THE UNIVERSITY OF CALIFORNIA

Santa Barbara

On the evolution of the animal phototransduction cascades

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy in

Ecology, Evolution & Marine Biology

by

David C. Plachetzki

Committee in charge:

Professor Todd H. Oakley

Professor Susan J. Mazer

Professor Scott A. Hodges

June 2009

The dissertation of David C. Plachetzki is approved.

_____

Scott A. Hodges

_____

Susan J. Mazer

_____

Todd H. Oakley

June 2009

On the evolution of the animal phototransduction cascades

by

David C. Plachetzki

Acknowledgements

My graduate experience in the department of Ecology, Evolution and Marine Biology at UCSB has been an enjoyable and transformative experience. During the past five years I have benefited greatly from the diversity of research perspectives and backgrounds that are represented in our department. I am foremost grateful to my graduate advisor Professor Todd H. Oakley. Todd has served as an excellent mentor and lab head, always open to new ideas with a basic interest in pushing the boundaries of evolutionary biology. I am also very grateful to Oakley lab personnel that I have had the privilege of working with including Jeanne Serb, Anja Rivera, Ann Syme, Anne Lindgren and Bjørn Östman. I am especially thankful to Sabrina Pankey, who has been both a collaborator and a useful foil to some of my most silly ideas. I am also thankful to Professors Susan J. Mazer and Scott A. Hodges for serving on my dissertation committee and for providing crucial feedback on earlier external funding proposals. I acknowledge Professor Bill Rice and members of Rice lab for laboring through numerous practices of my symposium and job talks over the years. Thanks also to Bill for offering statistical guidance on some aspects of my graduate research. I am also grateful to Dr. Tristan Long of Rice lab for offering guidance and material resources on some of the behavioral experiments that we have developed in the hydra. Tristan has always been willing to help in any capacity and our research has benefited from this openness. One of the best experiences that I have had as a grad student in EEMB has been my involvement in organizing the departmental seminar series. I am thankful to Professor Jonathan Levine who served

as the faculty advisor for the series and also to Shawna MacMahon and Robin Pelc who co-organized the seminar series during my tenure. Another formative experience that I have had during my time as a grad student was my involvement with the evodevo course at Friday Harbor Laboratories in Washington State. Here, I am most indebted to Professors Billie J. Swalla (University of Washington) and Kenneth M. Halanych (Auburn) who have provided expert guidance and support over the years. I am also grateful to Professor Gretchen Hoffmann for giving me the "hard sell" on EEMB roughly five years ago. Gretchen was correct, EEMB was the perfect opportunity for me and I am glad to have taken her advice. Finally, I am thankful to my parents Chuck and Sally Plachetzki for their encouragement and enthusiasm throughout the years.

CIRRICULUM VITA
DAVID C. PLACHETZKI

Education

Ph.D. (Candidacy, 11/2006) Ecology, Evolution and Marine Biology (EEMB)
University of California at Santa Barbara (UCSB) -*Graduation: 6/2009*

Master's Degree of Biological Science (Emphasis on Molecular Biology)
2000, Northern Michigan University

Bachelor's Degree of Biological Science (Emphasis on Ecology and Evolution)
1995, Northern Michigan University

Fellowships and Grants

2008, Grant in Aid of Research (UCSB Affiliates) *$3000*

2008, Dean's Dissertation Fellowship (Division of Graduate Studies, UCSB) *$16,000*

2008, Graduate Student Travel Grant (EEMB/UCSB) *$500*

2007, Graduate Mentorship Program Fellowship (Division of Graduate Studies, UCSB) *$16,000*

2007, Dissertation Improvement Grant, DEB-0710406 (National Science Foundation) *$12,000*

2006, Travel Grant (European Molecular Biology Laboratories) *$500*

2005, Tuition Fellowship (Friday Harbor Laboratories) *$800*

2005, Fee Fellowship (Division of Graduate Studies, UCSB) *$500*

2004, Block Grant Fellowship (Division of Graduate Studies, UCSB) *$11,000*

1999, Travel Grant (Northern Michigan University) *$600*

1998, Development Fund Grant Award (Northern Michigan University) *$1,200*

1998, Spooner Grant for Student Research (Northern Michigan University) *$600*

Awards and Honors

2008, Honorable Mention for Best Student Presentation Award – *DSEB (SICB)*

2008, Ernst Mayr Award Finalist -*Society for Systematic Biology*

2008, The Buschbaum Prize for Excellence in Photomicrography –*American Microscopical Society.*

2007, Graduate Student Poster Award -Society for Molecular Biology and Evolution.

Professional Experience

2004-09 Graduate Student (Advisor: Todd H. Oakley) EEMB, *UC Santa Barbara*

2003-04 Research Technician (PI: Aaron Fox) Committee on Neurobiology, *University of Chicago*

2003 Research Technician III (PI: Nadean Brown) Division of Developmental Biology, *University of Cincinnati*

2002-03 Research Technician II (PI: Nadean Brown) Developmental Systems Biology Core, *Northwestern University*

2000-02 Research Technician (PI: Sara Szuchet) Department of Neurology, *University of Chicago*

Teaching Experience

2008 Teaching Assistant. Biol 533 (Evolution and Development of the Metazoans) Friday Harbor Laboratories, *The University of Washington*

2006 Laboratory instructor. EEMB 108 (Comparative Vertebrate Anatomy) Department of Biology, *UC Santa Barbara*

2005-07 Discussion Group Leader, EEMB 102 (Macroevolution) Department of Biology, *UC Santa Barbara*

2006 Section Leader, EEMB 3L (Introductory Biology) Department of Biology, *UC Santa Barbara*

2000 Laboratory Instructor and Lecturer, CH 454 (Biochemical Techniques: PCR and Molecular Cloning) Department of Chemistry, *Northern Michigan University*

1999-00 Laboratory Instructor, CH 111 and 112 (General Chemistry)
Department of Chemistry, *Northern Michigan University*

1999 Laboratory Technician and Lecturer, BI 418 (Molecular Biology)
Department of Biology, *Northern Michigan University*

Workshops and Summer Coursework

9/2006 EMBO Practical Course on Molecular Approaches to Evolution and Development. *Stazione Zoologica Anton Dohrn. Naples, Italy.*

7/2005 Evolution and Development of the Metazoans, Friday Harbor Laboratories *University of Washington. Friday Harbor, WA*

Professional Service

2008 Search Committee Member – EEMB Comparative Genomics, *UC Santa Barbara*

2008 Session Chair "Genomics and Speciation" – *Evolution* 2008, Minneapolis MN.

2007-08 Graduate Student Mentor and Senior Honor's Thesis Advisor (*Matt Harms, Caitlin Fong*).

2006-07 Graduate Student Mentor. UCSB California Alliance for Minority Participation (CAMP).

2005-07 EEMB Seminar Series Coordinator. *UC Santa Barbara*

2004-09 Science Fair Mentor. Santa Barbara County Science Fair. Santa Barbara, CA.

1999 Science Olympiad Coordinator and Judge. *Science Olympiad*, Marquette, MI.

Reviewed manuscripts for:

Molecular Phylogenetics and Evolution
Systematic Biology
Frontiers in Bioscience
American Malacological Bulletin
Evolution: Education and Outreach
The Biological Bulletin

Selected Press Coverage of Ph.D Dissertation Research

Press Releases: UCSB Press Office, National Science Foundation. Print: McClatchy News Service (Miami Herald, Sacramento Bee, others). Santa Barbara Independant. SEED Magazine. Martha Stewart Living. Science Illustrated. Radio: BBC World Service "Science in action". KCLU NPR. Internet News Outlets: Yahoo News, United Press International, News Daily, Xinhoa. Internet Blogs and Science Digests: Popular Mechanics, Astrobiology Magazine, The Scientist, PLoS ONE Picks.

Professional Memberships

Society for Molecular Biology and Evolution
Society for Integrated and Comparative Biology
Society of Systematic Biologists
The American Microscopical Society

Publications

Blackburn, D. C., K. W. Conley, D. C. Plachetzki, K. Kempler, B. A. Battelle, and N. L. Brown. 2008. Isolation and expression of *Pax6* and *atonal* homologues in the American Horseshoe Crab, *Limulus polyphemus. Developmental Dynamics* 237(8): 2209-2219.

Oakley, T. H., D. C. Plachetzki and A. S. Rivera. 2007. Furcation, field-splitting, and the evolutionary origins of novelty in arthropod photoreceptors. *Arthropod Structure & Development* 36: 386-400

Plachetzki, D. C., Degnan B. M, and T. H. Oakley. 2007. The origins of novel protein interactions during animal opsin evolution. *PLoS ONE* 2(10): e1054. doi:10.1371/journal.pone.0001054

Plachetzki, D. C., and T. H. Oakley. 2007. Key transitions during animal phototransduction evolution: Novelty, tree thinking, co-option and co-duplication. *Integrative and Comparative Biology*, doi:10.1093/icb/icm050

Harzsch, S., K. Diploma, D. C. Plachetzki, N. Brown, R. Melzer, K. Kempler and B. Battelle. 2006. Evolution of arthropod visual systems: development of the eyes and central visual pathways in the horseshoe crab Limulus polyphemus *Developmental Dynamics.* 20;235(10):2641-2655. (*cover*)

Plachetzki, D. C., Serb, J. and T. H. Oakley. 2005. New Insights in to the Evolutionary History of Photoreceptor Cells. *Trends in Ecology and Evolution.* 20(9): 465-466.

Szuchet, S., D. C. Plachetzki, M. A. Seeger, M. S. Domowicz and F. G. Szele. 2003. NOVOcan: a molecular link among selected glial cells. *Biophysical Chemistry*. 108 (1-3): 245-258.

Szuchet, S., D. C. Plachetzki and R. Karialukas. 2001. Oligodendrocytes express an α/β-Interferon-susceptible Mx gene: Molecular characterization of the encoded protein. *Glia*. 37(2) 183-189.

Szuchet, S., D. C. Plachetzki and K. Eaton. 2001. Oligodendrocyte transmembrane protein: a novel member of the glutamate-binding protein subfamily. *Biochemical and Biophysical Research Communications*.18 283(4):900-907.

Selected Oral Presentations

Plachetzki, D.C. 2009. The Origins and Evolution of the Animal Phototransduction Cascades. Departmental Seminar. Center for Population Biology. *UC Davis*, Davis CA.

Plachetzki, D.C. and T. H. Oakley. 2009. The Origins and Evolution of Metazoan Phototransduction Pathways: A History of Paraphyly and Derivation. *Society for integrative and Comparative Biology*, Boston MA. – Honorable Mention, Division of Systematic and Evolutionary Biology

Plachetzki D.C. 2008. The origin and evolution of the animal phototransduction cascades. *The University of Chicago,* Chicago IL.

Plachetzki D.C. 2008. The origin of the phototransduction cascades and the evolutionary history of a uniquely metazoan sensory modality. *UC Berkeley*, Berkeley CA.

Plachetzki D.C. 2008. Eye evolution from the bottom up: the origins and diversification of the animal phototransduction networks. *Friday Harbor Laboratories*, *University of Washington*, Friday Harbor WA.

Plachetzki D.C. 2008. The origins and evolution of novel photosensitivity pathways in animals. *Evolution*, Minneapolis MN. -Ernst Mayr symposium.

Plachetzki D.C. and T. H. Oakley. 2007. The evolution of animal photosensitivity. *EEMB Graduate Student Symposium, UC Santa Barbara*. Santa Barbara CA.

Plachetzki, D.C. and T.H. Oakley. 2007. New insights into the evolutionary history of animal photoreceptors. *Society for Integrative and Comparative Biology*. Phoenix AZ.

Plachetzki, D.C. and T. H. Oakley. 2006. Analysis of Cnidarian Genomes Reveals a New Class of Metazoan Opsin. *Neurobiology Research Institute* annual symposium, UC Santa Barbara. Santa Barbara CA.

Plachetzki, D.C. and T. H. Oakley. 2006. The whole EnChelata: a phylogenomics approach to the problem of arthropod relationships. *EEMB Graduate Student*

Symposium, UC Santa Barbara. Santa Barbara CA.

Plachetzki, D.C. 2005. Chaetognath relationships: a longstanding phylogenetic enigma comes to a head. *Friday Harbor Laboratories, University of Washington.* Friday Harbor WA.

Poster Presentations

Plachetzki D., Degnan B. M., and T. H. Oakley. 2007. Adaptive evolution of a physiological novelty: opsin phylogeny and the dawn of animal phototransduction cascades. *Society for Molecular Biology and Evolution.* Halifax, CA -Best Student Poster Award Winner.

Plachetzki, D. and T. H. Oakley. 2006. The dawn of animal opsins. *EEMO Practical Course on Molecular Approaches to Evolution and Development. Stazione Zoologica Anton Dohrn*. Naples, Italy.

Plachetzki, D. and T. H. Oakley. 2006. A new class of metazoan opsin from *Hydra* and *Nematostella*. *Integrating Evolution, Development, and Genomics Conference. UC Berkeley*. Berkeley, CA.

Plachetzki, D. and T. H. Oakley. 2006. A new clade of metazoan opsin from *Hydra* and *Nematostella*. *Society of Molecular Biology and Evolution.* Arizona State University. Tempe, Az

Conley, K. W., D.C. Blackburn, D. Plachetzki, K. Kempler, J. Currie, B.A. Battelle and N.L. Brown. 2006. Pax6 and Atonal expression during embryonic eye formation in the American Horsehoe crab, *Limulus polyphemus. Society for Developmental Biology Annual Meeting*. Ann Arbor, MI. *Developmental Biology* 295(1): 417.

Plachetzki, D., B. Swalla, K. Halanych and T. Oakley. 2006. The fine structure of the chaetognath nervous system in selected developmental stages. *Graduate Student Symposium. UC Santa Barbara*. Santa Barbara, CA.

Plachetzki D., D. Blackburn, K. Kempler, B. A. Battelle and Nadean L. Brown. 2003. Investigation of eye development in the American horseshoe crab. *Development Basis for Evolutionary Change*. The University of Chicago. Chicago, IL

Szuchet, S., D. Plachetzki and M. Domowicz. 2001. NOVOcan: a Novel Oligodendrocyte Proteoglycan. *Chicago Chapter Society for Neuroscience, Annual Meeting*. Chicago IL.

Szuchet, S., M. Domowicz and D. Plachetzki. 2001. NOVOcan: a Putative Matrix Receptor. *International Society for Neurochemistry (ISN) and the*. Buenos Aries,

Argentina. *Journal of Neurochemistry*. 78: 29-29 Suppl. 1,

Szuchet, S., D. Plachetzki, M. Domowicz and F. Szele. 2001. NOVOcan: Its Structure and Immunohistochemical Localization in Rat Brain. *American Society for Cell Biology annual meeting*. Washington, D.C. *Molecular Biology of the Cell*. 12: 63A-63A 343 Suppl.

Manuscripts In Progress

Rivera, A., D.C. Plachetzki, M.S. Pankey and T. H Oakely. *Daphnia pulex* genome sequence reveals gene duplication and loss dynamics of eye developmental and phototransduction loci.

Plachetzki, D.C., C. Fong and T.H. Oakley. Evidence that the ancestral animal phototransduction cascade utilized a cyclic nucleotide gated ion channel.

Plachetzki, D.C., C. Fong and T.H. Oakley. Nematocytes integrate light information into the firing response.

Plachetzki, D.C., M.S. Pankey and T.H. Oakely. The functional diversification of the animal opsins.

Plachetzki,D.C. and A. Heyland. Studies on the neuroanatomy and neuroethology of the chaetognath *Sagitta elegans* .

Abstract

On the evolution of the animal phototransduction cascade

By

David C. Plachetzki

The origination of biological complexity is a fundamental issue in evolutionary biology. Such issues are addressed in science through the study of models. The animal phototransduction cascade underlies all visual and many non-visual photosensitivity phenotypes in animals and represents a useful natural model for the study of the evolution of complexity.

Animal phototransduction cascades are mediated by the sensory receptor protein opsin. Using a comparative phylogenomics approach, we investigate a global phylogeny for opsin by including sequence data from the genomes of early branching animal lineages. We find that opsin proteins originated prior to the last common ancestor of cnidarians and bilaterians, but were not present in earlier branching lineages such as sponges or animal outgroups. Our phylogeny also provides an analytical scaffold for investigations into the molecular basis for phototransduction diversification and the ancestral state of these cascades. Using various statistical measures, we provide compelling evidence that changes in two amino acid residues in particular have underlined the diversification of the various classes of opsin, a finding supported by previous biochemical studies. We further explore the mechanisms of animal phototransduction cascade diversification and describe the macroevolutionary

dynamics of gene duplication and functional co-option that have each flavored the evolutionary history of animal phototransduction.

In order to polarize the wealth of existing data on the composition of bilaterian phototransduction cascades we require an understanding of the ancestral state of this pathway. Focusing on specific ion channel components of this cascade, our studies of gene expression and photobehavior in the cnidarian *Hydra magnipapillata* provide strong evidence that phototransduction in the early branching cnidarians relies on the activities of Cyclic Nucleotide-Gated ion channels. This finding renders cnidarian phototransduction as similar to the *ciliary* pathway used by vertebrates and other taxa for vision, in contrast to the *rhabdomeric* pathway common to insects and other invertebrates. We use these data to investigate ancestral composition of the animal phototransduction. Our results force recognition that the ciliary pathway represents the ancestral state of animal phototransduction, whereas the rhabdomeric pathway is a derived condition.

Our studies have rested heavily on the recently completed genome sequences of numerous basal animal taxa including the cnidarians *H. magnipapillata* and *Nematostella vectensis*. Both of these animals lack eyes but display a rich corpus of photosensitive phenotypes. In what capacity do these animals integrate photosensitivity into their behavior? We show that opsin and other phototransduction components are expressed in the cnidarian-specific stinging cells known as nematocytes. We further show that light intensity acts to attenuate nematocyte discharge. This finding adds a new dimension to our understanding of basic nematocyte biology and offers a novel context for exploring nematocyte function.

Table of Contents

Chapter 1

On the evolution of the animal phototransduction cascades

"It seemed to me from the start of my observations that knowledge of the remarkable properties of the polyps could bring pleasure to the inquisitive and contribute something to the progress of natural history." -Trembly, 1744

Biologists are amazed by the intricacy and complexity of biological interactions between molecules, cells, organisms, and ecosystems. Yet underlying all this biodiversity is a universal common ancestry. How does evolution proceed from common starting points and go on to generate the biodiversity we see today? The primary goal of my thesis research is to shed light on this *novelty* problem by using animal phototransduction system as a model for the evolution of complexity. At the core of visual function in metazoans are specialized biochemical signal transduction cascades that translate individual photons from the physical environment into nerve impulses. These phototransduction cascades are implicated in primary vision and various non-visual phenotypes in a broad range of animal lineages that have been studied to date and represent ancient evolutionary modules whose components have duplicated and diversified over evolutionary time and are amenable to phylogenetic study. The purpose of my dissertation research is to unravel the evolutionary history gene duplication and network diversification that has lead to the present diversity of animal visual pathways. Before I explicate my approach to this question, some

background on the biochemical composition of the phototransduction pathways is required.

An Overview of Animal Phototransduction

All animal phototransduction cascades are mediated by the visual pigment opsin (Terakita 2005). Opsins are seven-transmembrane receptor proteins of the G protein-coupled receptor (GPCR) class. Upon interaction with light, opsins change in structural conformation in a manner that leads to the activation of downstream signaling components. All phototransduction pathways exhibit compositional similarities that are consistent with the fact that they are based on GPCR signaling. For instance, upon activation of opsin by light the next step in all phototransduction cascades is the activation of a G protein. Specific amino acid interactions between activated opsin and the G protein facilitate this interaction (Marin et al. 2000). In addition, the ultimate outcome of phototransduction is the firing of a photoreceptive neuron such as the rod and cone cells of the vertebrate retina. This firing is accomplished through the opening or closing of ion channels, a process that changes the cell's electrochemical potential. However, despite these generalities differences in the composition of the phototransduction cascades are known from a variety of animal lineages.

Two modes of phototransduction have been described: a *ciliary* mode common to the visual systems of vertebrates and best understood in the rod and cone

cells of the vertebrate retina, and a so called *rhabdomeric* mode of phototransduction common to protostomes and well-studied in the compound eyes of flies and annelids (Arendt 2003) (Figure 1.1). These modes of phototransduction are mediated by homologous opsins of different paralogy classes. The ciliary cascade is mediated by c-opsin whereas the rhabdomeric cascade is initiated by r-opsin. The G proteins involved in these two modes of phototransduction are also individuated based on paralogy. For instance, the ciliary mode of phototransduction involves the activation of a $G_{i/o}$ class of G proteins whereas the rhabdomeric mode utilizes a G_q class of G proteins (Hardie and Raghu 2001). Once initiated, the different modes of phototransduction diverge both in the intermediary effectors that comprise the cascades and in the ion channels that give rise to the cell-physiological response to light. Ciliary phototransduction cascades include the enzyme phosphodiesterase (PDE) and the second messenger cyclic guanidine monophosphate (cGMP) for intermediate signaling and a Cyclic Nucleotide-Gated ion channel (CNG) for physiological response (Pifferi et al. 2006). Alternatively, the rhabdomeric phototransduction cascade utilizes the enzyme protein lipase C (PLC) and lipid-based signaling molecules for intermediate steps of the pathway which culminates in the opening of a Transient Receptor Potential ion channel (TRPC) in eliciting the cellular response (Venkatachalam and Montell 2007). Both signaling pathways are attenuated by the actions of arrestin proteins. A third mode of animal phototransduction referred to as G_o-coupled phototransduction has been described by some authors (Kojima et al. 1997). This pathway is in most respects similar to the ciliary mode of signaling and

for our purposes we consider G_o-coupled signaling to be a form of ciliary phototransduction.

Present data suggest that the contrasting modes of phototransduction mediate primary vision in manner that recapitulates the two major lineages of bilaterian animals (Figure 1.2). Ciliary phototransduction cascades mediate primary vision in all cases known from deuterostomes, the clade of animals that includes vertebrates and other chordates, cephalochordates, echinoderms and hemichordates. Conversely, rhabdomeric cascades mediate vision in protostomes such as insects and other arthropods, mollusks, annelids and an assortment of additional invertebrate phyla. While ciliary and rhabdomeric phototransduction cascades underlie primary vision in deuterostomes and protostomes respectively, it is also known that both major lineages of bilaterian animals posses both pathways (Arendt 2001; Plachetzki et al. 2005; Velarde et al. 2005). Rhabdomeric phototransduction has been identified in a population of intrinsically photosensitive ganglion cells of the vertebrate retina (Melyan et al. 2005; Panda et al. 2005) and ciliary opsins have been identified in the brains of insects and annelids (Arendt 2004; Velarde et al. 2005). The evolutionary events that have lead to this arrangement are unclear as are the functional consequences of this dichotomy. In order to address these issues we must first gain an appreciation for the deeper evolutionary history of the phototransduction cascades by exploring these systems in earlier branching non-bilaterian animals such as cnidarians and sponges.

Animal Phototransduction as a Model for the Origination of Complexity in Evolution.

One approach to understanding the evolution of a complex trait is to understand the evolutionary histories of the components that comprise the trait in question using phylogenetic approaches. The diversification of the animal phototransduction cascades offers a useful model for the evolution of complexity as each of the components that comprise the phototransduction systems are discrete proteins that are amenable to phylogenetic study.

My investigations have focused on the following questions. First, when did the animal phototransduction cascades originate in evolution? Can we identify the node of evolutionary origins of these visual and photosensitivity pathways? Are these systems specific to animals – *are they metazoan synapomorphies*- or can their origins be traced to clades that existed prior to the evolution of metazoans? Second, what is the molecular basis for the diversification of the animal phototransduction networks? This question is addressed both on the level of individual amino acid substitutions and in the larger scale dynamic of gene duplication and loss for phototransduction loci. Next I ask, what was the composition of the ancestral phototransduction network? Data on the timing and the molecular basis for phototransduction diversification provide important new insights on the evolution of the visual pathways, however it is also important to understand the ancestral state of the phototransduction cascade upon its origination so that the changes that have occurred

in evolution can be polarized in an evolutionary context. Finally, my investigations have revealed interesting findings on the possible function of opsin-mediated phototransduction pathways in the basal animal phylum Cnidaria. In a chapter directed at understanding the organismal biology of cnidarians, we explore the role of photosensitivity in mediating nematocyte firing in cnidarians?

Chapter Overview

In chapter 2 we use comparative genomics and phylogenetics to explore the evolutionary history of opsin genes. Because all animal phototransduction cascades are initiated by opsins, an appreciation of their evolutionary relationships and timing of origins is an important first step in our larger goal of understanding the diversification of the phototransduction networks. We find compelling evidence that opsins originated prior to the last common ancestor of cnidarians and bilaterians (a clade referred to as Eumetazoa (see Figure 1.2) but not in earlier branching lineages (Plachetzki et al. 2007). These results are important for the following reasons. First, while previous phylogenetic treatments of animal opsins have been reported (Arendt et al. 2004; Koyanagi et al. 2005; Terakita 2005) ours was the first to include data from early branching animal taxa. This study identifies for the first time a new clade of animal opsins from the cnidarians that we have christened *cnidops*. The discovery of cnidops and its placement in animal opsin evolution provides key data for the examination of the changes that have occurred in the evolutionary history of the

phototransduction cascades. In addition, our phylogeny falsifies previous hypotheses of a direct evolutionary linkage between the visual systems of animals and those of other taxa that originated prior to the animal lineage such as fungi, choanoflagellates and other animal outgroups (Gehring 2001).

A common thread my dissertation research is the use of phylogenetic methods to test hypotheses regarding the diversification of complex traits. Our phylogeny for animal opsins provides a tool for understanding the molecular basis of phototransduction diversification. In addition to our report of a new view of animal opsin phylogeny, chapter 2 also examines the molecular basis for the changes opsin-G protein interactions that characterize the ciliary and rhabdomeric pathways. Using a range of comparative statistical methods, we find strong evidence that two amino acid positions in opsins co-vary with G protein interaction class across opsin phylogeny. Importantly, these two residues have specifically been shown to play a role in opsin-G protein interactions in previous biochemical studies (Marin et al. 2000), thus providing functional support for our phylo-informatic findings.

Chapter 3 further explores the molecular basis for the diversification of the animal phototransduction pathways, but from the vantage point of gene duplication and divergence. Gene duplication and divergence has been suggested as a mechanism for the evolution of novel and complex traits (Ohno 1970; Lynch 2007). More recently, evolutionary change by co-option of pre-existing components into novel functional contexts has received much attention (Carroll 2005; Williams et al. 2008). Using our new phylogeny for animal opsins, we test the utility of these

contrasting paradigms for explaining the evolution of animal phototransduction systems. Not unexpectedly, we provide evidence that both processes have been active in the diversification of phototransduction systems. Our approach introduces a novel, but simple phylogenetic methodology for discriminating between gene duplication and divergence, and co-option phenomena in the evolution of complex traits and is useful for a broad range of questions (Plachetzki 2007).

Our phylogenetic results are also useful in resolving the ancestral state and compositional history of the animal phototransduction cascades. Because the particular types of ion channels utilized in the ciliary and rhabdomeric pathways are the key determinants of the physiological differences that characterize these two pathways (e.g., the closing of CNG ion channel leads to hyperpolarizing potentials in ciliary photoreceptors whereas the opening of TRP ion channels facilitate depolarizing potentials in rhabdomeric photoreceptors) (Hardie and Raghu 2001), understanding which type of ion channel functioned in the ancestral phototransduction pathway is vital to our understanding of the history and function of these systems. In Chapter 4 I provide strong evidence based on character mapping, gene expression and behavioral-pharmacological studies that the ancestral animal phototransduction cascade utilized a CNG ion channel in signaling. This result polarizes existing data and allows recognition that the vertebrate phototransduction pathway resembles the ancestral state of phototransduction whereas the rhabdomeric pathway that functions in the visual systems of insects and other protostomes represents a derived condition.

