^{-1} cm^{-2}). e, Intensity coding. The white-light stimulus for top trace was one log unit more intense than for bottom trace. f, Absence of photosensitivity in untransfected cells (top trace) and in cells expressing EGFP but not melanopsin (bottom trace). g, h, Voltage-clamp recordings. g, The light stimulus (horizontal bar) elicits tonic inward current and increased current noise (middle trace). The same trace at lower gain (top trace) shows responses to voltage ramps (bottom trace) imposed before and during light response. h, Current–voltage relations of light-evoked current in g (stimulated minus dark current) (top trace). Absence of light-evoked current in untransfected cell (bottom trace).

Figure 2

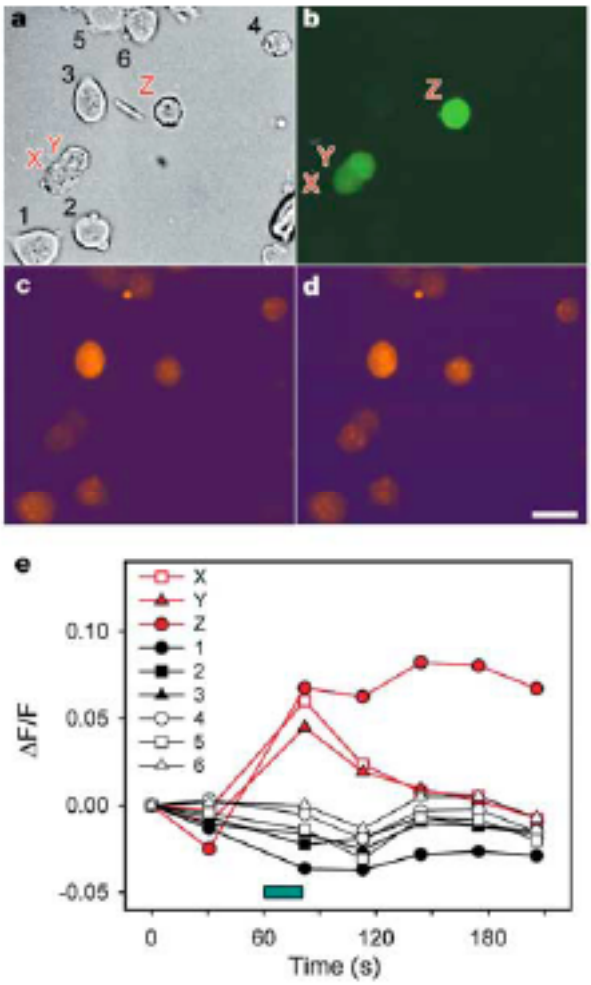


Figure 2 Light evokes calcium responses. Brightfield image (a) and EGFP fluorescence (b) of live cells. Of nine cells, three (X, Y and Z) were transfected (EGFP+). c, d, Pseudocolour images of Rhod-2-AM fluorescence before (c) and after (d) exposure to a light stimulus (500 nm, 10^{15} photons s^{-1} cm^{-2}). Only transfected cells exhibited increased calcium signals. Scale bar, 20 μm. e, Normalized change in fluorescence over time for transfected cells (red lines) and untransfected cells (black lines). The green bar marks the stimulus.

Figure 3

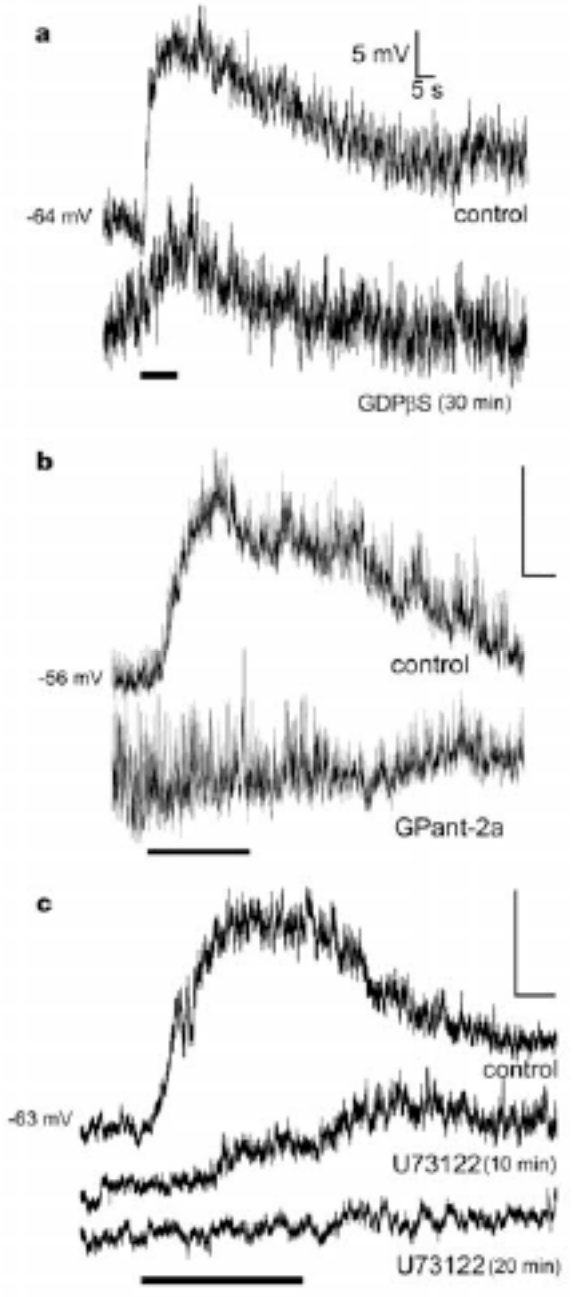


Figure 3 Phototransduction cascade. Voltage responses to bright white-light stimuli (horizontal bars) before and during drug application. Photoresponses were reduced or blocked by internal application of GDPS (a; 2 μM), a non-specific antagonist of G proteins, or of GPant-2a (40 μM), a Gq/11 antagonist (b). c, Bath application of the PLC antagonist U73122 (10 μM) eliminated the light response (c); the inactive analogue U73343 had no effect (Supplementary Fig. 2a). Calibration bars, 5 mV, 5 s.

Figure 4

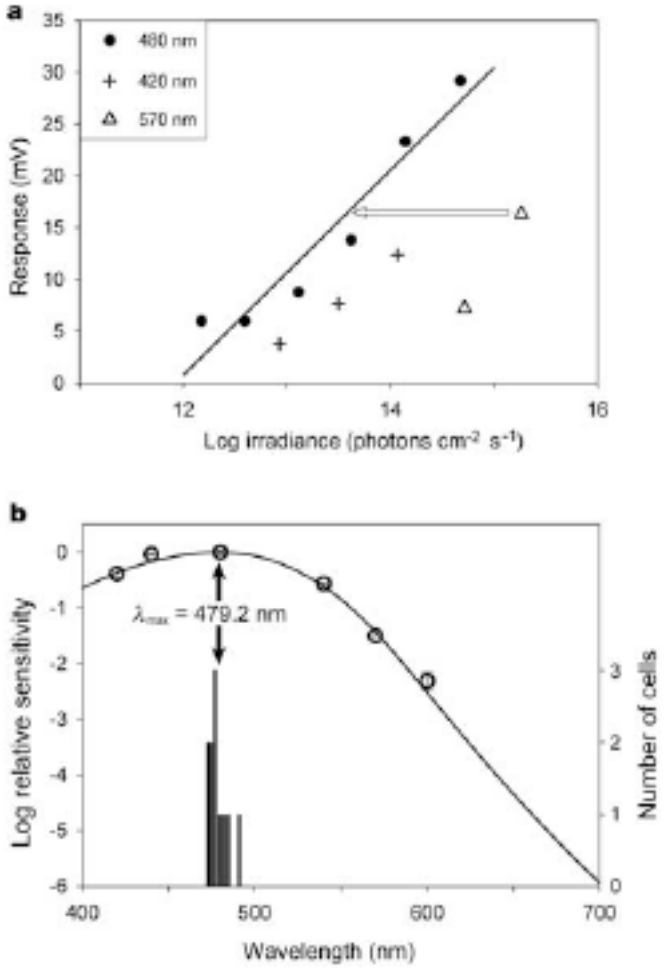


Figure 4 Spectral sensitivity. a, Plot of peak depolarization evoked in one cell by stimuli of various wavelengths. The solid line shows the least-squares linear fit to 480-nm data (solid circles). The horizontal displacement of other points from this line (open arrow) estimates sensitivity at corresponding wavelength relative to 480 nm. b, The histogram shows the distribution of the estimated optimal wavelength (λ_{max}), determined for each cell from the best-fitting retinaldehyde template. Mean λ_{max} = 479.2 nm ± 1.5 nm (n = 12). Above the histogram, each point represents the mean value of relative sensitivity at that wavelength among all cells (error bars, barely visible, are s.e.m.). The curve is the least-squares best-fitting retinaldehyde template function; λ_{max} (479.2 nm) has an ordinate value of 0. Medium supplemented with 11-cisretinaldehyde.

Supplementary Data:

TRPC3 forms the light-activated channels of HEK293-TRPC3 cells

The light-activated current in these cells appears to pass through the TRPC3 channels they express. Both the reversal of the light-activated current near 0 mV and its pronounced outward rectification (Fig. 1H) resemble TRPC3-mediated cationic currents previously characterized in this and other cell types. Further, the light response was blocked by substitution $LaCl_3$ (100 μM) for $CaCl_2$ in the bath (Supplementary Fig. 2B) or external application of SK96365 (100 μM), both known to non-selectively block TRPC-mediated currents. A closely related cell line lacking the TRPC3 protein (HEK-tsA) never exhibited detectable light responses when transfected with the melanopsin expression vector (n=10; data not shown). The Gq-PLC-TRPC3 cascade to which melanopsin couples in this study closely resembles that previously characterized for endogenous M1 muscarinic and purinergic receptors in this cell line. Supplementary Figure 2C provides direct evidence for such sharing of signalling components by showing that the inward current induced by the muscarinic agonist carbachol (200 μM) substantially occluded the light-evoked current (n=3).

Effects of omitting or altering the retinoid chromophore

Melanopsin-expressing HEK293-TRPC3 cells exhibited photosensitivity even when supplementary retinaldehyde was omitted from the culture medium as well as from the recording bath (data not shown). We suspect that this is because retinoids and retinoid binding proteins are present in the serum added to the culture medium.

There were no obvious differences in the form of the light response between cells provided all-*trans* retinaldehyde after transfection and those provided with 11-*cis* retinaldehyde. Spectral analysis of cells provided with all-*trans* rather than 11-*cis* retinaldehyde revealed an action spectrum very similar in form to that in Fig. 4A, but peaking at a slightly shorter wavelength (~470 nm). Multiple retinaldehyde isomers arise from exposure of all-*trans* retinal to light (Maeda et al., 1978) and we do not know which of these served as the chromophore for melanopsin when all-*trans* was the supplied retinoid.

Additional control studies for spectral analysis

Because there has been speculation that melanopsin might be a bistable pigment, we considered the possibility that prior exposure to certain narrow-band stimuli might affect the cell's sensitivity through differential reisomerization (photoreversal). However, a post-hoc analysis of our data indicates that sensitivity to a standard stimulus (480 nm) was not affected by the spectral composition of the immediately preceding stimulus.

