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Sensing in Nature

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PREFACE

Rose, thou art sick!
The invisible worm
That flies in the night,
In the howling storm,

Has found out thy bed
Of crimson joy:
And his dark secret love
Does thy life destroy.

—William Blake (1794)

All organisms ranging from the simplest unicellular form to the most advanced multicellular organism possess the capability to detect different signals in their surroundings. Cells live in a complex environment and can sense light, physical parameters, chemical cues and biological signalling molecules from other cells. They also receive information about the internal state of the cell. It seems that evolution selected internal representations that symbolize states that are more important for cell survival and growth. Discrimination between self and externally generated signals is very important for extracting relevant information of the environment for survival. The adequate sensor must be both specific and sensitive. Sensory cell, organs and evolutionary mechanisms that detect a variety of stimuli as single-photons, single molecules, temperature changes, small fluctuations of electromagnetic fields, etc., have been described in different organisms.

Responses to extracellular changes directly confer survival fitness by means of complex regulatory networks. Despite their complexity, the networks must be evolvable because of changing ecological and environmental pressures. Signal transduction networks are designed to rapidly respond to changes in the environment and may utilize multilayer receptors located in the cell membrane to perform computations on numerous input stimuli. For instance, it has been described that some bacteria possess more than

100 different sensors used to form a picture of their environment. These sensors can evaluate relevant environmental parameters such as the presence of nutrient substrates, oxygen, temperature, light and gradients of chemical stimuli as well (chemotaxis). Thus, any given chemoreceptor cell can have a combination of receptors, each of which may respond to different chemical molecules.

In 1995, John Maynard Smith tried to explain in his book *The Major Transition in Evolution* how structural complexity and evolutionary novelties are associated with adaptive radiations in new ecological territories. New structures (sensor organs) require the evolution of new developmental programs. Therefore, to understand the origin of the morphological novelties, we must look to the genetic control of development. One of the most important biological discoveries of the past two decades is that most animals share specific families of genes that regulate major aspects of body patterns. In several instances, shared aspects of development and regulatory gene expression reflect the evolution of pre-existing ancestral structures. Cell signalling pathways are constructed from a limited number of component types that rely upon a small number of discrete mechanisms of action. The discovery of this universal genetic toolkit for an animal's development has had important impacts. Evolution appears to have converged on the same network motifs on different systems, suggesting that they were selected because of their functions. We can extend these to the evolution of sensor systems. One example is the basic mechanism underlying chemoreception and the interaction of a chemical stimulus with membrane cell receptors. In fact the primary visual sensors for insects and vertebrate are G-protein-coupled receptors (GPCRs) expressed by sensory receptor cells that initiate intracellular signal transduction cascades in response to appropriate stimuli. Furthermore, taste and smell are mediated in part by similar receptors. The identification of sensory GPCRs and their related downstream transduction components from a variety of species have provided an essential tool for understanding the molecular evolution of sensory systems.

The ability of animals to distinguish such a large diversity of natural chemical stimuli resides in the ability of the central nervous system to recognize the signalling patterns of large groups of cells**.** In addition to the development of sense organs, one outstanding achievement during evolution has been its integration with the rest of the information flow in the central nervous system to guide appropriate responses in terms of motor outputs. Scientists think neurons and synapses first appeared on Earth more than 600 million years ago in *cnidarians.* The nervous system, similar to the immune system, consists of complex networks that have been known to be closely interrelated, sharing mechanisms of gene regulation, signalling and cell communication. Arranged in circuits, neurons open up new behavioural possibilities for an animal. Electrical conduction via axons is faster and more precise than the diffusion of chemical signals, enabling quick detection and a coordinated response to threats and opportunities.

Cephalization is the process in animals by which nervous and sensory tissues become concentrated in the "head." Centralized nervous systems must have originated multiple times in multiple bilaterian lineages. The Neocortex is an important novelty of the mammalian brain that has been enlarged in primates evolution and is characterized by new functions, including those of cortical networks devoted to vision and motor processing. In humans, the neocortex occupies 80% of the volume of the brain. The fundamental future challenge is to decipher the neural wiring (connectome) diagram associated with complex behaviors and functions as perception, emotions and *self-knowledge.*

Biological systems are an emerging discipline that may provide integrative tools by assembling the hierarchy of interactions among genes, proteins and molecular networks involved in sensory systems. The aim of this volume is to provide a picture, as complete as possible, of the current state of knowledge of sensory systems in nature. The presentation in this book lies at the intersection of evolutionary biology, cell and molecular biology, physiology and genetics. *Sensing in Nature* is written by a distinguished panel of specialists and is intended to be read by biologists, students, scientific investigators and the medical community.