In Chapter 5 I explore an interesting aspect of cnidarian organismal biology that was suggested by my investigations into the expression of phototransduction genes in the cnidarian *Hydra magnipapillata.* In studying the expression of opsin and other phototransduction genes in the hydra it became clear that were expressed in the battery cell complexes that house nematocytes, the stinging cells common to all cnidarians. We have developed a simple nematocyte discharge assay from earlier methods (Watson and Hessinger 1989). By conducting these trials under different light regimes we have obtained evidence that nematocyte firing is attenuated by information from the light environment. Earlier work has shown that nematocyte function is attenuated by both mechanosensory and chemosensory information from the environment (Watson and Hessinger 1994). Ours is the first study to implicate photosensitivity in this behavior as well. In light of these data the cnidarian nematocyte can be viewed as an emerging model for the integration of complex sensory information into a single cellular context. How these signals are transdused and integrated into behavioral responses remains an attractive area of future research. Further, the nematocyte itself represents a fascinating model for the evolution of complexity and novelty in nature.

In my closing chapter I discuss the impact that the work presented here has had in fostering a new understanding of the evolution of animal visual systems. I also draw into chapter 6 a discussion of recent findings on cnidarian phototransduction from other researchers that largely support and augment our findings. I identify key

questions for future research that are raised by the current data on phototransduction evolution and the cnidarian visual cycle.

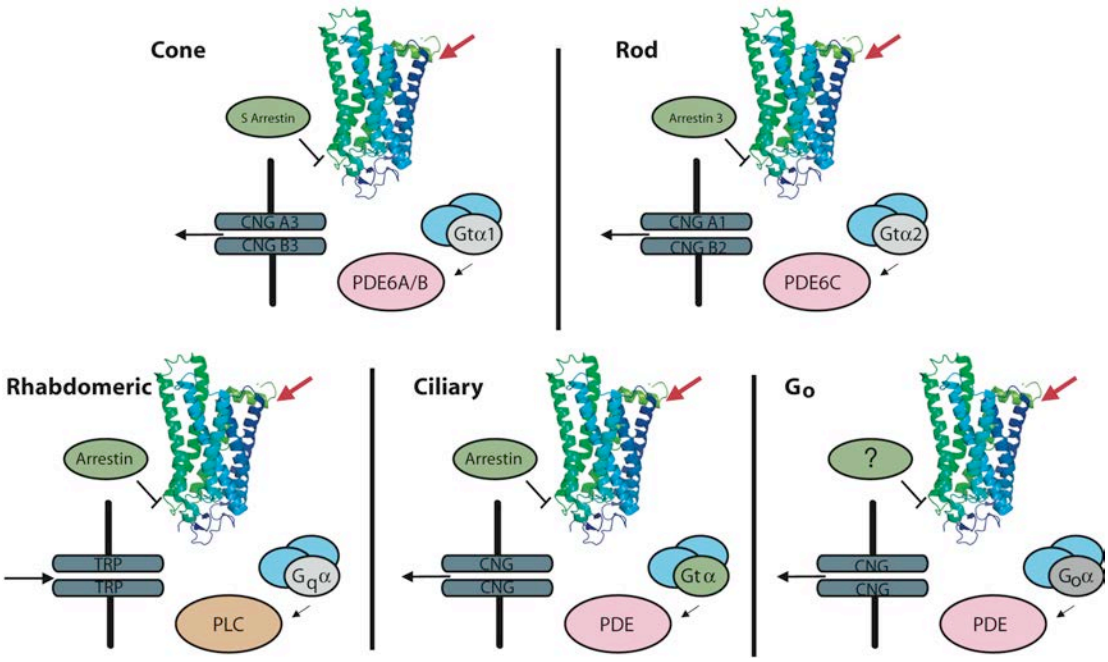

Figure 1.1. Simplified phototransduction cascades of the rod and cone photoreceptors of the vertebrate retina and the more general rhabdomeric, ciliary and G_o signaling pathways found in animals. The rod and cone phototransduction cascades are members of the ciliary class. The phototransduction cascade is initiated by light (arrow) ultimately leading to the efflux (left-pointing arrow) or influx (right-pointing arrow) of K^+ and Na^+ ions. Rod-specific and cone-specific paralogs of $G_t\alpha$, PDE, CNG and arrestin are known to have arisen by segmental genome duplications (Nordström et al. 2004). PDE, phosphodiesterase; Gα, G α subunit of the G protein; CNG, cyclic-nucleotide-gated ion channel; TRP, transient-receptor-potential ion channel. From Plachetzki and Oakley (2007).

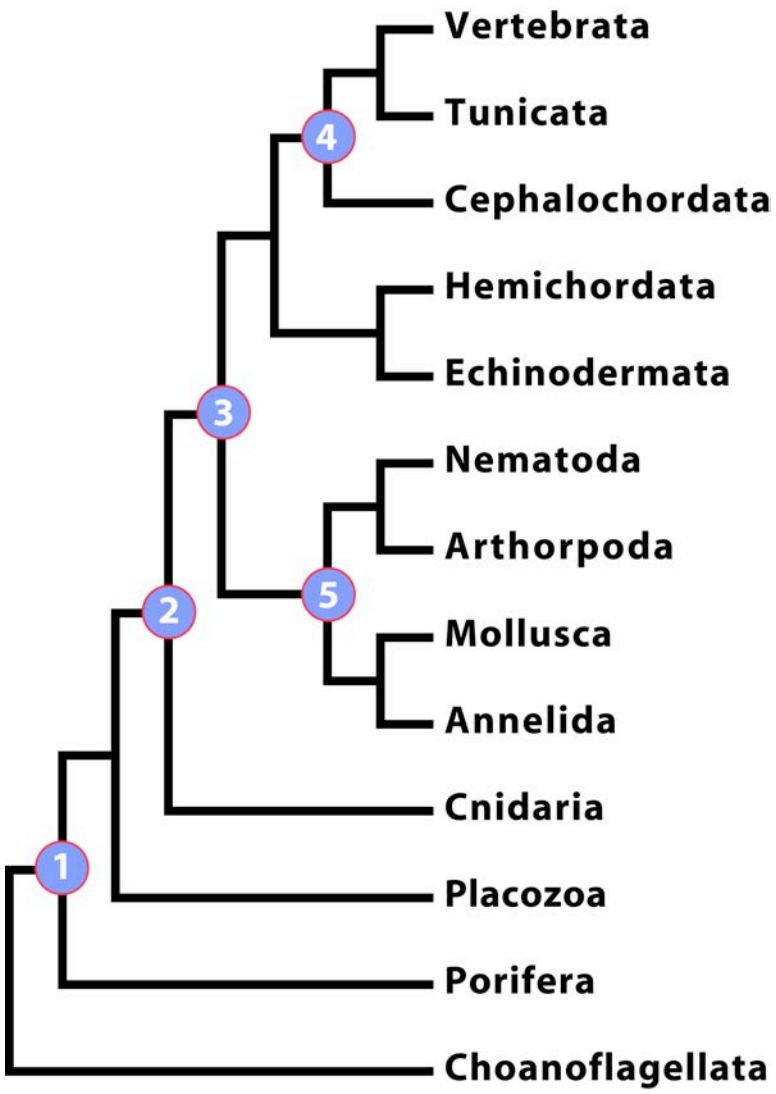

Figure 2.2. A current view of animal phylogeny. Terminal taxa are phyla for which at least one genome sequence is available. Major lineages are indicated numerically: 1, Metazoa; 2, Eumetazoa; 3, Bilateria; 4 Protostomia; 5, Deuterostomia. Earlier branching basal metazoa are represented by the phyla Cnidaria, Placozoa and Porifera. The Choanoflagellata represent the closest extant animal outgroup. Tree based on Halanych (2004), Dunn et al. (2008) and Bourlat et al. (2006).

Chapter 2

The origins of novel protein interactions during animal opsin evolution

Plachetzki, D. C., Degnan B. M, and T. H. Oakley. 2007. The origins of novel protein interactions during animal opsin evolution. *PLoS ONE* 2(10): e1054. doi:10.1371/journal.pone.0001054

Abstract

Biologists are gaining an increased understanding of the genetic bases of phenotypic change during evolution. Nevertheless, the origins of phenotypes mediated by novel protein-protein interactions remain largely undocumented. Here we analyze the evolution of opsin visual pigment proteins from the genomes of early branching animals, including a new class of opsins from Cnidaria. We combine these data with existing knowledge of the molecular basis of opsin function in a rigorous phylogenetic framework. We identify adaptive amino acid substitutions in duplicated opsin genes that correlate with a diversification of physiological pathways mediated by different protein-protein interactions. This study documents how gene duplication events early in the history of animals, followed by adaptive structural mutations, increased organismal complexity by adding novel protein-protein interactions that underlie different physiological pathways. These pathways are central to vision and other photo-reactive phenotypes in most extant animals. Similar evolutionary processes may have been at work in generating other metazoan sensory systems and other physiological processes mediated by signal transduction.

Introduction

Documenting the specific genetic changes driving phenotypic evolution is a fundamental goal of current biology. Genetic changes are known to modify

phenotype during evolution by altering the interactions between a protein and its ecological or biochemical environment (Yokoyama 2000; Zhang et al. 2002; Spaethe and Briscoe 2005; Bridgham et al. 2006), by modulating existing protein-protein interactions (Turner and Hoekstra 2006), or by changing protein-DNA interactions through regulatory mutations (Shapiro et al. 2004; Bachner-Melman et al. 2005; Rockman et al. 2005; Tishkoff et al. 2007). However, the specific genetic changes that give rise to the evolutionary origins of novel protein-protein interactions have rarely been documented in detail (Lynch et al. 2004).

Animal phototransduction pathways offer great opportunity for elucidating the genetic basis for evolutionary novelty for a number of reasons. First, a diversity of presumably ancient phototransduction pathways exists in animals (Arendt 2003). Second, the composition of these cascades has been the subject of numerous functional biochemical studies. This is especially important because experimental demonstration that specific mutations cause phenotypic changes is often the most difficult aspect of a full documentation of the causal genetic changes driving phenotypic evolution (Hoekstra and Coyne 2007). Third, the proteins of animal phototransduction are amenable to phylogenetic study.

Fundamental to animal phototransduction pathways are the opsin visual pigment proteins, which bind to light reactive chromophores. As members of the G protein-coupled-receptor (GPCR) family [6], the various clades of animal opsins activate alternative G-proteins, resulting in three major phototransduction networks: ciliary, rhabdomeric, and G_o–coupled (Arendt 2001; Terakita 2005; Raible et al.

2006). Ciliary opsins initiate signaling through binding of a $G_{i/t}$ α subunit of the G-protein (Terakita 2005), rhabdomeric opsins utilize a G_q α subunit in signaling (Hardie and Raghu 2001) and class-specific G_o α has been identified in the G_o-coupled opsin signaling pathway (Kojima et al. 1997; Koyanagi et al. 2002; Koyanagi et al. 2005). Another class of opsins, including Retinal G-protein-coupled Receptor (RGR) and retinochromes, probably do not signal through any G-protein. Instead they are involved in the re-activation of the light reactive chromophore.

Here, we couple specific functional knowledge about opsin's role in signal transduction with new phylogenetic analyses. These analyses elucidate specific genetic changes that were likely involved in the origins of the different animal phototransduction networks, which mediate various light responses of animals. Our phylogenetically based analyses indicate a significant correlation between opsins' G-protein binding phenotypes and amino acid positions in the fourth cytoplasmic loop of opsin, especially positions homologous to 310 and 312 of bovine rhodopsin. Previous biochemical analyses demonstrate that these same amino acid positions are involved in G-protein binding function. Our additional analyses indicate that G-protein binding phenotypes likely diversified at the time of opsin gene duplication events before the origin of bilaterians, and the specific amino acid changes involved retain a pattern consistent with purifying selection.

Results

Multiple opsin genes are present in cnidarians but absent in a demosponge

Screens for opsin genes in the genome trace data from the cnidarians *Hydra magnipapillata* and *Nematostella vectensis* produced multiple unique opsins which all lack introns. Six of these cnidarian opsins were found in public Expressed Sequence Tag (EST) databases (Table S1). Consistent with opsin status, Hydra2 is expressed in the nerve net of *Hydra* based on in situ hybridization and includes sequence motif hallmarks of opsin (figure 2.1).

While multiple opsins were found in the cnidarian genomes, our screens for opsins in trace data from the poriferan *Amphimedon queenslandica* did not produce any putative opsins. These screens did produce several non-opsin, rhodopsin-class GPCR genes from *Amphimedon* (data not shown). We were also unable to obtain opsin sequences from the trace genome data of the placozoan *Trichoplax* or the choanoflagellate *Monosiga*. Animal (type II) opsins are also unknown from numerous fungal genomes (Dunlap 2005).

A new class of cnidarian opsins helps resolve phylogenetic relationships

In our discussion of unrooted phylogenies, we refrain from using the common terms “sister group” and “clade” in favor of the terms “adjacent group” and “clan” as the former terms imply a rooting hypothesis *a priori* (Wilkinson 2007).

Our phylogenetic analyses reveal a new clan of opsins known only in cnidarians, which we have named “cnidops”. Together, metazoan opsins form two major clans in unrooted analyses. One clan unites rhabdomeric and RGR/G_o–coupled opsins in an adjacent group and the second consists of the newly identified cnidops family plus the ciliary opsins, which we here confirm by phylogenetic analysis to include a non-bilaterian representative (Nematostella4; figure 2.2).

Without non-opsin outgroups, our analyses of metazoan opsin phylogeny yielded well supported topologies. In order to root our tree, we used a combination of likelihood comparisons, reconciled tree analyses and parametric bootstrapping (summarized in figure 2.3 and described in Methods). The majority of available evidence indicates that opsin phylogeny is best rooted between the ciliary and non-ciliary clades (figure 2.4A).

Timing of origin of major opsin clades

Using our rooted opsin phylogeny, reconciled tree analyses (RTA) provide new information on the timing of origin of the various opsin clades (figure 2.5). RTA indicates that the ciliary and cnidops lineages originated by gene duplication at or before the cnidarian-bilaterian common ancestor (Eumetazoa), approximately 600 million years ago (Peterson et al. 2004; Peterson and Butterfield 2005).

Of the two ancient metazoan opsin lineages, one is represented in extant taxa by the newly described cnidops opsins of cnidaria, RGR/G_o and rhabdomeric opsins. Our results concur with previous minima for the origins of RGR/G_o and rhabdomeric

opsins at the protostome-deuterostome common ancestor (Urbilateria) (Arendt 2001; Arendt et al. 2004) and provide a new maximum for the origin of these clades by a gene duplication event younger than the eumetazoan ancestor (figure 2.5). A second ancient opsin lineage survives as the ciliary opsins and includes one cnidarian opsin (Nematostella4) and those of the vertebrate rods, cones, pineal and parapineal, and of invertebrate extra-ocular cells (Arendt et al. 2004; Velarde et al. 2005). Despite finding the *Nematostella* gene, we did not find any *Hydra* genes from this ciliary opsin clade.

The early evolution of G protein binding partners

Our phylogeny provides a framework for understanding key transitions in the evolution of animal phototransduction pathways. Opsins interact with their corresponding G proteins, in part through binding at conserved sequence motifs located in opsin's fourth cytoplasmic loop (Marin et al. 2000). We obtained clear reconstructed ancestral states for most of the residues in a conserved tri-peptide motif for the ciliary, rhabdomeric and RGR/G_o nodes. For the most part, the remainder of the residues in the fourth cytoplasmic loop can be unequivocally reconstructed to the level of Dayhoff classes (i.e., C/HRK/FYW/DENQ/LIVM/GATSP) (Hrdy et al. 2004) (figure 2.6). In addition, many of the residues of this region significantly co-vary with G protein α subunit interaction on our phylogeny. Two of the highest scoring residues in the co-variation analysis, residues 310 and 312 corresponding to positions 1 and 3 of the G protein-binding tripeptide motif (Marin et al. 2000),

possessed a signature of selection using algorithms in DIVERGE2 (Zheng et al. 2007) (figure 2.7).

We also explored the character history of G protein binding interactions by reconstructing their ancestral states across our phylogeny (figure 2.6). While we cannot conclusively resolve the G protein interaction in the ancestor of rhabdomeric+G_o opsins or the ancestor of ciliary+rhabdomeric+G_o clades, our reconstructed ancestral G protein interaction states for ciliary, rhabdomeric and G_o are highly significant (P>0.95 for each) and are congruent with previous empirical biochemical studies (Hardie and Raghu 2001; Koyanagi et al. 2002; Su et al. 2006) (figure 2.6).

Discussion

Our findings provide several new insights into the evolution of animal phototransduction cascades. First, we identify a new class of opsins that appears to be unique to cnidarians. Second, we find no evidence of any opsins in genomes of a demosponge, a parazoan, a choanoflagellate or fungi. We conclude that opsin-mediated phototransduction cascades originated early in eumetazoan evolution, after this lineage diverged from the demosponge lineage. The rhabdomeric and G_o-coupled cascades appear to be bilaterian innovations. Third, the origins of novel phototransduction cascades, as defined by the evolution of alternative opsin-G protein interactions, co-occur on our phylogeny with the gene duplications that have given

rise to the major opsin classes. Fourth, the specific mutational events that have lead to the functional diversification of the major opsin lineages are suggested by co-variation analyses and tests for selection. These results demonstrate how gene duplications, together with structural mutations that lead to novel protein-protein interactions, can contribute to the evolution of novel physiological traits. Each of these points is discussed further below.

The timing and relationships of animal opsins

The inter-relationships of animal opsins reported here are based on thorough phylogenetic analyses that included standard phylogenetic tree estimation, comprehensive likelihood comparisons, RTA, and parametric bootstrapping (figure 2.3). Taken together, the majority of evidence favors the hypothesis depicted in figure 2.4A, although the trees in figures 2.4B and C remain viable alternative hypotheses. However, because these latter hypotheses require additional gene duplication and loss events to explain the rooted topology, and because parametric bootstrapping simulations indicate that long branch lengths can contribute to the erroneous resolution of specifically these topologies, we favor the former hypothesis (figure 2.4A). Given present sampling and the assumption of monophyly of major opsin clades, any other phylogenetic hypotheses are significantly rejected by the data. Even if figure 2.4A does not represent the true history of opsin, the two viable alternative hypotheses (figures 2.4B and C) do not drastically alter our interpretation of the timing of origins of animal opsin in RTA analysis. Irrespective of which

hypothesis we consider, animal opsins originate at the same node (Eumetazoa). If we assume the hypothesis depicted in figure 2.4B our conclusion that RGR/G_o-coupled and rhabdomeric opsins are bilaterian synapomorphies also remains intact, but requires an additional loss of cnidops prior to Bilateria. The latter is also true of the topology shown in figure 2.4C.

A maximum date for the origin of animal opsins (when a GPCR first bound a light reactive chromophore) is equivocal because we did not obtain any opsins in the genome sequence of an earlier branching animal, the demosponge *Amphimedon queenslandica*, or from any non-animal genomes. While the larvae of *Amphimedon* are capable of phototaxis, studies of spectral sensitivity have suggested the activities of a flavin-based photoreceptor in this behavior (Leys et al. 2002), as opposed to retinal as used by opsins. Further, we uncovered no animal (type II) opsins from bioinformatic screens of the placozoan *Trichoplax* or the choanoflagelate *Monosiga* genomes and no true homolog of type II opsins have been described outside of Metazoa (Spudich et al. 2000). While we cannot rule out an earlier opsin origin coupled with loss in lineages leading to the species with genome sequences, we strongly favor the hypothesis that animal opsins were present in the eumetazoan ancestor of cnidarians and bilaterians, but not at earlier nodes in eukaryote phylogeny. Since porifera, *Monosiga*, and fungi seem to lack opsins, yet possess rhodopsin-class GPCR proteins without the Lysine residue to bind a light reactive chromophore, one possibility is that a rhodopsin-class GPCR gained the ability to bind a chromophore by substitution to a Lysine residue during animal evolution.

Our results contrast with previous studies on opsin relationships that placed ciliary and G_o-coupled opsins as sister groups (Arendt 2001), or adjacent groups (Terakita 2005), and those that placed ciliary and rhabdomeric opsins as sister groups (Arendt 2003). However, in lacking data from non-bilaterian animals, these previous works were able to include only three of the four animal opsin lineages discussed here. The new clade of opsins from the Cnidarian, "cnidops", has aided in the resolution of the overall opsin phylogeny, especially because non-opsin outgroups seem to destabilize ingroup topology.

The presence of an opsin from the cnidarian *Nematostella* that in our analyses groups within the ciliary clade has been foreshadowed by previous morphological descriptions of ciliary photoreceptors (Eakin and Westfall 1962; Eakin 1979), but no previous opsin data from the group have confirmed this hypothesis. Our ability to find only cnidops and not ciliary opsins in *Hydra* probably indicates that ciliary clade opsins were lost during this species' evolutionary history.

The genetic basis for opsin-G protein interactions.

Multiple lines of evidence support the hypothesis that amino acid substitutions in the 4th cytoplasmic loop of duplicated opsins were involved in the origins of novel opsin-G protein interactions. First, specific amino acid states significantly co-vary with G-protein activation phenotypes during opsin phylogeny (figure 2.7). Because we used a Bayesian approach that estimated the posterior probability of all possible trees, the estimated correlation accounts for uncertainty in opsin phylogeny. The

highest p-values in these correlation analyses correspond to residues formerly demonstrated by site-directed mutagenesis to mediate opsin-G protein interaction in ciliary opsins (i.e., residues 310 and 312 (Marin et al. 2000)).

These amino acid changes retain the signature of selection, as given by site-specific shifts in evolutionary rates, a statistical test that uncovers significant conservation within gene clades compared to diversification between gene clades (Zheng et al. 2007) (figure 2.7). Although this pattern cannot be used to determine if the mutations were originally fixed by natural selection, conservation after an ancient diversification indicates a long period of stasis, consistent with purifying selection on adaptive, alternative amino acid states. The combination of correlation, functional tests, and a pattern consistent with purifying selection strongly suggests that substitutions in residues homologous to bovine opsin 310 and 312 contributed to the origin of novel phototransduction pathways with alternative G-protein binding partners.

The pattern of early divergence followed by conservation is also evident in our estimates of ancestral opsin-G protein binding phenotypes. Using available data, we were able to unequivocally resolve the ancestral G-protein usage phenotypes for the ciliary, rhabdomeric and RGR/G_o-coupled opsin clades (figure 2.6). That we can resolve the G protein binding specificities at the base of each of these clades, but not at deeper nodes, suggests an early diversification of G protein binding specificities, coupled with relative stasis of protein interactions once the major opsin clades were established.

Because we find that opsins were present in extant eumetazoans but not in animals representing earlier branching lineages (i.e. demosponges), the evolution of novel phototransduction cascades in animals probably proceeded by evolving specialized protein-protein interactions between new opsin paralogs and existing G protein signaling intermediaries, an evolutionary process previously described as molecular exploitation (Bridgham et al. 2006). Each of the G α subunit paralogs known to interact with opsins in bilaterians has also been described from plants, fungi, protists, cnidarians and sponges indicating a long pre-animal history for the different G-proteins (Suga et al. 1999; O'Halloran et al. 2006).

While our inclusion of new data from the Cnidaria has greatly enhanced our ability to reconstruct opsin phylogeny, functional or phenotypic data from the newly reported cnidops clade do not exist. Further studies on the biochemistry of signaling by cnidops are required if we are to better understand the history of opsin-G protein interactions in this important family of opsins. Despite this missing data, P-values for our ancestral state reconstructions for the remaining opsin classes suggest that our results are robust.

Implications for early animal sensory systems

Our finding of an adaptive, early partitioning of opsin-mediated signaling into discrete functional classes in the course of their evolution provides insight into the organismal properties of the ancestor of cnidarian and bilaterian animals. The activities of ciliary, rhabdomeric and G_o-coupled opsins are known to exist in close

proximity (Gomez and Nasi 2000), if not the same cell (Su et al. 2006), in numerous taxa. The early, adaptive evolution of the different photosensory pathways suggested by our analyses may be an indication of the functional necessity to transduse these signaling cascades through non-overlapping channels in the eumetazoan ancestor, an animal that by our analysis possessed ancestral ciliary and ancestral cnidops+rhabdomeric+RGR/G_o-clade opsins. Such early functional requirements could have included the need to delineate light information used for vision from that used to entrain the circadian clock cycle (Gehring and Rosbash 2003) or to differentiate between light of differing wavelengths, two core processes accomplished by specific cell types in bilaterian animals (Arendt 2003).

The proposed relationship between gene duplication and biological complexity has been invoked and re-invoked at various points throughout the past century of biological investigation (Taylor and Raes 2004), and was most famously articulated by Ohno (Ohno 1970). Despite the great level of interest in models for the evolution of complexity based on gene duplication, data demonstrating how gene duplication can be implicated in the origins of novel protein-protein interactions are not common. Here we begin to fill this gap and show that a group of familiar signaling pathways, animal phototransduction cascades, provides a useful model for the study of the origins of novel phenotypes.

Materials and Methods

Data mining

Publicly available trace genome sequence data from *Hydra magnipapillata*, *Nematostella vectensis* and *Amphimedon queenslandica* (http://www.ncbi.nlm.nih.gov/Traces) were subjected to tblastn (Altschul et al. 1997) searches using a wide range of homologous opsin proteins from bilaterian animals (Table S1). We implemented an in-house assembly pipeline, where BLAST hits were extended to their largest possible contigs under stringent parameters, using only clean trace data as determined using PHRAP (Ewing and Green 1998) and an 85% identity cut off for joining trace fragments. Genes were predicted from genomic contigs using GenomeScan (Yeh et al. 2001). A combination of BLAST searches, phylogenetic analyses and sequence motif analyses were used to establish the authenticity of opsin sequences recovered from genome analyses. In particular, the residue homologous to the bovine lysine 296, which binds a light reactive chromophore (Palczewski et al. 2000), has been used to validate opsin identity (figure 2.1). We corroborated the existence of our predicted proteins by using EST databases at either NCBI (http://www.ncbi.nlm.nih.gov/), for *Hydra*, or the Joint Genome Institute, for *Nematostella* (http://www.jgi.doe.gov/). Homologous proteins and outgroup data from bilaterian animals used in phylogenetic analyses were also obtained from NCBI (Table S1).

Animal culture and in situ hybridization

Hydra magnipapillata (UC Irvine strain) were reared in the laboratory under standard conditions at 18° C. The expression of one opsin, HM2, was investigated in *Hydra magnipapillata* using in situ hybridization following Grens et al. (Grens et al. 1996). The following modifications were made: A pCRII vector (Invitrogen) containing HM2 opsin cDNA (~750 bp) was used as template for probe synthesis. DIG-labeled probes were synthesized using T3 and T7 polymerase (Roche). Both sense and anti-sense probes (0.3 μg/ml) were hybridized at 55° C for 48 to 60 hours. Sense probes produced no detectable signal.

Phylogenetic approaches

For phylogenetic analyses, only the 7-transmembrane region including intervening inter- and extra-cellular domains was included (330 amino acids), as it was difficult to ascertain homology of N- and C- termini due to sequence length variation and lack of conservation across genes. Taxon/gene selection was done in a manner that enhanced both the taxonomic representation across the Metazoa and the inclusion of well-studied subfamilies (i.e., arthropod Rh opsins and vertebrate visual opsins), but also allowed for thorough computational analyses. Protein sequences were aligned using T-COFFEE under default parameters (Notredame et al. 2000) and alignment manipulations were done using Seaview (Galtier et al. 1996). Phylogenetic analyses were conducted using unweighted Maximum Parsimony (MP) implemented in PAUP* 4.0b10 (Swofford 2002), Maximum Likelihood (ML) implemented in

PHYML v2.4.4 (Guindon and Gascuel 2003), and Bayesian Markov Chain Monte Carlo (BMCMC) implemented in MrBayes 3.1 (Ronquist and Huelsenbeck 2003). Support for internal nodes was assessed with 1000 bootstrap replicates for MP and ML, and posterior probability for BMCMC analyses. ML and BMCMC approaches assumed best-fit models of protein evolution, as determined using ProtTest (Abascal et al. 2005). For ML tree calculations, both the proportion of invariant sites and the α parameter of the gamma distribution were estimated and both tree topology and branch lengths were optimized in PHYML. Bayesian analyses were conducted for 20 million generations using default priors and heating parameters for each of four chains. For the monitoring of progress in BMCMC runs, we assumed convergence of the Markov Chains when standard deviation of split frequencies (SDSF) fell below 0.01, indicating that the two independent runs resulted in similar phylogenies (Ronquist and Huelsenbeck 2003). Following the BMCMC runs, burnin was assessed using Tracer (Rambaut and Drummond 2003) and by the SDSF of the two independent BMCMC runs. For un-rooted analyses, the initial 11 million generations with an SDSF of 0.0015 or greater were discarded. For rooted analyses the first 12 million generations with an SDSF of 0.0024 or greater were removed prior to consensus tree calculation.