We considered the possibility that the EGFP co-expressed by the cells might distort the action spectrum by acting as a screening pigment, absorbing stimulus light in a spectrally dependent manner. However, quantitative analysis revealed no detectable difference in transmittance through cells that expressed EGFP and those that did not. Nor would light emitted by fluorescing EGFP have distorted the action spectrum because by our estimates our spectral stimuli would have triggered fluorescence irradiances at least 7 log units below the threshold of the phototransduction mechanism in melanopsin-expressing HEK293-TRPC3 cells. The close agreement between the

data and the retinaldehyde template function (Fig. 4B) would not be expected if EGFP absorption or emission substantially distorted the action spectrum.

We also considered the possibility that using irradiance-response data at 480 nm as the standard for evaluating sensitivity at other wavelengths might somehow have biased the estimated λ_{max} toward 480 nm. A post hoc analysis of the data determined that this was not the case by confirming that λ_{max} values were essentially unchanged if data at 480 nm were excluded from the analysis and another wavelength was used as the benchmark.

Supplementary Methods:

Cell culture. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; with 4.5 g/L G glucose, L-glutamine, 110 mg/L sodium pyruvate; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, and 100 units/ml penicillin, 100ug/ml streptomycin at 37°C in the presence of 5% CO2 and diluted twice weekly. Control recordings in cells lacking the TRPC3 protein were made in T-cell surface antigen 201 HEK cells ("HEK-tsA").

Functional expression of melanopsin. The mouse melanopsin (*opn4*) open reading frame (ORF; GenBank accession number NM_013887) was PCR amplified (AmpliTaq Gold Polymerase: Applied Biosystems; Foster City, CA) from a pCRII-TOPO vector (Invitrogen) that contained the mouse melanopsin ORF. PCR primers were generated from the melanopsin sequence with restriction enzyme sequences appended to the 5' end to facilitate directional cloning (IPF320, 5'-agcgctgctagcatggactctccttcaggacca-3'; IPB335, 5'-gtcgaccccgggctacagatgtctgagagtcacatcatc-3'; IPB378, 5'-

ctcgagctacagatgtctgagagtcacatcatc-3'). PCR products were digested with the appropriate restriction enzymes (*NheI*, *XmaI*, and *XhoI*) before ligation into the vectors. Vector melanopsin sequences were confirmed by dideoxy terminator sequencing. After transfection by the calcium phosphate method (Chen and Okayama, 1987), EGFP fluorescence appeared in cells within 24h. Retinaldehyde was added to the medium from a stock dissolved in dimethyl sulfoxide (DMSO) to a final medium concentration of 2 μM. Control vectors included the empty pIRES-EGFP vector lacking melanopsin and a pcDNA3.1(+)-melanopsin vector lacking EGFP.

Electrophysiology. Cells were seeded on coverslips coated with poly-D-lysine (100 μg/ml; Sigma, St. Louis, MO) and mounted in a chamber (Warner Instruments, Hamden, CT; RC-26GLP). The bath of Tyrodes solution consisted of (in mM) NaCl 140, KCl 5, CaCl2 2, glucose 15, HEPES 10, pH 7.4 (~299 mosM). The upright epifluorescence microscope used for recordings was a Nikon E600 equipped with cooled CCD camera (DAGE-MTI 300T), integrating frame grabbers (DAGE-MTI IFG; 10 bit) and Scion model LG3-16 [8 bit], and video monitor.

Micropipettes were pulled from borosilicate glass on a Flaming/Brown puller (P-97; Sutter, Novato, CA). Whole-cell or perforated patch recordings were made with an Axopatch 1D or Multiclamp 700A amplifier and PClamp software (Axon Instruments, Union City, CA), with digitization rates >4 times the low-pass cutoff (1-4 kHz). In a few cells, for perforated patch recordings were made with pipettes containing (in mM) K-gluconate 140, NaCl 5, KCl 4, HEPES 10, glucose 10 and amphotericin B 100 μg/ml, pH 7.3 (~283 mOsm).

Most pharmacological agents were dissolved in Tyrodes solution and bath-applied. GPant-2a (Mukai et al., 1992) and GDPβS were dissolved in the pipette solution. GDPβS, GPant-2a, U73122 and U73340 were obtained from Biomol (Plymouth Meeting, PA); pertussis toxin from Calbiochem (San Diega, CA); 11-*cis* retinal from Dr. Rosalie Crouch, and all other compounds from Sigma.

Photic stimulation and spectral analysis. Light stimuli from a 100W tungsten source were filtered (neutral density and narrow-band interference filters; 10 nm width; Oriel, Stratford, CT), gated by shutter (Uniblitz VS35; Vincent Associates, Rochester, NY) and calibrated by a radiometer (S370, UDT Instruments, Baltimore, MD). The irradiance of the unfiltered ("white") stimulus was (in photons·sec^{-1}·cm^{-2}) 4 x 10^{12} at 400nm, 6 x 10^{13} at 500nm and 1 x 10^{14} at 600nm.

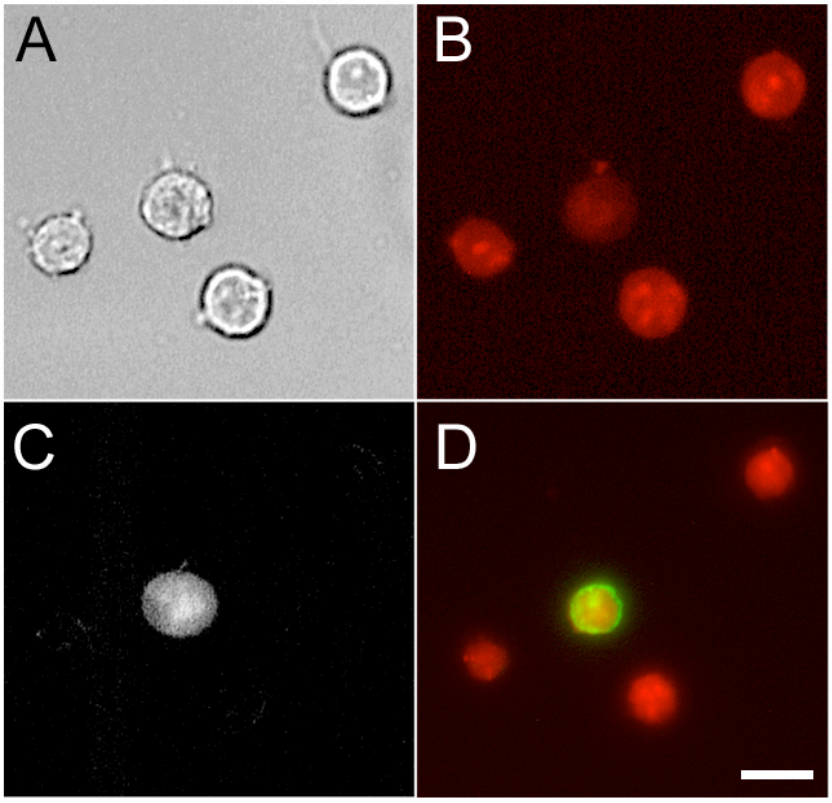
For the spectral studies, individual cells were first tested with light of 480 nm at 3-4 intensities within the dynamic range of the photoresponse. Two or three other wavelengths were then tested once each at a single intensity, followed by retesting at 480 nm at a single intensity to confirm the stability of sensitivity. The other wavelengths were then retested in reverse order, usually at a new intensity, followed by a retest at 480 nm. The process was repeated until reduced sensitivity at 480 nm signalled rundown or until the recording was lost. This interleaved, counterbalanced design controlled for possible effects of stimulus order on apparent sensitivity. Such effects might result from gradual rundown of responsiveness or from wavelength-dependent depletion or replenishment of the pool of available photopigment, as occurs in bistable opsins. Nine cells were tested with a set of three wavelengths (480nm, 420nm and 570nm), and 3 cells were tested with a largely distinct set of 4 wavelengths (480nm,

440nm, 540nm and 600nm). In individual cells, we tested an average of 4.3 intensities at 480nm and 2.3 intensities for other wavelengths. Each intensity was tested an average of 1.4 times.

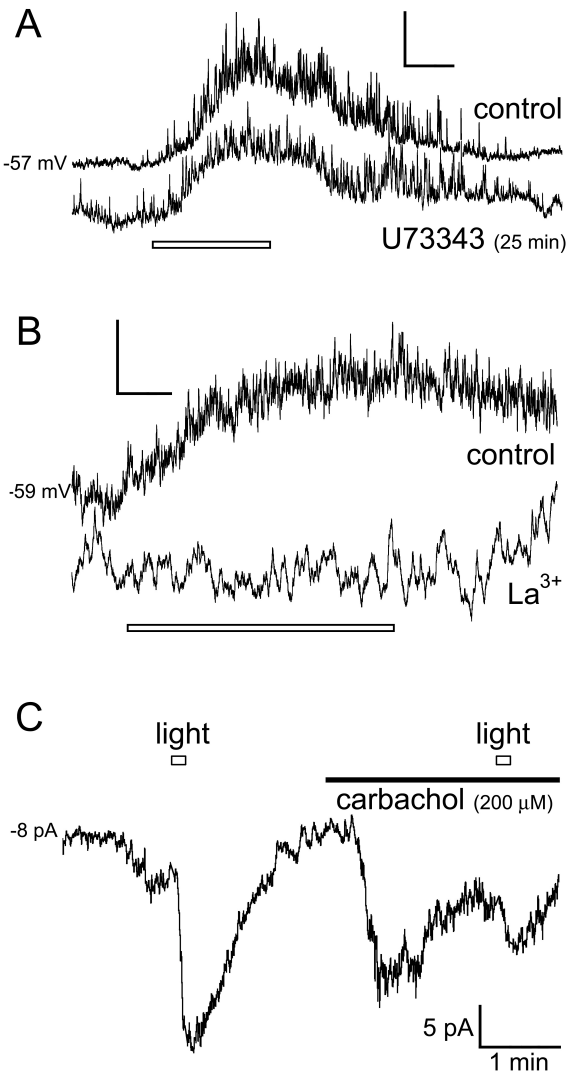
In analyzing the irradiance-response data for individual cells, we considered the possibility that a Michaelis-Menten function might better fit the 480 nm data than did the linear regression illustrated in Fig. 4A and might yield a different spectral sensitivity curve. The action spectrum derived from this alternative analysis was essentially unchanged, with the optimal wavelength (λ_{max} = 478.8 nm ±1.38 nm S.E.) within 1nm of that derived when using the linear fit. We opted for the linear fit because the Michaelis-Menten method yielded more variable results, probably in part because our data did not routinely sample the saturating portion of the dynamic range of the light response.

Calcium imaging. We selected the non-ratiometric, long-wavelength Ca^{2+} indicator Rhod-2-AM (Minta et al., 1989; Molecular Probes, Eugene, OR) for its spectral separation from melanopsin absorbance and from EGFP excitation and emission. Cells loaded with Rhod-2-AM (4-6 μM in Tyrodes, 30 min, 37 °C) were superfused with Tyrodes as for electrophysiology. For Fig. 4F-I, a reference cell near the imaged field was filled by a patch pipette with the ultraviolet dye Fluorogold to permit alignment of the immunofluorescence and calcium images.

