The evolution of signalling systems in animals: insights from vision and chemokines

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General Abstract

Try to summarize key findings for the three main chapters..

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# Chapter 1

## General Introduction

### General Introduction

#### The origin of multicellularity: a major evolutionary transition

The astonishing diversity of life on Earth showcases the profound impact of evolution over billions of years. Underpinning the complexity of life forms are major evolutionary transitions, landmark events which have drastically shaped the trajectory of life and paved the way for the rich biodiversity we observe today (Smith and Szathmary 1997). Major evolutionary transitions include for instance the origin of eukaryotes from the merging of an archaeal host and a bacterial endosymbiont (McInerney et al. 2015; Zaremba-Niedzwiedzka et al. 2017; Donoghue et al. 2023) and the emergence of multicellularity (Ruiz-Trillo and Nedelcu 2015). Multicellularity has arisen several times independently in various eukaryotic lineages resulting in a diverse set of complex multicellular organisms, including brown algae, red algae, green algae and land plants, fungi, and animals (Ruiz-Trillo and Nedelcu 2015). The characteristics of the ancestral unicellular eukaryote and the mechanisms driving the emergence of multicellularity vary between lineages and remain subjects of ongoing research (Ruiz-Trillo and Nedelcu 2015).

The origin of animals through multicellularity has seen various hypotheses, each centred around the nature of the unicellular ancestor. This has been recently reviewed by Brunet and King (Brunet and King 2022) and is here summarized. Prior to the establishment of molecular phylogenies, proposed ancestral lineages spanned a range from amoebozoans (Haeckel 1876) to choanoflagellates (Metchnikoff 1886) and ciliates (Saville-Kent 1882). This lack of consensus throughout the 19th and 20th centuries, was amplified by both technical and conceptual limitations. A notable point of contention was the debate over animal monophyly. Some researchers questioned the relatedness of sponges to other animals, postulating the possibility of distinct ancestors for sponges (choanoflagellates) and the remainder of animals (ciliates) (Saville-Kent 1882). Contemporary molecular phylogenies unequivocally support the monophyly of animals and choanoflagellates as their sister group, together forming the clade Choanozoa, within the broader Holozoan clade (Wainright et al. 1993; Lang et al. 2002; Ruiz-Trillo et al. 2008). Choanozoa is corroborated by morphological and biochemical evidence: the collar complex surrounding the flagellum, a defining feature of choanoflagellates, is not only found in sponge choanocytes but across various animals and is composed of cytoskeletal filaments that are homologous among choanoflagellates, sponges, and other animals (Nerrevang and Wingstrand 1970; Lyons 1973; Rieger 1976; Brunet and King 2017; Colgren and Nichols 2020). While the choanoflagellate-like ancestor hypotheses is now the most widely accepted, the specific mechanisms behind the evolution of animals from such an ancestor remain to be clarified. Theories have revolved around the two hypotheses of aggregative and clonal multicellularity, with the latter currently gaining wider acceptance (Brunet and King 2017). However, a recent theory posits that the mutual ancestor of animals and choanoflagellates presented a complex life-cycle, including transitions between amoeboid and flagellate phenotypes, similar to the cell types present in modern sponges (Arendt et al. 2015; Brunet and King 2017; Brunet et al. 2021; Brunet and King 2022). These alternative phenotypes were temporally segregated into different cells in the ancestor, however, following a process of clonal multicellularity these different phenotypes became spatially rather than temporally segregated. This combined with division of labour and innovation lead to the evolution of animals (Brunet and King 2017). The notion that living choanoflagellates present multiple phenotypes including sessile, swimming, and colonial forms, plus the fact that other closely related holozoans such as ichthyosporeans and filastereans also assume diverse cellular forms (Suga and Ruiz-Trillo 2013; Hehenberger et al. 2017; Parra-Acero et al. 2018; Brunet et al. 2019; Parra-Acero et al. 2020; Tikhonenkov, Hehenberger, et al. 2020; Tikhonenkov, Mikhailov, et al. 2020), support this line of investigation, that is currently topic of active research, driven by the emergence of holozoans as model organisms (Booth and King 2022).