The evolutionary relationships between members of the larger GPCR class of proteins remain poorly understood. Because of this, our outgroup selection in rooted analyses was empirical. We selected the shortest-branched sequences from a pool of

rhodopsin class GPCRs (Spudich et al. 2000) from a variety of taxa (Table S1) after non-bootstrapped ML and shorter (i.e., 3-5 million steps) BMCMC analyses.

Tests of phylogenetic hypotheses

In order to address the lack of resolution among the major opsin clades encountered in phylogenetic analyses that included outgroups (figure S2.2), we tested the significance of a wide range of competing phylogenetic hypotheses for metazoan opsin. First, we assumed monophyly of each of the four major opsin clades (cnidarian opsin, ciliary, RGR/G_o and rhabdomeric). This assumption is supported by the results of unrooted MP, ML and BMCMC analyses (figure S2.2) and by previous studies on opsin phylogeny (Arendt 2003; Terakita 2005). Our topology contains four opsin clades that can be rearranged in 15 possible rooted binary trees. Second, we calculated the likelihood for each possible tree using the "resolve multifurcations" function in TREEFINDER (Jobb et al. 2004). Finally, we used CODEML, as included in PAML (Yang 1997), to assess the significance of each of these trees. These results were further analyzed using CONSEL (Shimodaira and Hasegawa 2001). A similar approach was used to assess alternative topologies in unrooted trees where having four major clades involves three possible unrooted topologies (figure 2.4).

Parametric bootstrapping

Given the ambiguous results obtained when including non-opsin outgroups, we suspected that long branch-attraction (LBA) artifacts (Felsenstein 1978), where the cnidops sequences were pulled to the base of the tree by long branch outgroups, were confounding our analyses. We tested this hypothesis by using Huelsenbeck's (Huelsenbeck 1997) method of parametric bootstrapping. Here, a tree including a non-opsin outgroup was constrained to reflect the best topology consistent with Tree 1B in figure 2.3. Assuming this tree and the best-fit model of opsin molecular evolution from prot-test analysis (Abascal et al. 2005) , we simulated 100 data sets using SeqGen (Rambaut and Grassly 1997). Each of these simulated data sets was subjected to ML and MP analyses. Since the data were simulated, we expected to recover the assumed phylogeny unless the topology/model combination is sensitive to artifacts like long branch-attraction (Huelsenbeck 1997). For ML analyses we found that the simulated cnidarian genes are incorrectly attracted to the base of the tree in 45% of replicates, whereas the correct, simulated topology was recovered in only 35% of the replicates. In MP analyses, the cnidarian opsin genes were incorrectly attracted to the outgroup in 49% of the replicates, while the simulated phylogeny was again recovered in only 35% of cases. These simulations show that analyses including opsin outgroups are sensitive to artifacts that induce erroneous topology estimates. Our results are similar to those reported for analyses of strepsipteran relationships, a well-known case of LBA (Huelsenbeck 1997).

Reconciled tree analysis

The number of gene duplication and loss events that are implied by a given rooting hypothesis can be used to assess the chances that a given hypothesis is tenable (Thornton and DeSalle 2000). We compared the number of duplications and losses implied by each possible root position of our unrooted phylogeny using NOTUNG (Chen et al. 2000; Durand et al. 2006). For these analyses we assumed a conservative species-level phylogeny for the major taxa included in our analyses: Hydrozoa, Anthozoa, Cephalochordata, Urochordata, Vertebrata, Annelida, Mollusca, and Arthropoda (Telford 2006) .

Character mapping and tests of co-variance

We scored G-protein interaction phenotypes for the opsins in our phylogenetic analysis as a discrete state character. Although these phenotypes are unknown for many opsins, especially those from non-model organisms, our analysis includes interaction data for 48% of the opsin sequences represented on our phylogeny (figures 2.6, S2.1).

Ancestral state reconstructions and tests for correlated character evolution were conducted using Bayesian mutational mapping (Nielsen 2001) as implemented in SIMMAP (Bollback 2006). Reconstructions were integrated over 18,000 trees that were sampled from our unrooted BMCMC analyses. Ancestral state reconstructions were conducted with 10 realizations sampled from the prior and 10 realizations sampled for each tree. We set an equal prior on the bias parameter but did not

abstract a prior for the rate parameter, instead using branch lengths from BMCMC trees as relative rates. As there is no *a priori* reason to assume different prior parameters, we chose this model, as it is the least parameterized model for morphological evolution possible in SIMMAP (Bollback 2006). We also tested a range of alternative prior settings and obtained similar P-values in these analyses (results not shown). Tests for selection were conducted in DIVERGE2 (Zheng et al. 2007) using our amino acid alignment, rooted phylogeny and the structure of bovine opsin previously calculated by x-ray crystallography (Li et al. 2004). Our estimate for site specific rate shift (functional divergence) between ciliary and cnidops+RGR/G_o + Rhabdomeric was significantly larger than 0: $\theta_1 = 5.82 \pm 0.05$. Site-specific posterior probabilities for residues 310 and 312 were 0.45 and 0.37 respectively.

Acknowledgments

We thank members of the Oakley lab for comments and ideas and H. Bode for providing *Hydra*. Thanks also to T. Cronin, M. Porter and M. S. Pankey for valuable comments on an earlier draft.

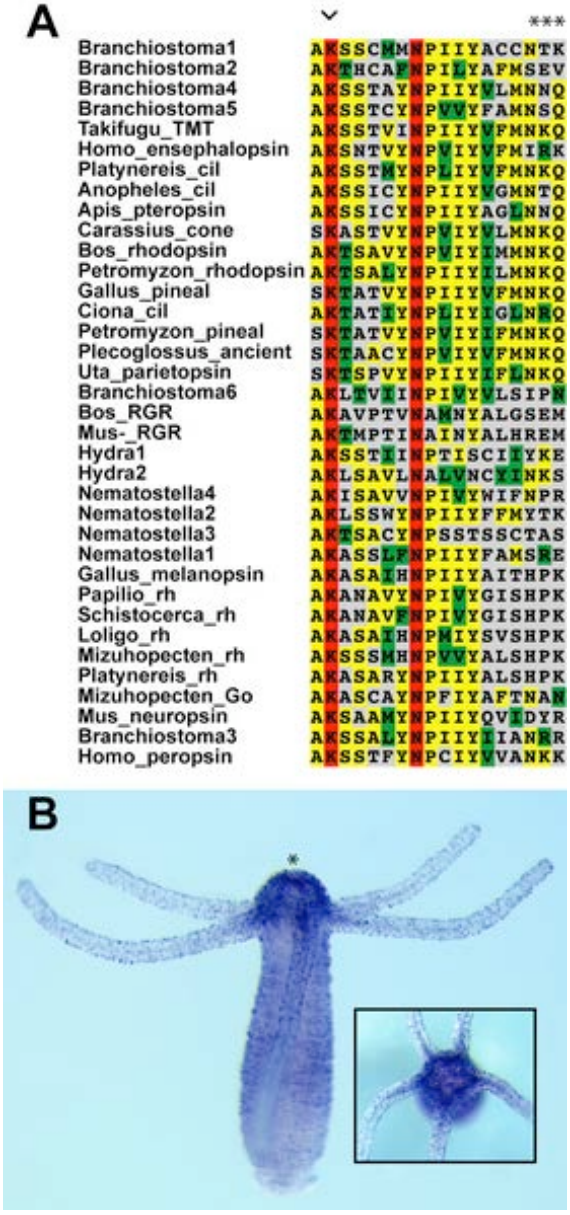

Figure 2.1. Sequence motifs and expression of cnidarian opsin in the nerve net of *Hydra magnipallata*. (A) Sequence alignment of 4th cytoplasmic loop region of animal opsins used in this study indicating the Lys 296 chromophore binding site (arrowhead) and the G protein-binding tripeptide (asterisks). (B) In situ hybridization with Hm2 cnidops probe. Asterisk denotes the hypostome. Opsin is expressed most strongly in a ring of sensory neurons that surround the mouth. Inset, oral view.

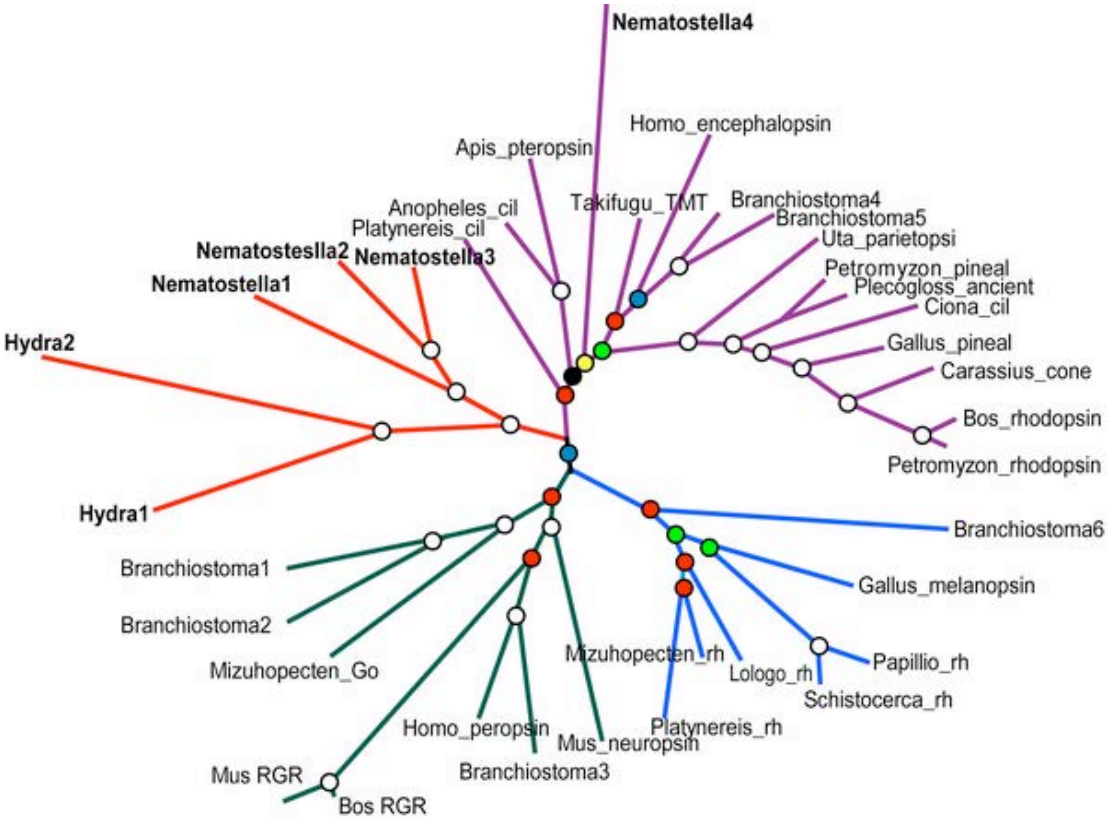

Figure 2.2. Unrooted metazoan-wide phylogeny of opsins, new cnidarian genes in bold, branches proportional to substitutions per site. Opsin parology classes denoted by branch color (Purple = ciliary; Blue = rhabdomeric; Green = G_o-coupled; Red = cnidops). Circles at nodes indicate Bayesian posterior probabilities (White = 1.0, Red>0.90, Blue>0.80, Green>0.70, Yellow>0.60, Black>0.50). cil = ciliary, rh = rhabdomeric.

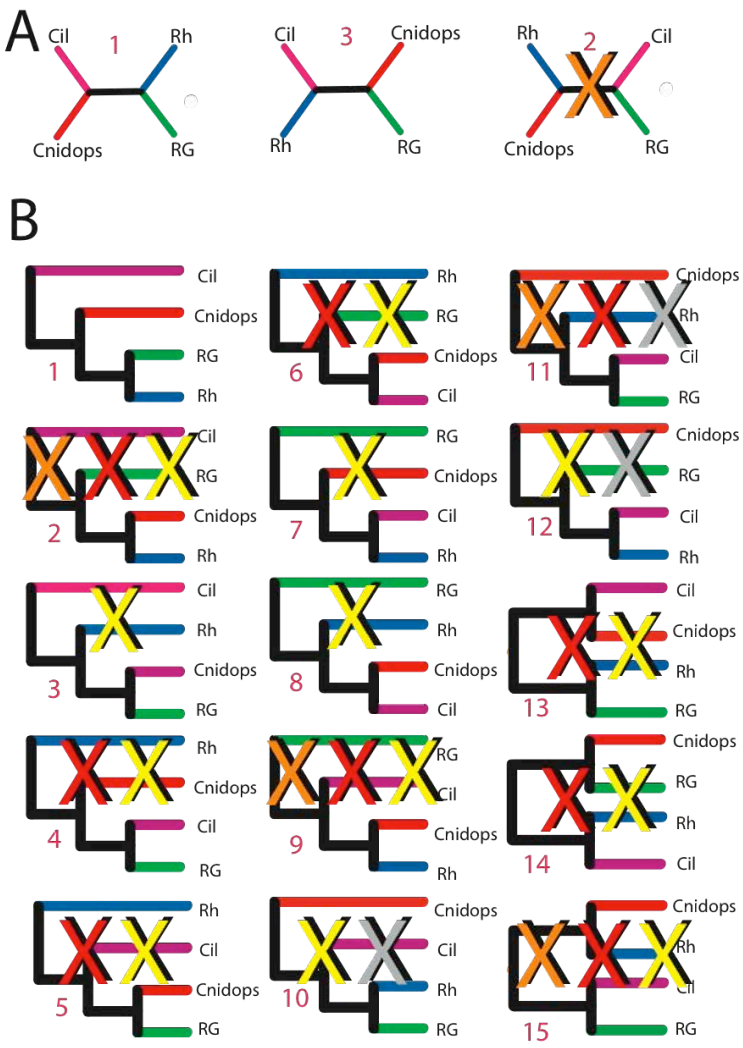

Figure 2.3. Summary of analyses to determine and root opsin phylogeny. Illustrated is each possible unrooted and rooted hypothesis, assuming monophyly of four major opsin clades. Trees in (A) 1-3 correspond to possible unrooted topologyies and those in (B) 1-15 represent all possible rooted trees, see Table 1. Orange X indicates that tree had significantly lower likelihood in opsin-only dataset. Red X indicates tree had significantly lower likelihood in opsin+outgroup dataset. Yellow X indicates that tree implies additional gene duplication/loss events compared to minimum (tree 1 is minimum with 2 duplications 0 losses implied). Grey X indicates tree with cnidops as earliest branching opsin group – a result inferred in parametric bootstrap analyses, which incorrectly grouped cnidops+outgroup opsins because of long branch-attraction. Cil= ciliary; Rh=rhabdomeric; RG=Go/RGR.

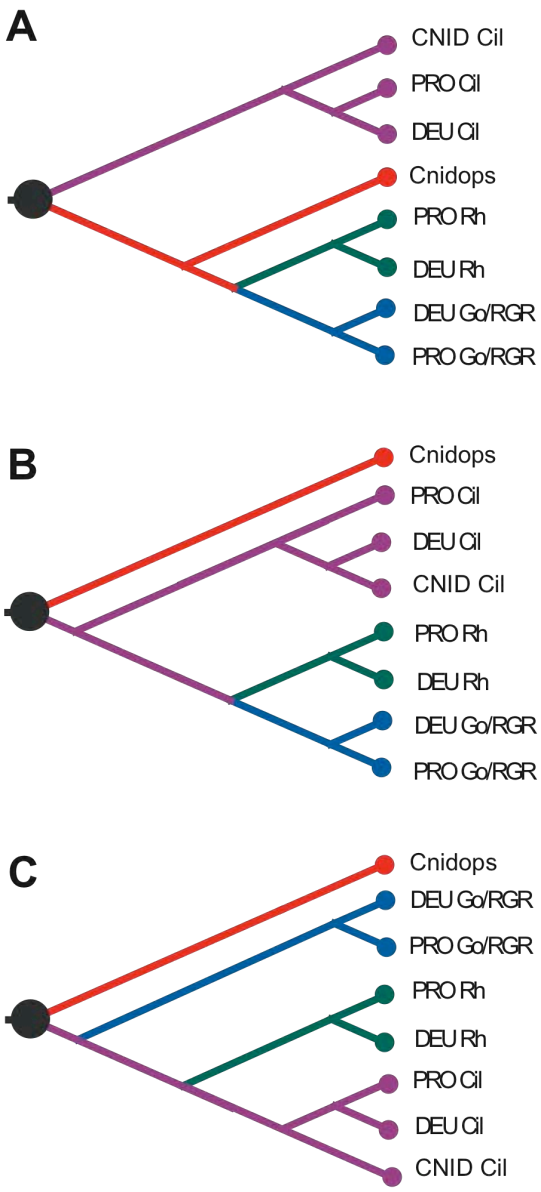

Figure 2.4. Three hypotheses for metazoan opsin relationships. These are statistically indistinguishable when considering only the likelihood of observed amino acid sequences. (A) Our preferred phylogenetic hypothesis where ciliary opsins are an outgroup to other opsin clades and cnidops is the sister to the rhabdomeric+G_o clade. This hypothesis minimizes the number of gene duplication and loss events required to explain the evolutionary history of metazoan opsins and is consistent with morphological data (Eakin and Westfall 1962). (B and C) Phylogenetic hypotheses in which cnidops represents the outgroup to other opsins. Given our finding of a ciliary opsin in *Nematostella*, these hypotheses require the additional loss of ciliary opsin in the *Hydra* lineage and an additional loss of cnidops in bilaterian animals.

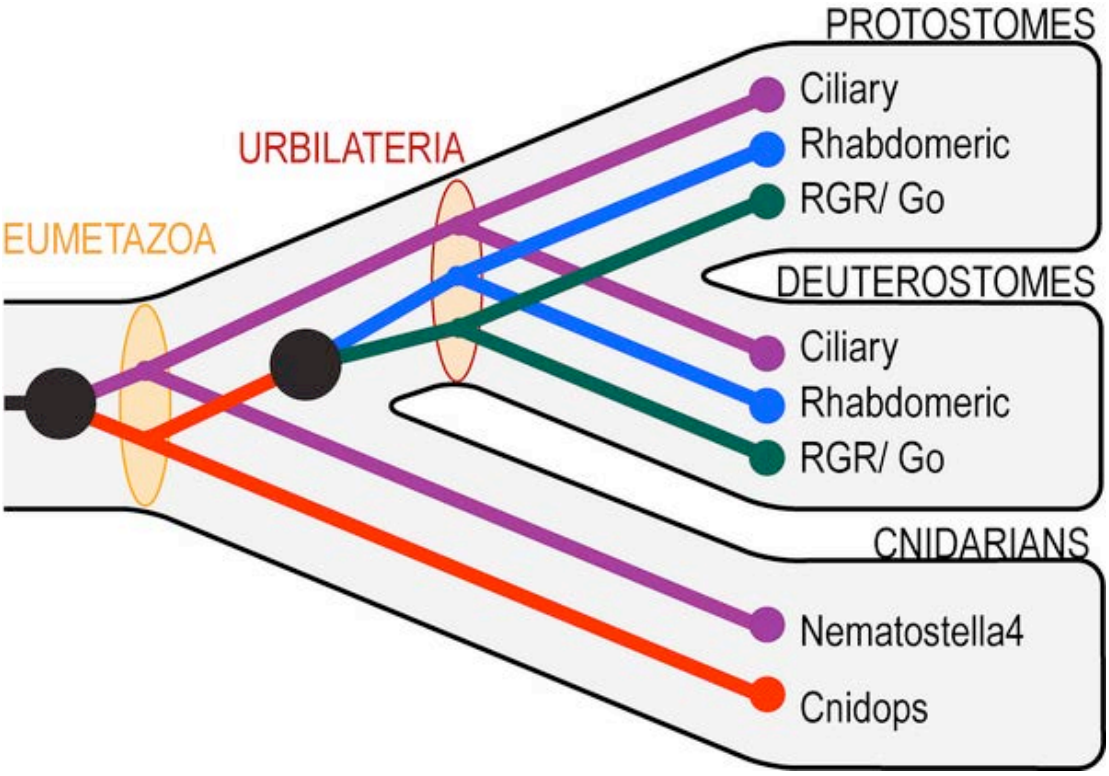

Figure 2.5. Our preferred hypothesis for opsin phylogeny (figure 2.4A) reconciled to a conservative view of metazoan phylogeny. Black circles indicate gene duplication events. Tan ovals indicate the opsin complement at key nodes in metazoan phylogeny. By this hypothesis, both ciliary and cnidops opsins were present in the eumetazoan ancestor of Cnidaria+Bilateria while rhabdomeric and G_o opsins evolved by gene duplication prior to the evolution of bilaterian animals, but not prior to earlier lineages.

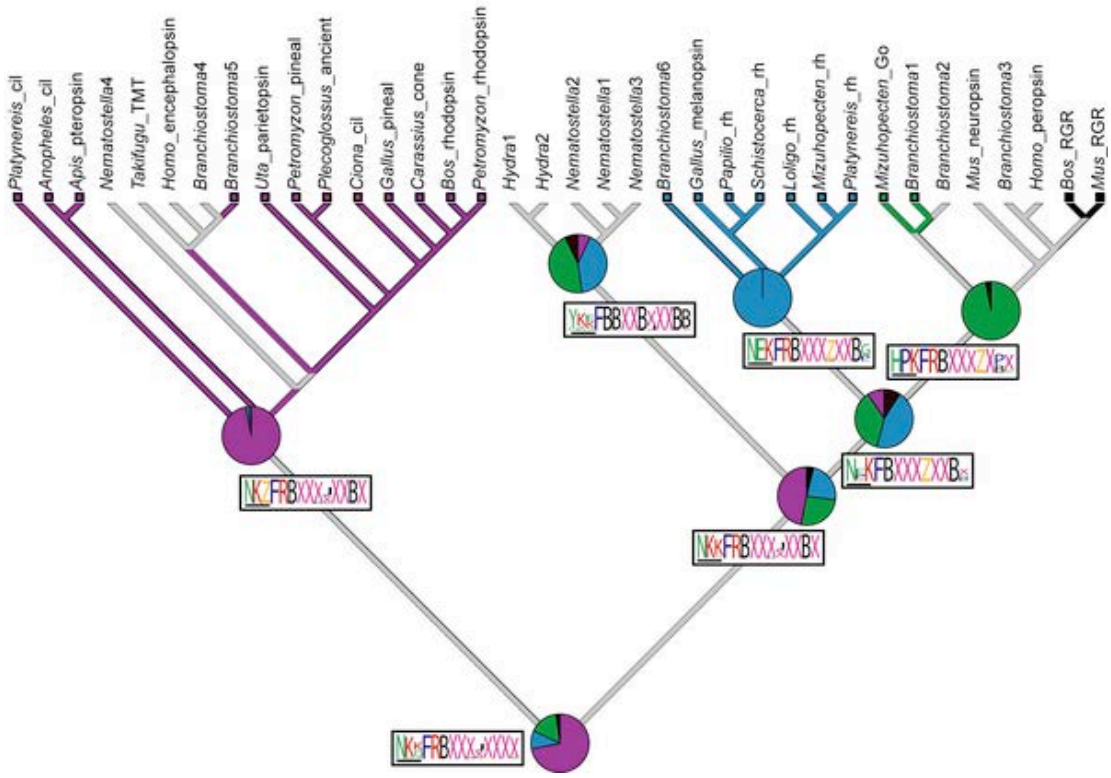

Figure 2.6. Ancestral state reconstruction of G protein-binding interactions for each metazoan opsin-mediated phototransduction cascade obtained by simulated mutational mapping (Bollback 2006) (see methods). For each class of opsin, the P value of the reconstructed ancestral G α interactions is represented in pie graphs. Ancestral G protein interactions in phototransduction cascades mediated by ciliary, rhabdomeric and G_o opsins can be significantly resolved (P>0.95) but the ancestral states of the rhabdomeric+G_o, and cnidops clades are equivocal. ML state reconstructions shown for each node as colored branches. Purple, G_i; Blue, G_q; Green, G_o; Black, no G protein interaction (as is the case for RGR/Retinochrome opsins); Grey, equivocal reconstruction from ML. Reconstructed ancestral amino acid motifs of the 4th cytoplasmic loop region of opsin are shown along branches in logos. Maximum vertical height scales to P =1.0. We obtained clear reconstructed states for most of the residues in a conserved tripeptide motif (residues 310, 311 and 312, horizontal bar) for the ciliary, rhabdomeric and G_o /RGR nodes. For the most part, the remainder of the residues in can be unequivocally reconstructed to the level of Dayhoff classes. B=HRK, X= LIVM, J=GATSP, Z=DENQ.

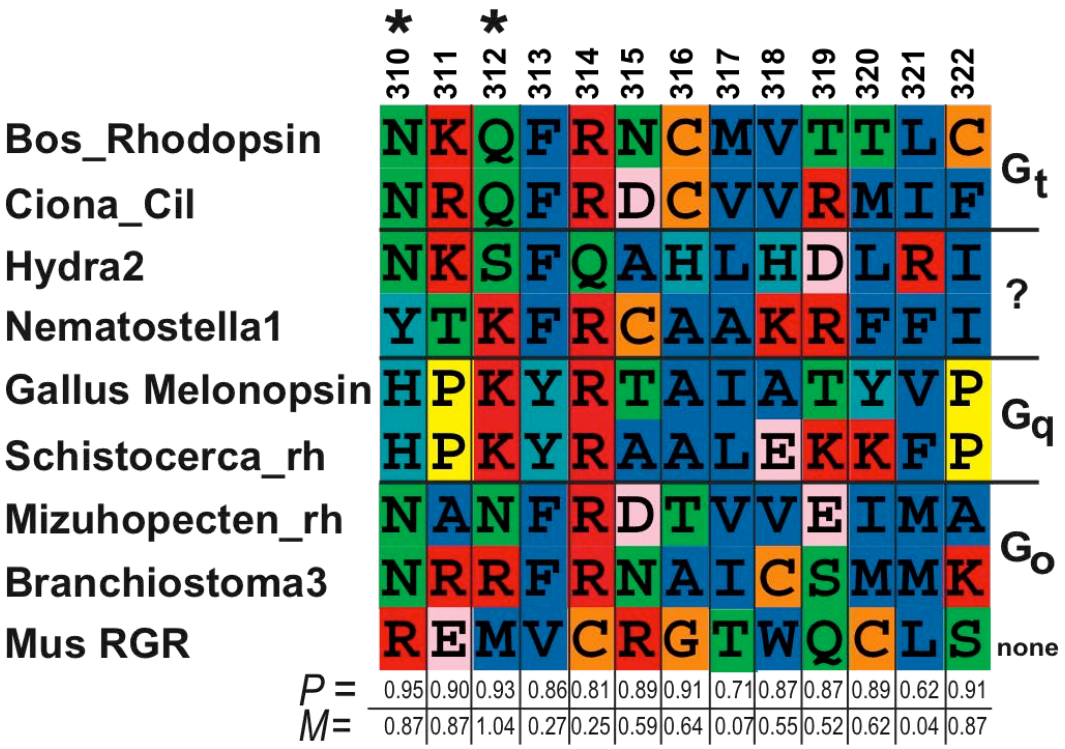

Figure 2.7. Summary of co-variation analyses and tests for selection. Previously described G protein binding states, together with amino acid sequences of the 4th cytoplasmic loop regions from our opsin dataset, were used to test the hypothesis that the two character sets co-vary across our opsin phylogeny. Co-variation was assessed using mutual information content (MIC). Shown here is a representation of these results. Predictive P-values (Bottom axis) based on MIC for co-variation between each residue in the 4th cytoplasimic loop and their respective G protein interactions (Right axis) are shown. These analyses were conducted using Bayesian mutational simulation mapping in SIMMAP (Bollback 2006). We also tested possibility that these residues had evolved under a selective regime using the criterion of site specific rate heterogeneity as implemented in DIVERGE2 (Zheng et al. 2007). The two highest scoring residues from co-variation analyses (310 and 312) also retain the signature of selection (asterisk). See table S1 for citations. *P*= predictive P-value, *M*= the M statistic given by MIC.