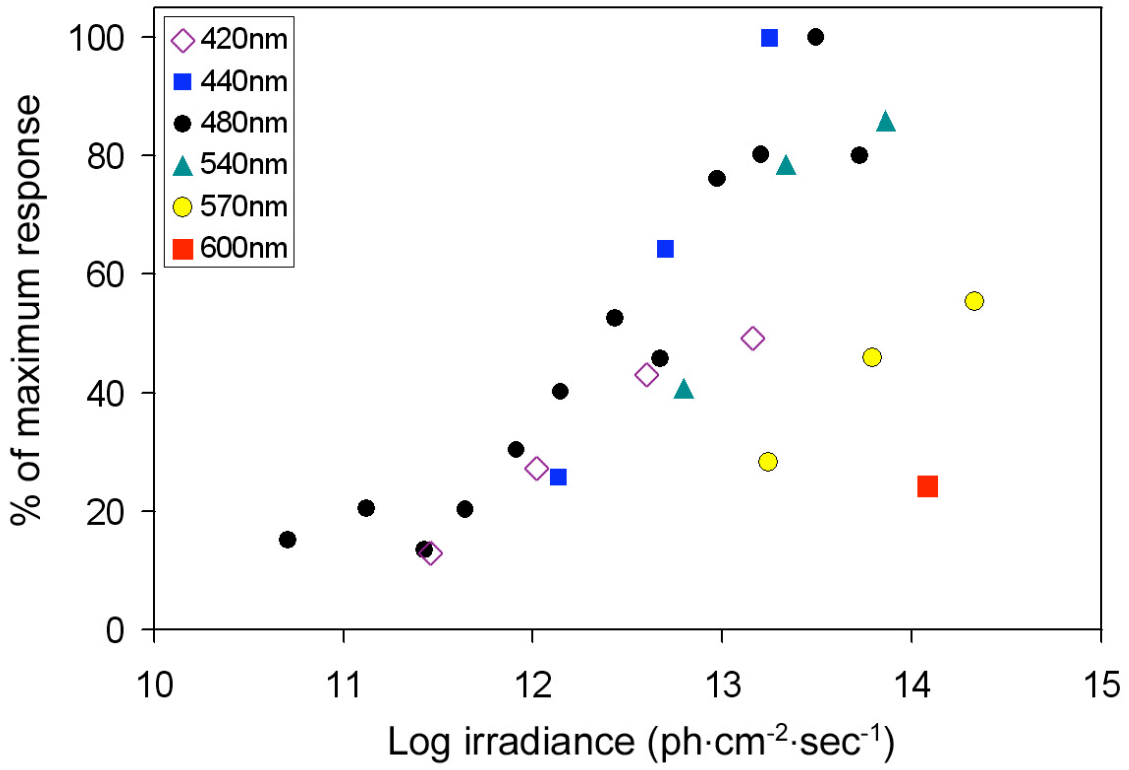
SUPPLEMENTARY FIGURES



Supplementary Figure 1 - Light evokes calcium responses in cells expressing melanopsin but not EGFP. Brightfield (A) and basal Rhod-2 fluorescence (B) live-cell images reveal 4 cells. Grayscale difference image (C; evoked minus basal fluorescence) shows that only one of these exhibited a calcium response to light (480 nm; 9 x 10^{14} photons·sec^{-1}·cm^{-2}). D: Anti-melanopsin immunofluorescence (green) overlaid on the Rhod-2 fluorescence (red) visible after processing; there is some histological shrinkage. Only the photosensitive cell in C exhibits melanopsin-like immunoreactivity. Scale bar: 20 µm.



Supplementary Figure 2 - A: Bath application of U73343 (10 μM), the inactive analog of the phospholipase C (PLC) antagonist U73122 (see Fig. 3C), failed to block light-evoked depolarization in a transfected HEK293-TRPC3 cell. B: Bath application of lanthanum, a cationic channel blocker effective against TRP channels, abolished the light response. C: Phototransduction cascade in transfected HEK293-TRPC3 cells shares signalling components with the M1 muscarinic pathway. Voltage-clamp recording shows inward current evoked by a standard flash (white bars) is reduced in the presence of inward current evoked by the muscarinic agonist carbachol (black bar). Calibration bars: A,B: 5 mV, 5 sec; C: 5 pA, 1 min.



Supplementary Figure 3 - Cumulative intensity-response data. Each point represents the mean normalized response, expressed as percentage of the maximal response at 480 nm, among all cells tested at that intensity and wavelength.

Melanopsin Triggers the Release of Internal Calcium Stores in Response to Light

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ABSTRACT

Melanopsin is the photopigment that confers photosensitivity upon intrinsically photosensitive retinal ganglion cells (ipRGCs). This subset of retinal ganglion cells comprises less than 2% of all RGCs in the mammalian retina. The paucity of melanopsin-positive cells has made studies on melanopsin signaling difficult to pursue in ipRGCs. To address this issue, we have established several cell lines consisting of a transformed human embryonic kidney cell line (HEK293) stably expressing human melanopsin. With these cell lines, we have investigated the intracellular rise in calcium triggered upon light activation of melanopsin. Our human melanopsin-expressing cells exhibit an irradiance-dependent increase in intracellular calcium. Control cells expressing human melanopsin, where the Schiff-base lysine has been mutated to alanine, show no responses to light. Chelating extracellular calcium has no effect on the light-induced increase in intracellular calcium suggesting that calcium is mobilized from intracellular stores. This involvement of intracellular stores has been confirmed through their depletion by thapsigargin, which inhibits a subsequent light-induced increase in intracellular calcium. Addition of the non-selective cation channel blocker, lanthanum does not alter light-induced rises in intracellular calcium, further supporting that melanopsin triggers a release of internal calcium from internal stores. HEK293 cells stably expressing melanopsin have proven to be a useful tool to study melanopsin-initiated signaling.

INTRODUCTION

The relatively recent identification of the photopigment melanopsin has added a new level of complexity to the long-standing concepts of phototransduction within the mammalian eye. For decades, it has been assumed that the rods and cones are solely responsible for all ocular photoreception. However, the discovery of melanopsin (Provencio et al., 1998b) has led to the identification of intrinsically photosensitive retinal ganglion cells (ipRGCs) (Berson et al., 2002), a new atypical photoreceptor class with a multitude of functions (Gooley et al., 2003; Kumbalasiri and Provencio, 2005; Hattar et al., 2006). The melanopsin containing ipRGCs are a small population of broadly distributed cells that represent 1-2% of retinal ganglion cells (Hattar et al., 2002; Morin et al., 2003; Sollars et al., 2003). ipRGCs project to various irradiance processing sites in the brain that are known to participate in non-visual light responses such as circadian photoentrainment, the pupillary light reflex, and regulation of sleep-wake states (Hattar et al., 2002; Gooley et al., 2003; Morin et al., 2003; Dacey et al., 2005; Hattar et al., 2006). Additionally, melanopsin has been implicated in the regulation of rod- and cone-mediated visual pathways (Hankins and Lucas, 2002; Belenky et al., 2003; Dacey et al., 2005; Barnard et al., 2006).

While the central projections of ipRGCs can provide insight into potential roles of this novel photoreceptor class, some of the functions of ipRGCs have been elucidated experimentally through gene knock-out studies. Mice null for melanopsin can photoentrain activity rhythms but demonstrate deficiencies in light-induced phase shifting of circadian locomotor rhythms (Panda et al., 2002; Ruby et al., 2002), the pupillary light reflex (Lucas et al., 2003), and the acute inhibition of locomotor activity by

light (negative masking) (Mrosovsky and Hattar, 2003). In the absence of functional rods and cones, melanopsin-null mice completely fail to photoentrain circadian rhythms, exhibit no pupillary light reflex, are incapable of photically regulating the melatonin biosynthetic pathway, and show no masking responses to light (Hattar et al., 2003; Panda et al., 2003).

Since its initial characterization, melanopsin has proven to be quite different from the opsin-based photopigments of rods and cones. At the amino acid level, it shares greater homology with the invertebrate opsins than the vertebrate visual opsins (Provencio et al., 1998b). This invertebrate-like character is also evident in predicted functional domains of the protein and the signal transduction cascade initiated by photoactivation of melanopsin (Isoldi et al., 2005; Panda et al., 2005; Qiu et al., 2005).

Unlike the cGMP-mediated phototransduction pathway of rods and cones, melanopsin triggers a phosphoinositide signaling cascade. For example, light-induced melanosome dispersion in cultured amphibian melanophores is a melanopsin-dependent phenomenon (Rollag et al., 2000) that can be pharmacologically blocked by inhibitors of phospholipase C (PLC) and protein kinase C (PKC) (Isoldi et al., 2005). In addition, chelation of intracellular calcium blocks light-induced melanosome dispersion (Isoldi et al., 2005). Heterologous expression systems have also indicated that melanopsin can activate phosphoinositide signaling resulting in transient increases in intracellular calcium concentrations (Panda et al., 2005; Qiu et al., 2005). There are many different signaling mechanisms by which a rise in calcium can occur. Furthermore, whether such increases arise from internal calcium stores, influx of

external calcium, or through a combination of both of these mechanisms still remains to be determined.

In this study, we have used the calcium sensitive fluorophore Rhod2-AM to investigate the source of melanopsin-initiated intracellular calcium increases in our heterologous expression system. Human embryonic kidney cell lines (HEK293) stably expressing human melanopsin have provided us a semiquantitative system with a robust signal-to-noise ratio to study calcium mobilization. We show that when stably transfected with melanopsin, HEK293 cells produce an irradiance-dependent intracellular increase in calcium. Overexpression of TRPC3 channels does not alter the calcium response in these cells, however depletion of intracellular calcium stores prevents subsequent light-mediated increases in intracellular calcium. Taken together, these data indicate that the rise in intracellular calcium is primarily from the mobilization of calcium from internal stores.

MATERIALS AND METHODS

HEK293 Cells

Cells were cultured in Dulbecco's modified Eagle medium (DMEM, containing 4,500 mg/L D-glucose, 584mg/L L-glutamine, 110 mg/L sodium pyruvate; Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 400 µg/ml G418, and 100 nM all-*trans*-retinaldehyde. The cells were grown at 37°C and 5% CO_2. HEK293 cell lines stably expressing the canonical *transient receptor potential channel 3* (TRPC3) were a gift from Mike Xi Zhu (Ohio State

University). HEK293 cells not overexpressing TRPC3 were purchased from BD Clontech (catalog #630903).

Construction of Expression Vectors

The human melanopsin open reading frame (GenBank accession number NM_033282) was digested with the restriction enzyme *EcoR*I from a pCR II-TOPO vector (Invitrogen, Carlsbad, CA, USA, 45-0640) containing the entire human melanopsin open reading frame (PZEO-hOPN3.29.05.10). The digested product was ligated into the multicloning site of the vector pcDNA3.1/Zeo (+) (Invitrogen, Carlsbad, CA, USA, V860-20), which had also been linearized with *EcoR*I. Following ligations and confirmation of the vector, the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA, 200518) was used to introduce the mutation of the lysine-340 residue to an alanine. The nucleotide sequences of the vectors were confirmed by dideoxy terminator sequencing (BigDye Terminator, Applied Biosystems, Foster City, CA, USA).

Stable transfection

Vectors were linearized with the restriction enzyme *Sca*I and purified by ethanol precipitation. Cells were transfected using the calcium phosphate method as described by Kingston, Chen, and Okayama (2003). Twenty-four hours after transfection, cells were harvested, diluted, and treated with medium supplemented with 500ug/ml zeocin in order to isolate and expand the individual clones. Stably transfected clones were subsequently confirmed by anti-melanopsin immunocytochemistry (Provencio et al., 2002).