We are truly grateful to all of the authors for their expertise contribution.

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CHAPTER 1

THERMOSENSORSY STEMS IN EUBACTERIA

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Abstract: Four different mechanisms have evolved in eubacteria to comply with changes in the environmental temperature. The underlying genetic mechanisms regulate gene expression at transcriptional, translational and posttranslational level. The high temperature response (HTR) is a reaction on increases in temperature and is mainly used by pathogenic bacteria when they enter their mammalian host. The temperature of 37°C causes induction of the virulent genes the products of which are only needed in this environment. The heat shock response (HSR) is induced by any sudden increase in temperature, allows the bacterial cell to adapt to this environmental stress factor and is shut off after adaptation. In a similar way the low temperature response (LTR) is a reaction to a new environment and leads to the constant expression of appropriate genes. In contrast, the cold shock response (CSR) includes turn off of the cold shock genes after adaptation to the low temperature. Sensors of temperature changes are specific DNA regions, RNA molecules or proteins and conformational changes have been identified as a common motif.

INTRODUCTION

In their natural environment, bacteria are exposed to a variety of environmental insults including sudden changes in osmolarity, in external pH, reactive oxygen species, limitations in nutrient supply and up- and downshifts in temperature.[1] Each stressful situation typically induces a stress response resulting in a characteristic change in the pattern of gene expression. This stress response helps the bacterial cells to restore cellular homeostasis, to protect vital processes and to increase the cellular resistance against subsequent stronger similar stress challenges.

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The habitat niches on earth vary considerably in temperature and therefore, many biological processes are optimized for different temperatures and the physiology of organisms are adapted to their cognate environments. Additionally, the particular niche or lifestyle of many bacteria may be subjected to regular, but sudden, variations in temperature. This reasoning applies for bacteria adjusting their activities according to seasonal variations and certainly for pathogens that circulate between the environment and warm-blooded hosts. Thus, temperature regulation of genes has been the focus of much research and how the temperature signal is sensed and transduced to the biosynthesis machinery has been studied extensively. Here, four different temperature-dependent regulation mechanisms can be distinguished, the heat shock response (HSR), the high temperature response (HTR), the cold-shock response (CSR) and the low-temperature response (LTR). While the first two recognize sudden increases in temperature, the other two respond to a sudden decrease. Furthermore, the heat- and cold-shock responses are transient and include a shut-off after adaptation has occurred even if cells are still exposed to the high or low temperature. The high and low temperature responses are constitutive and persist as long as the bacterial cells are exposed to that temperature. The high temperature response plays an important role for pathogenic bacteria to recognize their mammalian host, where exposure to 37°C induces the virulence genes, which are not needed outside this environmental niche. All four responses are based on genetic programs, which consist of three major steps:

1. Registration of the stress factor by a sensor molecule.
2. The sensor molecule directly or indirectly leads to the induction of a subset of genes called stress genes specific for the inducing stress factor.
3. In the case of a heat- or cold-shock response, expression of the stress genes is reduced after adaptation through a feedback inhibition loop.

How does the sensor register changes in the environmental temperature? Since temperature changes can affect the conformation of virtually any biomolecule, the underlying principle of temperature sensing is based on such conformational changes. Three different thermosensory biomolecules have been described so far: DNA, RNA and proteins. The purpose of this chapter is to describe how these three thermosensors sense temperature changes, thus controlling gene expression at the transcriptional, translational and posttranslational level. Several recent review articles have dealt with one or the other aspect of bacterial thermosensors.[2-6]

DNA ACTING AS THERMOSENSOR

Three different principles have been described involving DNA as thermosensor: DNA supercoiling, promoter-curvature and nucleoid-associated proteins.