Table 1. Likelihood comparisons for alternative hypotheses of opsin evolution

Tree	-ln[*L*]	Δ-ln[*L*]	+- SE –ln[*L*]	AU	pKH	pSH	pRELL	Reconciled Trees	
								Dup	Loss
A1	-15637.857	0.000	0.000	0.660	-1.000	1.000	0.581	-	-
A2	-15642.642	-1.842	7.349	0.420	0.401	0.467	0.395	-	-
A3	-15642.642	-4.785	4.032	0.076	0.118	0.301	0.024	-	-
B1	-16190.354	-0.553	1.696	0.518	0.372	0.854	0.131	0	0
B2	-16195.124	-5.324	4.625	0.038	0.125	0.345	0.002	0	$1^{1, 2, 3}$
B3	-16192.821	-3.020	5.982	0.302	0.307	0.600	0.065	0	$1^{1, 2, 3}$
B4	-16196.016	-6.215	4.921	0.045	0.103	0.274	0.001	0	$1^{1, 3}$
B5	-16193.309	-3.508	6.185	0.150	0.285	0.552	0.013	3	$2^{1, 3}$
B6	-16193.576	-3.775	3.272	0.070	0.124	0.540	0.003	0	1^{1}
B7	-16192.200	-2.399	6.672	0.302	0.360	0.657	0.057	0	$3^{1, 2, 3}$
B8	-16192.081	-2.280	4.176	0.352	0.293	0.708	0.084	1	$3^{1, 2, 3}$
B9	-16194.972	-5.172	5.481	0.048	0.173	0.364	0.002	2	1^{1}
B10	-16189.801	0.000	0.000	0.643	1.000	1.000	0.275	1	2^{2}
B11	-16193.990	-4.189	3.832	0.081	0.137	0.489	0.008	0	1^{2}
B12	-16190.299	-0.498	5.686	0.621	0.465	0.857	0.247	1	$3^{1, 2, 3}$
B13	-16190.591	-0.791	1.387	0.311	0.284	0.837	0.032	0	1^{2}
B14	-16191.817	-2.016	6.722	0.464	0.382	0.709	0.078	0	$3^{1, 2, 3}$
B15	-16196.016	-6.215	4.921	0.045	0.103	0.274	0.001	0	1^{2}

Likelihood comparison tests for trees in figure 2.3. Results were calculated under the WAG+I+G model. *L*, likelihood; AU, Approximately Unbiased Test (Shimodaira); KH, Kishino-Hasegawa test (Kishino and Hasegawa 1989); SH, Shimodaria-Hasegawa test (Shimodaira and Hasegawa 2001); pRELL, resampled log-likelihood bootstrap percentage (Yang and Rannala 1997) .

[1] Requires Cnidarians Lost RGR/Go Clade
[2] Requires Cnidarians lost Rhabdomeric
[3] Requires Bilaterians lost cnidops

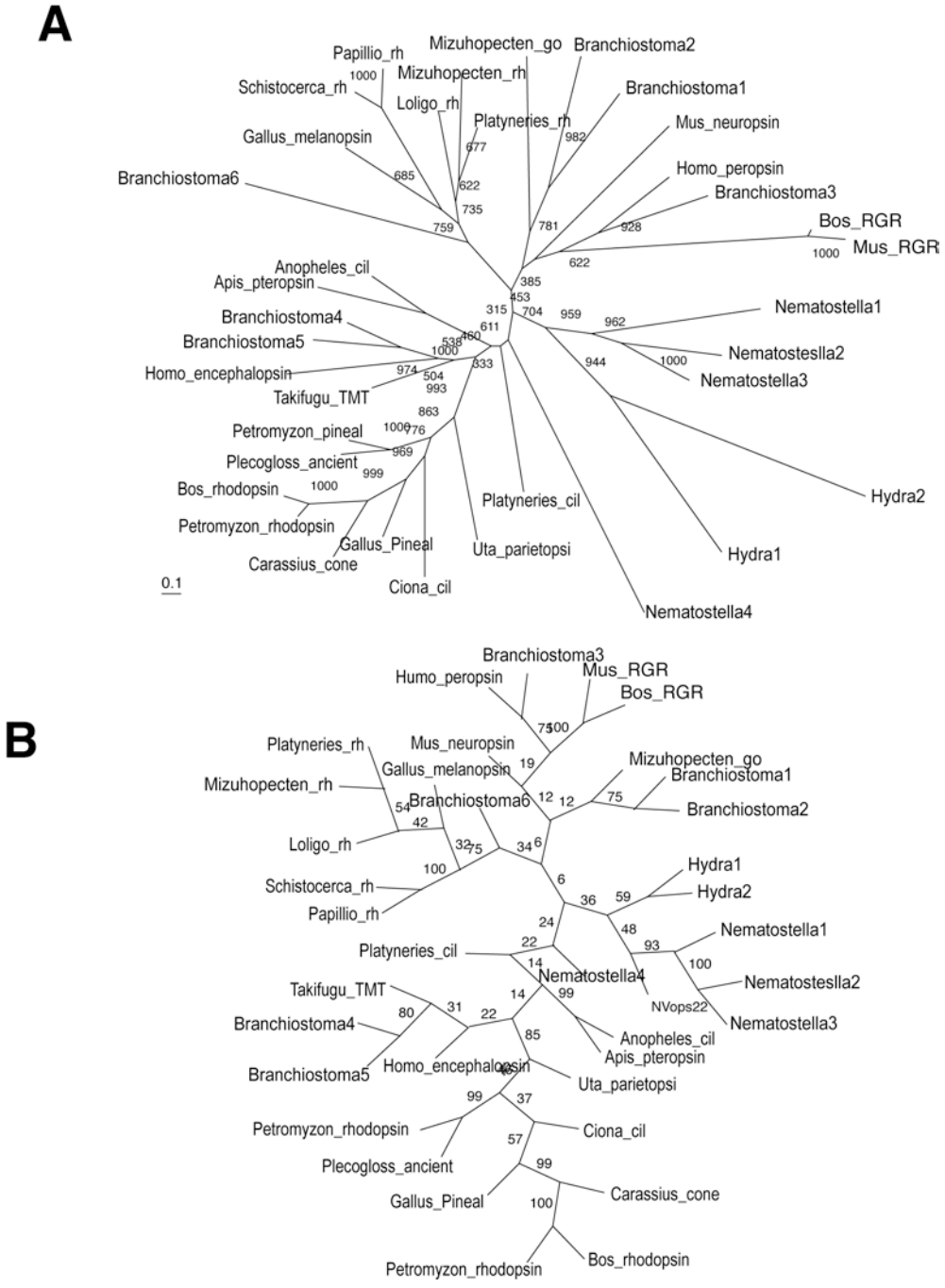

Figure S2.1. Phylogenetic analyses of opsins without an outgroup **A**. ML analyses were conducted assuming the WAG +I+G+F model. **B**. Unweighted MP. Node numbers represent bootstrap proportions out of 1000 replicates.

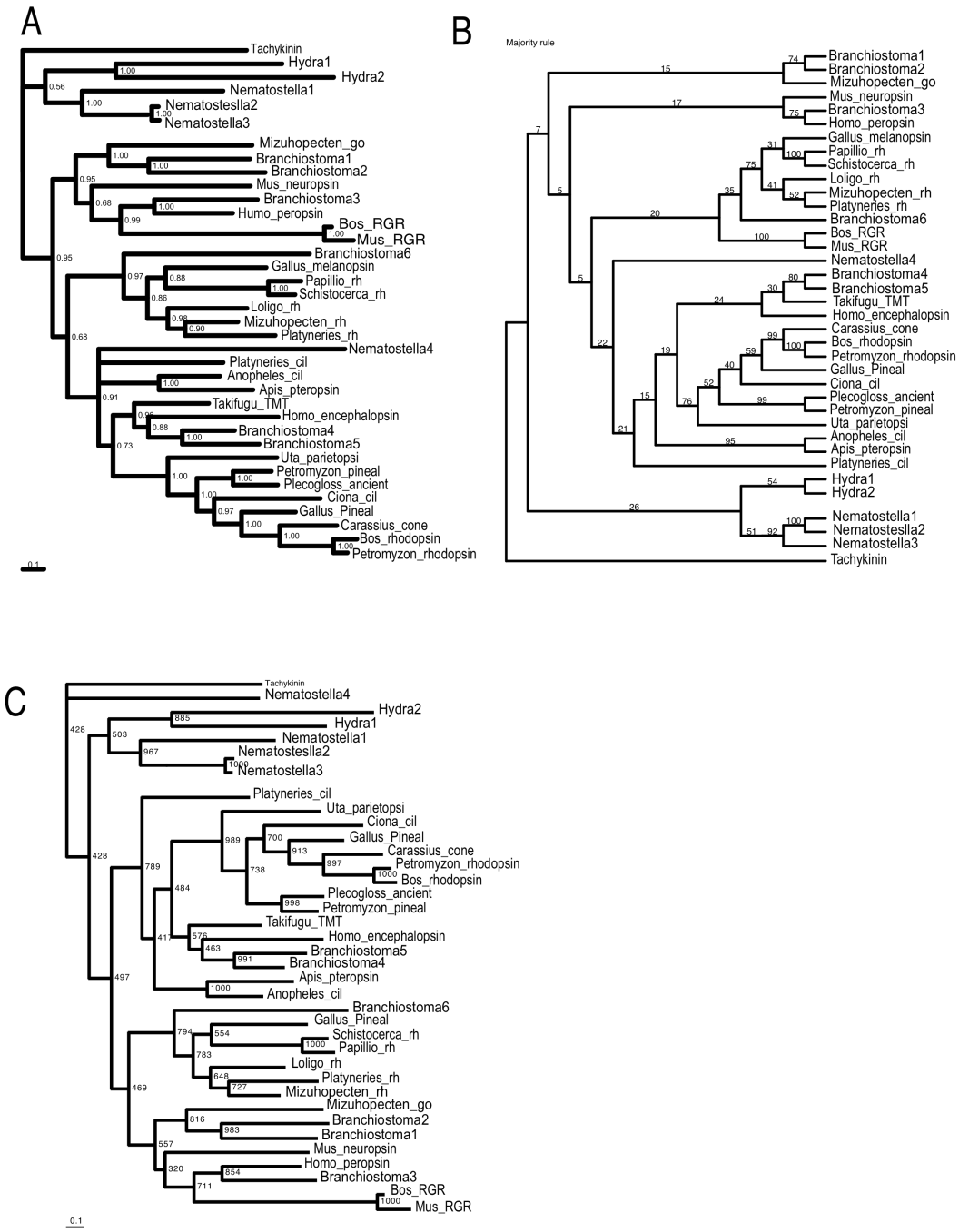

Figure S2.2. Results of analyses when including non-opsin outgroup. **A**. BMCMC, **B**. MP, and **C**. ML. BMCMC and ML analyses were conducted under the WAG+I+G+F model. Similar analyses with other outgroups produced qualitatively similar results (not shown).

Chapter 3

Key transitions during the evolution of animal phototransduction: Novelty, "tree-thinking", co-option and co-duplication

Plachetzki, D. C., and T. H. Oakley. 2007. Key transitions during animal phototransduction evolution: Novelty, tree thinking, co-option and co-duplication. *Integrative and Comparative Biology*, doi:10.1093/icb/icm050

Abstract

Biologists are amazed by the intricacy and complexity of biological interactions between molecules, cells, organisms, and ecosystems. Yet underlying all this biodiversity is a universal common ancestry. How does evolution proceed from common starting points to generate the riotous biodiversity we see today? This "novelty problem" - understanding how novelty and common ancestry relate - has become of critical importance, especially since the realization that genes and developmental processes are often conserved across vast phylogenetic distances. In particular, two processes have emerged as the primary generators of diversity in organismal form: duplication plus divergence and co-option. In this paper, we first illustrate how phylogenetic methodology and "tree-thinking" can be used to distinguish duplication plus divergence from co-option. Second, we review two case studies in photoreceptor evolution – one suggesting a role for duplication plus divergence, the other exemplifying how co-option can shape evolutionary change. Finally, we discuss how our tree-thinking approach differs from other treatments of the origin of novelty that utilized a "linear-thinking" approach in which evolution is viewed as a linear and gradual progression, often from simple to complex phenotype, driven by natural selection.

Introduction

"Novelties come from previously unseen association of old material. To create is to recombine. " -Jacob (1977)

"Gene duplication emerged as the major force of evolution." -Ohno (1970)

Two different, but interdependent, processes likely contribute to the majority of novel evolutionary changes and each will leave different signatures that are distinguishable in comparative analyses. Some novel traits have their mutational origins in duplication, subsequently followed by differential divergence. Gene duplications are perhaps the best-studied duplication events, but "duplication" (either by copying or by fission) happens at other levels of biological organization, including protein domains, groups of interacting proteins, chromosomes, genomes, cells, organs, castes, species, and ecosystems. Other novel traits originate as new combinations of existing traits, a process sometimes termed bricolage, or tinkering (Jacob 1977). As with duplication, recombinational novelties occur at multiple levels of biological organization: domains fuse to form new proteins, proteins gain new interactions with other proteins, cells/species merge as in the endosymbiotic origin of eukaryotes, and new ecological interactions emerge by dispersal of species. Recognizing and distinguishing duplication and co-option requires a historical perspective.

This historical or "tree-thinking" perspective (Ohara 1997), which focuses on duplication and co-option, has not always been the dominant mode of thinking about the origin of novelties. Many evolutionists have instead suggested linear histories for the origin of complex traits, whereby the evolution of traits is viewed as a linear and gradual progression, often from simple to complex phenotype, and driven by natural selection. For example, Salvini-Plawen and Mayr constructed linear histories of eye evolution, which they termed "morphological sequences of differentiation" by collecting examples of eyes of differing complexity from closely related species (Figure 3.1a). Additionally, Nilsson and Pelger constructed a linear conceptual model for the gradual origin of lens eyes (Figure 3.1b). These linear histories are important for understanding how, and how quickly, natural selection might incrementally mold complexity; yet at the same time linear thinking discourages questions about the origins of novelty. Under linear models, all that is needed to proceed in a gradual and linear fashion from simple to complex traits is nondescript heritable variation and selection. As such, heritable variation – which is abundant in nature – is taken for granted and the focus is placed almost exclusively on selection. When considering linear, gradual models, little attention is therefore paid to how variations originate or to whether certain types of variation (e.g. duplication or co-option) are more common fuel for evolution. In contrast, the possibility of identifying the different types of variation that have been involved in the origins of novelties is precisely what a historical, tree-thinking perspective can add.

Duplication/divergence and co-option in the origin of novelties

Certainly, many novelties originate by duplication and subsequent differential divergence. In fact, duplication-and-divergence has become widely accepted as the primary means of origin of new genes (Zhang et al. 2003; Taylor and Raes 2004). The mutational mechanisms causing duplication are well understood, and include retrotransposition, segmental and genome duplications (Hurles et al. 2004). The original mutational events, however, are only part of the story, the other part being the subsequent differential divergence of the newly arisen gene copies. Divergence may involve natural selection on one or both copies, or neutral processes that fix complementary loss of gene functions (Zhang et al. 2003). Besides genes, duplication may occur also at other levels of biological organization. For example, co-expressed gene networks can be duplicated (Conant and Wolfe 2006). In this case, the mechanism of duplication may often be whole genome duplications, unless all genes in the network are copied independently or co-expressed genes are genomically adjacent, thereby allowing segmental duplication of the network. In yeast, differential divergence of duplicated co-expression networks has occurred by differential loss of network interactions (Conant and Wolfe 2006). Duplication also occurs in tissues and organs. For example, serial homologs like vertebrae, teeth, segments, limbs, and eyes may be considered duplicates (Arthur et al. 1999; Minelli 2000). The mechanistic origins of duplicated organs are not as well understood as are duplications that occur at the genetic level. One likely mechanism is fission of a

formerly homogeneous field of cells into multiple fields, a developmental mechanism involved in the duplication of arthropod eyes (Friedrich 2006).

In addition to duplication-plus-divergence, evolutionary novelties often originate as new combinations of existing genes, structures, or species. For example, many new genes originate as novel combinations of existing protein domains. As one of many examples, the nervous-system-specific transcription-factor gene *Pax-6* originated as a fusion of a *Paired* domain and a *Homeodomain* (Catmull et al. 1998). The mechanism of domain fusion likely involves the duplication of those domains, thus illustrating one way that duplication and co-option are inter-related. New combinations of existing elements also occur at other levels of biological organization. Gene-interaction networks gain new interactions with existing genes (Olson 2006). In addition, whole gene networks are often co-opted to function in new contexts (Gompel et al. 2005). Mechanistically, this often occurs by changes in gene regulation. In laboratory experiments, changes in regulation of transcription factor genes that lead to new sites of expression can cause ectopic production of organs like eyes (Halder et al. 1995). Ectopic expression of structures during evolution has been termed heterotopy (Haeckel 1866; West-Eberhard 2003). Interestingly, a co-option event at the level of the developmental regulatory network that leads to ectopic organ expression can be considered a mechanism for the duplication of organs, illustrating another relationship between co-option and duplication. Namely, co-option at one level may be viewed as duplication at another level of biological organization. Finally, co-option is a process that may act at the species level. Fusions of existing

species may have led to novel species, for example by endosymbiosis (Thompson 1987; Margulis and Sagan 2002; Kutschera and Niklas 2005).

Although analogous processes of duplication and co-option generalize across numerous levels of biological organization, we focus our attention here on protein-interaction networks. In protein networks - like other biological levels - duplication-divergence and co-option lead to different patterns that can be detected by phylogenetic analyses (Abouheif 1999; Geeta 2003; Serb and Oakley 2005). Despite the focus here on networks, the general approach can be easily translated to other levels of biological organization. Before examining the evolutionary origins of specific phototransduction networks below, we will outline the phylogenetic patterns in protein networks expected to result from origins of novelty by duplication and co-option.

Phylogenetic patterns following duplication and co-option

If two protein networks originated by duplication of an ancestral network, then multiple components of the descendent networks will show patterns of co-duplication. In other words, simultaneous duplication of multiple genes of an ancestral network is a pattern consistent with the origin of the descendent networks by duplication (Figure 3.2a). In contrast, the components of a novel protein network that evolved by co-option will have originated by gene duplications that occurred at different times (Figure 3.2b). Co-duplication and co-option need not be considered discrete alternatives. Instead, some genes of a network may co-duplicate, whereas

others may be co-opted, an intermediate situation that will often be true, especially when examining the evolution of particular networks at multiple time scales, and when examining networks at increasing spatial scales by increasing the number of interactions considered.

Differentiating co-duplication from co-option requires determining the relative timing of duplications for the genes involved in the network. Co-duplication may be viewed as a null hypothesis, which can be rejected by different hypothesis-testing procedures, in favor of the alternative hypothesis of co-option. A primary means of testing gene co-duplication is by reconciled tree analysis (RTA), the comparison of gene phylogenies to a species phylogeny (Goodman et al. 1979; Page and Charleston 1998). RTA determines a phylogenetic interval for gene duplication events, with upper bounds at one speciation event and lower bounds at another speciation event. For example, if two sister species possess duplicate genes and an outgroup species possesses only one such gene, we might infer that a gene duplication occurred prior to the origin of the sister species, but after their divergence with the outgroup. Algorithmic approaches are available for more complicated situations (Page and Charleston 1998; Page 2002). If duplications giving rise to the origins of genes in a protein network map to different nodes on a species phylogeny, the null hypothesis of co-duplication is rejected in favor of network assembly by recombination of protein interactions (co-option). Another means of testing a co-duplication hypothesis is by explicitly estimating divergence times of the nodes leading to the network genes, for example by relaxed molecular clock analysis

(Britton et al. 2002; Sanderson 2002; Thorne and Kishino 2002). Such an analysis may offer an additional test after RTA, especially in situations where RTA maps co-duplication events to a large phylogenetic interval due to lack of sampling or extinction of species. Conceptually analogous statistical tests for co-speciation in host and parasite phylogenies also have been described (Huelsenbeck et al. 2000).

Once the evolutionary patterns of co-duplication and co-option have been established for a protein network, additional questions can be addressed. To what extent do co-duplicated genes respond to natural selection as a unit of interacting proteins rather than as individual genes? Are rates of evolution in co-duplicated genes concordant? Does one descendent network evolve at a different rate than the other? Are duplicated networks sub-functionalized (Conant and Wolfe 2006)? Can we determine the cis-regulatory changes responsible for co-option events (Gompel et al. 2005)? Do rates of evolution change after co-option? Does selection act to allow co-opted genes to specialize to their new roles? Next, we propose that metazoan phototransduction will provide a valuable model for investigating co-duplication, co-option, and the evolutionary outcomes of these novelty-generating mechanisms.

Tree-thinking and the evolution of animal phototransduction cascades

Multiple different signaling pathways enable much of life to respond to, and interact with, the light environment (Spudich et al. 2000). The dominant signaling pathways found in animals are phototransduction cascades initiated by opsin, a member of the G-protein-coupled-receptor (GPCR) class of proteins. Variations of

opsin pathways mediate a wide range of acuity for sensing light, from simple photosensitivity to vision (Land and Nilsson 2002). In this section we explore the evolution and diversification of the animal phototransduction cascade with the purpose of highlighting instances where duplication and co-option may have contributed to key transitions in the evolution of these pathways. Namely, we will focus on two examples. First, we review evidence that the different phototransduction pathways of vertebrate rods and cones originated primarily by duplication. Second, we suggest that the evolutionary origins of two primary transduction pathways of animals (ciliary and rhabdomeric) involved extensive co-option.

Duplication and the origin of vertebrate rod and cone phototransduction cascades

The dual presence of rods and cones allow vertebrate retinas to overcome an inherent trade-off between acuity and sensitivity: whereas rods are specialized for sensitivity in dim light (scotopic vision), cones are specialized for acuity in bright light (photopic vision). This duplicity theory (Schultze 1866) was first supported by the observation of two morphological classes of vertebrate photoreceptor cells and is now widely accepted and is supported by additional physiological and molecular data. How did the novelty of dual cell types originate in the evolution of the vertebrate retina? In this section, we will first discuss a hypothesis framed in linear thinking. Next, we will take a tree-thinking perspective and argue that rod and cone phototransduction pathways originated largely by duplication. Establishing

duplication of pathways requires two things. First, the elements of the pathways must differ. Second, the origin of the elements of the pathway must be coincident in time. Based on available data, both hold true for rod and cone transduction pathways.

One perspective on the origin of rods and cones is that a subset of one cell type gradually transformed into another cell type. In a timeless classic, *The Vertebrate Eye and its Adaptive Radiation*, Gordon Walls invoked one such linear model of transformation from primitive to derived stating "They [rods] were derived quite simply from cones by the enlargement of the outer segment and by an increase in the number of visual cells connected to each nerve cell" (Walls 1942). That vertebrate cones are more ancient than rods is in fact supported by current knowledge that rod opsins are derived within paraphyletic cone opsin clades (Okano et al. 1992). However, Walls' astute ascertainment of ancestry, entrenched in the linear mode of thinking, tells us little about the types of evolutionary processes or the types of variation that could have given rise to the novelty of vertebrates' rod cells. In fact, if rods and cones evolved by some means other than by gradual transformation, the linear framework would obscure this phenomenon.

Another perspective is that rods and cones represent evolutionary duplicates or paralogs: just as genes duplicate within lineages, so too may cell types duplicate. Such hypotheses of duplication are supported by the similarity of components. The similarity of amino-acid components of proteins supports duplication of the proteins. In an analogous way, the similarity of expressed protein components of cell types may support duplication of the cell types (Arendt 2003). Although the entire

repertoire of expressed proteins in rods and cones is not yet known, we can begin to address a duplication hypothesis for the cell types by investigating their phototransduction pathways. Rods and cones use similar, but different, phototransduction pathways, which may have originated by duplication.

More specifically, rods express one set of paralogous cell-type-specific phototransduction proteins and cone cells express another (Hisatomi and Tokunaga 2002; Nordström et al. 2004). Rod-specific and cone-specific paralogs include those of opsin, the G protein α subunit (Gα), phosphodiesterase (PDE), cyclic-nucleotide-gated ion channels (CNGs) and arrestin (Hisatomi and Tokunaga 2002; Nordström et al. 2004). From a tree-thinking perspective, we can ask if these data indicate duplication or co-option in the origin of rod and cone phototransduction. If the rod phototransduction pathway is a duplicate of the cone pathway, then most components of those pathways should be co-duplicated. In contrast, if one pathway originated largely by co-option, then the components should have originated by duplication at different times. The co-duplication of phototransduction pathway components can be treated as a null hypothesis that could be rejected in favor of co-option.

For the phototransduction pathways of rods and cones, the null hypothesis of co-duplication currently cannot be rejected. Paralogs for each rod-specific and cone-specific gene originated through large-scale segmental duplication of the genome (Nordström et al. 2004). RTA indicates that each of these duplications pre-dates the origin of gnathostomes (jawed vertebrates) and post-dates the split of cephalochordates/urochordates and vertebrates (Nordstrom and others 2004; Figure

3.4a). As such, the duplication of multiple genes in these pathways maps to the same phylogenetic interval and is consistent with co-duplication. This phylogenetic interval, however, is large and encompasses branches leading to two extant groups – agnathans [assumed monophyletic (Takezaki et al. 2003)] and chondrichthyans – that could be used to further refine the timing of the gene duplications. If agnathans and chondrichthyans both possess rod and cone paralogs of multiple phototransduction genes, then the phylogenetic interval – thus inferred to be pre-vertebrate - for these duplications would be reduced, providing a more stringent test of the co-duplication null hypothesis.

Unfortunately, data for phototransduction genes are largely absent from early branching vertebrates. Nevertheless, there is some evidence that differentiated rod and cone phototransduction cascades were present at the origin of vertebrates. Namely, multiple opsin genes are known from agnathans. The most in-depth phylogenetic treatment of the problem of the evolution of basal vertebrate opsin concluded that rod-class and cone-class opsins are present in agnathans (jawless vertebrates), and that rod-opsins branch from within paraphyletic cone-opsin clades (Collin 2006; Pisani et al. 2006). From this we can conclude that the duplication of the gene that gave rise to the rod-specific opsin class took place prior to the last common ancestor of living vertebrates. One is tempted to argue that this finding alone necessitates that the origin of rod and cone pathways pre-date living vertebrates. However we still lack data on the rod- and cone-class paralogy assignments for the other phototransduction proteins in agnathans. It remains

possible that rod opsin duplicated earlier than the rest of the rod-specific phototransduction genes. For example, in early branching vertebrates, rod opsins and cone opsins could interact with the same genes in the rest of the pathway, which duplicated later. In addition, there is the potential for rod- and cone-specific genes to have duplicated coincidentally, perhaps by genome duplication, within the same phylogenetic interval of rod and cone cell type origins, which were later entrained to their respective cell type-specific pathways. However, this possibility would require a complicated assortment of reciprocal loss of gene expression events. We do not advocate either of these alternative hypotheses for the evolution of vertebrate rods and cones; rather we favor the co-duplication hypothesis. Nevertheless, we recognize that co-duplication can be subjected to additional tests.

The realization that rod and cone phototransduction pathways represent evolutionary duplicates suggests two areas for future work. First, the mode of differentiation of the duplicate pathways may be examined. Was natural selection involved in the differentiation? To what extent does selection act on the entire biochemical pathway as compared to the individual genes? At least one interesting study has been conducted that bears on these questions. Carleton and coworkers (2005) examined opsin genes and found that there are no amino acids that uniquely define the rod-opsin clade. This result indicates that the origin of the rod pathway probably was not accompanied by a change in the biochemical function of opsin. As such, if selection acted to differentiate all genes in the rod pathway, including opsin, it must have involved changes in rod-opsin expression and not its biochemical

interaction with other proteins. Another possibility is that selection acted on other components of the pathway, but was not involved in the maintenance of the duplicated rod opsin.

A second area for future study is to extend our interpretation to the next level of biological organization above the pathway, to the entire cell type. How have the dual processes of co-duplication plus divergence and co-option contributed to the evolution of rod and cone cells? In order to answer this question we must be able to link the cell's morphological phenotype with its physiological phenotype in an evolutionary explanation (Figure 3.4a). In the case of rods and cones, we can begin to make some preliminary connections. It has been shown that the transcription factor Nr2e3 serves as a switch for the terminal differentiation and maintenance of rod cells in the vertebrate retina (Chen et al. 2005; Peng et al. 2005). Comparisons between wild-type and Nr2e3 mutants have revealed that this transcription factor up-regulates rod-specific phototransduction genes in rod-transcriptional contexts and at the same time down-regulates cone-specific genes in cone-specific contexts. In the course of development, vertebrate rod and cone cells - together with other retinal cell types - are derived from a pool of multi-potent progenitor cells (Turner and Cepko 1987; Wetts and Fraser 1988). At some point prior to the evolution of rods and cones, Nr2e3 must have been co-opted to serve a role in either the inhibition or activation of each phototransduction cascade.