Immunocytochemistry

Cells were washed with phosphate buffered saline (PBS; pH 7.4) and fixed for one hour with 4% EM-grade paraformaldehyde in PBS at 4^{o}C. Following fixation and washing with PBS, the cells were incubated in a 6% goat serum block in PBS for one hour. The cells were then placed in a 1:4000 dilution of anti-melanopsin primary antiserum (UF028) for an overnight incubation at 4^{o}C. This rabbit-generated polyclonal antiserum was made against a peptide representing the 15 N-terminal amino acids of the predicted human melanopsin sequence. Following the overnight incubation, cells were placed in a 1:500 dilution of the goat anti-rabbit IgG secondary antibody conjugated to Alexa Fluor 555 (Invitrogen, Carlsbad, CA, USA, A21429) for 1 hour at room temperature. Vectashield containing the nuclear stain DAPI (Vector Laboratories, Burlingame, CA, USA, H-1200) was added and the cells were coverslipped. Laser scanning confocal photomicrographs were taken with the 63x water objective of a Zeiss LSM 510 META.

Calcium Imaging

Cells were seeded 48 hours prior to the experiments on circular coverslips precoated with polyethylineimine (PEI; 25ug/ml) (Vancha et al., 2004). To observe changes in intracellular calcium, the long-wavelength excitation/emission calcium indicator Rhod-2AM (Invitrogen, Carlsbad, CA, USA, R-1244) was used. To improve loading and increase retention of the indicator, Probenecid (Invitrogen, Carlsbad, CA, USA, P36400), an inhibitor of organic-anion transporters was added. Cells were placed in the

dark at least 4 hours prior to the experiment. Loading and handling prior to the experiment was carried out under dim red light. Hanks Buffered Saline Solution (HBSS; Invitrogen, Carlsbad, CA, USA, 14175-095) was used to load the Rhod-2AM and Probenecid (5μM and 2.5mM, respectively; 30min; 37°C). After incubation, cells were washed twice with HBSS and placed into the imaging chamber with HBSS containing or lacking calcium (Invitrogen, Carlsbad, CA, USA, 14025-092), depending on the experimental protocol. Cells were viewed with the 63x water objective of the Zeiss LSM 510 META. The fluorophore was monitored at a wavelength of 561 nm at an excitation wavelength of 488 nm at the specified intensities. For specific experiments, Thapsigargin (1μM; Sigma, St. Louis, MO, USA, T-9033) and Lanthanum Chloride (200 μM; Sigma, Sigma, St. Louis, MO, USA, L4131) were added to the well.

Data Analysis

Nine 40 x 40μm areas from each field of view were measured for fluorescence intensity as a function of time for each experiment. There were approximately 3-5 cells per area measured. Images were captured every 1.5 seconds. The ratio of change in fluorescence divided by baseline fluorescence ($\Delta F/F$) was calculated to normalize the data. This data was analyzed and plotted with GraphPad Prism 4 software.

Real Time PCR

Total RNA was extracted with *Tri-Reagent-LS* (1ml/75cm^2 confluent cell flask, Sigma, St. Louis, MO, USA) according to manufacturer's instructions. The RNA *pellet* was re-suspended in H_2O/DEPC (diethyl-pyrocarbonate, Ambion, Austin, TX, USA).

The RNA samples were treated with DNAse according to manufacturer's instructions (turbo-*DNA-Free*^{TM}, Ambion Inc.), and RNA concentration (OD260) was determined in a spectrophotometer.

The RNA was reverse transcribed with 1µg of RNA, 1µl of random *primers* (0.2µg/µl), 1µl of dNTPs (10mM, Invitrogen, Carlsbad, CA, USA), and H_2O/DEPC to complete the volume of each sample to 13µl, followed by incubation at 65^{o}C for 5min. 7µl of a solution containing 1µl of ribonuclease inhibitor (40U/µl), 1µl of DTT (0.1M), 1µl of Superscript III and 4µl of PCR 5X buffer (all from Invitrogen, Carlsbad, CA, USA) were added to the tubes after centrifugation and cooling. The tubes were then incubated for 5min at 25^{o}C, 50min at 50^{o}C, and the reaction was inactivated by 15min at 70^{o}.

The real-time (quantitative) PCR reactions were performed with a pair of primers specific for human TRPC3 channel: forward: 5'-CAA AGC TTC ATT AAG TCG TGT CAA A-3'; backward: 5'-CAA GAG CTG CTG CTG GCA-3', spanning the second intron (GenBank access number NW_922217) designed by Primer Express program (Applied Biosystems, Foster City, CA, USA), synthesized by Invitrogen, Carlsbad, CA, USA, in 96 well-plates. The 18S ribosomal RNA (forward: 5'-CGG CTA CCA CAT CCA AGG AA-3'; backward: 5'-GCT GGA ATT ACC GCG GCT-3') was utilized as a control to normalize the values of TrpC3 channel mRNA. The reaction mixes were prepared with *primers* (300nM for TrpC3 channel, 50nM for RNA 18S), iQ™ SYBR® Green Supermix 2X (100mM of KCl, 40mM of Tris-HCl, 1.6mM of dNTPS, iTaq DNA polymerase 50U/ml and 6mM of $MgCl_2$, BioRad Laboratories, Hercules, CA, USA) and H_2O to the final volume of 24µl/well. The reactions were carried out in the iCycler thermal-cycler (BioRad Laboratories, Hercules, CA, USA), with the following conditions: 1 cycle at

95°C for 7min; 50 cycles of 95°C for 10sec, 60°C for 1min. The data were compared by analysis of variance (ANOVA) followed or not by Student-Newman-Keuls test. The difference was considered significant when $p<0.05$. The specificity of the reaction was confirmed by a subsequent melting point analysis.

RESULTS

Generation of Stable lines

We generated three stably transfected cell lines for these studies. The hOPN4 line was created by stably transfecting human melanopsin into a HEK293 parental cell line that does not overexpress the TRPC3 channel. This parental cell line was also used to generate our control cell line (hOPN4-K340A), which stably expresses a mutant human melanopsin. In this mutant melanopsin, the seventh transmembrane domain Schiff-base lysine necessary for chromophore linkage is replaced with an alanine residue thereby rendering it incapable of covalently binding retinoid chromophore. The hOPN4-TRPC3 cell line was created by stably transfecting human melanopsin into the HEK293-TRPC3 parental cell line, which overexpresses the TRPC3 channel and has been used previously for melanopsin expression studies (Qiu et al., 2005). All three cell lines were immunopositive for melanopsin whereas the untransfected parental HEK293 cell line and the TRPC3 cell line (overexpressing TRPC3 but not expressing melanopsin) were immunonegative for melanopsin (Fig. 1). Melanopsin appeared properly targeted to the plasma membrane in all cell lines stably transfected with melanopsin as labeling was primarily found in the periphery of the cell. Although membrane targeting cannot be visualized at the light microscopy level, scanning

confocal optical sections of 1 μm reveal melanopsin immunoreactivity closely associated with the membrane boundary. In the generation of transfectants, three clones were produced for the hOPN4 and hOPN4-K340A stable transfected cell lines. Each of these clones was also tested for anti-melanopsin immunoreactivity and all were positive for melanopsin. No clonal differences were observed in the immunoreactivity among the sets of stable cell lines (data not shown).

Light-evoked Calcium Responses

In the melanopsin expressing cells, light (488 nm) evoked a transient increase in intracellular calcium (Fig. 2A). This light-induced response was tested in three clones stably expressing melanopsin. All three clones demonstrated similar response to the light pulse, indicating that there were no clonal specific differences due to varying integration of melanopsin. In the melanopsin mutant controls, light did not trigger a rise in the intracellular calcium (Fig. 2B). Again, three clones of the mutant control were tested and there were no clonal differences identified.

To confirm that all cells were viable and capable of triggering calcium responses, thapsigargin, an inhibitor of the sarco/endoplasmic reticular calcium ATPase (SERCA) (Inesi et al., 2005), was applied to the cells, following light pulses. SERCA pumps normally function to counterbalance the persistent leak of calcium from ER stores, and when inhibited, calcium is no longer pumped back into intracellular stores. In all of our cells, thapsigargin triggered a robust increase in the intracellular calcium concentration (Fig. 3A-E). Light responses prior to the thapsigargin were only observed in the hOPN4 and hOPN4-TRPC3 cell lines (Fig. 3A and C, respectively).

TRPC3 Expression

Real-time PCR was used to assay expression of TRPC3 in the cultured cells. RNA was extracted from each of the cell lines in the study and cDNA was then generated from each of the RNA extracts for the assay. Using oligonucleotide primer pairs specific for TRPC3 cDNA, real time PCR was performed. Melting point analysis revealed that all primer pairs produced a single amplicon with a uniform melting curve.

Data was analyzed relative to the expression levels in the hOPN4 cells. HEK293 cells endogenously express TRPC3 channels. This endogenous expression is similar in both the melanopsin expressing cells (hOPN4) and untransfected control cells with no significant difference between these cell lines (Fig. 4). Both the hOPN4 cells and the TRPC3cells that are stably transfected with the TRPC3 channel, had an approximately 1000-fold increase in TRPC3 expression. There were also significant differences between the hOPN4-TRPC3 and TRPC3 cells. The cells expressing TRPC3 alone show significantly greater expression than the cells stably expressing both melanopsin and TRPC3. This difference in expression may be due to the simultaneous overexpression of two integral membrane proteins, which may result in the observed attenuated expression of TRPC3.

Irradiance Dependent Response

The irradiance: response relationship was established by applying five seconds of varying irradiances at a wavelength of 488 nm to the field of cells. The irradiance required for a half maximal response for the hOPN4 was 5.08×10^{12} photons $sec^{-1}cm^{-2}$

(Fig. 5A) and for the hOPN4-TRPC3 cells was $9.27x10^{12}$ photons $sec^{-1}cm^{-2}$ (Fig. 5C). The presence of the overexpressed TRPC3 channels did not result in any differences in the light-evoked calcium response of cells. The control cells did not respond to light, even at maximal irradiances (Fig. 5B, D, and E).

Internal Calcium Stores

In the hOPN4 cells, the light induced increase in intracellular calcium could be triggered repeatedly over time with slight decreases in amplitude with subsequent stimulations (Fig. 6A). This type of responsiveness could also be elicited in media without calcium and supplemented with the calcium chelator EGTA (0.5μM), demonstrating that this response could persist even with the external calcium reduced to a minimal concentration (Fig. 6B). However, a subsequent calcium response could not be elicited following the liberation of internal stores by thapsigargin (Fig. 6C), indicating that the light-stimulated intracellular calcium increases require functional internal calcium stores. Furthermore, the addition of the nonspecific cation blocker lanthanum (200 μM), which could block endogenous TRPC channels, did not alter the calcium response of the cells (Fig. 6D).

DISCUSSION

Several studies have shown that melanopsin triggers a transient increase in intracellular calcium in response to light, however, the specifics of this calcium response has remained unclear (Sekaran et al., 2003; Isoldi et al., 2005; Melyan et al., 2005; Panda et al., 2005; Qiu et al., 2005; Warren et al., 2006). Here we report that in

response to light, human melanopsin expressed in HEK293 cells triggers a release of calcium from internal calcium stores.