DNA Supercoiling

Plasmids from mesophilic and hyper-thermophilic bacteria can undergo a reversible change in their supercoiling level depending on the temperature.[7] A heat shock introduces a transient increase in positive supercoiling leading to plasmid relaxation mediated by DNA gyrase and topoisomerase I.[8] Recovery to the normal supercoiling level is observed within 10 min after the heat shock and is dependent on DNA gyrase, the nucleoid-binding

protein HU and the molecular chaperone DnaK.[7] On the contrary, a cold-shock decreases plasmid supercoiling and recovery to the original supercoiling level occurs after about 60 min and may involve DNA gyrase and the HU protein.[9] Since transcription efficiency can be influenced by the DNA topology,[10] the level of DNA supercoiling acts as an important parameter in temperature-dependent gene regulation.

Promoter-Curvature

Another important DNA element being able to respond to temperature changes are intrinsic bends. It has been shown that intrinsically curved DNA regions characterized by AT-tracts[11] located upstream of a promoter influence binding of the RNA polymerase.[12] Temperature-induced changes in the topology within these regions directly influence gene expression. One example is the *plc* gene of *Clostridium perfringens* coding for phospholipase C. At low temperature, the altered curvature upstream of its promoter leads to the induction of *plc*. Here, low temperature increases the bending of the AT-tracts thus enhancing the binding affinity for the RNA polymerase.[13,14]

Shigella flexneri is a facultative intracellular pathogen and some genes required for pathogenicity are located within a 31 kb region of the 230 kb plasmid pINV.[15,16] *Shigella* cells are able to penetrate into and replicate within human colonic epithelial cells. Both chromosomal virulence (*vir*) genes and the plasmid pINV are involved in expression of the pathogenicity phenotype in *S. flexneri*.[17] Expression of the invasive phenotype is regulated by the growth temperature.[18] Bacteria growing at 37°C are virulent and able to invade epithelial cells, whereas the same cells are non-invasive when grown at 30°C. Using the method of transposon mutagenesis, a gene has been identified being responsible for the growth-dependent phenotype. When inactivated, cells become virulent even at the low temperature.[19] This gene codes for the H-NS (**h**eat-stable **n**ucleoid-**s**tructuring) protein and silences expression of *virF* coding for a transcriptional activator, which in turn triggers a regulatory cascade involving the activation of other regulatory genes.

At the *virF* promoter, H-NS binds to two sites separated by a region of DNA curvature. Binding to these regions occurs co-operatively at temperatures below 32°C but not at 37°C and bent DNA might act as a sensor of temperature.[20] Experiments have revealed that the intrinsic bent located between the two H-NS binding sites melts abruptly at around 32° allowing the formation of a productive transcription complex[21] (Fig. 1A). Taken together, all experimental data support the hypothesis that the curved DNA tract within the *virF* promoter acts as a thermosensor.

Nucleoid-Associated Proteins

Nucleoid-associated proteins exert genome structuring functions in bacteria. Binding of these proteins to DNA does not only influence its conformation, but also DNA replication, recombination and transcription.[22,23] The best characterized nucleoid-associated protein present in different enteric bacteria is H-NS, which serves as the paradigm of a globular modulator exerting its effect, mostly negative termed silencing, in response to different environmental signals including temperature.[23] H-NS prefers AT-rich sequences and is itself subject to temperature control. While formation of higher-order oligomers and the DNA-binding capacity are reduced at 37°C,[24] the H-NS to DNA ratio increases three- to four-fold during growth at low temperature.[25] Temperature-modulated accessibility of promoter regions occupied by H-NS at low temperature plays a key role of virulence gene