#### Expansion of signal transduction systems in animals

Regardless of the precise mechanisms behind the origin of multicellularity in animals, this major transition has had profound implications. Obligate multicellular organisms such as animals must interact with the environment as a whole entity rather than as individual cells and this requires complex mechanisms for internal communication and coordination amongst cells. Consequently, cells must undergo subspecialisations for different tasks, whilst contemporarily maintaining the ability to collaborate with each other (Ruiz-Trillo et al. 2007). Ultimately this paved the way for the vast diversity of animal forms, ranging from relatively simple to extremely complex organisms with intricate systems for self-coordination and interaction with the non-self, such as the nervous and immune systems (Bich et al. 2019; Jékely 2021; Jékely et al. 2021).

From a genetic perspective, we expect the emergence of novel genes to accompany the evolution of animals in response to these new challenges. Indeed, research into genes originating at the stem of metazoa point towards an increase in new genes for nucleic acid binding molecules, transcription factors and molecules involved in cell signalling (Paps and Holland 2018). Cell signalling plays a pivotal role in facilitating biological processes requiring communication amongst cells. Typically, it involves chemical messages or ligands—either endogenous or exogenous—that engage cellular receptors. This activation triggers a sequence of intracellular events, the signal transduction, involving second messengers and various effectors (Foreman et al. 2010). Ultimately, this allows cells to detect and react to extracellular cues either deriving from other cells, like hormones, neurotransmitters, and neuropeptides, or from external stimuli such as light (Elphick et al. 2018; Moroz et al. 2021; Oteiza and Baldwin 2021). There are many different types of receptors that generally fall within the categories of ligand-gated ion channels, enzyme linked receptors, G-protein-coupled receptors (GPCRs) and even intracellular receptors (Foreman et al. 2010). GPCRs, in particular, play a key role in numerous signalling pathways in animals, from neural communication, light reception and other sensory systems and immunity. Given the importance of cell signalling for animals, it is not surprising that one of the categories of gene families that was found to have significant emergence of new genes in the stem of metazoa is signalling molecules (Paps and Holland 2018). Additionally, even when there has not been a *de novo* origin of novel genes, there can be expansions within existing gene families effectively introducing novel genes that are often associated with new functions. This seems to have been the case for GPCR receptors. GPCR signalling is ancient, being present throughout eukaryotes, however, a huge expansion of this gene family occurs in animals. This is not seen neither in close relatives of animals nor in other multicellular organisms (e.g., plants have a comparatively limited set of GPCRs) (de Mendoza et al. 2014). This dramatic increase of GPCRs in animals is likely linked to their heightened need for rapid responsiveness to their environment.

Given the centrality of these receptors in orchestrating myriad biological processes, they have long been a primary subject of research, with a particular focus in deciphering their evolution to gain insights into the fundamental biological processes that they govern (Fredriksson et al. 2003; Foster et al. 2019). Understanding the evolution of these molecules, sheds light on animal evolution, especially during its early stages when critical adaptations were likely to have occurred following the transition to the novel multicellular lifestyle. Similarly, unravelling the evolutionary histories of other molecules involved in GPCR signalling, such as the second messengers and effectors, is also important in understanding the evolution of cell signalling in animals.

### General Aims of the Thesis

During my PhD, I was interested in investigating the evolution of signalling systems in animals. For this, I focused my attention on two different biological processes that rely on signal transduction systems. The first is vision, a widespread phenomenon in animals fundamental for the response to external light stimuli (Land and Nilsson 2012). The second is chemokine signalling, best known for its role in immunity but also involved in other physiological and developmental processes that require internal organismal communication (Murphy 2023). Each presented unique challenges but were both primarily addressed with phylogenetic methods and in some cases with additional bioinformatic approaches such as single cell sequencing analyses. In this short General Introduction, I will delineate the basic background and aims for both systems studied. In the next chapter, General Methods, I will introduce the basics of the methodologies used. Further details about both the background and the methodologies are then provided in the respective chapters.