The role of internal calcium stores has been previously implicated in the melanopsin response to light in both the melanopsin-dependant melanosome dispersion (Isoldi et al., 2005) and the different heterologous melanopsin expression systems (Melyan et al., 2005; Panda et al., 2005; Qiu et al., 2005). However, the contribution of the internal calcium stores is not supported by all studies of melanopsin phototransduction. In the light-sensitive RGCs of mouse *rd/rd cl* retina, it is reported the presence of cadmium, the calcium channel blocker, resulted in a loss of the light-induced calcium fluxes in the cells, although the data are not shown (Sekaran et al., 2003). The findings from that study suggest the role of external calcium influx rather than any significant contribution of internal calcium store release in the response to light. While the source of this discrepancy is unclear, it should be noted that in that study, cadmium did not block the response in all the cells measured and the response in those cells could have been masked by the relatively low signal-to-noise ratio, indicating that the role of internal calcium stores cannot be completely dismissed in the light-sensitive RGCs. Furthermore, in Neuro-2a cells transiently expressing melanopsin, the light induced current was unaltered by the presence of cadmium (Melyan et al., 2005).

Cytoplasmic calcium concentration is under tight cellular regulation. Two receptor classes mediate the release of calcium from the endoplasmic reticulum: the inositol 1,4,5-trisphosphate (IP_3) receptors and the ryanodine receptors (Berridge, 2002). Melanopsin-initiated phototransduction, like that of the invertebrate opsins, has

been shown to involve a G_q or a related G protein that subsequently activates the phosphoinositide pathway (Isoldi et al., 2005; Panda et al., 2005; Qiu et al., 2005). In the HEK293 cells stably expressing melanopsin, the activation of PLC cascade and the lack of ryanodine receptors (Tong et al., 1999) strongly implicates the IP_3 receptor as mediating the release of internal calcium stores in response to light.

While the activation of IP_3 receptors and release of internal calcium was suggested from previous studies, the internal calcium stores acting as the sole source of the rise in intracellular calcium in our cells was somewhat surprising because there exist several opportunities for the contribution of external calcium. The canonical transient receptor potential (TRPC) channels originally identified in *Drosophila* (Cosens and Manning, 1969; Montell, 2005b) have been of particular interest in the studies of melanopsin signaling. Because of melanopsin's similarity to the invertebrate phototransduction cascade, the mammalian TRPC channels have become interesting candidate terminal effectors. At least three mechanisms have been proposed for the activation of the TRPC channels. First, TRPC channels may function as store operated channels, being activated by transient increases in intracellular calcium concentrations arising from the release of calcium from internal stores. Second, metabolites of the phosphoinositide signaling pathway such as diacylglycerol or polyunsaturated fatty acids may directly activate TRPC channels. Third, TRPC channels may simply be activated by being translocated into the plasma membrane in response to a stimulus (Montell, 2005b).

Overexpression of TRPC3 channels is necessary for light-evoked electrophysiological responses in HEK293 cells (Qiu et al., 2005). Coexpression of

melanopsin and TRPC3 channels in *Xenopus* oocytes modifies photoinduced currents (Panda et al., 2005). However, we did not find the overexpression of TRPC3 channels to alter the melanopsin-dependent intracellular calcium signal. In our system, the TRPC3 channels may not be participating significantly in the intracellular increase in calcium, but rather, TRPC3 channels may mediate the sodium current required for light-evoked membrane depolarization. The TRPC3 channel's selectivity ratio for calcium over sodium is 1.6 (Kamouchi et al., 1999). Furthermore, in the melanopsin expressing *Xenopus* oocyte, the light-induced inward current was inhibited by the replacement of the extracellular sodium with the nonpermeant cation NDMG (Panda et al., 2005). While we have also previously found that the addition of the nonspecific cation blocker lanthanum resulted in the loss of the light activated current in melanopsin expressing HEK293 cells (Qiu et al., 2005), the rise in intracellular calcium was unaffected by the addition of lanthanum in these cells. Therefore, although we did not observe intracellular calcium dynamics altered by the overexpression of TRPC3, the TRPC3 channels could be mediating sodium influx, which would not be evident by calcium imaging, but would be observed electrophysiologically as shown in the previous studies.

With the stable transfection of human melanopsin into the HEK293 cells, we generated a stable system that allowed us to further dissect and understand the components of melanopsin phototransduction. Although we are not studying melanopsin in its natural context, ipRGCs, the HEK293 cells provide the necessary environment for proper trafficking and activation of melanopsin. Furthermore, despite that HEK293 cells were originally isolated from primary human embryonic kidney cells and transformed by adenovirus 5 DNA, these cells are found to express proteins

characteristic of neuronal cells (Shaw et al., 2002). In addition, HEK293 suspension cells are capable of expressing functional rhodopsin (Brueggemann and Sullivan, 2002) and some of the HEK293 cell lines contain the visual cycle retinoid processing proteins found in the retinal pigment epithelium (Ma et al., 1999; Chen et al., 2003). If our HEK293 cells are like HEK293-F cells, which do not express retinoid processing proteins (Redmond et al., 2005), melanopsin may also function as a bistable pigment with intrinsic photoisomerase capabilities not requiring the retinoid processing proteins (Koyanagi et al., 2005; Melyan et al., 2005; Panda et al., 2005). Therefore, HEK293 cells provide the necessary components for observing responses to light within the dynamic range typical for excitation of the ipRGCs. Finally, the irradiance: dependent calcium responses observed in our stable transfectants indicate that this system represents a useful semiquantitative tool that may be used in future experiments for the functional screening and characterization of melanopsin mutants.

FIGURES

Figure 1: Stable Transfection

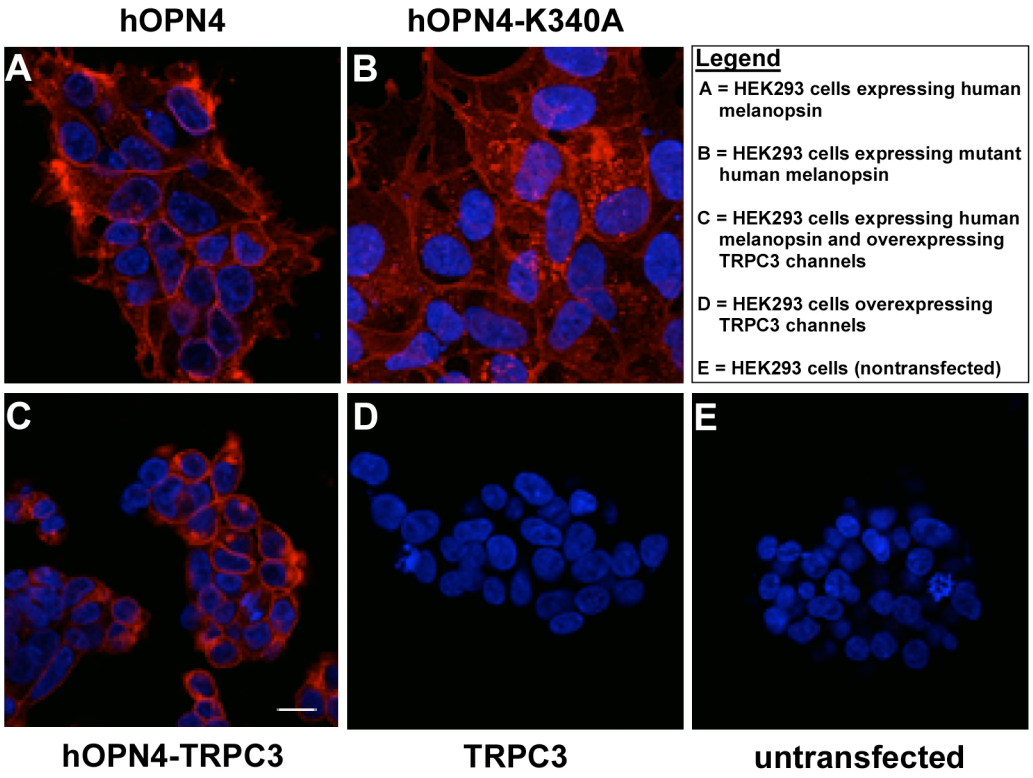


Figure 1: Confocal photomicrographs of HEK293 cell lines stained with human melanopsin (red; UF028 and Secondary Antibody Alexa555) and dapi nuclear stain (blue). hOPN4 cells stably express melanopsin (A) and hOPN4-K340A stably expressing mutant melanopsin (B), both stain positive for melanopsin and dapi. hOPN4-TRPC3 (C) stains positive for both melanopsin and dapi. TRPC3 without melanopsin (D) and nontransfected cells stain positive for dapi only. Scale Bar = 20μm.

Figure 2: Light induced Calcium Flux

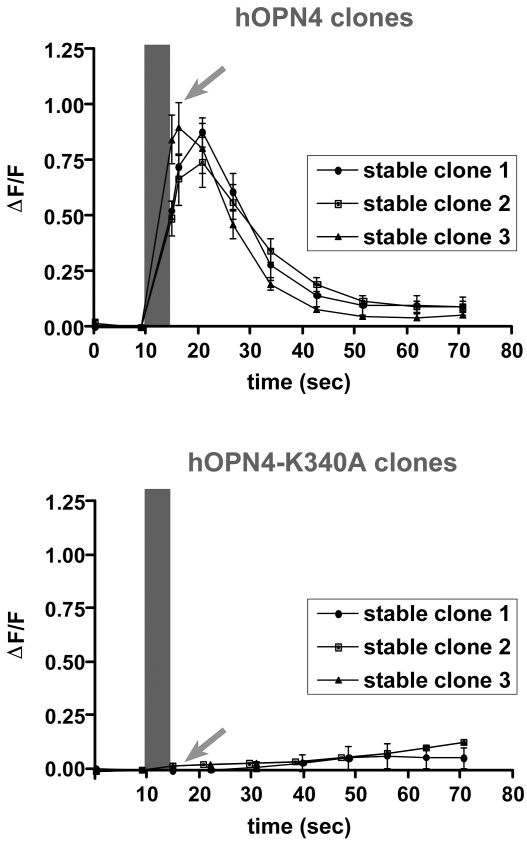


Figure 2: Cells were exposed to 5 seconds of 488nm light at 3.8 x 10^{12} photons $sec^{-1}cm^{-2}$ (represented by black bar). Three clones of each of the stable transfections were tested. Arrow directs towards the presence or the lack of calcium flux in response to light in each of the clones. All three melanopsin expressing clones, the HEK293-hOPN4 display increases in fluorescence following light exposure. The three melanopsin mutant expressing clones, the hOPN4-K340A clones, showed no increase in fluorescence following the light pulse. Each point represent n=9 and SEM.