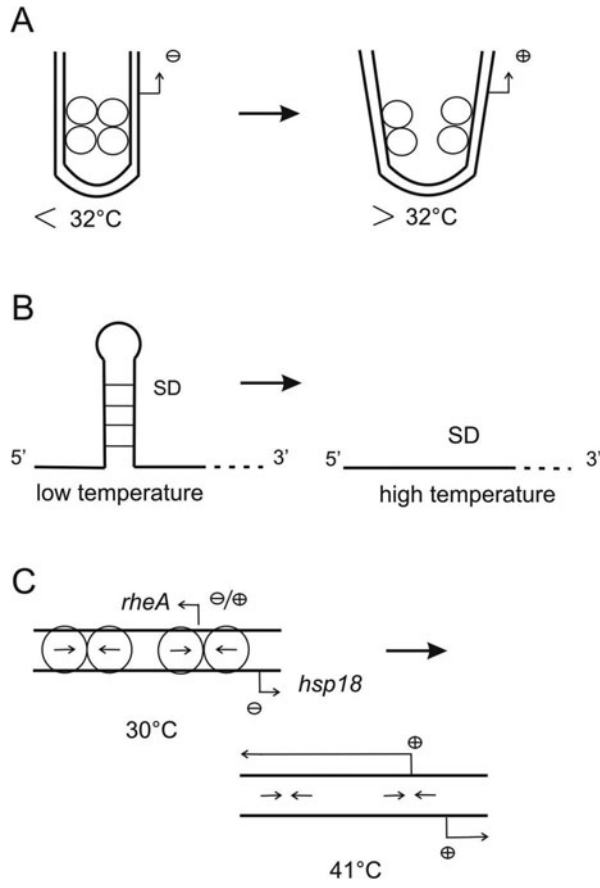

Figure 1. A) Histone-like proteins such as H-NS bind to two different sites on a chromosome or plasmid. At temperatures below 32°C, the DNA is bent in such a way to allow interaction between the two complexes and thus prevent binding of RNA polymerase. At temperatures above 32°C, the bend is reduced in such a way that the two complexes loose interaction and the RNA polymerase can now access the promoter and start transcription. B) At low temperature, the mRNA forms a stem-loop structure sequestering the SD sequence and the ribosomes do not recognize the SD sequence. High temperature will lead to melting of the stem-loop structure allowing access of the ribosomes to the SD sequence. C) At 30°C, the RheA repressor of *Streptomyces albus* binds to two sites as a homodimer thereby preventing expression of the gene *hsp18* coding for a small heat shock protein and regulating its own expression. At 41°C, the repressor undergoes a conformational change causing its dissociation from both sites leading to increased production of the RheA protein itself and of transcription of the *hsp18* gene.

expression in many human pathogens, like *E. coli*, *Salmonella* and *Shigella flexneri*.[26-29] This will be illustrated by two different examples.

Pap pili, encoded by the **p**yelonephritis-**a**ssociated **p**ili (*pap*) operon, are expressed by uropathogenic *E. coli* cells and facilitate the attachment to uroepithelial cells and subsequent colonization of the host upper urinary tract. Pap pili transcription is regulated in response to the growth temperature.[30] Optimal expression occurs at 37°C, with a 52-fold reduction in *papBA* transcription at 23°C[31] and this regulation occurs at the level of transcription.[32] Two proteins have been identified to play an important role in the regulation of transcription of

the *papBA* operon, H-NS and RimJ. H-NS prevents transcription at the low temperature[33,34] by binding within the *pap* regulatory region at 23°C but not at 37°C.[31] RimJ is an N-terminal acetyltransferase of the ribosomal protein S5[35] and deletion of the *rimJ* gene leads to a loss of thermoregulation resulting in equivalent *papBA* transcript levels at both 37°C and 23°C.[36] The mechanism by which RimJ represses *papBA* transcription is unknown.

One of the major virulence factors in *Salmonella enterica* is a Type III secretion system (T3SS) encoded in the *Salmonella* pathogenicity island 2 (SPI-2). This horizontally acquired genomic island contains genes whose products activate and assemble the T3SS that is required during intracellular infection and that injects into host cells the effector proteins required for intracellular survival.[37,38] Cells grown at 30°C or lower have been shown to be unable to express the T3SS. Here, virulence gene expression is controlled by Hha and H-NS, two nucleoid proteins silencing the virulence genes at temperatures below 30°C.[39] While H-NS silences expression of the response regulator SsrR, which activates a set of genes responsible for the host infection, Hha silences the SPI-2 gene transcription.

RNA ACTING AS THERMOSENSOR

RNA thermometers have evolved to sense and transduce ambient temperature signals to the translation machinery and most of them are located in the 5′-untranslated region (UTR) of bacterial heat shock and virulence genes (*cis*-acting RNA thermometers), while a few described so far act in *trans* through a small RNA interacting with the appropriate mRNA. At low temperature, the Shine-Dalgarno (SD-) sequence is trapped in a hairpin structure and increasing temperature destabilizes that structure in such a way that the SD-sequence becomes available to the ribosomes allowing translation initiation (Fig. 1B). RNA thermosensors register even subtle changes in temperature and adjust gene expression accordingly. All known RNA thermometers control translation. They control several responses such as the HSR.[40-43]