Co-option and the major animal phototransduction networks

Animals possess two major classes of photoreceptor cells, ciliary and rhabdomeric. Like rods and cones (which are both ciliary photoreceptor cells), the two major animal classes were first distinguished on the basis of physiological and morphological differences (Eakin 1963). The presence of these major classes has been further supported by molecular data (Arendt 2003). In this section, we first briefly discuss a linear perspective on the origins of the major photoreceptor cell types. We next take a tree-thinking perspective on the origins of ciliary and rhabdomeric cells, and address the null hypothesis of duplication. As discussed below, unlike the origins of rods and cones, co-duplication can be clearly rejected in favor of co-option in the origins of ciliary and rhabdomeric cells.

One perspective on the origins of ciliary and rhabdomeric cells is a gradual linear model. Salvini-Plawen and Mayr (1977) in a landmark paper, include a steady infusion of linear explanations throughout their analysis. Based on morphological data available at the time (see references therein and also Eakin 1979 for a response), these authors provided a scheme of linear modifications that are possible in ciliary and rhabdomeric photoreceptor cells in the course of evolution. Their scheme for ciliary photoreceptors portrays 14 different morphologies from disparate metazoan phyla, each derived by transition from a common prototypical ancestor. Their explanation for diversity among ciliary photoreceptors is displayed as a carousel of varying photoreceptor morphologies revolving around a central ancestral state, each separated by a single linear transition (figure 9, Salvini-Plawen and Mayr 1977).

Tree-thinking provides another perspective on animal photoreceptor origins. Instead of treating photoreceptor cell type as a single morphological unit and providing descriptive accounts of possible modes of transitions between types, we can examine separately the individual components of the cells, and quantify the timing of their origins. Although the entire suite of expressed proteins in photocells is unknown, especially in non-model organisms, we can investigate the phototransduction pathways of different cell types as we have done above for rods and cones. A null hypothesis of duplication for the origin of ciliary and rhabdomeric cells has been described previously (Arendt 2001; Plachetzki et al. 2005). As we will next describe, ciliary and rhabdomeric cell origins reject this null hypothesis of duplication in favor of co-option, providing a counter-example to duplicated rods and cones.

Many similarities exist in the signaling networks of phototransduction in all animals studied to date. In bilaterian animals, three alternative phototransduction cascades are known – two of these cascades correspond to the ciliary and rhabdomeric morphological cell classes (Arendt 2001). All three of these general pathways are based on canonical GPCR signaling, yet they differ in important ways, including the sub-class of the opsin protein that initiates each cascade. In one class, photoreceptors with ciliary morphology (such as rods and cones in the example above) generally utilize ciliary opsins ($G_t\alpha$ and PDE) in cell signaling. Ciliary phototransduction leads to the closing of CNGs, the reduction of cation concentration and the hyperpolarization of the cell. Second, the rhabdomeric phototransduction

cascade (such as that present in an insect's ommatidia) is also initiated by a class-specific opsin paralog, but it utilizes $G_q\alpha$ and phospholipase C (PLC) in cell signaling. Upon activation of the rhabdomeric phototransduction cascade, transient-receptor-potential (TRP) ion channels open allowing the influx of cations and the depolarization of the cell.

An additional class of phototransduction in bilaterian animals has been proposed, based on the presence of a third clade of opsin proteins (known from mollusks, cephalochordates and vertebrates) that utilize $G_o\alpha$ in cell signaling. This phototransduction pathway has been studied in the dual-retina mantle eye of the scallop *Patinopecten* (Kojima et al. 1997; Gomez and Nasi 2000), the parietal eye of the reptile *Uta* (Su et al. 2006) and from the cephalochordate *Branchiostoma* (Koyanagi et al. 2002). These few investigations have left many unanswered questions. For instance, similar to the ciliary pathway, the G_o-mediated pathway in both the vertebrate parietal eye and the scallop retina manifest their physiological signal through CNG ion channels. However, unlike existing data from ciliary and rhabdomeric phototransduction cascades, it would appear that G_o phototransduction displays some evolutionary plasticity in the physiological outcome it specifies. As a first example, in the vertebrate parietal eye, G_o-signaling leads to a depolarization of the cell but in the scallop this pathway causes hyperpolarization (Su et al. 2006). As another example of G_o plasticity during evolution, some members of the G_o class of opsins appear to have lost their role in phototransduction altogether and instead play

important enzymatic functions as photoisomerases (Shen et al. 1994; Chen et al. 2001), enzymes that regenerate the light-reactive chromophore that binds with opsin.

If ciliary, rhabdomeric, and G_o phototransduction cascades originated by duplication, one would expect to find a simultaneous origin by gene duplication of the four major components: opsin, G-protein, PLC/PDE, and ion channel. Testing this co-duplication null hypothesis requires dating the origin of each component separately.

To begin testing the co-duplication hypothesis, we have recently estimated dates for the origin of opsin clades using RTA (Plachetzki and others, in review). Opsins of the three major clades (ciliary, rhabdomeric, G_o) were already known to predate bilaterians (Arendt 2003; Terakita 2005). However, no analysis had included opsins from early-branching animals, thus precluding a specific upper bound for the origins of opsin. Our study reported opsins discovered by screening trace genome-sequence data from the cnidarians *Hydra magnipapillata* and *Nematostella vectensis* and the poriferan *Amphimedon queenslandica* (Plachetzki and others, in review), allowing us to date the origins of the three opsin clades. This study had three major results. First, we identified a new class of opsin genes present only in cnidarians. Second, we uncovered ciliary opsins from cnidarians. Third, although our search did uncover opsin-like GPCR's, we were unable to find any true opsins in the genome of the demosponge *Amphimedon queenslandica*. Based on the best-supported phylogeny, using RTA allowed us to conclude that the ciliary class of opsin genes has an ancient origin in the Eumetazoan ancestor of cnidarians + bilaterian animals, while

the rhabdomeric and G_o sub-families are bilaterian innovations with a single sister group of opsins in cndiarians. Based on this timing of the origins of major opsin clades, we will be able to test for co-duplication of opsin and the other components of the signaling cascade.

If G proteins co-duplicated with opsins at the origin of the three animal phototransduction cascades, they should map by RTA to the same phylogenetic interval as the opsins. As we shall discuss, the co-duplication of G-proteins and opsins can be rejected. Assuming co-duplication, the opsin results above allow a specific prediction for the timing of origin of the other transduction components of the animal phototransduction cascade. Under co-duplication, $G_o\alpha$ and $G_q\alpha$ (the G_o and rhabdomeric G-proteins) would be the result of a bilaterian-specific duplication and $G_t\alpha$ (the ciliary G-protein) would date from a duplication preceding the Eumetazoan ancestor. In contrast to this prediction, the individual G-protein classes are known to pre-date animals, thus rejecting the co-duplication hypothesis. A previous survey (Suga et al. 1999) identified the full complement of $G\alpha$ paralogs known from animal phototransduction from the demospongae, indicating pre-animal origins. Therefore, existing $G\alpha$ proteins gained new interactions with opsin proteins during animal evolution. Co-option, not co-duplication was responsible for the origins of the interactions between opsins and G-proteins in the first ciliary, rhabdomeric and G_o pathways (Figure 3.4b).

A similar approach can be taken to test the null hypothesis of co-duplication between opsins and PLC/PDE and similarly between opsins and TRP/CNG ion

channels. In these cases, co-option is evident immediately as the ciliary cascade uses PDE and the rhabdomeric cascade uses PLC, two non-homologous genes. Co-option is also evident in the origin of the rhabdomeric phototransduction cascade, the only pathway to use TRP ion channels. Despite the differences in how these signaling networks mediate their physiological effects, CNG ion channels are known to play a role in both ciliary and G_o phototransduction networks (Su et al. 2006). It may be that the presence of CNG ion channels in ciliary and G_o phototransduction indicates the condition of the ancestral phototransduction pathway. CNG ion channels thus represent a conserved component of ciliary and G_o pathways, networks that otherwise changed through an opsin-duplication event.

In testing the null hypothesis of co-duplication as a mutational mechanism for the origin of the animal phototransduction pathways, we are confronted with the attendant challenges of dealing with vast phylogenetic time scales. Issues relating to our ability to reconstruct evolutionary events that occur at deep time intervals remain a general problem for phylogenetics. As such, it is possible that co-duplication was, in fact, an important factor in the origins of animal phototransduction pathways, but that this signal has been lost in the course of evolution. We advocate the common solution of applying additional data to the problem. Finally, further examination of the phototransduction pathway in early-branching metazoans might provide additional clues to the origins of these pathways.

Summary

Tree-thinking provides a general framework for understanding the types of variation present at the origins of novel traits. Understanding these elements is central if we are to construct more complete evolutionary narratives and a more expansive model for the evolutionary process. In some ways, the linear approach has been an important heuristic for understanding the directionality and rate of evolution, but the linear mode falls short of illuminating processes in evolution that have given rise to novel traits. This is precisely where tree-thinking approaches excel. We have used the evolution of the phototransduction cascade as a model protein-interaction network to explore the utility of tree-thinking in explaining some of the key transitions in the evolution of this pathway. This approach, however, is quite amenable to analyzing other levels of biological organization above the molecular level. Not only can the evolution of elements at a single level be better understood using tree-thinking, but interactions between levels of organization can also be addressed. One important outcome of expanding our understanding of the dual novelty-generating processes of duplication plus divergence and co-option to other levels of biological organization will be to gain insight into how varying levels interact in evolutionary processes (Buss 1987). For instance, can gene duplications provide a selective context for cell type duplications to occur? Can co-option by a given cell type of an alternative signaling or developmental pathway reinforce the evolution of novel tissues, such as vertebrate or molluscan retinas? Much of current evolutionary biology seeks to

address the “novelty problem”. We argue that the tree-thinking framework can better facilitate our understanding of both the patterns and the processes of evolution.

Acknowledgements

We are grateful to Bernd Schierwater and Rob DeSalle for organizing the symposium “Key transitions in animal evolution” at the 2007 SICB meeting. We are also thankful to members of the Oakley lab and two anonymous reviewers for comments on the manuscript. This work is supported by grant DEB0316330 to T.H.O from the NSF .

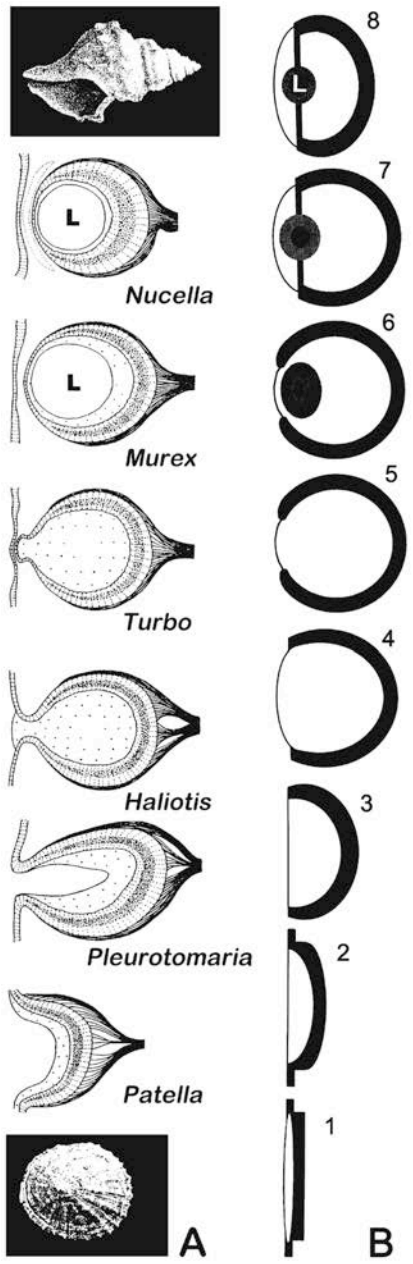

Figure 3.1. Linear models of eye evolution. A. Salvini-Plawen and Mayr (1977) provided several "morphological series of differentiation", such as the gastropod eyes depicted here. They viewed eye evolution as a gradual and linear transformation from simple to complex eyes, as illustrated by these living gastropod eyes. B. (Nilsson and Pelger 1994) constructed a conceptual model of the evolution of lens eyes that allowed them to estimate the time necessary to evolve such an eye. This model assumes a gradual and linear progression from simple to complex eye. Figure taken from (Kutschera and Niklas 2004).

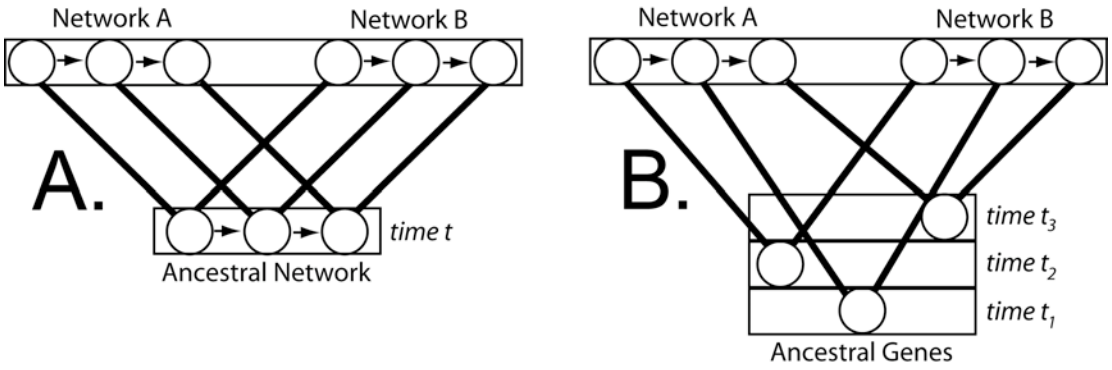

Figure 3.2. Co-duplication and co-option can be understood in terms of the phylogenetic interval within which the interacting elements originate. A. Co-duplication and divergence is characterized by a shared temporal interval of element originations. B. Co-option occurs when interactions between elements is de-coupled from their temporal origins.

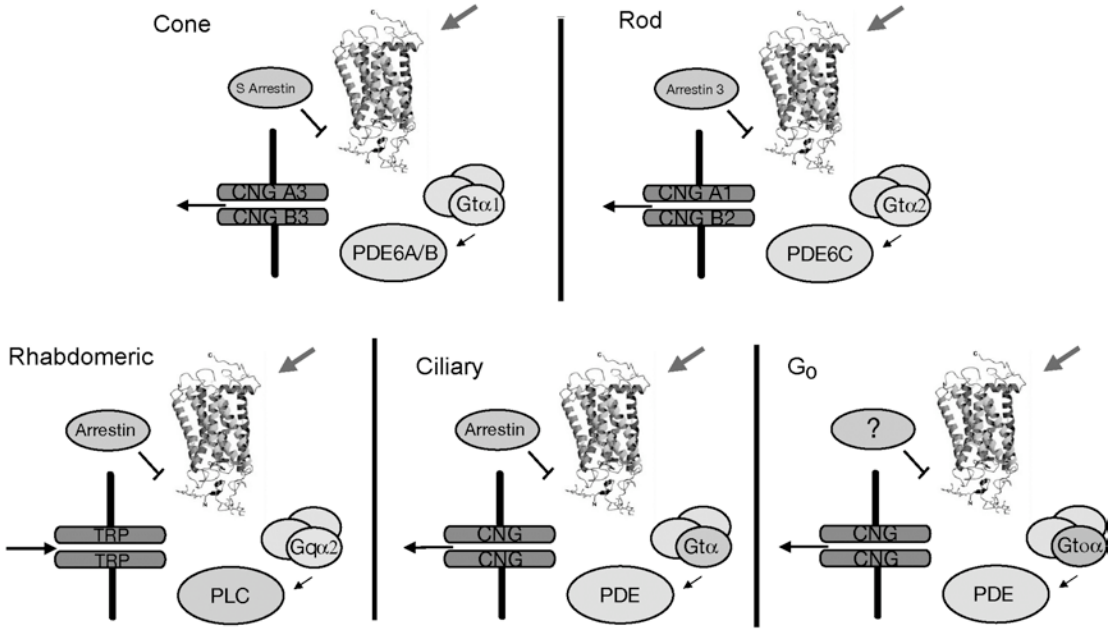

Figure 3.3. Simplified phototransduction cascades of the rod and cone photoreceptors of the vertebrate retina and the more general rhabdomeric, ciliary and G_o signaling pathways found in animals. The rod and cone phototransduction cascades are members of the ciliary class. The phototransduction cascade is initiated by light (arrow) ultimately leading to the efflux (left-pointing arrow) or influx (right-pointing arrow) of K^+ and Na^+ ions. Rod-specific and cone-specific paralogs of $G_t\alpha$, PDE, CNG and arrestin are known to have arisen by segmental genome duplications (Nordström et al. 2004). PDE, phosphodiesterase; $G\alpha$, G α subunit of the G protein; CNG, cyclic-nucleotide-gated ion channel; TRP, transient-receptor-potential ion channel.

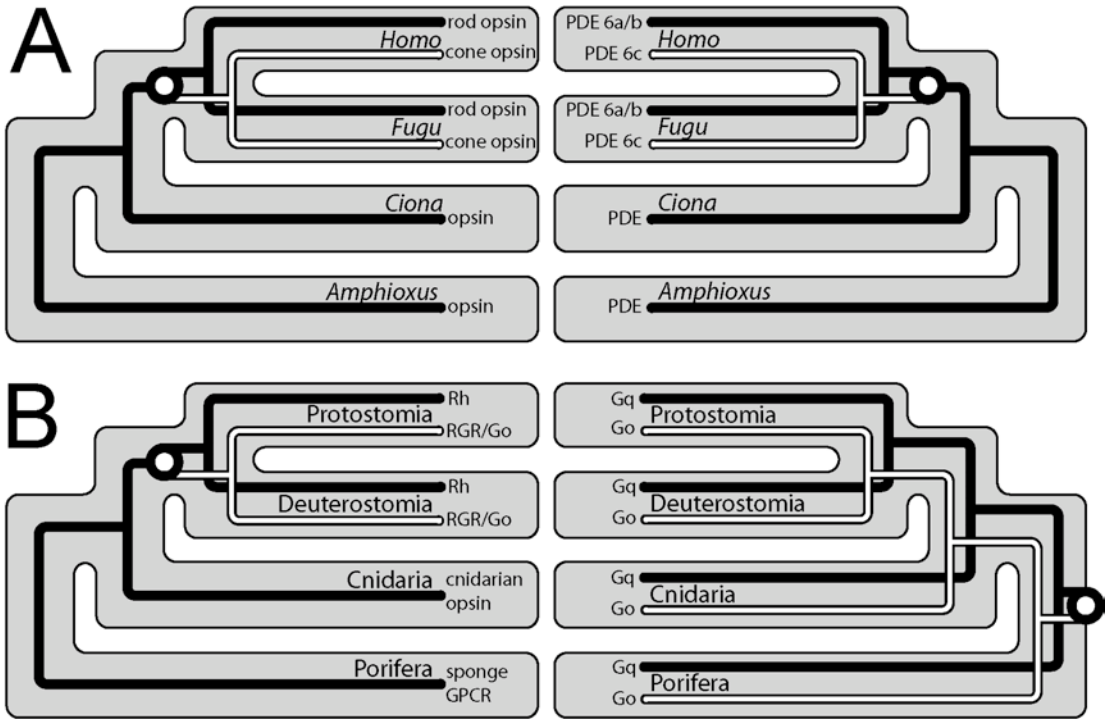

Figure 3.4. The co-duplication and co-option modes of variation can be understood using tree-thinking. A. The evolution of vertebrate rod and cone cells may be an example of co-duplication and divergence. Rod (black) and cone (white) opsins duplicated within the same phylogenetic interval as their PDE-binding partners (PDE6C, white; PDE6AB, black) (Nordstrom et al 2005). B. The origin of the animal phototransduction cascade itself involved co-option. The origin of opsin must have occurred after G-protein α subunits from existing GPCR pathways had diversified (Suga et al. 1999). The G-protein α subunit involved in cnidarian opsin signaling remains unknown. PDE, phosphodiesterase; Rh, rhabdomeric opsin; RGR/G_o, RGR/G_o, opsin; GPCR, G-protein-coupled receptor.

Chapter 4

Evidence that cyclic nucleotide gated ion channels functioned in the ancestral animal phototransduction cascade

Abstract

The evolutionary origins of phototransduction-based light sensitivity have remained obscure since Darwin first proposed the animal sensory systems as a model for the evolution of complexity (Darwin 1859), but can now be revealed using paleogenomic approaches. Phototransduction proteins shared between cnidarian (e.g. hydra, anemones, and box jellies) and bilaterian animals (e.g. humans and flies) were likely present at the origin of opsin-mediated photosensitivity phenotypes in animals. In addition to opsin, bilaterian phototransduction is known to utilize multiple protein components, including specific ion channel proteins that control the functional neurophysiologic response to light. However, until recently components of cnidarian phototransduction were unknown. Here, we show that a cyclic nucleotide gated (CNG) ion channel functions in cnidarian phototransduction. One such CNG protein is specifically co-expressed with opsin in neurons of *Hydra magnipapillata*. Further, cis-diltiazem, a drug that inhibits CNG function, ablates a stereotyped hydra photobehavior. We combine these results with existing data on ion channel complement from other animal photosystems using explicit ancestral state reconstruction statistics to infer that CNG ion channels functioned in the ancestral animal phototransduction cascade. Our results illustrate how pre-existing and novel components are combined during evolution to allow for the origin of novel, integrated and complex phenotypes, such as the phototransduction cascade –the cornerstone of animal vision.

"[We] must not go back to first stock of all animals, but merely to classes where types exist for if so it will be necessary to show how the first eye is formed –how one nerve becomes sensitive to light… is impossible." Darwin, 1837, 21

Introduction

A fundamental question in biology is how complex, integrated traits originate. In animals, opsin-mediated phototransduction cascades are complex signaling pathways that mediate all known visual phenotypes (Terakita 2005) and present a useful model for the study of complex trait evolution (Plachetzki 2007). Two modes of phototransduction have been described in animals: A *ciliary* mode of phototransduction, which utilizes a cyclic-nucleotide second messenger system and involves Cyclic Nucleotide-Gated ion channels (CNGs) (Matulef and Zagotta 2003) and a *rhabdomeric* pathway, in which signal transduction is accomplished through a lipid-based signaling molecule (Leung et al. 2008) and Transient Receptor Potential (TRP) ion channels (Hardie and Raghu 2001; Venkatachalam and Montell 2007). Both ciliary and rhabdomeric pathways were present prior to the origin of bilaterian animals (Arendt et al. 2004; Velarde et al. 2005) and are each utilized in different organismal contexts, forming a pattern that recapitulates the two major divisions of bilaterian animals. Primary vision is mediated by ciliary pathways in deuterostomes and by rhabdomeric pathways in protostomes (Arendt 2003; Plachetzki et al. 2005).

Although well understood on the functional biochemical level, the evolution

of the phototransduction systems remains little understood and has been the subject of both classical puzzlement among evolutionists (Darwin 1859) and cynicism among anti-scientific theorists (Behe 1996). In the opening passage Darwin notes with incredulity in his notebook D (Darwin 1837) that understanding how animal photosensitivity *per se* could have evolved (e.g., how the first light sensitive neuron arose) was "impossible". In our epigraph, Darwin grapples with the problem of complex trait (e.g., eye) origination but argues that his mechanism for trait diversification in evolution (e.g., natural selection) can be understood, even without understanding the process of trait origination itself (e.g., variation) in depth.

A modern approach to this question of complex trait evolution is to analyze individually the components that define a complex structure, to determine which – if any – predate the evolution of the integrated trait in question. The evolutionary history of these genetic components can be revealed by comparative paleogenomics of animals that posses the trait and their closest relatives that lack it. An assessment of which components were incorporated into the integrated trait upon its origination can come by correlating pre-existing components with a view of the trait's reconstructed ancestral composition. Here, we use this strategy to explore the compositional history of CNG and TRP ion channel usage in the evolutionary history of the animal phototransduction cascades. We provide strong evidence that a CNG ion channel functions in cnidarian phototransduction. By analyzing these and previous data under an explicit likelihood framework for trait evolution we infer that the ancestral animal phototransduction pathway was also based on CNG signaling.

Results

Phylogenomic analysis reveals putative CNG ion channels in basal taxa.

In order to understand the deep evolutionary history of the phototransduction pathways, we first examined genome sequences of bilaterian and non-bilaterian animals, and of animal outgroups, for the presence of putative phototransduction ion channels. In all, genome data from 20 animal taxa were analyzed including data from the basal animal lineages the cnidarians *Hydra magnipapillata* and *Nematostella vectensis*, the placozoan *Tricoplax adhaerens*, the poriferan sponge *Amphimedon queenslandica* and an animal outgroup the choanoflagellate *Monosiga bevicolis*. Loci belonging to both the CNG and TRP ion channel gene families were recovered from each animal genome (Supplementary Table 1).

Our phylogentic analyses reveal a diversity of functional classes of both CNG (Figure 4.1) and TRP (Figure 4.2) loci from each of the early branching animal genomes. However, loci homologous to previously described CNG or TRP phototransduction ion channels were recovered from basal taxa only for the CNG class. Ciliary phototransduction in vertebrate rod and cone cells is accomplished using CNG ion channels that function as heterodimers that are comprised of paralogous A and B subunits. We recovered CNG loci from each of the basal animal lineages that fall out in a monophyletic clade with the bilaterian CNG A and B classes. Our analyses suggest that these basal CNG homologs are descendents of an ancestral genetic locus that predated the duplication event that gave rise to bilaterian

A and B CNG subunits. Our phylogenetic analyses also uncovered numerous TRP loci, but none of these are homologous to the TRPC class of ion channel known for its involvement in rhabdomeric phototransduction (Hardie and Raghu 2001). The functional attributes of animal TRP classes are not well understood, but the TRP loci that we recovered from the early branching taxa that are most closely related to phototransductive TRPCs appear fall out with TRP2 ion channels (Venkatachalam and Montell 2007).

Opsin and CNG ion channels are co-expressed in the same cell types.

A single CNG locus from the phototransductive clade, *hmCNG*, was recovered from the genome sequence of the hydra. Due to the absence of TRPC loci from the genomes of this and other basal taxa, we hypothesized that *hmCNG* might play a role in cnidarian phototransduction. We first tested this hypothesis through studies of gene expression. In hydra, *hmCNG* is specifically co-expressed with a previously described (Plachetzki et al. 2007) cnidarian opsin *hmOps2*. Cells expressing both *hmOps2* and *hmCNG* were distributed throughout the animal, but were concentrated in the oral hypostome and in cells associated with the stinging cells of cnidarians known as nematocytes (Figure 4.3).

Behavioral pharmacologic studies support CNG function in cnidarian phototransduction.

A diversity of photosensitivity phenotypes has been described in Cnidarian taxa that range from diurnal migration in pelagic species (Mackie 1999) to phototaxis in benthic taxa (Ewer 1947). Due to its reproducibility in an experimental setting, one previously described photobehavior in hydra –the contraction response- has been the subject of much investigation. Upon stimulation with bright light, dark-adapted hydras display a series of postures that culminate in a tight retraction of the animal into its most condensed state (Passano and McCullough 1962, 1965; Rushforth 1973) (Figure 4.5, see also supplementary movie). We assayed the ability of the CNG inhibitor cis-diltiazem to ablate this crumple response in hydra. Upon stimulation with a bright (3,500 lux) blue light (520 nm) 23 out of 29 hydra subjects displayed the contraction response within 20 minutes. However, in the presence of the CNG inhibitor, this response rarely occurred (seven responses in 35 trials). The difference between our two bright light trials is significant (P=0.004, Fisher's exact test). However, under dim (5 lux) light conditions only eight out of 45 trials resulted in a response under control conditions and similarly, 6 out of 42 animals responded in the presence of the CNG inhibitor. Data resulting from the two dim light trials were not significantly different (P=0.77). In all cases the test animals were responsive to mechanical stimuli before and after behavioral trials were conducted, thus eliminating the possibility that treatment with cis-diltiazem had inhibited non-photo dependent nervous system function. Together with our finding that both *hmCNG* and *hmOps2*

co-localize to the same cell types, these results strongly implicate CNG ion channel function in cnidarian phototransduction.