#### The origin and evolution of vision in animals

Vision is an example of a sensory system that functions through GPCR signalling. It is a quintessential feature of animals, deeply influencing their ecology and behaviour (Nilsson 2009). At its core, vision consists of a photo-sensitive molecule coupled to a signal transduction machinery within a highly specialised photoreceptor cell. The photo-sensitive molecule is an opsin, a GPCR of class A, bound to a derivative of vitamin A, the retinal (Terakita 2005). When the retinal is hit by light it changes conformation (from 11-cis to all-trans), inducing a structural change of the opsin which in turn triggers the G alpha protein it is coupled with activating a signal transduction pathway called phototransduction. There are two major types of phototransduction, rhabdomeric and ciliary, depending on the type of opsins that initiate them, but both culminate in the modulation of ion channels initiating electrical signalling of the photoreceptor cell (Hardie and Juusola 2015; Lamb 2020).

Photoreceptor cells (PRCs) are classified based on the type of opsins and phototransduction pathway employed (Arendt 2003). A general peculiarity of PRCs is the enlargement and folding of the membrane surface to increase the area with the photopigment and therefore enhance light sensitivity. This characteristic membrane folding is present within the cilia of ciliary PRCs of vertebrates, while in rhabdomeric PRCs of insects such as *Drosophila melanogaster* this folding is in the apical surface of the cell forming the rhabdomere (Arendt 2003). Historically, these morphological differences dictated PRC classification. It was believed that rhabdomeric PRCs were characteristic of the protostome (e.g. insects) lineage of Bilateria, while ciliary PRCs were specific to deuterostomes, including vertebrates (Eakin 1979). However, it is now known that ciliary PRCs are present within protostomes (Arendt et al. 2004; Passamaneck et al. 2011; von Döhren and Bartolomaeus 2018) and rhabdomeric PRCs within deuterostomes (Hattar et al. 2002; Ullrich-Lüter et al. 2011). Therefore, molecular definitions offer a more accurate classification, especially for non-bilaterian animals. While complex visual structures, such as eyes, are believed to have evolved independently on multiple occasions (Land and Nilsson 2012; Picciani et al. 2018), their fundamental units—photoreceptor cells—stem from a limited number of subtypes that may share a common ancestral cell type. This suggests that, despite variations in phototransduction machinery, there may exist a core set of regulatory genes defining this broad cell type (Arendt 2008; Arendt et al. 2016), consistent across all animal photoreceptor cells.

Beyond the phototransduction machinery and photoreceptor cells, vision encompasses another layer of molecular complexity. After the retinal is isomerized from it cis to its trans state by light, it must return to its cis state in order to be receptive to new light stimuli. This recycling occurs through a series of enzymatic reactions occurring as part of the retinol metabolism (Palczewski and Kiser 2020). As the opsin alone cannot carry out the visual function, this pathway that allows constant replenishment of the cis-retinal is just as essential part of the molecular assembly of vision.

Photoreceptor cells are present even in some early-branching animals, such as cnidarians (Nordström et al. 2003; Kozmik et al. 2008; Picciani et al. 2018) and potentially ctenophores (Horridge 1964; Jékely et al. 2015; Tamm 2016), suggesting that vision must have originated early in animal evolution. Some molecular components underpinning it, such as core signal transduction elements, likely trace back more anciently, while others, such as the regulatory genes involved in photoreceptor cell identity, may be animal innovations. Unravelling the evolutionary history of all these molecular players, identifying key innovations and major family expansions, can not only elucidate the emergence of vision but also enrich our understanding of animal evolution more broadly.