RNA Thermometer and the HSR

The alternative sigma factor σ32 acts as a key regulator of the HSR in *E. coli*.[44] While at low temperature, cells contain very little sigma-32 (10-30 molecules at 30°C), 5 min after a temperature upshift to 42°C, the amount of σ32 increases about 15-fold. This dramatic increase results from both changes in the stability (will be discussed later) and synthesis of σ32, where synthesis is regulated at the level of mRNA. At lower temperatures, the *rpoH* mRNA is folded into a secondary structure that occludes the SD-sequence and the initiation codon. Here, almost the entire secondary region of the transcript is located in the coding region and not in the 5′-UTR. Two segments called A and B form an extensive RNA secondary structure thus blocking entry of the ribosomes to the SD-sequence. Exposure of cells to the high temperature disrupts the secondary structure and liberates the SD-sequence.[40]

Another RNA thermosensor called ROSE (for **r**epression **o**f heat **s**hock gene **e**xpression) element was discovered in *Bradyrhizobium japonicum*[45] and has been described later in different *Rhizobium* species and in *Agrobacterium tumefaciens*.[41,46] All ROSE elements are located in the 5′-UTR transcripts coding for small heat shock genes, are 70-120 nucleotides long, acquire a complex structure comprising 2-4 stem loops, where the 3′-proximal hairpin contains the SD-sequence and in some cases the AUG start

codon as well. Short internal loops and bulges in the computer-predicted final structure are assumed to create a thermolabile structure that melts at increasing temperatures.

A third RNA thermometer is the fourU element. This unusually short thermosensor consists of only 52 nucleotides folding in two hairpins. It was initially described controlling expression of the small heat shock gene *agsA* in *Salmonella*.[47] It consists of two hairpins, where hairpin I might play a structural role during cotranscriptional folding and hairpin II is blocked by a consecutive stretch of four uridine residues used to base-pair with the SD-sequence. Temperature-dependent opening of hairpin II allows binding of the ribosomes to the SD-sequence. A similar structure of four U residues that pair with the SD-sequence has been predicted upstream of the *lcrF* gene in *Y. pestis*.[48] This gene codes for a transcription factor, which is responsible for inducing the expression of plasmid-encoded virulence genes in response to temperature.

RNA Thermometers and the CSR

In *E. coli* and other bacteria, the expression of cold shock genes becomes specifically enhanced or induced de novo during the growth lag following a temperature-downshock from 37°C to 15°C.[49] One of the cold shock genes, *cspA*, codes for the major cold shock protein CspA.[50] CspA and its homologues destabilize secondary structures in both RNA and DNA and are therefore referred to as nucleic acid chaperones.[51] While the *cspA* transcript is unstable at 37°C with a half-life of about 10 sec,[52] it becomes highly stable upon a shift to 15°C. Three-base substitutions around the SD-sequence in the 159-bp 5′-UTR region stabilize the transcript 150-fold, resulting in constitutive expression of *cspA* at 37°C. It has been suggested that at 37°C, the *cspA* transcript adopts a secondary structure which is recognized by RNase E, while it folds into a different secondary structure at 25°C not recognized by this endoribonuclease.[53] Taken together, the *cspA* RNA serves as a cold-shock sensor.

A completely different mechanism has been suggested for cold shock induction of the *pnp* gene of *E. coli* coding for a 3′ to 5′ exonuclease. A more than 10-fold increase in the amount of the *pnp* transcript has been described to occur within the first hour upon a cold shock.[54] While at 37°C only the monocistronic *pnp* transcript is present, a bicistronic mRNA including the coding region of the downstream gene *deaD* encoding a DEAD-box RNA helicase predominates. A Rho-dependent termination site present within the coding region of *pnp* is suppressed upon a cold shock.