Studies of character evolution suggest a role for CNG ion channels in the ancestral phototransduction cascade.

Opsin phylogeny provides a framework for understanding the compositional history of the phototransduction cascades. Recently, the deep phylogenetic history of the opsins has been explored using studies in comparative genomics that included data from the earliest branching extant animal lineages. These studies are in agreement that animal opsins originated prior to the ancestor of cnidarians and bilaterians but were not present in the earlier branching animal lineages (e.g., sponges and placozoans), or in animal outgroups (Plachetzki et al. 2007; Kozmik et al. 2008; Suga et al. 2008). These studies have also revealed a new clade of animal opsins from the cnidarian genomes that represents a critical element for unraveling the origins and compositional trait history of the animal phototransduction cascades. In order to assess the ancestral and intervening states of ion channel usage across the diversification of animal phototransduction cascades, we mapped data reported here, together with all existing functional data, onto a phylogeny for animal opsins (Plachetzki et al. 2007) under an explicit statistical model for character evolution. Our results clearly suggest (posterior probability = 0.99) that the ancestral animal phototransduction cascade utilized a CNG ion channel for signaling (Figure 4.4).

Discussion

By integrating data from gene expression, behavior and trait evolution, our results effectively triangulate a first glimpse into the composition and function of the ancestral phototransduction pathway that is based on an explicit statistical treatment. We show that opsin and CNG transcripts are co-expressed in specific cell types in the cnidarian *Hydra magnipapillata*. Studies of hydra photobehavior using the inhibitory drug cis-diltiazem further implicate CNG function in cnidarian phototransduction systems. Finally, character mapping reveals with strong probability that the ancestral animal phototransduction cascade itself utilized CNG ion channels to elicit signaling.

Our phylogenomic approach identified both CNG and TRP loci from each of the basal lineages included in this study including cnidarians, poriferans and placozoans. However loci closely related to phototransductive ion channels were only found for CNGs. Neither CNGs nor TRPs of the phototransductive TRPC class were recovered from the genome sequence of a choanoflagellate, an animal outgroup. Previous studies have indicated that animal opsins originated prior to Eumetazoa, the ancestor of cnidarians and bilaterian animals, but opsin loci were not recovered from earlier animal lineages such as poriferans or placozoans (Plachetzki et al. 2005; Suga et al. 2008). The current data suggest that the CNG ion channel machinery for animal phototransduction was in place prior to the origins of opsin. This finding mirrors earlier findings that G proteins and the enzyme Phosphodiesterase (PDE), both components of animal phototransduction cascades, had diversified prior to metazoa

(Koyanagi et al. 1998; Suga et al. 1999). The origination of animal phototransduction can therefore be seen as a process involving the co-option of previously evolved elements into a new cascade founded by the origination of the opsin lineage of sensory receptors (Plachetzki 2007).

The studies of opsin and CNG gene expression reported here are consistent with an extensive literature demonstrating that each of these loci function in a neuronal context (Terakita 2005; Pifferi et al. 2006). Opsin expression has been described in the nervous systems of numerous cnidarian taxa (Plachetzki et al. 2007; Koyanagi et al. 2008; Kozmik et al. 2008; Suga et al. 2008) but the present study represents the first time ion channel expression has been assayed in this group.

Upon stimulation with bright light, dark-adapted hydra display a stereotypical contraction response. The adaptive explanation for this behavior may relate to the necessity of expelling digested gastric contents at the beginning of the light cycle, as cnidarians lack a through gut (Kass-Simon G 2002). We use this behavior as a probe for investigating the phototransduction system in a cnidarian model. Cis-diltiazam is an L type ion channel blocker that is commonly used to inhibit CNG ion channel function (Stern et al. 1986) and has been deployed in other studies that have dealt with a range of bilaterian taxa including nematodes (Ward et al. 2008) and vertebrates (Peng et al. 2005). While it is possible that cis-diltiazem could inhibit a range of effectors underlying such a complex trait as the contraction response in hydra, for the following reasons, we feel that our data reflect a specific inhibition of phototransduction in the hydra: First, a single CNG locus was recovered from

informatic screens of the hydra genome, thus ameliorating any suggestion that cis-diltiazem was acting on some other non-phototransductive CNG channel. Second, CNG transcripts specifically localize to opsin expressing cells. This result alone does not denote a functional relationship between opsin and CNG, but TRP ion channels of the type used in rhabdomeric phototransduction (e.g., TRPCs) are not present in the cnidarian genomes. Finally, nervous system function in general is not ablated in our drug treatments as subjects retain responsiveness to mechanical stimuli before and after trials. Thus, our experiments faithfully represent a direct inhibition of phototransduction.

Our conclusion from studies of character evolution that the ancestral animal phototransduction pathway utilized CNG ion channels is consistent with recent studies that have implicated other intermediary components of the ciliary mode of phototransduction. Recently, both PDE (Kozmik et al. 2008) and a cyclic nucleotide second messenger (Koyanagi et al. 2008) have been implicated in cnidarian phototransduction.

Phototransduction pathways that utilize CNG ion channels are associated with receptor neuron hyperpolarization (Chen 2005), whereas those involving TRP ion channels lead to the depolarization of the cell (Hardie and Raghu 2001). While it is not possible to test this hypothesis directly, our findings suggest that the ancestral photoreceptive animal neuron hyperpolarized upon stimulation with light and that a switch to a depolarizing electrophysiological mode likely occurred at the origin of the rhabdomeric pathway.

Methods

Data Mining

Publicly available gene models for all but the sponge *Amphimedon queenslandica* were compiled using BlastDB and subjected to tblastn (Altschul et al. 1997) searches using a wide range of bilaterian CNG and TRP loci as bait. For *A. queenslandica* trace genome data were mined (http://www.ncbi.nlm.nih.gov/Traces). Sponge CNG and TRP gene predictions were obtained as previously described (Plachetzki et al. 2007). A combination of BLAST searches and phylogenetic analysis was used to identify ingroup CNG and TRP loci and to establish outgroup loci.

Animal Culture and in situ hybridization

Cultures of *Hydra magnipapillata* were maintained using standard methods (Lenhoff 1982; Plachetzki et al. 2007). A partial transcript for *hmCNG* was cloned by reference to an expressed sequence tag (Genbank accession # DT606755). The expression of *hmOps2* and *hmCNG* was studied using multi channel in situ hybridization. RNA probes were synthesized using DIG (*hmOps2*) and fluorescein (*hmCNG*) (Roche). Hybridization was conducted as described by Grens et al. (Grens et al. 1995) with the following modifications. The final wash was extended to eight 20-minute washes in PBST (PBS + 0.1% TWEEN 20). Post hybridization animals were blocked for 2 hours using the Roche western blocking reagent (5x). Primary antibodies goat α-DIG and donkey α-fluorescein (Roche) were diluted in blocking

reagent 1:1000 and applied overnight at 4° C. Animals were washed eight times for 20-minutes in PBST again blocked again for 2 hours at room temperature. Fluorescent secondary antibodies Alexa Fluor 488-conjugated chicken α-goat (Invitrogen) and Alexa Fluor 546-conjugated goat α-donkey were added in 5x blocking reagent (1:400) overnight at 4° C. Eight final washes for 20-minutes were done prior to data collection using a Fluoview 500 confocal microscope.

Phylogenetic analyses

Amino acid sequences for CNG and TRP datasets were aligned using TCOFFEE (Notredame et al. 2000) under default parameters and alignment manipulations were done using Seaview (Galtier et al. 1996). Phylogenetic analyses were conducted using Maximum Likelihood (ML) as implemented in RaxML (Stamatakis et al. 2005) and Bayesian Markov Chain Monte Carlo (MCMC) approaches using PhyloBayes (http://www.lirmm.fr/mab/article.php3?id_article=206). Support for internal nodes was assessed with 1000 bootstrap replicates for ML and posterior probability for BMCMC analyses. ML analysis assumed the best-fit model WAG+I+G determined using ProtTest (Abascal et al. 2005). BMBMC analyses were conducted under the default CAT approach. Convergence of BMCMC chains was determined to have occurred once the mean likelihood difference between chains fell below 0.01. Ancestral state reconstructions were done in SIMMAP using stochastic mutational mapping (Bollback 2006). Character states were coded as morphological characters with flat priors.

Photobehavior Assay

Individual hydras were tested in 20-mL pyrex dishes in 5-mL of hydra medium (Lenhoff 1982). Subjects were staged for a minimum of 20 minutes in total darkness to allow dark-adaptation and recovery from any transportation shock. After 20 minutes, individual subjects were exposed to blue light of either 5 lux for dim light trials, or 3500 lux for bright light trials. Light of a sharply defined spectrum peaking at 520 nm was emitted from an LED array (SuperBright LEDs). Light intensity as given in lux as measured using as Smart Luxmeter (Aquatic Ecosystems, Inc.). Hydra were tested for the contraction response (Passano and McCullough 1962, 1965) in individual arenas so that only one individual was tested at a time. The time until first complete contraction was recorded and experiments were terminated after 20 minutes if the animal did not contract. For pharmacologic treatments, the cis-diltiazem was used at a concentration of 1 μM in standard hydra medium (Lenhoff 1982).

Figure 4.1. Phylogeny of animal CNGs. A single CNG *hmCNG* locus was recovered from the genome of *Hydra magnipapillata* (bold). This gene falls out at the base of the *B* clade of CNGs which function in ciliary phototransduction. Nodal support, as given by posterior probability is shown at nodes. Tree rooted with a sponge CNG homolog.

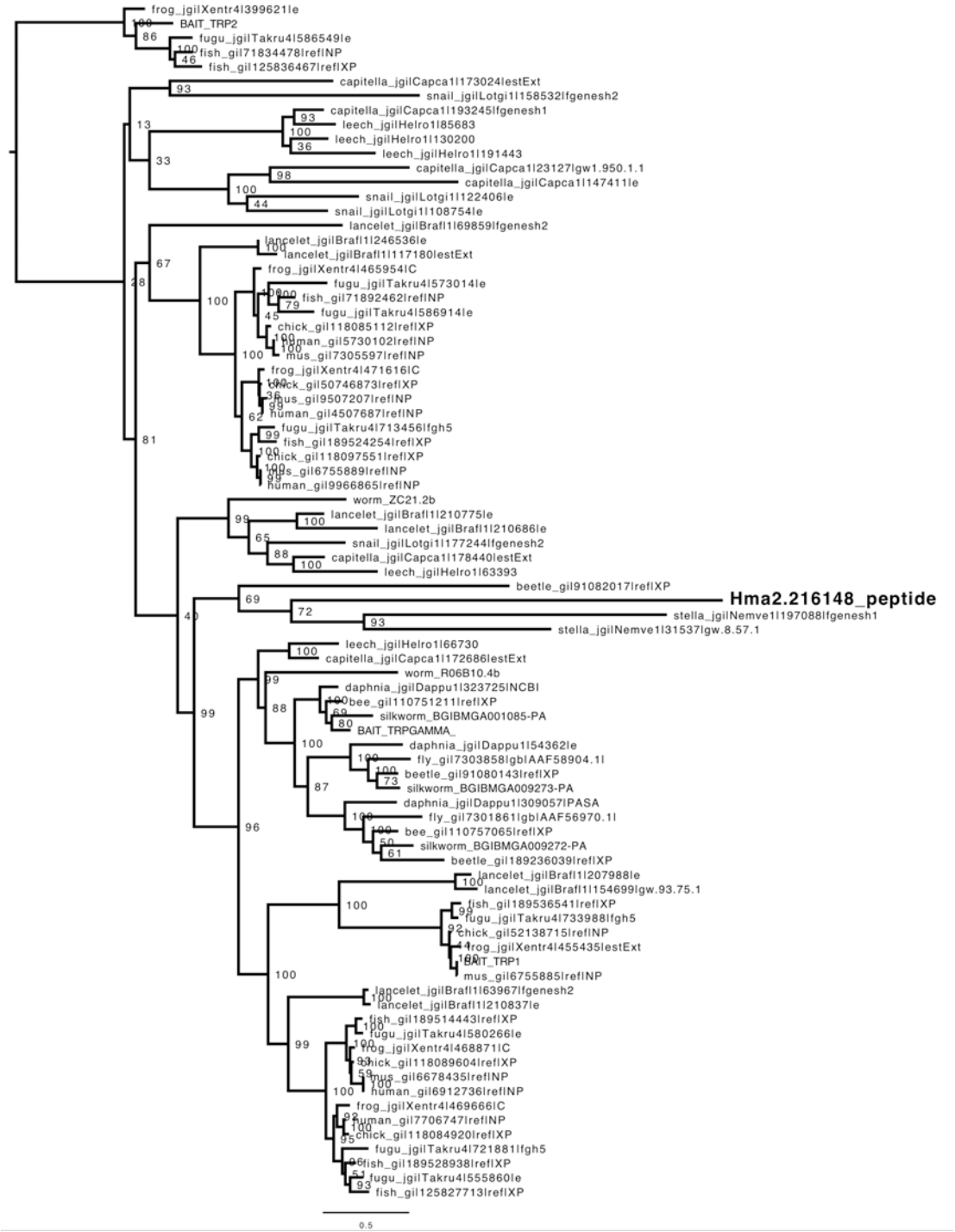

Figure 4.2. Phylogeny of animal TRPs. A single TRP locus was recovered from the genome of *Hydra magnipapillata* (bold). This gene is positioned outside of a clade of TRPs known to play a role in rhabdomeric phototransduction. Nodal support, as given by bootstrapping percentages is shown at nodes.

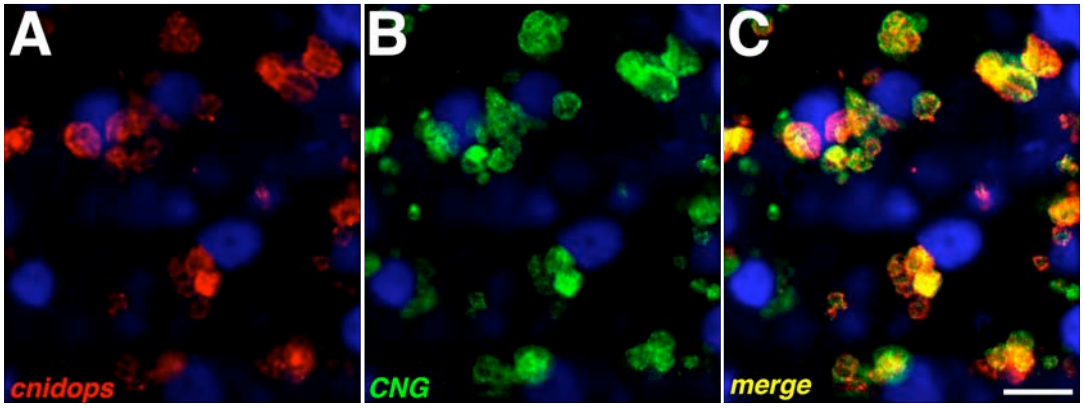

Figure 4.3. Opsin and CNG transcripts co-localize to the same cell types in *H. magnipapillata*. Data represent a single confocal section of 0.2 μm thick. Blue, cell nuclei and nematocyst capsules; Red, cnidops; Green, CNG; Yellow, channel merge. Scale bar = 50μm.

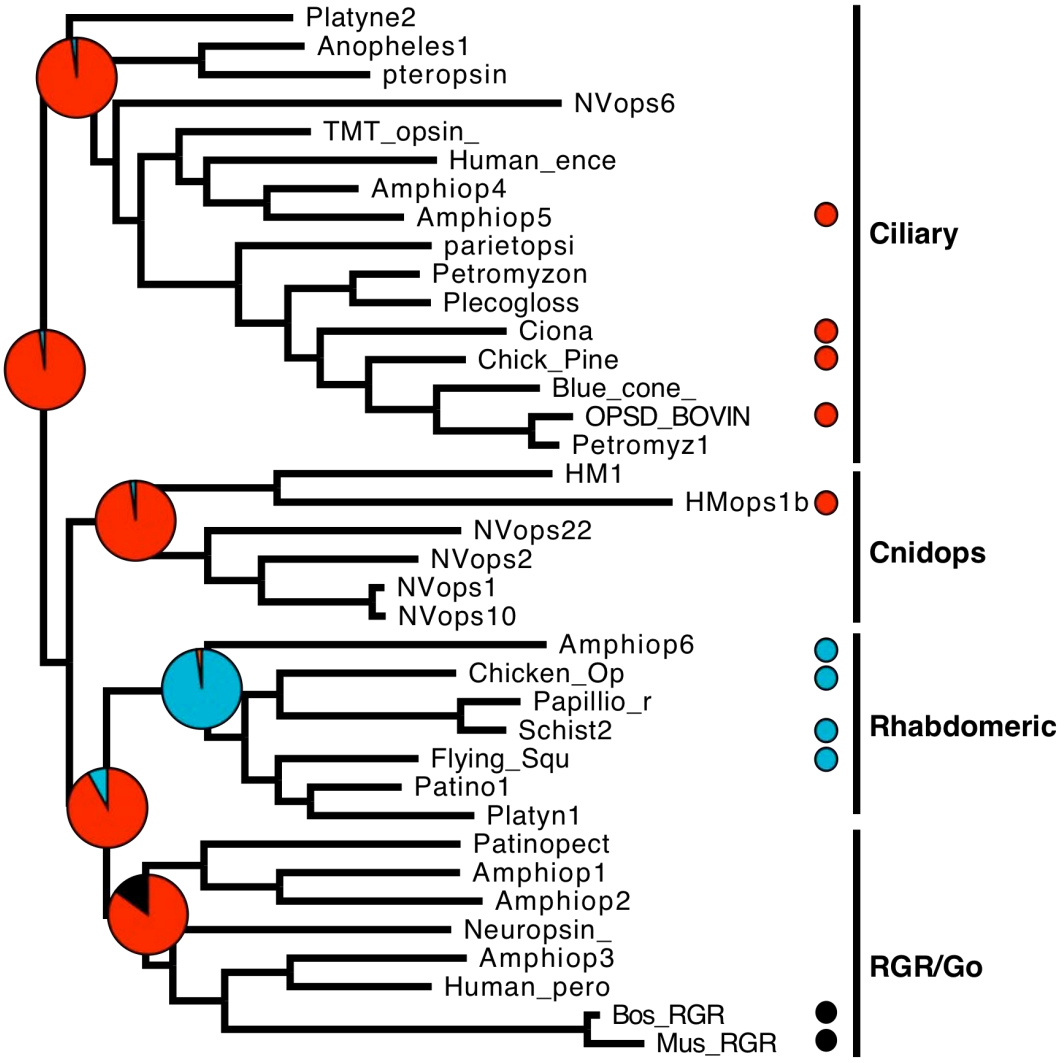

Figure 4.4. Reconstructed ancestral ion channel usage states on opsin phylogeny. Small circles indicate data points collected from the literature and the current study. Pie graphs indicate posterior probabilities of reconstructed ion channel states in opsin evolution. Opsin phylogeny from (Plachetzki et al. 2007). Red, CNG; Blue, TRP; black; no ion channel.

Figure 4.5. Montage of hydra contraction response. Upon stimulation with bright light, dark adapted hydra display a stereotyped contraction response culminating in the tight contraction of the animal into a sphere. This animal had been staged in total darkness for 30 minutes prior to recording. See also supplementary movie.

Chapter 5

Evidence that light information is integrated into the nematocyte firing response

Abstract

Nematocytes, the synapomorphic stinging cells of cnidarians, are known to integrate both chemo- and mechano-sensory information into their discharge response. Here we provide evidence that light information is also involved in modulating nematocyte firing. We report that several components of the phototransduction cascade including opsin, cyclic nucleotide gated ion channel (CNG) and arrestin are specifically expressed in cells of the nematocyte lineage. We further show that the magnitude of nematocyte discharge co-varies as a function of light intensity. Fewer nematocytes are recovered from a nematocyte discharge assay under conditions of bright light than are recovered in dim light conditions. These results provide compelling evidence that nematocyte firing involves the integration of light information, in addition to previously described chemo- and mechano-sensory integration. Our study is the first to identify a sensory receptor involved in nematocyte discharge and provides further evidence on the sensory poly-modal nature of nematocyte discharge.

Introduction

Nematocytes, the stinging cells of cnidarians, are a fascinating model for the evolution of complexity (Lenhoff 1988). Present only in the cnidarian lineage, nematocytes display a diversity of form and function that has captivated biologists since the 19th century (Ehrenberg 1836). The functional components of nematocytes

are complex secretory products known as cnidae (also referred to as nematocysts). *Hydra magnipapillata* possesses four types of cnidae (Figure 5.1). These include the desmonemes, which act to ensnare prey, the stenoteles, which penetrate and inject venom into ensnared prey, the atrichous isorhizas, which are used in locomotion and the holotrichous isorhizas, which function in defense (Kass-Simon G 2002). Nematocytes bearing each type of cnidae are housed in battery cell complexes, which are arranged in an orderly arrangement across the length of the tentacle (Eakin and Westfall 1962).

In the past two decades it has become clear that nematocyte discharge is modulated by the integration of both chemical and mechanical cues from the environment. Based on studies of behavior, it was demonstrated that N-acetylated sugars and low molecular weight amino acids (chemicals common to cnidarian prey items like micro-crustaceans) were capable of tuning the nematocyte firing response for higher efficiency by inducing alterations in length of mechano-sensitive cilia that serve as triggers (Watson and Hessinger 1989). Weather or not this response to chemical cues from the environment is based on cell-autonomous sensory properties of the nematocytes themselves, by information relayed through the cnidarian nervous system, or by a combination of the two, remains unclear (Eakin and Westfall 1962). This lack of understanding stems largely from our present ignorance of the specific sensory receptor proteins that mediate nematocyte responses to environmental stimuli. To date, no sensory receptor of any type has been correlated with nematocyte function.

Animal vision and many non-ocular photosensitivity phenotypes are predicated on the action of an opsin-mediated phototransduction cascade (Terakita 2005). Recent studies have identified elements of the animal phototransduction pathway in the hydra (Plachetzki et al. 2007; Plachetzki 2009) and other cnidarians (Koyanagi et al. 2008; Kozmik et al. 2008; Suga et al. 2008). An emerging view of the phototransduction system in cnidarians suggests that its composition is similar to the ciliary type of phototransduction known from vertebrate rod and cone cells (Koyanagi et al. 2008; Kozmik et al. 2008; Plachetzki 2009). This, in contrast to the rhabdomeric phototransduction system whose components seem to be lacking in cnidarians (Plachetzki et al. 2007; Koyanagi et al. 2008; Plachetzki 2009). Ciliary phototransduction is mediated by a class specific c-opsin, a sensory receptor protein with membership of a larger class of proteins called G protein coupled receptors (GPCRs). In the vertebrate retina, c-opsin initiates a cell signaling pathway that ultimately gives rise to the closing of CNG ion channels (Pifferi et al. 2006). Recently, CNG expression and function has been demonstrated to mediate photobehavior in the hydra (Chapter 4) (Plachetzki 2009). Arrestin proteins attenuate phototransduction by effectively quenching activated opsin and are ubiquitous in animal phototransduction systems (Hardie and Raghu 2001).

Nematocyte function is best understood in the fresh water polyp hydra (Ewer 1947; Hessinger 1988; Kass-Simon G 2002). These animals also possess a stereotypical photobehavior that includes positive phototaxis. Because a certain type of nematocyte (atrichous isorhiza) have been previously implicated in locomotion

(Ewer 1947), we hypothesized that photosensitivity is an additional mode of sensation, in addition to chemoreception and mechanoreception, that is integrated into the nematocyte discharge response. Previous studies by our group into the molecular basis for photobehavior in hydra have revealed that the photo pigment opsin is expressed in battery cell complexes in the hydrozoan *Hydra magnipapillata*. Here we show using in situ histochemistry (ISH) that opsin and other phototransduction components including arrestin and cyclic nucleotide gated ion channel (CNG) are expressed in non-stenotele nematocytes of the battery cell complex. Further, data from a nematocyte discharge assay revealed that the quantity of nematocytes that are recovered co-vary as a function of light intensity. These results provide the first demonstration of the modulation of nematocyte discharge by light information and the first sensory receptor gene to be identified on the molecular level to function in nematocyte firing. In addition, our findings add an interesting new dimension to our understanding of opsin function in animals.

Results

Optical dissection of battery cell morphology

If light information plays a role in modulating nematocyte discharge it is reasonable to suspect that components of the animal phototransduction cascade would be expressed in these nematocytes or in closely apposed cells that share synaptic connectivity (Westfall 1988). We first examined the fine scale cellular architecture of

H. magnipapillata so as to provide a morphological context for our investigations. Confocal microscopy of hydra was conducted using three spectrally non-overlapping stains: phalloidin, which stains F actin containing cells such as muscle; anti acetylated α tubulin, an antibody directed against cells of a neuronal lineage such as nematocytes and other neurons; and DAPI, which stains cell nuclei and nematocyst capsules (Szczepanek et al. 2002). Consistent with previous results (Eakin and Westfall 1962), we observe that nematocytes and the battery cell complexes that house them are arrayed in a regular fashion across the length of the tentacle in the hydra (Holstein 1988) (Figure 5.2a). Closer examination reveals the fine structure of the battery cell complexes (Figure 5.3b). These structures are characterized by a single large stenotele nematocyte at center, surrounded by smaller nematocytes of the remaining isorhiza, holotrichous isorhiza or desmoneme varieties (Wood 1998; Kass-Simon G 2002). A schematic of the battery cell complex is given in Figure 5.2c.

Phototransduction genes are expressed in components of the battery cell complex

Cnidarian opsin (*hmOps1*) and CNG had been cloned from *H. magnipapillata* as previously reported (Plachetzki et al. 2007; Plachetzki 2009). A partial sequence of the arrestin gene was cloned using data from bioinformatic screens of the genome sequence for *H. magnipapillata*. Phylogenies for opsin and CNG have been previously reported (Plachetzki et al. 2007; Plachetzki 2009). Our phylogenetic results for arrestin are given in Figure 5.3. The genome of *H. magnipapillata*

contains a single arrestin locus, which falls out at the base of a clade containing the visual arrestin known to function in vertebrate ciliary signaling.

The cellular localization of opsin in the tentacles of hydra was achieved using whole mount ISH, cryo-sectioning and confocal microscopy. Our results suggest that opsin transcripts are expressed in the accessory cells that lie directly adjacent to the large stenotele nematocytes (Figure 5.4).

We examined the expression of opsin, CNG and arrestin in the tentacles of hydra using FISH. We hypothesized that the phototransduction components CNG and arrestin would co-localize with opsin in the accessory cells that flank the large stenotele nematocytes. Our data confirm this hypothesis (Figure 5.5). While these methods are unable to resolve the specific types of nematocytes involved, opsin and the other phototransduction components CNG and arrestin are specifically co-expressed in accessory cells that flank the stenotele nematocyte. These cells correlate to the locations of the remaining non-stenotele nematocyte types (e.g., atrichous isorhiza, holotrichous isorhiza, or desmoneme).

Evidence that light information is integrated in the nematocyte discharge response

Our results from studies of phototransduction gene expression suggest that light information might be involved in the modulation of nematocyte firing. We next tested the response of nematocyte function under different light regimes. Using gelatin coated probes to capture discharged nematocytes, our data suggest that the ambient light environment can affect the magnitude of nematocyte firing. We find

that a higher number of nematocytes are recovered under low light conditions (5 lux, 520 nm) compared with similar assays conducted in bright light conditions (3500 lux, 520 nm). Under dim light 184 nematocytes were recovered from 55 trials while in bright light 84 nematocytes were recovered in 40 trials. These data depict a trend that is nearly significant (fisher's exact test p= 0.06).

Discussion

Our results provide a new understanding of the basic biology of nematocyte function. First, we provide detailed morphological information on the cellular architecture of the tentacles and battery cell complex in the hydrozoan *H. magnipapillata*. Next, we report the co-expression of opsin and the additional phototransduction components CNG and arrestin in these battery cell complexes and put forth the hypothesis that light information is integrated into the nematocyte firing response. This hypothesis receives support from behavioral assays of nematocyte firing under different light conditions.