Figure 3

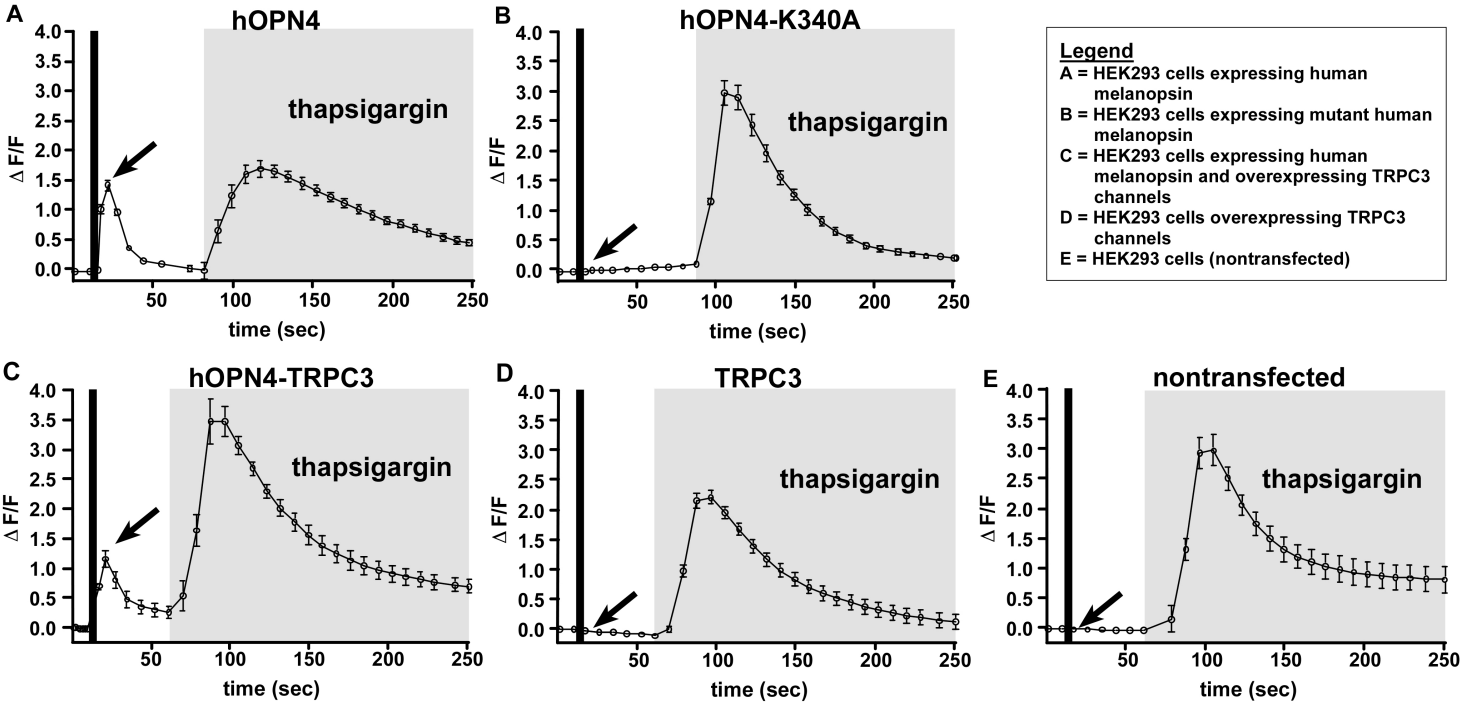


Figure 3: Cells were exposed to 5 seconds of 488nm light at 3.8 x 10^{12} photons $sec^{-1}cm^{-2}$ (represented by black bar). Following light exposure, thapsigargin was added to the cells to a concentration of 1μM in the well (gray region). The hOPN4 (A) cells show increases to both light and thapsigargin. The hOPN4-K340A (B) show increases to calcium only with the application of thapsigargin. The hOPN-TRPC3 cells (C) show increases to both light and thapsigargin. TRPC3 cells (D) and the nontransfected cells show an increase in intracellular calcium for thapsigargin only. Each point represents n=9 and SEM.

Figure 4: TRPC3 mRNA

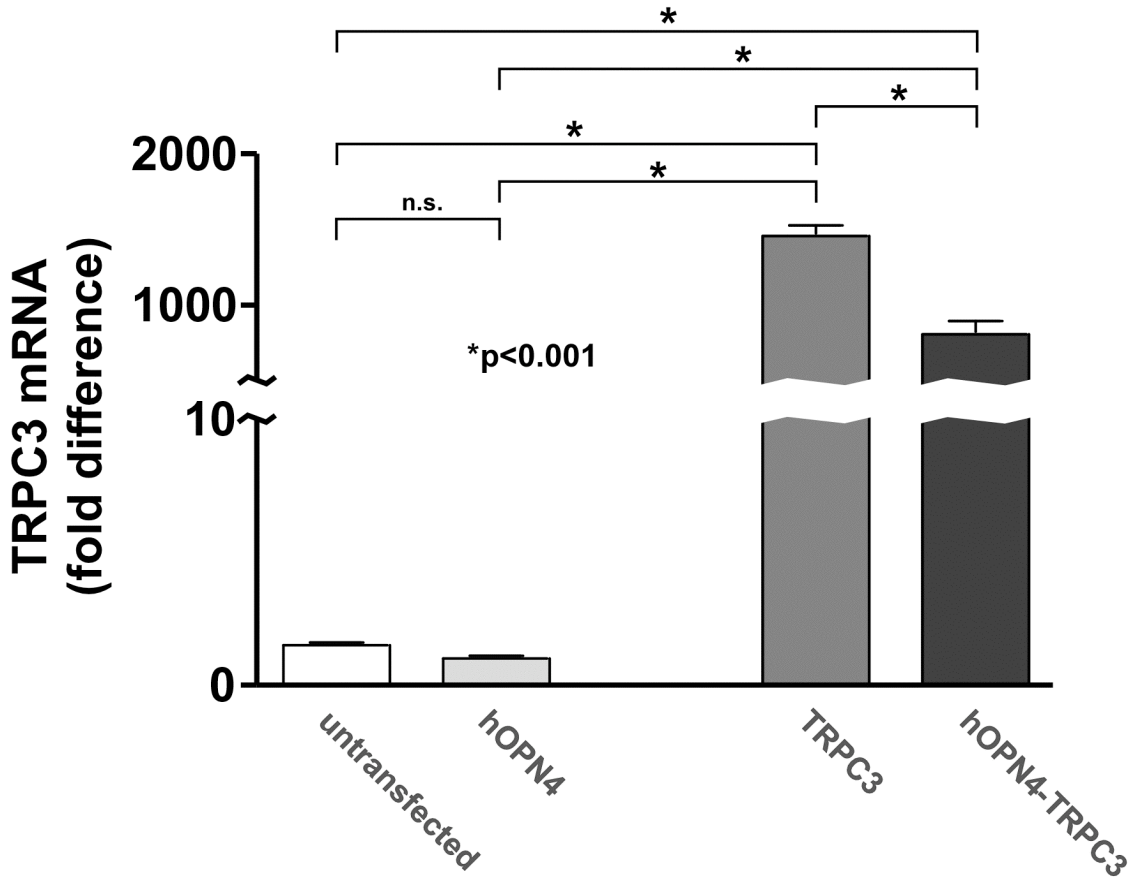


Figure 4: The HEK293 cells have endogenous expression of TRPC3. This basal level of expression is relatively the same when cells are stably transfected with human melanopsin. Cells stably transfected with the TRPC3 channels display an overexpression with 1000-fold increase compared to the endogenous TRPC3 levels. Stable transfection of human melanopsin and TRPC3 results in decreased overexpression of TRPC3.

Figure 5: Fluence Response

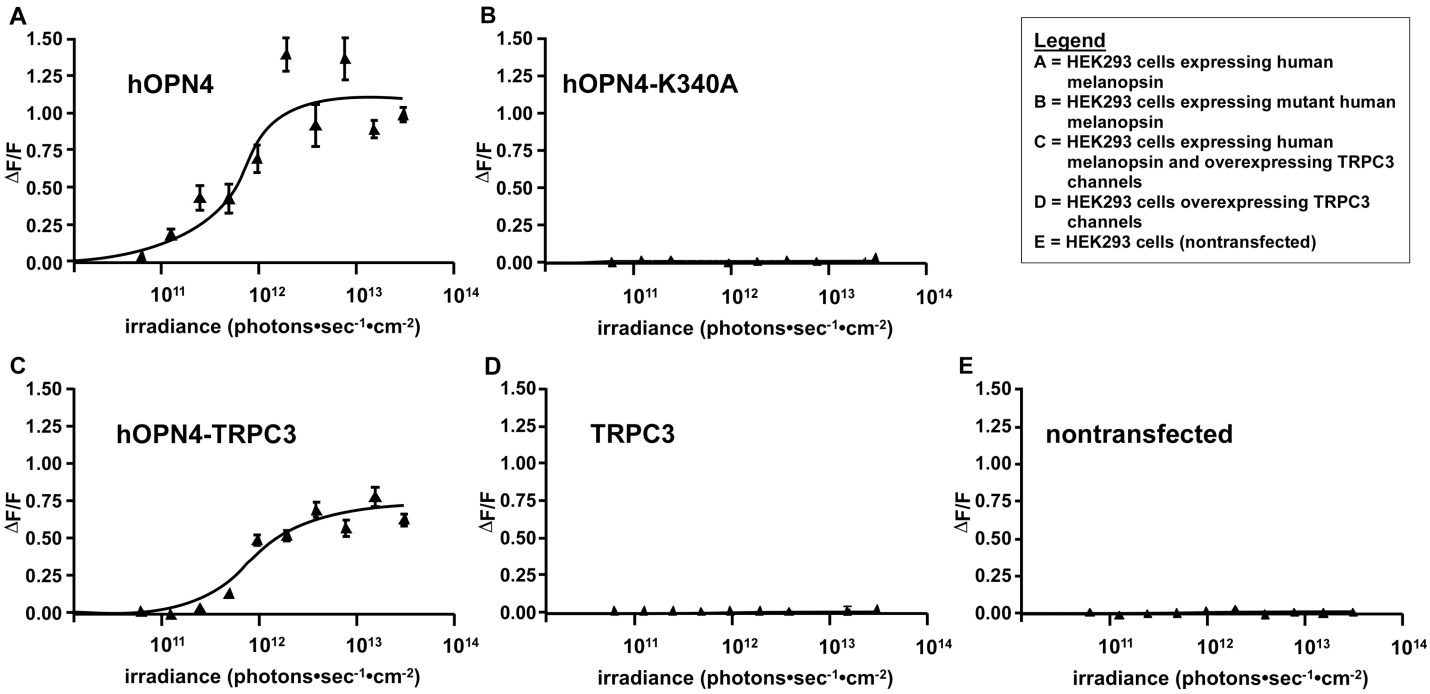


Figure 5: Cells were exposed to increasing intensities of 488nm light. The calcium flux in response to five-second light stimulus was measured for each group of cells. Light intensity for each pulse was given in photons $cm^{-2}s^{-1}$. A sigmoidal response curve was fitted to the data. hOPN4 (A) and hOPN4-TRPC3 (C) show a dose response to the increasing light intensities. hOPN4-K340A, TRPC3, and nontransfected cells (B, D, and E respectively), did not display any calcium flux in response to light. Bars indicate SEM (n = 9).