In *Borrelia burgdorferi*, the causative agent of Lyme disease, the alternative sigma factor σS plays a central role in the regulation of virulence-associated major outer surface proteins. Translation of the *rpoS* mRNA is stimulated at 37°C by the small DrsA RNA. At 23°C, this noncoding RNA folds into a stable secondary structure, which does not allow base-pairing with the *rpoS* mRNA. It has been suggested that the higher temperature leads to melting of the secondary structure of the DsrA RNA, which is now able to interact specifically with the anti-SD sequence of the *rpoS* transcript. This in turn would stimulate ribosome interaction with the SD-sequence under virulence conditions.[55]

RNA and the LTR

Bacteriophage λ belongs to the group of temperate phages, which have to make a decision whether to enter the lytic or the lysogenic pathway about 10 min after infection. Here, the gene cIII product plays an important role in this decision. It does so by binding

to the ATP-dependent FtsH protease, which degrades the cII protein, a transcriptional activator of central importance in the lysogenic pathway.[56,57] High concentrations of cIII promote stabilization of cII thus favouring lysogeny. Two alternative structures of the cIII transcript were first predicted and later verified by structure probing in vitro and in vivo.[43] While one secondary structure sequesters part of the SD-sequence and the start codon, the alternative structures leaves the translation initiation region accessible to the ribosomes to allow translation of cIII. The equilibrium between both structures is temperature-dependent. At high temperature (45°C), the start codon and the SD-sequence are sequestered in a hairpin structure largely preventing synthesis of cIII. This in turn leads to a degradation of cII and the lytic cycle is initiated by these bacterial cells. Under physiological temperature (37°C), the equilibrium is shifted toward the alternative secondary structure in which the ribosome binding site become available leading to the synthesis of cIII followed by initiation of the lysogenic pathway. In the present case, the *cis*-acting RNA thermometer switches on translation with decreasing temperature and does not operate by gradual melting of the secondary structure as in the case of the *rpoH* mRNA. It alternates between two mutually exclusive conformations. What might be the biological reason for temperature control of cIII translation? Phage λ tends to enter the lytic cycle when the host cells are healthy and a sufficient amount of nutrients is available. On the contrary, if the growth conditions are poor, it prefers to integrate its genome into the host chromosome. But under life-threatening conditions such as a severe heat shock (45°C), it might be beneficial for the phage to escape from the host cells.

The small DsrA RNA is an example for a *trans*-acting RNA thermosensor by controlling translation of the *E. coli rpoS* mRNA. In *E. coli* the *rpoS* gene codes for the general stress sigma factor RpoS (σS), the expression of which is controlled at the levels of transcription, translation and protein stability. The amount of active RpoS is adjusted in response to various environmental signals and each step of *rpoS* expression can be affected by one or several environmental stimuli.[58] One of the environmental cues that increase translation of the *rpoS* transcript is low temperature (below 37°C). Here, the small RNA DrsA plays an important role.[59] This *trans*-acting RNA pairs with the leader region of the *rpoS* mRNA to allow a more efficient translation.[60] Temperature affects both the rate of transcription initiation of the *dsrA* gene and the stability of its transcript.[61] The net effect is a 25-fold decrease in full-length *dsrA* transcript at 37°C compared to 25°C. What mechanism is responsible for temperature regulation at the *dsrA* promoter? It could be shown that the sequence of the −10 element and the spacer region are essential elements for the thermal response of the *dsrA* promoter.[62]

PROTEINS ACTING AS THERMOSENSOR

Protein-based thermosensors can either involve temperature-dependent changes in the conformation of the protein itself or in assembly of protein complexes consisting either of identical or different subunits. Protein sensors described so far include transcriptional and translational regulators, molecular chaperones and proteases.

Protein Thermosensors and the HTR

TlpA was the first documented case of a temperature-sensing gene regulator and was presumed to be an ideal sensor of environmental signals. The TlpA protein is encoded

by the 96 kb pSLT virulence plasmid of *Salmonella enterica*[63] and characterized by a remarkable long α-helical coiled-coil motif.[64] The N-terminus of TlpA is a sequence specific DNA-binding domain acting as an autoregulatory repressor. TlpA is present in a temperature-dependent two-state equilibrium, between unfolded monomers and highly α-helical coiled-coil oligomers. At physiological temperatures transcription of *tlpA* is low by the repressing activity of TlpA, which in its dimeric and folded coil-coiled conformation is able to bind to the *tlpA* operator. Elevated temperature leads to a shift in the equilibrium that favours the nonfunctional unfolded monomeric form resulting in increased transcription.[64-66] The function of TlpA is unknown, but it does not seem to play a role in the pathogenicity of *Salmonella* per se, but imply an alternative function which is not directly involved in the virulence of *Salmonella*.[67] It might negatively regulate genes to be identified.