Morphological analyses

Our studies of the cellular morphology of the tentacles of hydra using confocal microscopy are consistent with earlier analyses, but have several advantages over earlier approaches (Eakin and Westfall 1962). We are able to discern individual cell types using specific and spectrally non-overlapping stains (Figure 5.2).

Nematocytes are derived from the interstitial stem cells known from hydrozoans and as such are members of the neuronal lineage (Bosch 2009). We demonstrate that a common antibody against animal neurons, acetylated α tubulin, provides a useful stain for nematocytes as well. Our data resolve both the nerve net and the battery cell complexes in the tentacles of hydra. In addition, confocal microscopy offers data from a wide spatial range of focal planes to be collected in focus, providing a sense of depth to the data. Together, these attributes provide a reliable morphological and spatial context for our examination of cell type identity in the hydra.

Phototransduction gene expression

Gene expression data reported here strongly suggest that the phototransduction loci CNG and arrestin co-localize with opsin in the accessory cells that surround the central stenotele nematocyte in the battery cell complexes of *H. magnipapillata* (Figures 5.4 and 5.5). Because these data alone cannot be used to discriminate between the types of nematocytes that opsin and the other phototransduction components are expressed in, these phototransduction gene expressing accessory cells could be either atrichous isorhiza, holotrichous isorhiza or desmoneme cells. Several lines of evidence support the idea that the genes for which we report expression data are involved in phototransduction. First, the opsin locus that we investigated possesses hallmark amino acid residues that are known to function in photosensitivity (Plachetzki et al. 2007). Second, both the CNG and arrestin loci that we report expression data for represent singleton genes in the

genome of *H. magnipapillata* (Plachetzki 2009). Because CNG and arrestin have been implicated in ciliary phototransduction in all previously described cases (Womack et al. 2000; Pifferi et al. 2006), we feel it is unlikely that these single copies of CNG and arrestin are involved in specifically non-phototransductive functions. Further, the arrestin locus that we describe is a member of a clade that includes other homologs that are known to function in ciliary phototransduction, e.g. vertebrate visual arrestin (Figure 5.3). Finally, we have previously demonstrated that the hydra CNG ion channel is involved in another photobehavior, the contraction response (Plachetzki 2009). Together, these data strongly implicate the expression of functional phototransduction genes in the nematocytes of hydra.

Nematocyte function in the context of light intensity

Our nematocyte discharge assay was adapted from previous reports (Watson and Hessinger 1989) and utilizes a gelatin-coated length of fishing line as a probe. When placed in contact with starved hydra, nematocytes discharge and become lodged in the gelatin layer of the probe. In many cases, the entire post-discharge nematocyte is recovered by this method, which allows the number of discharged nematocytes to be quantified by counting under light microscopy. When these trials are conducted in differing light environments we obtain results that co-vary with the light regime being tested. One possible limitation to our experimental approach is that stenotele nematocytes may be disproportionately recovered by our discharge assay due to the harpoon-like morphology of their cnidae (Figure 5.1). Despite this

possibility, the following conclusions can be made. First, the impact of light intensity that we observe on nematocyte discharge is presented as a phenotype of stenotele nematocytes. However, we have shown that stenotele nematocytes do not express opsin or other phototransduction components (Figure 5.5). This suggests that the response of nematocytes to light must not be a cell autonomous phenotype and is most likely accomplished by lateral inhibition of stenotele nematocytes by neighboring cell types that express phototransduction genes. Based on morphological studies Westfall (1988) first proposed that nematocyte discharge physiology could involve the integration of signals from either neighboring nematocytes and/or the underlying nervous system. Recent data has demonstrated a physiological linkage between the nematocytes of the hydrozoan *Stauridiosarsia* where mechanical stimuli of one nematocyte was found to alter the electrophysiological potential of neighboring cells (Brinkmann 1996). Our data suggest that light information may also be transduced between cells of the battery cell complex. In our experiments, light intensity was inversely proportional to nematocyte discharge, suggesting that opsin-mediated signaling plays an inhibitory role.

Implications for nematocyte function

Because hydras are most active during the day, the inhibitory nature of opsin-mediated phototransduction could serve to reduce discharge in non-feeding contexts such as locomotion. These and other possibilities exist for the adaptive significance of photosensitivity in nematocyte firing, however without data on the specific types

of nematocytes that express this sensory modality, we are left with only conjecture. We must also recall that both chemosensitivity and mechanosensitivity have been implicated in nematocyte firing, so whatever the role of photosensitivity in discharge, is likely to act in conjunction with other environmental cues.

The opsin-mediated phototransduction cascade is most often equated with the visual phenotypes of animals but knowledge of a rich diversity of non-visual phenotypes is emerging (Gotow and Nishi 2007). Photosensitivity in the freshwater polyp hydra has been well known since the 18th century and indeed, this capacity was one of the characteristics that lead to the classification of the hydra as an animal, not a plant (Trembly 1744). However, the role of photosensitivity in modulating nematocyte function has to our knowledge not been proposed. Despite the fact that both chemoreception and mechanoreception have been faithfully demonstrated in nematocyte firing, our description of opsin-mediated photosensitivity in these cells represents the first sensory receptor gene to be correlated with nematocyte function. These findings further enforce our recognition of the nematocyte as one of nature's most complex animal cell types (Lenhoff 1988) and adds another sensory modality to the complicated dynamic that is nematocyte behavior.

Methods

Immunohistochemistry and FISH

Animals were starved prior to staining for two days. Prior to fixation, animals were relaxed in 2% urethane (Sigma) in hydra medium (HM; 1.0 mM $CaCl_2$, 1.5 mM $NaHCO_3$, 0.1 mM $MgCl_2$, 0.008 mM $MgSO_4$, 0.03 mM KNO_3; pH 8.0). Animal were fixed overnight at 4°C in 4% paraformaldehyde (Sigma). Animals were washed five times for five minutes in PBST (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, 0.1% Tween 20, pH 7.4) and blocked for two hours in PBST + 20% normal goat serum (NGS; Sigma) at room temperature. Animals were then incubated with primary antibody anti-acetylated α tubulin (1:500) in blocking solution over night at 4°C. Following primary antibody, animals were washed 5 times for 5 minutes in PBST and blocked as before. Secondary antibody, Cy2-conjugated anti mouse Ig (Jackson), was added over night at 4°C in blocking reagent. Samples were then washed five times for five minutes in PBST and a 1:1000 of DAPI stock + Alexa Fluor 488-labeled phalloidin (Invitrogen) was added for one hour. Samples were then washed five times for five minutes and mounted in glycerol. RNA probe construction (*hmCNG* and *hmArr*) and FISH microscopy were conducted as previously reported (Plachetzki 2009).

Phylogenetic Analysis

A phylogeny for animal CNG was previously described. Arrestin the dataset was aligned using TCOFFEE (Notredame et al. 2000) under default parameters and alignment manipulations were done using Seaview (Galtier et al. 1996). Phylogenetic analyses were conducted using Maximum Likelihood (ML) as implemented in RaxML (Stamatakis et al. 2005) and Bayesian Markov Chain Monte Carlo (MCMC) approaches using PhyloBayes (http://www.lirmm.fr/mab/article.php3?id_article=206). Support for internal nodes was assessed with 1000 bootstrap replicates for ML and posterior probability for BMCMC analyses. ML analysis assumed the best-fit model WAG+I+G determined using ProtTest (Abascal et al. 2005). BMBMC analyses were conducted under the default CAT approach. Convergence of BMCMC chains was determined to have occurred once the mean likelihood difference between chains fell below 0.01.

Nematocyte Discharge Assay

Our nematocyte discharge assay was based on the method of Wagner and Hessinger (Watson and Hessinger 1989), but we used 2 lb test fishing line (Cabelas) in place of glass rods. Lengths of fishing line (ten cm) were dipped in 20 % poly lysine (Sigma) and left to dry for five. Probes were then dipped twice in 20% gelatin (Knox) that had been dissolved in HM and heated to 65° C. Probes were used within 24 hours of their fabrication. For nematocyte discharge trials, healthy responsive animals were placed in either dim blue light (5 lux, 520 nm) or bright blue light (3500

lux, 520 nm) for six hours prior to probing. Animals were touched once at the distal tips of tentacles for one second and the probe was withdrawn. The distal 1 cm of the probes were cut and mounted in glycerol. Nematocytes that were collected in the probes were counted by light microscopy at 60X.

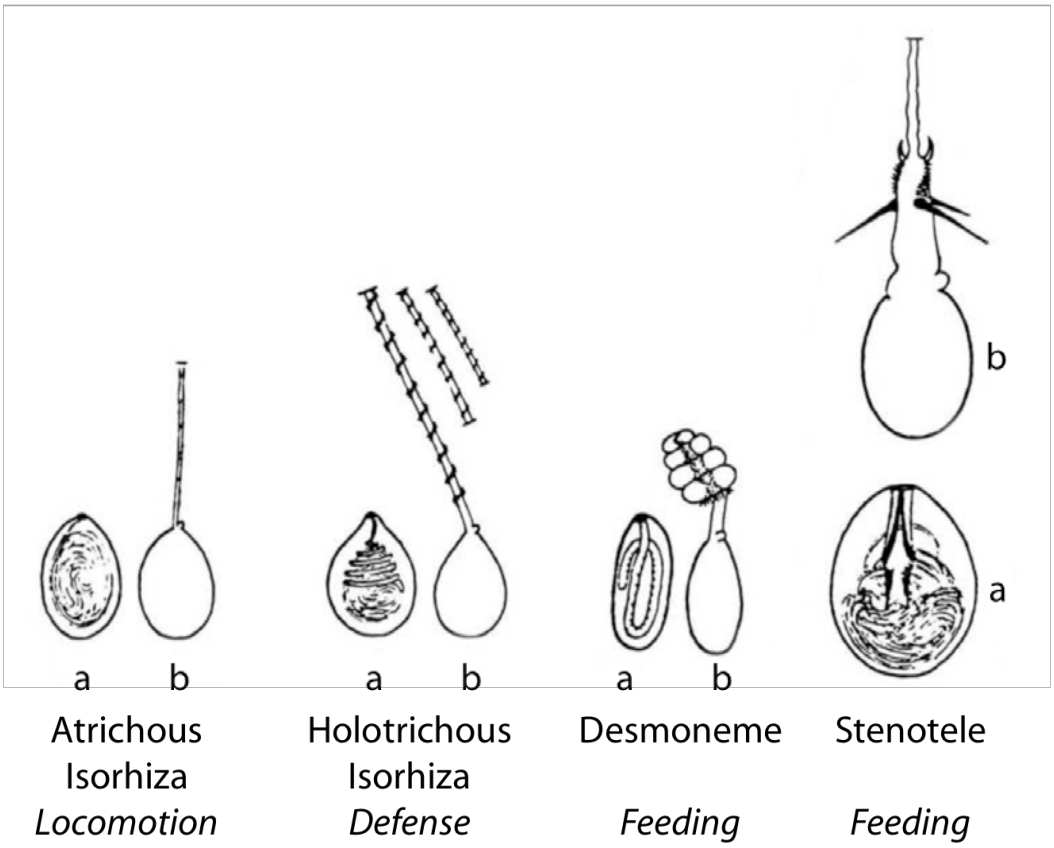

Figure 5.1. Morphological and functional diversity of the nematocytes of hydra. A, intact nematocyte. B, post-discharge nematocyte. Derived from Kass Simon (2002).

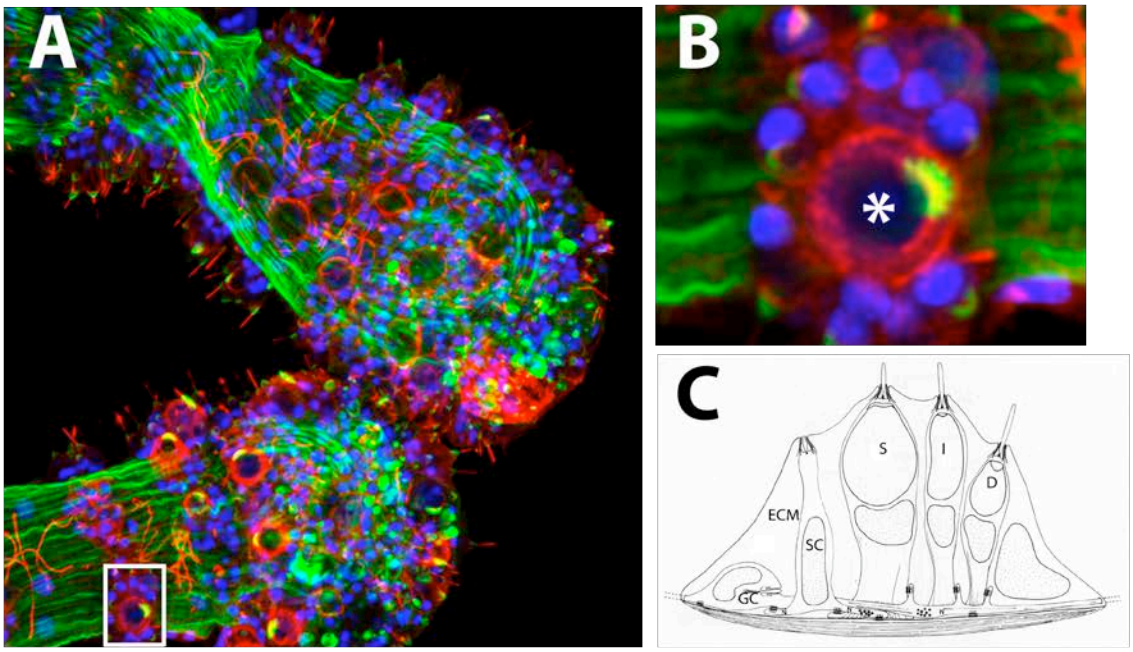

Figure 5.2. Tentacle arrangement and fine structure of the battery cell complex of *H. magnipapillata.* **A.**, confocal micrograph showing to tentacle bulbs (tips). Nematocytes are arranged in a linear fashion across the width of the tentacles (red). White demarcation indicates area shown in **B**. **B.**, Magnification of battery cell complex. **C.**, schematic of battery cell complex from side view. Red, nematocytes and neurons stained with anti-acetylated α tubulin; Green, musculature and F actin positive fibrils stained with phalloidin; Blue, cell nuclei and nematocyte capsules. Asterisk indicates stenotele nematocyte. ECM, extracellular matrix; I, isorhiza nematocyst, D, desmoneme nematocyst; S, stenotele nematocyst; N, neurite. Panel C adapted from Westfall (1988). Width of demarcation in **A** = 50 μm.

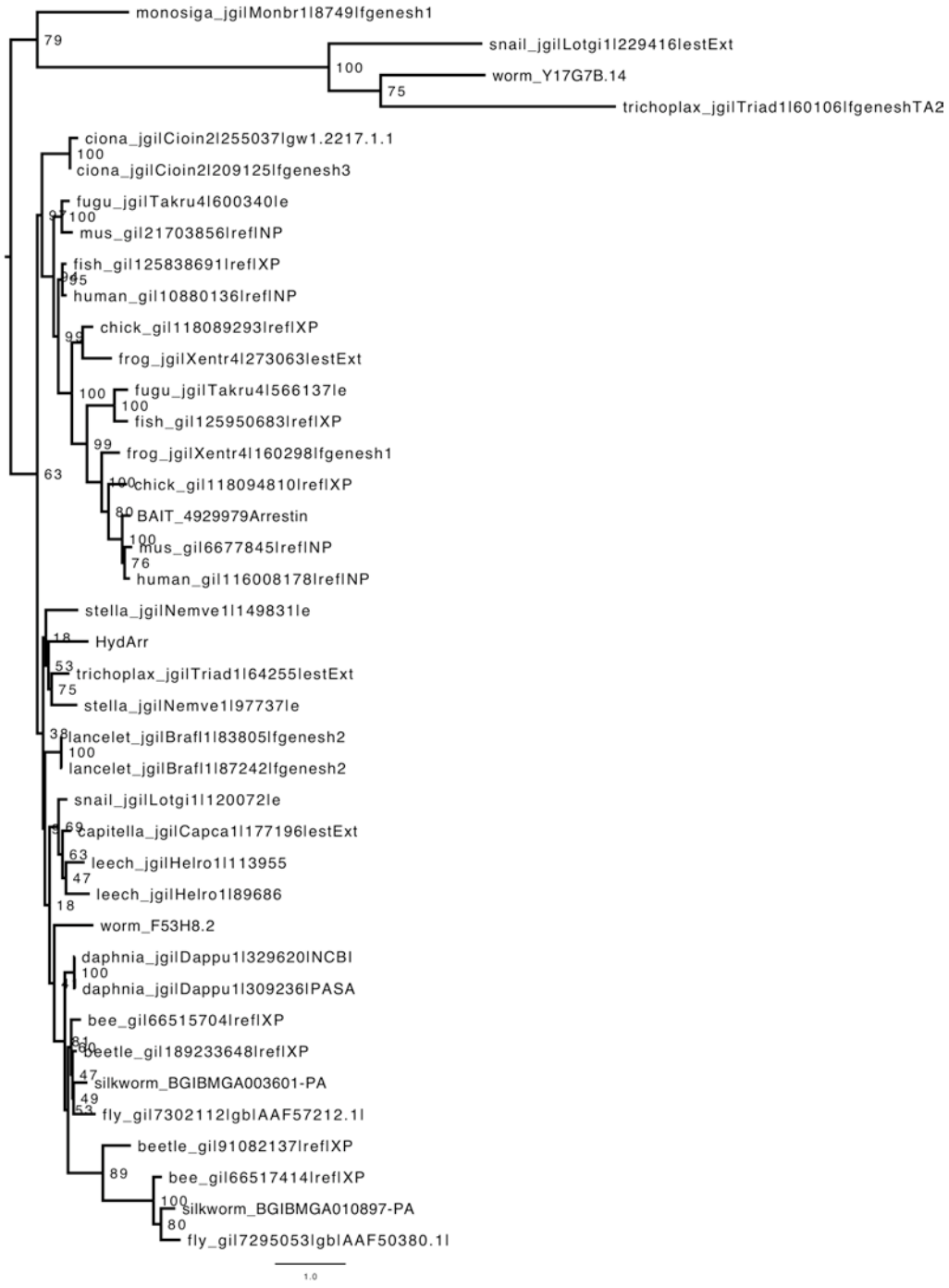

Figure 5.3. Maximum likelihood phylogeny of animal arrestins. A single arrestin locus (HydArr) was recovered from the genome sequence of *H. magnipapillata* (arrow). This sequence lies at the base of the protostome arrestins. Tree rooted with arrestins from the choanoflagellate *Monosiga* an animal outgroup. Branch support is given by bootstrap percentages.

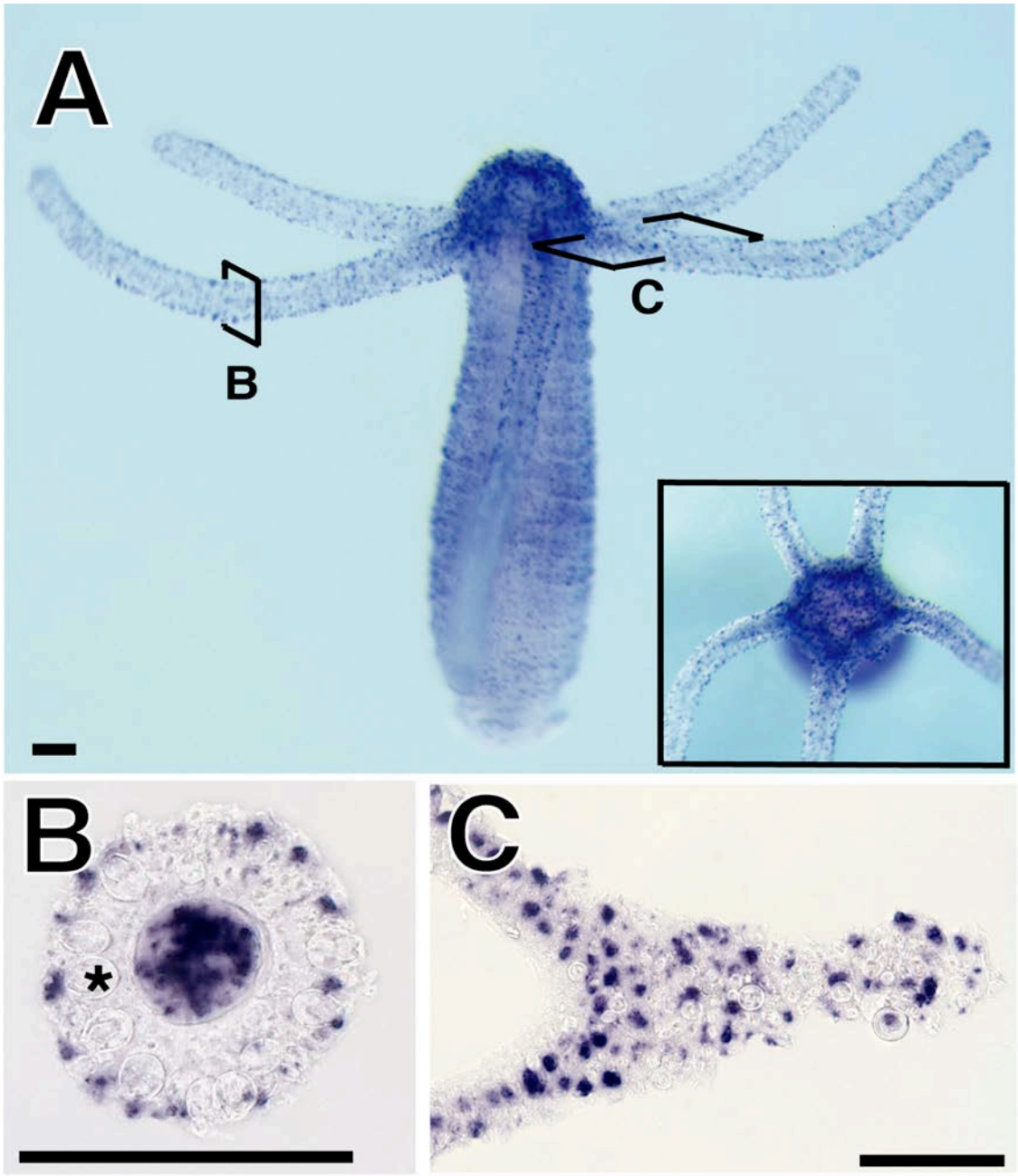

Figure 5.4. Cnidops expression in *H. magnipapillata*. **A.**, Whole-mount *in situ* hybridization. Cnidops is expressed in the oral hypostome (head) of the animal as well as throughout the tentacles and body column. **B** and **C**, cryosections of tentacles of whole-mount corresponding to planes depicted in **A**. Cnidops is expressed in cells closely apposed to the teardrop shaped stenotele nematocysts, asterisk. Scale bars = 100 μm.

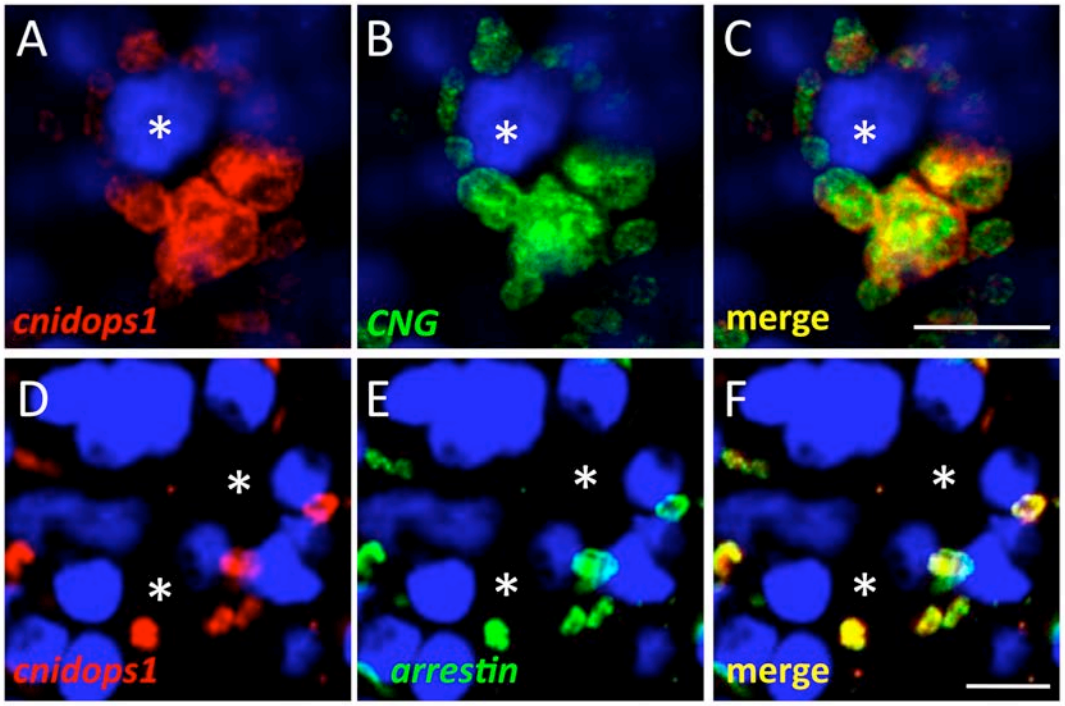

Figure 5.5. Cnidarian opsin cnidops and CNG transcripts co-localize to specific cells in the battery cell complex. Fluorescent *in situ* hybridization showing cnidops expression (**A**), CNG expression (**B**) and the co-localization of these expression profiles (**C**). Data represent a single confocal optical section of 0.2 μm. Asterisk = stenotele nematocyst (Compare with Figures 2 and 4, chapter 4). Red, cnidops; Green, CNG; Yellow, Merge; Blue, cell nuclei and nematocyte capsules. Scale bar = 50 μm.

Chapter 6

Future Directions

Since our first description of the cnidops class of cnidarian opsins (Plachetzki et al. 2007), numerous additional reports have addressed both the composition of cnidarian phototransduction and the deep relationships between animal opsins. These reports have detailed a diversity of cnidops loci from the hydrozoan jellies *Cladonema* and *Podocoryne* (Suga et al. 2008) and the cubozoans *Tripedalia* (Kozmik et al. 2008) and *Carybdea* (Koyanagi et al. 2008). Further, data on the expression of other cnidarian phototransduction genes not dealt with here has become available. Kozmic (Kozmik et al. 2008) reported the expression of one subunit of the intermediary enzyme phosphodiesterase (PDE) in the camera eye of Tripedalia (Kozmik et al. 2008). PDE functions in the ciliary cascade in vertebrates (see figure 1.1) (Hardie and Raghu 2001). In addition, a cyclic nucleotide second messenger has been implicated in the phototransduction cascade in *Carybdea* (Koyanagi et al. 2008). These data are consistent with our finding in Chapter 4 that a Cyclic Nucleotide-Gated (CNG) ion channels function in cnidarian phototransduction. Together, these data contribute to an emerging view that cnidarian phototransduction cascades are similar to the ciliary mode of transduction.

Animal phototransduction cascades are complex cell signaling systems. As such, a more comprehensive understanding of the cnidarian visual cascade could come by investigating other putative phototransduction loci including rhodopsin

kinase, guanylyl cyclase, adenylate cyclase and the remaining PDE subunits. One recent study indicated that a G_s G protein functioned in cnidops mediated signaling. However, this finding requires further study as the authors used cross-reactive antibodies of undemonstrated specificity to make this claim (Koyanagi et al. 2008). In the course of my research I obtained data that both $G_{i/o}$ and G_q also co-express with cnidops (data not shown). This important first step in phototransduction requires further investigation.

Animal visual phenotypes are a fascinating model for adaptive evolution. In particular, the evolution of color vision has received much attention (Briscoe and Chittka 2001). Cnidarians are known to exhibit a range wavelength-specific photobehaviors, but color vision *per se* has not been demonstrated. The discovery of cnidops provides a molecular context for future investigations into spectral tuning and the possibility of color vision in cnidarians.