Figure 6

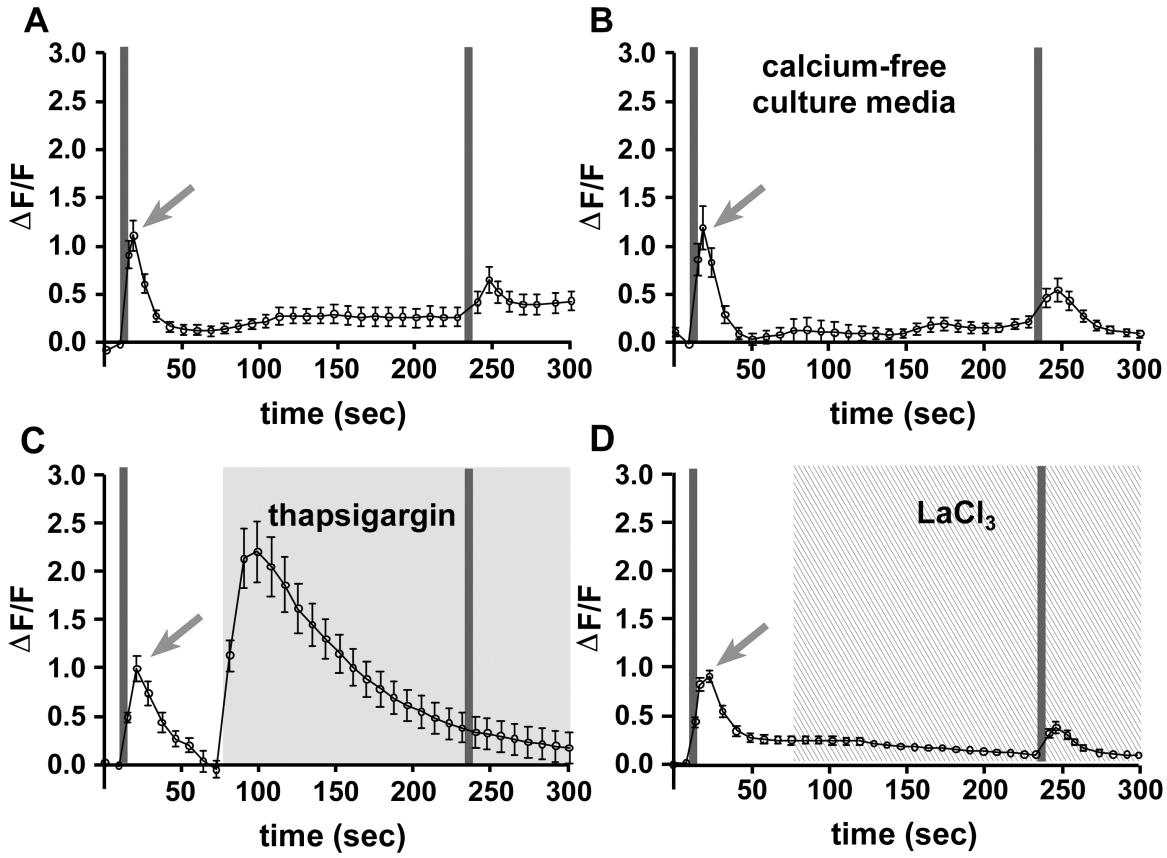


Figure 6: HEK293-hOPN4 cells were treated to two-5 second pulses of 488nm light at 3.8 x 10^{12} photons $sec^{-1}cm^{-2}$ (black bars) in different extracellular environments. In A, the cells were treated to the repeat light pulse in media supplemented with calcium. In B, the cells were in a media without calcium, supplemented with 0.1μM EGTA. In C, the cells are in media with calcium and in between the two light pulses, 500nM thapsigargin was added to the cells. In D, the cells are in media with calcium and 200 μM lanthanum chloride was added to the well. Each point represents n=9 and SEM.

DISCUSSION

Opsins are the protein moiety of photopigments that employ retinaldehyde as a chromophore (Spudich et al., 2000). They are G-protein-coupled heptahelical transmembrane receptors with many diverse functions, ranging from light-driven proton pumps to photopigments involved in vision. Interestingly, within the past decade, several non-classical opsins have been discovered: RGR, peropsin, melanopsin, encephalopsin, and neuropsin (Pandey et al., 1994; Blackshaw and Snyder, 1999; Provencio et al., 2000; Tarttelin et al., 2003). These opsins have been of great interest in light of emerging evidence of the existence of an ocular non-rod, non-cone photoreceptor responsible for mediating the synchronization of circadian rhythms to the astronomical day (Panda et al., 2002; Ruby et al., 2002). Although each of these non-classical opsins has been a possible candidate, as each one is found in the eye, mounting evidence implicated melanopsin as the leading candidate to function in this capacity as I began the studies in this dissertation (Kumbalasiri and Provencio, 2005). However, even with the compelling evidence supporting melanopsin as the photopigment of the ipRGCs, there were those who remained skeptical of the novel opsin's role as a true sensory photopigment (Bellingham and Foster, 2002). There was the concern that melanopsin was not functioning in a photosensory capacity, but rather assisting another yet to be identified photopigment by functioning as a photoisomerase. Therefore, the studies in this dissertation were designed to provide evidence that melanopsin is indeed a functional photopigment that confers photosensitivity to the ipRGCs and to elucidate the signaling cascade initiated upon melanopsin's activation by light.

Over the past decade, the race to elucidate the details of non-visual phototransduction has been underway and there have been several studies that have been published within the same time period demonstrating an emerging consensus concerning melanopsin activity. However, there are also several important differences and uncertainties that exist amongst the various studies, which remain to be clarified in future studies of melanopsin phototransduction. This discussion is aimed to focus on the results of the studies presented within this dissertation, providing perspective of how these data fit into our current understanding and to identify any inconsistencies that may exist within the current literature.

Because only 700-1000 retinal ganglion cells in the mouse retina express melanopsin (Hattar et al., 2002) , the model system of heterologous expression of melanopsin in the human embryonic kidney (HEK293) cells has been an invaluable tool for studying melanopsin activity. The HEK293 cell line was initially chosen as one of the four candidate cell lines for the expression system. The other cell lines initially selected and tested included the *Danio rerio* (zebrafish) embryonic cell line (ZEM-2S), the human retinal pigmented epithelia cell line (ARPE-19), a rat retinal ganglion cell line (RGC-5) (Krishnamoorthy et al., 2001) and the *Xenopus laevis* melanophore cell line from which melanopsin was originally cloned. The well-characterized HEK293 cell line was initially selected as one of the candidate cell lines primarily because of its successful use for transfection and study of proteins. In addition, the HEK293 cells were also interesting candidates due to their neuronal-like characteristics (Shaw et al., 2002) and expression of the retinoid processing protein RPE65 (Ma et al., 1999). Ultimately, the HEK293 cells

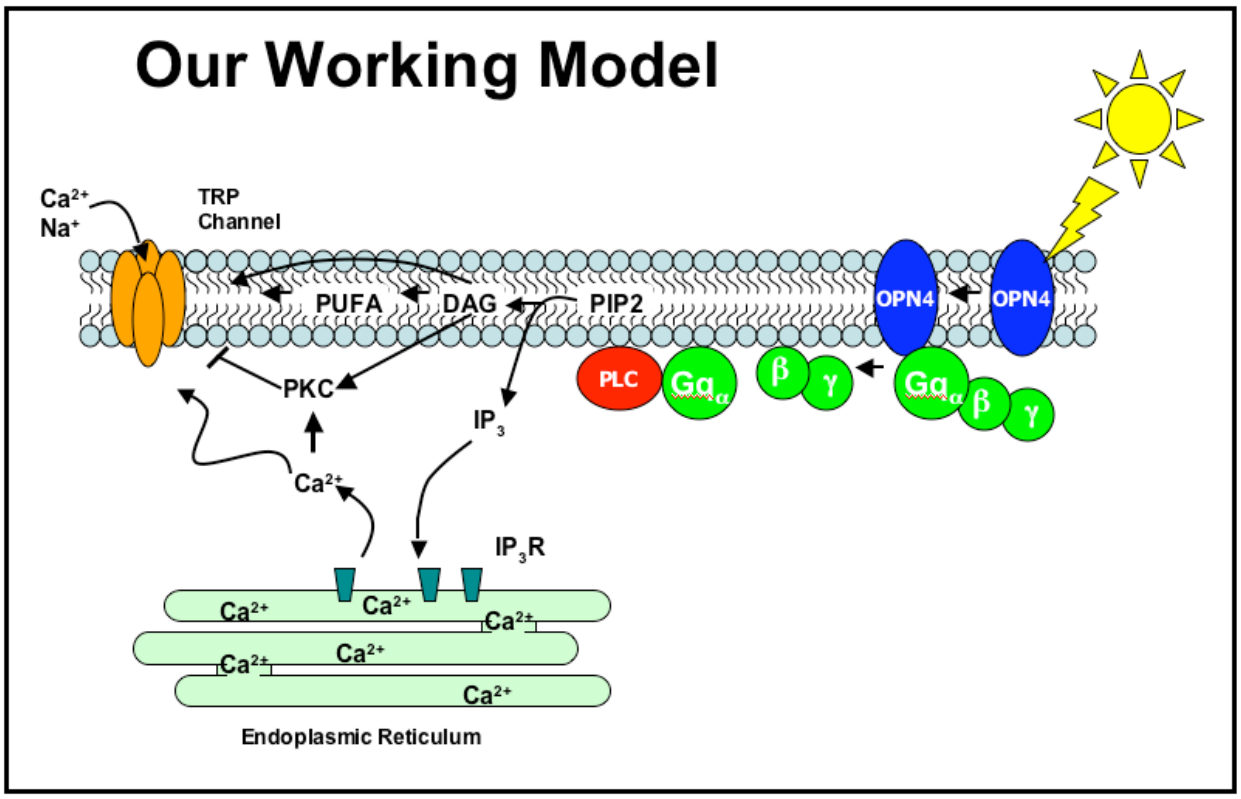
became the cell line selected for the studies due to their efficient transfection, proper trafficking to the membrane, and functional expression of melanopsin.

With the heterologous melanopsin expressing HEK293 cells, I was able to take the fundamental step to show that transfection of melanopsin induces photosensivity in the previously non-photosensitive cell line (Qiu et al., 2005). These data provided the necessary evidence that melanopsin does in fact form a functional photopigment. At the same time we published these data, two other heterologous systems were also presented (*Xenopus* oocytes and Neuro-2A cells), providing further evidence of melanopsin-induced photosensitivity (Melyan et al., 2005; Panda et al., 2005).

In the melanopsin expressing HEK293 cells, the action spectrum of melanopsin-mediated electrophysiological light responses indicated a peak sensitivity of 479 nm, similar to the 480 nm peak spectral sensitivity observed in *Xenopus* oocytes heterologously expressing melanopsin (Panda et al., 2005; Qiu et al., 2005). These values are consistent with the peak spectral sensitivities of rodent and primate ipRGCs (484 and 482 nm, respectively) (Berson, 2003; Dacey et al., 2005). They are also consistent with action spectrum studies of light induced circadian phase shifting in retinally degenerate mice, which implicate a photoreceptor with peak sensitivities in the blue wavelengths of 480 nm (Yoshimura and Ebihara, 1996). However, the results did conflict with two other studies that indicate a melanopsin peak spectral sensitivity between 360 and 430 nm (Newman et al., 2003; Melyan et al., 2005). The source of this incongruity remains unknown and may be due to the membrane environment of the cell line in which melanopsin is being expressed in these other heterologous expression systems.