Numerous studies have delved into the evolution of opsins, illuminating the vast diversity of these molecules across animals, including non-bilaterians (Feuda et al. 2012; Feuda et al. 2014; D’Aniello et al. 2015; Roberts et al. 2022; De Vivo et al. 2023; McCulloch et al. 2023). Such research has led to significant discoveries, including the identification of phylogenetically related placopsins in placozoans, a non-bilaterian phylum lacking neurons (Feuda et al. 2012). Nevertheless, comprehensive investigations into the evolution of all molecular components involved in vision remain sparse.

Thus, one of my PhD goals was to fill in some of these gaps by investigating the evolution of the complex molecular assembly of vision. For this, I identified two main aims:

##### Aim 1: Reconstructing the evolution of the molecular components of photoreceptor cells.

The first aim is to understand the evolution of the molecular setup of photoreceptor cells, including both the phototransduction machinery and the regulatory toolkit that define the cell type. The objectives of aim 1 are addressed in Chapter 3.

##### Aim 2: Reconstructing the evolution of the retinol metabolism.

The second major aim is to investigate the evolution of the retinol metabolism that includes enzymes involved in the recovery of the cis-retinal, discerning whether specific components may have undergone distinct evolutionary events in animals. Aim 2 is addressed in Chapter 4.

#### Evolution and molecular diversity of chemokine signalling systems.

The immune system exemplifies an organism-wide system necessitating cellular coordination to detect and counteract external invaders. Present across the animal kingdom, immune systems function through an intricate range of subsystems (Yuan et al. 2014). In vertebrates, the chemokine signalling system plays a fundamental role in both innate and adaptive immunity (Murphy 2023). Best known for the chemoattraction of leukocytes during host defence (Wong and Fish 2003; Blanchet et al. 2012); chemokine signalling is also implicated in homeostasis, development (Zlotnik and Yoshie 2000; Tran and Miller 2003; López-Cotarelo et al. 2017), and neuronal communication (Tran and Miller 2003; de Haas et al. 2007; Rostène et al. 2007). Failure of the system can lead to various diseases (Tran and Miller 2003; Blanchet et al. 2012), including cancer (Nagarsheth et al. 2017).

The chemokine system comprises two primary components: the chemokine ligands, small cytokines possessing chemotactic attributes, and the chemokine receptors, Class A GPCRs. Canonical chemokine ligands possess in their N-terminal portion characteristic cysteine patterns that can be used to classify them into subgroups. Canonical chemokine receptors are in turn classified based on the type of ligands they respond to, although there tends to be a high degree of promiscuity in the system (Zlotnik and Yoshie 2000; Nomiyama et al. 2011). Additionally, several other molecules have been implicated in the system. For example, ligands bearing varying degrees of sequence similarity to canonical chemokines have been found to activate some canonical receptors and/or have chemotactic properties (Zhang et al. 2018). Conversely, some so-called atypical chemokine receptors can bind canonical ligands, but do not trigger the signal transduction pathway necessary for chemokine function (Bonecchi and Graham 2016; Chen et al. 2018). Therefore, these additional players can be considered as “non-canonical” chemokine components. Yet, their relationship with the canonical components and amongst each other is unclear, hampering our understanding of the origin and evolution of the system. Applying evolutionary approaches can aid in clarifying the relatedness of these molecules and ultimately help clarify the evolution of the whole system. Thus, the second goal of my PhD was to explore the evolution of the chemokine system including both its canonical and non-canonical components. For this, three main aims were identified:

##### Aim 1: Uncovering the relationships among canonical and non-canonical components.

The first aim was to investigate the evolutionary relationships among all chemokine ligands and amongst all receptors, including all known canonical and non-canonical molecules. This served as a first step for subsequent phylogenetic analyses.

##### Aim 2: Reconstructing the evolution of all canonical and non-canonical ligands.

The following aim was to perform phylogenetic analyses for each ligand family identified to discern their evolution in animals.

##### Aim 3: Reconstructing the evolution of all canonical and non-canonical receptors.

Similarly, the last aim was to understand the evolutionary history of receptor groups.

The work addressing these aims is detailed in Chapter 5 and was carried out in collaboration with other members of the Feuda Group.

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