At present, all evidence suggests that a functional transition occurred at the origin of the rhabdomeric pathway that underlies the visual phenotypes of insects, arthropods and other protostomes. Our analyses in Chapter 2 and 3 suggests that co-option of pre-existing signal transduction components has been a major driver in the diversification of the animal phototransduction networks. It is possible that the rhabdomeric pathway evolved by co-opting an otherwise integrated signaling cascade, possibly from some other sensory modality like mechanotransduction. The molecular basis for mechanosensory phenotypes is poorly understood and there are likely numerous analogous systems in animals. However, the rhabdomeric

phototransduction cascade displays some interesting similarities with what *is* known about mechanotransduction. Both mechanotransduction and rhabdomeric phototransduction utilize TRP ion channels for signaling (Venkatachalam and Montell 2007). Rhabdomeric photoreceptor neurons are also specified in development by genes that underlie mechanosensory neuron specification (Brown et al. 2001). In order to test the fascinating hypothesis that rhabdomeric signaling evolved by co-opting mechanosensory cascades we must first gain a better understanding of metazoan mechanotransduction systems in general. Here, the cnidarian models, specifically their nematocytes that have been utilized to good effect in elucidating the evolution of phototransduction cascades, are strong candidates.

Literature Cited

Abascal F, Zardoya R, Posada D (2005) ProtTest: selection of best-fit models of protein evolution. Bioinformatics 21(9): 2104-2105.

Abouheif E (1999) Establishing homology criteria for regulatory gene networks: prospects and challenges. Novartis Found Symp 222: 207-221.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25(17): 3389-3402.

Arendt D (2003) Evolution of eyes and photoreceptor cell types. Int J Dev Biol 47(7-8): 563-571.

Arendt D, Tessmar-Raible K, Snyman H, Dorresteijn AW, Wittbrodt J (2004) Ciliary photoreceptors with a vertebrate-type opsin in an invertebrate brain. Science 306(5697): 869-871.

Arendt DaW, J (2001) Reconstructing the eyes of Urbilateria. Transactions of the Royal Society of London B(356): 1545-1563.

Arthur W, Jowett T, Panchen A (1999) Segments, limbs, homology, and co-option. Evol Dev 1(2): 74-76.

Bachner-Melman R, Dina C, Zohar AH, Constantini N, Lerer E et al. (2005) AVPR1a and SLC6A4 gene polymorphisms are associated with creative dance performance. PLoS Genet 1(3): e42.

Behe MJ (1996) Darwin's Black Box: Free Press.

Bollback JP (2006) SIMMAP: stochastic character mapping of discrete traits on phylogenies. BMC Bioinformatics 7: 88.

Bosch TC (2009) Hydra and the evolution of stem cells. Bioessays 31(4): 478-486.

Bourlat SJ, Juliusdottir T, Lowe CJ, Freeman R, Aronowicz J et al. (2006) Deuterostome phylogeny reveals monophyletic chordates and the new phylum Xenoturbellida. Nature 444(7115): 85-88.

Bridgham JT, Carroll SM, Thornton JW (2006) Evolution of hormone-receptor complexity by molecular exploitation. Science 312(5770): 97-101.

Brinkmann M, D. Oliver and U. Thrum (1996) Mechanoelectric transduction in nematocysts of a hydropolyp (Corynidae). J Comp Physiol A 178: 125-138.

Briscoe AD, Chittka L (2001) The Evolution of Color Vision in Insects. Annu Rev Entomol 46: 471-510.

Britton T, Oxelman B, Vinnersten A, Bremer K (2002) Phylogenetic dating with confidence intervals using mean path lengths. Mol Phylogenet Evol 24(1): 58-65.

Brown NL, Patel S, Brzezinski J, Glaser T (2001) Math5 is required for retinal ganglion cell and optic nerve formation. Development 128(13): 2497-2508.

Buss LW (1987) The Evolution of Individuality: Princeton University Press. 201 pp. p.

Carleton KL, Spady TC, Cote RH (2005) Rod and cone opsin families differ in spectral tuning domains but not signal transducing domains as judged by saturated evolutionary trace analysis. J Mol Evol 61(1): 75-89.

Carroll SB, Grenier, J. K., and Weatherbee, S. D (2005) From DNA to Diversity:Molecular Genetics and the Evolution of Animal Design. Oxford: Blackwell. 257 p.

Catmull J, Hayward DC, McIntyre NE, Reece-Hoyes JS, Mastro R et al. (1998) Pax-6 origins--implications from the structure of two coral pax genes. Dev Genes Evol 208(6): 352-356.

Chen CK (2005) The vertebrate phototransduction cascade: amplification and termination mechanisms. Rev Physiol Biochem Pharmacol 154: 101-121.

Chen J, Rattner A, Nathans J (2005) The rod photoreceptor-specific nuclear receptor Nr2e3 represses transcription of multiple cone-specific genes. J Neurosci 25(1): 118-129.

Chen K, Durand D, Farach-Colton M (2000) NOTUNG: a program for dating gene duplications and optimizing gene family trees. J Comput Biol 7(3-4): 429-447.

Chen P, Hao W, Rife L, Wang XP, Shen D et al. (2001) A photic visual cycle of rhodopsin regeneration is dependent on Rgr. Nat Genet 28(3): 256-260.

Collin S, Trezise, A. (2006) Response to Pisani et al. Current Biology 16(9): R320-R320.

Conant GC, Wolfe KH (2006) Functional partitioning of yeast co-expression networks after genome duplication. PLoS Biol 4(4): e109.

Conway Morris S, Cohen BL, Gawthrop AB, Cavalier-Smith T, Winnepenninckx B et al. (1996) Lophophorate phylogeny; discussion and reply. Science 272(5259): 282-283.

Darwin C (1859) On the origin of the species by means of natural selection, or, The preservation of favoured races in the struggle for life. London: John Murray ... ix, [1], 502, 532 , [501] folded leaf of plates p.

Darwin C (1987) Transmutation Notebooks B-D 1836-1844, transcriber Kohn D; Barrett PH GP, Herbert S., Kohn D, Smith S, editor. Ithica: Cornell University Press.

Dunlap JC, Loros, J. J. (2005) Neurospora Photoreceptors. In: Briggs WR, Spudich J. L., editor. Handbook of Photosensory Receptors. Weinheim: Wiley-VCH Verlag GmbH & Co. pp. 473.

Dunn CW, Hejnol A, Matus DQ, Pang K, Browne WE et al. (2008) Broad phylogenomic sampling improves resolution of the animal tree of life. Nature 452(7188): 745-749.

Durand D, Halldorsson BV, Vernot B (2006) A hybrid micro-macroevolutionary approach to gene tree reconstruction. J Comput Biol 13(2): 320-335.

Eakin RM (1963) Lines of evolution of photoreceptors. In: Mazia E, Tyler, A., editor. General physiology of cell specification. New York: McGraw-Hill. pp. 393-423.

Eakin RM (1979) Evolutionary Significance of Photoreceptors: In Retrospect. American Zoologist 19: 647-653.

Eakin RM, Westfall JA (1962) Fine Structure of Photoreceptors in the Hydromedusan, Polyorchis Penicillatus. Proc Natl Acad Sci U S A 48(5): 826-833.

Ehrenberg C (1836) Beobachtungen uber die organisation der Armpolypen. Mitt Naturf Ges Berlin 2: 27-29.

Ewer RF (1947) On the functions and mode of action of the nematocytes of hydra. Proc Zool Soc Lond 117: 365-376.

Ewing B, Green P (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res 8(3): 186-194.

Felsenstein J (1978) Cases in which parsimony or compatibility methods will be positively misleading. Systematic Zoology 27: 401-410.

Friedrich M (2006) Continuity versus split and reconstitution: exploring the molecular developmental corollaries of insect eye primordium evolution. Developmental Biology in press.

Galtier N, Gouy M, Gautier C (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci 12(6): 543-548.

Geeta R (2003) Structure trees and species trees: what they say about morphological development and evolution. Evol Dev 5(6): 609-621.

Gehring W, Rosbash M (2003) The coevolution of blue-light photoreception and circadian rhythms. J Mol Evol 57 Suppl 1: S286-289.

Gehring WJ (2001) The genetic control of eye development and its implications for the evolution of the various eye-types. Zoology (Jena) 104(3-4): 171-183.

Gomez MP, Nasi E (2000) Light transduction in invertebrate hyperpolarizing photoreceptors: possible involvement of a Go-regulated guanylate cyclase. J Neurosci 20(14): 5254-5263.

Gompel N, Prud'homme B, Wittkopp PJ, Kassner VA, Carroll SB (2005) Chance caught on the wing: cis-regulatory evolution and the origin of pigment patterns in Drosophila. Nature 433(7025): 481-487.

Goodman M, Czelusniak J, Moore G, Romero A (1979) Fitting the gene lineage into its species lineage, a parsimony strategy illustrated by cladograms. Syst Zool 28(2): 132-163.

Gotow T, Nishi T (2007) Involvement of a Go-type G-protein coupled to guanylate cyclase in the phototransduction cGMP cascade of molluscan simple photoreceptors. Brain Res 1144: 42-51.

Grens A, Mason E, Marsh JL, Bode HR (1995) Evolutionary conservation of a cell fate specification gene: the Hydra achaete-scute homolog has proneural activity in Drosophila. Development 121(12): 4027-4035.

Grens A, Gee L, Fisher DA, Bode HR (1996) CnNK-2, an NK-2 homeobox gene, has a role in patterning the basal end of the axis in hydra. Dev Biol 180(2): 473-488.

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52(5): 696-704.

Haeckel E (1866) Generelle Morphologie der Organismen: Allegmeine Grudzuge der organischen Formen-Wissenschaft, mecanisch begrundet durch die von Charles Darwin reformite Descendez-Theorie, 2 volumes. Berlin: Beorg Reimer.

Halder G, Callaerts P, Gehring WJ (1995) Inducion of ectopic eyes by targeted expression of the *eyeless* gene in *Drosophila*. Science 267: 1788-1792.

Hardie RC, Raghu P (2001) Visual transduction in Drosophila. Nature 413(6852): 186-193.

Hessinger DaL, HM (1988) The Biology of Nematocysts. San Diego: Academic Press INC. 600 p.

Hisatomi O, Tokunaga F (2002) Molecular evolution of proteins involved in vertebrate phototransduction. Comp Biochem Physiol B Biochem Mol Biol 133(4): 509-522.

Hoekstra HE, Coyne JA (2007) The locus of evolution: evo devo and the genetics of adaptation. Evolution 61(5): 995-1016.

Holstein T (1988) The ultrastructure of nematocytes. In: Hessinger DaHL, editor. The biology of nematocytes. San Deigo: Academic Press INC.

Hrdy I, Hirt RP, Dolezal P, Bardonova L, Foster PG et al. (2004) Trichomonas hydrogenosomes contain the NADH dehydrogenase module of mitochondrial complex I. Nature 432(7017): 618-622.

Huelsenbeck JP (1997) Is the Felsenstein zone a fly trap? Systematic Biology 46(1): 69-74.

Huelsenbeck JP, Rannala B, Larget B (2000) A Bayesian framework for the analysis of cospeciation. Evolution Int J Org Evolution 54(2): 352-364.

Hurles ME, Willey D, Matthews L, Hussain SS (2004) Origins of chromosomal rearrangement hotspots in the human genome: evidence from the AZFa deletion hotspots. Genome Biol 5(8): R55.

Jacob F (1977) Evolution and tinkering. Science 196(4295): 1161-1166.

Jobb G, von Haeseler A, Strimmer K (2004) TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol Biol 4: 18.

Kass-Simon G SAA (2002) The behavioral and developmental physiology of nematocysts. Can J Zool 80: 1772-1794.

Kishino H, Hasegawa M (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in hominoidea. J Mol Evol 29(2): 170-179.

Kojima D, Terakita A, Ishikawa T, Tsukahara Y, Maeda A et al. (1997) A novel Go-mediated phototransduction cascade in scallop visual cells. J Biol Chem 272(37): 22979-22982.

Koyanagi M, Terakita A, Kubokawa K, Shichida Y (2002) Amphioxus homologs of Go-coupled rhodopsin and peropsin having 11-cis- and all-trans-retinals as their chromophores. FEBS Lett 531(3): 525-528.

Koyanagi M, Ono K, Suga H, Iwabe N, Miyata T (1998) Phospholipase C cDNAs from sponge and hydra: antiquity of genes involved in the inositol phospholipid signaling pathway. FEBS Lett 439(1-2): 66-70.

Koyanagi M, Kubokawa K, Tsukamoto H, Shichida Y, Terakita A (2005) Cephalochordate melanopsin: evolutionary linkage between invertebrate visual cells and vertebrate photosensitive retinal ganglion cells. Curr Biol 15(11): 1065-1069.

Koyanagi M, Takano K, Tsukamoto H, Ohtsu K, Tokunaga F et al. (2008) Jellyfish vision starts with cAMP signaling mediated by opsin-G(s) cascade. Proc Natl Acad Sci U S A 105(40): 15576-15580.

Kozmik Z, Ruzickova J, Jonasova K, Matsumoto Y, Vopalensky P et al. (2008) Assembly of the cnidarian camera-type eye from vertebrate-like components. Proc Natl Acad Sci U S A 105(26): 8989-8993.

Kutschera U, Niklas KJ (2004) The modern theory of biological evolution: an expanded synthesis. Naturwissenschaften 91(6): 255-276.

Kutschera U, Niklas KJ (2005) Endosymbiosis, cell evolution, and speciation. Theory Biosci 124(1): 1-24.

Land MF, Nilsson D-E (2002) Animal Eyes; Willmer P, Norman D, editors. Oxford: Oxford University Press.

Lenhoff H (1982) Water, Culture Solutions and Buffers. In: Lenhoff H, editor. Hydra Research Methods. New York: Plenum. pp. 19-28.

Lenhoff HaL, SG (1988) How the animal nature of marine cnidarians was recognized and the nematocyte discovered In: Hessinger DaL, HM editor. The biology of nematocysts. San Diego: Academic Press INC. pp. 1-20.

Leung HT, Tseng-Crank J, Kim E, Mahapatra C, Shino S et al. (2008) DAG lipase activity is necessary for TRP channel regulation in Drosophila photoreceptors. Neuron 58(6): 884-896.

Leys SP, Cronin TW, Degnan BM, Marshall JN (2002) Spectral sensitivity in a sponge larva. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 188(3): 199-202.

Li J, Edwards PC, Burghammer M, Villa C, Schertler GF (2004) Structure of bovine rhodopsin in a trigonal crystal form. J Mol Biol 343(5): 1409-1438.

Lynch M (2007) The frailty of adaptive hypotheses for the origins of organismal complexity. Proc Natl Acad Sci U S A 104 Suppl 1: 8597-8604.

Lynch VJ, Roth JJ, Takahashi K, Dunn CW, Nonaka DF et al. (2004) Adaptive evolution of HoxA-11 and HoxA-13 at the origin of the uterus in mammals. Proc Biol Sci 271(1554): 2201-2207.

M. Wilkinson, J. O. McInerney, R. P. Hirt, P. G. Foster, and T. M. Embley (2007) Of clades and clans: terms for phylogenetic relationships in unrooted trees. Trends in Ecology and Evolution 22(3): 114-115.

Mackie GO (1999) Colenterate organs. Mar Freshw Behav Physiol 32: 113-127.

Margulis L, Sagan D (2002) Acquiring Genomes: A Theory of the Origins of Species. New York: Basic Books.

Marin EP, Krishna AG, Zvyaga TA, Isele J, Siebert F et al. (2000) The amino terminus of the fourth cytoplasmic loop of rhodopsin modulates rhodopsin-transducin interaction. J Biol Chem 275(3): 1930-1936.

Matulef K, Zagotta WN (2003) Cyclic nucleotide-gated ion channels. Annu Rev Cell Dev Biol 19: 23-44.

Melyan Z, Tarttelin EE, Bellingham J, Lucas RJ, Hankins MW (2005) Addition of human melanopsin renders mammalian cells photoresponsive. Nature 433(7027): 741-745.

Minelli A (2000) Limbs and tail as evolutionarily diverging duplicates of the main body axis. Evolution & Development 2(3): 157-165.

Nielsen RH, J. P. (2001) Detecting positively selected amino acid sites using posterior predictive p-values. Pacific Symposium on Biocomputing, Proceedings: World Scientific. pp. 576–588.

Nilsson DE, Pelger S (1994) A pessimistic estimate of the time required for an eye to evolve. Philisophical Transactions of the Royal Society of London B 256(1345): 53-58.

Nordström K, Larsson TA, Larhammar D (2004) Extensive duplications of phototransduction genes in early vertebrate evolution correlate with block (chromosome) duplications. Genomics 83(5): 852-872.

Notredame C, Higgins DG, Heringa J (2000) T-Coffee: A novel method for fast and accurate multiple sequence alignment. J Mol Biol 302(1): 205-217.

O'Halloran DM, Fitzpatrick DA, McCormack GP, McInerney JO, Burnell AM (2006) The molecular phylogeny of a nematode-specific clade of heterotrimeric G-protein alpha-subunit genes. J Mol Evol 63(1): 87-94.

Ohara RJ (1997) Population thinking and tree thinking in systematics. Zoologica Scripta 26: 323-329.

Ohno S (1970) Evolution by gene duplication. New York: Springer-Verlag.

Okano T, Kojima D, Fukada Y, Shichida Y, Yoshizawa T (1992) Primary structures of chicken cone visual pigments: vertebrate rhodopsins have evolved out of cone visual pigments. Proc Natl Acad Sci U S A 89(13): 5932-5936.

Olson EN (2006) Gene regulatory networks in the evolution and development of the heart. Science 313(5795): 1922-1927.

Page RDM (2002) Tangled trees: phylogeny, cospeciation and coevolution. Chicago: The University of Chicago Press.

Page RDM, Charleston MA (1998) Trees within trees: phylogeny and historical associations. Trends in Ecology and Evolution 13(9): 356-359.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H et al. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. Science 289(5480): 739-745.

Panda S, Nayak SK, Campo B, Walker JR, Hogenesch JB et al. (2005) Illumination of the melanopsin signaling pathway. Science 307(5709): 600-604.

Passano LM, McCullough CB (1962) The Light Response and the Rhythmic Potentials of Hydra. Proc Natl Acad Sci U S A 48(8): 1376-1382.

Passano LM, McCullough CB (1965) Co-Ordinating Systems and Behaviour in Hydra. Ii. the Rhythmic Potential System. J Exp Biol 42: 205-231.

Peng GH, Ahmad O, Ahmad F, Liu J, Chen S (2005) The photoreceptor-specific nuclear receptor Nr2e3 interacts with Crx and exerts opposing effects on the transcription of rod versus cone genes. Hum Mol Genet 14(6): 747-764.

Peterson KJ, Butterfield NJ (2005) Origin of the Eumetazoa: testing ecological predictions of molecular clocks against the Proterozoic fossil record. Proc Natl Acad Sci U S A 102(27): 9547-9552.

Peterson KJ, Lyons JB, Nowak KS, Takacs CM, Wargo MJ et al. (2004) Estimating metazoan divergence times with a molecular clock. Proceedings of the National Academy of Sciences of the United States of America 101(17): 6536-6541.

Pifferi S, Boccaccio A, Menini A (2006) Cyclic nucleotide-gated ion channels in sensory transduction. FEBS Lett 580(12): 2853-2859.

Pisani D, Mohun SM, Harris SR, McInerney JO, Wilkinson M (2006) Molecular evidence for dim-light vision in the last common ancestor of the vertebrates. Curr Biol 16(9): R318-319; author reply R320.

Plachetzki D, CR Fong and TH Oakley (2009) Evidence that a Cyclic Nucleotide Gated Ion Channel Functioned in the Ancestral Animal Phototransduction Cascade. in progress.

Plachetzki D, Oakley, T., H. (2007) Key transitions during the evolution of animal phototransduction: novelty, "tree-thinking," co-option, and co-duplication. Integrative and Comparative Biology 47: 759-769.

Plachetzki DC, Serb JM, Oakley TH (2005) New insights into the evolutionary history of photoreceptor cells. Trends Ecol Evol 20(9): 465-467.

Plachetzki DC, Degnan BM, Oakley TH (2007) The Origins of Novel Protein Interactions during Animal Opsin Evolution. PLoS ONE 2(10): e1054.

Raible F, Tessmar-Raible K, Arboleda E, Kaller T, Bork P et al. (2006) Opsins and clusters of sensory G-protein-coupled receptors in the sea urchin genome. Dev Biol 300(1): 461-475.

Rambaut A, Grassly NC (1997) Seq-Gen: An application for the Monte Carlo simulation of DNA sequence evolution along phylogenetic trees. Computer Applications in the Biosciences in press.

Rambaut A, Drummond A (2003) Tracer. MCMC Trace Analysis Tool. 1.0 ed. Oxford: University of Oxford.

Rockman MV, Hahn MW, Soranzo N, Zimprich F, Goldstein DB et al. (2005) Ancient and recent positive selection transformed opioid cis-regulation in humans. PLoS Biol 3(12): e387.

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19(12): 1572-1574.

Rushforth NB (1973) Behavioral modifications in Colenterates. In: W.C. Corning JADaAODW, editor. Invertebrate Learning. New York: Plenum. pp. 123-269.

Sanderson MJ (2002) Estimating absolute rates of molecular evolution and divergence times: A penalized likelihood approach. Molecular Phylogenetics and Evolution 19(1): 101-109.

Schultze M (1866) Anatomie und Physiologie der Retina. Achiv Fur Mikroskopisches Anatomie 2: 175-286.

Serb JM, Oakley TH (2005) Hierarchical phylogenetics as a quantitative analytical framework for Evolutionary Developmental Biology. Bioessays 27(11).

Shapiro MD, Marks ME, Peichel CL, Blackman BK, Nereng KS et al. (2004) Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. Nature 428(6984): 717-723.

Shen D, Jiang M, Hao W, Tao L, Salazar M et al. (1994) A human opsin-related gene that encodes a retinaldehyde-binding protein. Biochemistry 33(44): 13117-13125.

Shimodaira H (2002) An approximately unbiased test of phylogenetic tree selection. Syst Biol 51(3): 492-508.

Shimodaira H, Hasegawa M (2001) CONSEL: for assessing the confidence of phylogenetic tree selection. Bioinformatics 17(12): 1246-1247.

Spaethe J, Briscoe AD (2005) Molecular characterization and expression of the UV opsin in bumblebees: three ommatidial subtypes in the retina and a new photoreceptor organ in the lamina. J Exp Biol 208(Pt 12): 2347-2361.

Spudich JL, Yang CS, Jung KH, Spudich EN (2000) Retinylidene proteins: structures and functions from archaea to humans. Annu Rev Cell Dev Biol 16: 365-392.

Stamatakis A, Ludwig T, Meier H (2005) RAxML-III: a fast program for maximum likelihood-based inference of large phylogenetic trees. Bioinformatics 21(4): 456-463.

Stern JH, Kaupp UB, MacLeish PR (1986) Control of the light-regulated current in rod photoreceptors by cyclic GMP, calcium, and l-cis-diltiazem. Proc Natl Acad Sci U S A 83(4): 1163-1167.

Su CY, Luo DG, Terakita A, Shichida Y, Liao HW et al. (2006) Parietal-eye phototransduction components and their potential evolutionary implications. Science 311(5767): 1617-1621.

Suga H, Schmid V, Gehring WJ (2008) Evolution and functional diversity of jellyfish opsins. Curr Biol 18(1): 51-55.

Suga H, Koyanagi M, Hoshiyama D, Ono K, Iwabe N et al. (1999) Extensive gene duplication in the early evolution of animals before the parazoan-eumetazoan split demonstrated by G proteins and protein tyrosine kinases from sponge and hydra. J Mol Evol 48(6): 646-653.

Swofford D (2002) PAUP*. 4.1b10 ed. Sunderland, MA: Sinauer Associates.

Szczepanek S, Cikala M, David CN (2002) Poly-gamma-glutamate synthesis during formation of nematocyst capsules in Hydra. J Cell Sci 115(Pt 4): 745-751.

Takezaki N, Figueroa F, Zaleska-Rutczynska Z, Klein J (2003) Molecular phylogeny of early vertebrates: monophyly of the agnathans as revealed by sequences of 35 genes. Mol Biol Evol 20(2): 287-292.

Taylor JS, Raes J (2004) Duplication and divergence: the evolution of new genes and old ideas. Annu Rev Genet 38: 615-643.

Telford MJ (2006) Animal phylogeny. Curr Biol 16(23): R981-985.

Terakita A (2005) The opsins. Genome Biol 6(3): 213.

Thompson JN (1987) Simbiont-induced speciation. Biological Journal of the Linnean Society 32: 385-393.

Thorne JL, Kishino H (2002) Divergence time and evolutionary rate estimation with multilocus data. Syst Biol 51(5): 689-702.

Thornton JW, DeSalle R (2000) Gene family evolution and homology: genomics meets phylogenetics. Annu Rev Genomics Hum Genet 1: 41-73.

Tishkoff SA, Reed FA, Ranciaro A, Voight BF, Babbitt CC et al. (2007) Convergent adaptation of human lactase persistence in Africa and Europe. Nat Genet 39(1): 31-40.

Trembly A (1744) Memoires pour srvir a l'histore d'un genre de polypes d'eau douce a brasen forme de cornes. Leyden: J & A Verbeck.

Turner DL, Cepko CL (1987) A common progenitor for neurons and glia persists in rat retina late in development. Nature 328(6126): 131-136.

Turner LM, Hoekstra HE (2006) Adaptive evolution of fertilization proteins within a genus: variation in ZP2 and ZP3 in deer mice (Peromyscus). Mol Biol Evol 23(9): 1656-1669.

Velarde RA, Sauer CD, Walden KK, Fahrbach SE, Robertson HM (2005) Pteropsin: a vertebrate-like non-visual opsin expressed in the honey bee brain. Insect Biochem Mol Biol 35(12): 1367-1377.

Venkatachalam K, Montell C (2007) TRP channels. Annu Rev Biochem 76: 387-417.

Walls GL (1942) The vertebrate eye and its adaptive radiation. Bloomfield Hills, MI: Cranbrook Press.

Ward A, Liu J, Feng Z, Xu XZ (2008) Light-sensitive neurons and channels mediate phototaxis in C. elegans. Nat Neurosci 11(8): 916-922.

Watson GM, Hessinger DA (1989) Cnidocyte mechanoreceptors are tuned to the movements of swimming prey by chemoreceptors. Science 243(4898): 1589-1591.

Watson GM, Hessinger DA (1994) Evidence for calcium channels involved in regulating nematocyst discharge. Comp Biochem Physiol Comp Physiol 107(3): 473-481.

West-Eberhard MJ (2003) Developmental Plasticity and Evolution. Oxford: Oxford University Press. 794 p.

Westfall J (1988) Presumed neuronematocyte synapse and possible pathways controlling discharge of a battery of nematocysts in hydra. In: Hessinger DaL, HM, editor. The Biology of Nematocytes. San Diego: Academic Press. pp. 25-37.

Wetts R, Fraser SE (1988) Multipotent precursors can give rise to all major cell types of the frog retina. Science 239(4844): 1142-1145.

Williams TM, Selegue JE, Werner T, Gompel N, Kopp A et al. (2008) The regulation and evolution of a genetic switch controlling sexually dimorphic traits in Drosophila. Cell 134(4): 610-623.

Womack KB, Gordon SE, He F, Wensel TG, Lu CC et al. (2000) Do phosphatidylinositides modulate vertebrate phototransduction? J Neurosci 20(8): 2792-2799.

Wood R (1998) Survey of the ultastructure of cnidocytes. In: Hessinger DaL, HM, editor. The Biolgoy of the Nematocytes. San Diego: Academic Press.

Yang Z (1997) PAML: a program package for phylogenetic analysis by maximum likelihood. Comput Appl Biosci 13(5): 555-556.

Yang Z, Rannala B (1997) Bayesian phylogenetic inference using DNA sequences: a Markov Chain Monte Carlo Method. Mol Biol Evol 14(7): 717-724.

Yeh RF, Lim LP, Burge CB (2001) Computational inference of homologous gene structures in the human genome. Genome Res 11(5): 803-816.

Yokoyama S (2000) Molecular evolution of vertebrate visual pigments. Prog Retin Eye Res 19(4): 385-419.

Zhang J, Zhang YP, Rosenberg HF (2002) Adaptive evolution of a duplicated pancreatic ribonuclease gene in a leaf-eating monkey. Nat Genet 30(4): 411-415.

Zhang P, Gu Z, Li WH (2003) Different evolutionary patterns between young duplicate genes in the human genome. Genome Biol 4(9): R56.

Zheng Y, Xu D, Gu X (2007) Functional divergence after gene duplication and sequence-structure relationship: a case study of G-protein alpha subunits. J Exp Zoolog B Mol Dev Evol 308(1): 85-96.