Although melanopsin is a vertebrate opsin, it actually shares greater homology at the amino acid level with the invertebrate opsins (Provencio et al., 1998b). The invertebrate homology is present in domains, which are thought to be of functional importance in the invertebrates. In the third transmembrane domain, melanopsin has an aromatic residue, which is likely to be part of a network that functions to stabilize the Schiff's base linkage between the chromophore and opsin protein (Provencio et al., 1998b). This stabilization is a key feature of invertebrate opsins, which after light activation do not release the inactive chromophore, but rather perform *in situ* regeneration of the all-*trans*-retinaldehyde. Interestingly, mammailian melanopsin may in fact possess some photoisomerase activity in addition to its signaling role (Melyan et al., 2005; Panda et al., 2005). Furthermore, in the third cytoplasmic loop, melanopsin has an eight amino acid insertion not found in vertebrate opsins (Provencio et al., 1998b). This is important as the third cytoplasmic loop is implicated in determining the G-protein family that is activated by the opsin, thereby suggesting that melanopsin may activate a signaling cascade distinctly different than that of typical vertebrate opsins.

Melanopsin's invertebrate like characteristics therefore predicted melanopsin phototransduction to be more similar to that of the invertebrates. In fact, the studies presented in this dissertation, in agreement with most of the literature on melanopsin, have confirmed the invertebrate-like phototransduction cascade triggered by melanopsin activation. The following diagram is our current working model of melanopsin phototransduction. Following this model is the evidence for this cascade as well as any conflicting data presented from other studies.



Unlike classical vertebrate visual photoreceptors, which in response to light utilize the G protein transducin to indirectly regulate cyclic nucleotide gated channels to hyperpolarize the membrane, we found that photoactivation of melanopsin results in the signaling of a Gq or a related G protein family member and subsequent depolarization (Qiu et al., 2005). The presence of pertussis toxin did not inhibit melanopsin-initiated signalling, suggesting that Gi, Go, or transducin type G proteins were not involved. We confirmed the role of the Gq family in the melanopsin signaling cascade by the application of the Gq protein antagonist GpAnt-2A, which acts by binding part of the receptor-binding domain on the Gq protein and blocking activation (Mukai et al., 1992). In the presence of the GpAnt-2A, light induced photocurrents were no longer observed in our melanopsin expressing cells. Melanopsin signaling through the Gq or related

protein has also been demonstrated in the *Xenopus* oocyte expression system (Panda et al., 2005) as well as the melanopsin-mediated melanosome dispersion response of the *Xenopus* dermal melanophore system (Isoldi et al., 2005). Furthermore, melanopsin signaling does not appear to regulate the cyclic nucleotide gated channels for depolarization as increases in cGMP have shown to have no effect on the melanopsin responses in rat ipRGCs (Warren et al., 2006) or the *Xenopus* dermal melanophores (Isoldi et al., 2005). However, it should be noted that in the Neuro-2A cells expressing melanopsin, there was in fact evidence for the role of cGMP in the melanopsin cascade (Melyan et al., 2005). Futhermore, in COS cells, heterologous melanopsin expression was capable of activation of the G protein transducin (Newman et al., 2003). This conflicting data possibly may be explained by the potential promiscuity in G-protein coupling of melanopsin that is observed in multiple G proteins (Peirson and Foster, 2006).

The next step in this cascade is the activation of phospholipase C. The alpha subunit of the Gq protein has been shown to bind and activate phosphoinositide specific phospholipase C (PI-PLC) (Hubbard and Hepler, 2006). This type of Gq activation of the phosphoinositide signaling cascade is characteristic of the invertebrate opsins (Peirson and Foster, 2006). It also appears to be the pathway by which melanopsin functions as three independent studies have shown that melanopsin triggers the activation of the classical phosphoinositide signaling cascade (Isoldi et al., 2005; Panda et al., 2005; Qiu et al., 2005). The downstream products of PLC signaling were also shown to increase in response to light in the *Xenopus* dermal melanophores. In these cells increases in inositol triphosphate (IP_3) as well as protein kinase C (PKC) phosphorylation were

observed (Isoldi et al., 2005). However, again in the melanopsin expressing Neuro-2A cells, they found no direct role for PLC or PKC in the light response (Melyan et al., 2005).

This signaling cascade ends with the light activation of melanopsin resulting in a transient increase in intracellular calcium and depolarization of the cell (Berson et al., 2002; Sekaran et al., 2003; Warren et al., 2003; Isoldi et al., 2005; Melyan et al., 2005; Panda et al., 2005; Qiu et al., 2005; Warren et al., 2006). While the first parts of the cascade have been similar to that of the invertebrate opsin cascades, the mechanics of this last portion of the cascade remains unclear. Predictions could be made from the invertebrate cascades, but the final portions of the invertebrate cascade vary as well. For example, *Limulus* photoreceptors are believed to utilize the cyclic nucleotide gated channels resulting in membrane depolarization (Garger et al., 2004), while *Drosophila* photoreceptors utilizes the canonical transient receptor potential (TRPC) channels, which open to cause the influx of calcium into the photoreceptor cells (Montell, 2005a). As for melanopsin, we have shown that the depolarization in response to light activation is blocked by the nonselective cation channel blocker lanthanum, which is a frequently used blocker of TRPC channels (Panda et al., 2005; Qiu et al., 2005; Warren et al., 2006) although we have also shown in work herein that melanopsin-mediated intracellular calcium increases in HEK293 cells heterologously expressing melanopsin are not blocked by lanthanum. This discrepancy between the sensitivity of the electrophysiological response to lanthanum blockade and the lack of lanthanum inhibition of the calcium response remains to be explained. One possibility is that the calcium increase is largely, if not exclusively, through the liberation of calcium from

intracellular stores thereby not being sensitive to extracellular lanthanum blockade. The electrophysiological response, however, is likely to result from ionic currents passing through the membrane, such as calcium or sodium passing through TRPC channels. While these relatively small currents are likely to be sufficient to depolarize the membrane and likely to be blocked by lanthanum, it is unlikely that they will contribute significantly to the light-induced calcium signature of the cell. Finally, as previously mentioned, CNG channels do not appear to contribute to the melanopsin cascade (Isoldi et al., 2005; Warren et al., 2006). Therefore, evidence supports a *Drosophila*-like cascade with a role for TRPC channels in the light activated melanopsin cascade.

The source of the intracellular rise in calcium in response to light activation of melanopsin has been ill-defined. Rises in intracellular calcium have been demonstrated in both the heterologous expression systems (Melyan et al., 2005; Panda et al., 2005; Qiu et al., 2005) as well as the natural melanopsin expressing cells, the ipRGCs and the *Xenopus* dermal melanophores (Sekaran et al., 2003; Warren et al., 2003; Isoldi et al., 2005; Sekaran et al., 2005; Warren et al., 2006). In mice, this light sensitive calcium flux in the ipRGCs is present from birth (P0) (Sekaran et al., 2005). In the rat ipRGCs, depolarization in response to light is blocked by the addition of calcium chelators placed within the cell (Warren et al., 2006), and in the *Xenopus* dermal melanophores, melanosome dispersion was also inhibited by the presence of the membrane permeable calcium chelator BAPTA-AM (Isoldi et al., 2005), indicating that the intracellular calcium increase significantly contributes to the final response of the signaling cascade. A clear understanding of the specifics of this calcium response is fundamental to understanding the cascade. Identifying whether melanopsin triggers a release of internal calcium

stores, an influx of external calcium, or a combination of the two will begin to provide the insight necessary for unraveling the last steps of the melanopsin cascade.

In order to determine the source of the melanopsin-mediated intracellular increase in calcium, I optimized my expression system and measurement setup in order to attain higher signal-to-noise ratios in the calcium signals of these cells. To optimize expression, HEK293 cells were stably transfected, eliminating the variable transfection efficacy and presence of high external calcium left from remnants of the calcium phosphate-based transfection reagents. Several clones were generated and tested, showing similar responsiveness, indicating no clonal differences or variability due to variability of transgene integration into the genome. The calcium monitoring system was altered to measure more rapid responses, which was important as the calcium increase typically begins within seconds of the light pulse.

In the HEK293 cells stably expressing melanopsin, it was determined that increases in intracellular calcium came from the release of internal calcium stores. The calcium response was unaltered by the lack of extracellular calcium, overexpression of TRPC3 channels, and the presence of lanthanum, the non-selective cation blocker. The release of internal calcium stores was not surprising because melanopsin has been shown to trigger a PLC signaling cascade, which results in the production of IP_3 that is capable of binding to receptors on the endoplasimic reticulum and causing the release of the internal calcium stores. However, the internal calcium stores acting as the sole source for the rise in intracellular calcium was somewhat surprising since there exist several opportunities for the contribution of external calcium through the TRPC channels. It should also be noted that Sekaran et al. (2003) provided evidence for the

role of extracellular calcium as the source for intracellular calcium increases. In their studies of the ipRGCs, they found that the addition of cadmium, another non-selective cation blocker, resulted in the loss of light induced elevation in intracellular calcium. However, the response was not lost from all the cells treated with cadmium and there was a great deal of background that may have masked the calcium response. Therefore, although our data conflicts with the studies by Sekaran et al. (2003), the robust and consistent responsiveness of my system provides strong evidence for the role in internal calcium stores.

Although I found that the TRPC channels did not contribute to the calcium flux in response to light, it does not mean that TRPC channels do not play a functional role in the cascade. The overexpression of the TRPC channels was necessary for the measurement of an electrophysiological response in the HEK293 cells (Qiu et al., 2005). In *Xenopus* oocytes the exogenous expression of TRPC3 channels was not necessary for light induced current, but the exogenous expression of the channels did modify the photoinduced current (Panda et al., 2005). As mentioned above, although calcium currents through the TRPC channels do not appear to contribute to light-evoked elevation of calcium in our cells, the nonselective cation channels may be a source of sodium influx into the cell necessary for membrane depolariztion or enhancement of the cascade in response to light.

The specific steps for activation of the TRPC channels by melanopsin still have yet be determined. There currently exist three proposals for the activation of the mammalian TRPC channels: activation via store operated calcium entry, increased diacylgylcerol (DAG), or regulated translocation of the channels to the membrane

(Montell, 2005b). All three are possible mechanisms, which could occur in our system and also in the ipRGCs. We have shown that light activated melanopsin activates a Gq or a related protein, that activates PLC (Qiu et al., 2005) and results in the increase in IP_3 (Isoldi et al., 2005), which should be accompanied by the production of DAG. Therefore, according to this data, melanopsin most likely activates the TRPC channels by either increased intracellular calcium from IP_3 activated release of internal calcium stores or direct DAG mediated activation. Recently, immunohistochemical experiments have identified the expression of TRPC6 in many of the RGCs, including those expressing melanopsin (Warren et al., 2006). Therefore, a candidate TRPC channel has been localized in the ipRGCs to mediate this response, but how this channel is activated and how it interacts with the rest of the signaling cascade remains to be determined.

Our stably transfected cells respond with characteristics similar to that of the ipRGCs, making our stable expression system an ideal model for studying melanopsin phototransduction. Along with the model system, the measure of light activated calcium responses has provided a robust semi-quantitative technique that can be utilized for future studies of melanopsin activity. This system can be used to resolve whether melanopsin is a photoisomerase as well as sensory photopigment by designing a proper protocol for the application of various wavelengths to elucidate such activity. This system can also be used to study the effects of single nucleotide polymorphisms (SNPs) of the melanopsin gene on melanopsin protein function, such as the generation of the lysine control mutant described in the second chapter of this dissertation. The

understanding of non-visual light perception has exploded in the past decade, but even with this explosion there still remains a great deal left for further studies to uncover.

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APPENDIX