The second example of a temperature-sensing autorepressor is the RheA protein identified in *Streptomyces albus*.[68] It negatively regulates expression of *hsp18* coding for a small HSP. While the RheA repressor reduces transcription of its own gene and prevents that of *hsp18* at 30°C, transcription occurs at 41°C (Fig. 1C). Circular dichroism spectroscopy revealed a temperature-dependent transition between an active and an inactive form of RheA.[69]

The *ymoA* gene codes for a small histone-like protein and is involved in thermoregulation of the Type III secretion system (T3SS) of *Yersinia pestis*, which is needed at 37°C, the host temperature, but not at low temperatures. The YmoA protein is highly stable at low temperature and unstable at 37°C. At that temperature, it will be degraded predominantly by the Lon protease and ClpXP acting as a backup system (if Lon is deficient).[70] Since the Lon protease is present and active at all temperatures, degradation might include a conformational change in YmoA at 37°C thus increasing its susceptibility to Lon or ClpXP degradation. Alternatively, an accessory protein might be induced or become activated at 37°C that modifies or targets YmoA for degradation.

Bordetella pertussis, the etiological agent of whooping cough, uses a two-component system comprised of the sensor kinase BvgS and the response regulator BvgA to control expression of virulence genes.[71] Temperature plays an important role in activation of BvgA and may be modulated by sulphate ions and nicotinic acid. Following induction of *bvgAS* at the mammalian body temperature of 37°C, phosphorylation by BvgS allows BvgA binding to promoter regions of virulence genes, such as the adhesin *fimX*.[72] It has been suggested that the transmembrane domain of BvgS senses temperature changes.

The most evolved temperature-sensing protein is HtrA (for **h**igh **t**emperature **r**equirement) of *E. coli* and also called DegP. This protein was initially identified in *E. coli* as a serine protease belonging to the trypsin clan SA.[73] SA proteases are characterised by a two-domain structure with each domain forming a six-stranded β barrel. The functional unit of HtrA appears to be a trimer forming a funnel-like shape with the proteolytic domain located at its top and the two PDZ domains protruding to the outside. The PDZ domains are highly mobile swinging around to capture substrate proteins and preferentially bind to the C-terminal 3-4 residues of their target proteins. When digestion of β-casein is followed, almost no proteolytic activity is detected below 20°C. At temperatures above 30°C, the proteolytic activity rapidly increases in a nonlinear fashion.[74] As a chaperone, HtrA was shown to refold periplasmic amylase MalS and the artificial substrate citrate synthase. As a protease, HtrA processively degrades misfolded proteins into peptides of defined size by employing a molecular ruler comprised of the PDZ domain 1 and the proteolytic site.[75] In a first step, the C-terminus of an unstructured protein is bound to PDZ domain 1.

In a second step, the first proteolytic cut is introduced by a neighbouring proteolytic site yielding the first product. Next, PDZ domain 1 binds to the new C-terminal end of the remaining substrate and performs a second cut about 12-17 residues into the substrate. This process is repeated until the substrate protein is completely digested.

Protein Thermosensors and the HSR

Two different classes of proteins have been described so far acting as thermosensors upon a sudden heat shock, molecular chaperones and proteases acting at the level of activity and stability, respectively.

One example is the already mentioned σ32 of *E. coli*. Besides being regulated at the level of translation, the sigma factor itself is controlled at the level of activity by DnaK and DnaJ and furthermore at the level of stability by the ATP-dependent metalloprotease FtsH.[76] It has been observed that σ32 is highly unstable at 30°C with a half-life of ~1 min. After a heat shock, σ32 is transiently stabilized with a half-live of ~5 min. Why the sigma factor is unstable at low temperature and by which mechanism it becomes transiently stabilized after a heat shock? Recently, two distinct sites in σ32 have been identified as binding sites for DnaK and DnaJ. DnaJ binding destabilizes a distant region of σ32 in close spatial vicinity of the DnaK-binding site and DnaK destabilizes a region in the N-terminal domain. These conformational changes in the native protein convert it into a substrate for the FtsH protease.[77]

The second example is the HrcA-GroE system of *Bacillus subtilis*. Here, the GroEL chaperone modulates the activity of the HrcA repressor protein. This regulatory protein controls expression of the heptacistronic *dnaK* and the bicistronic *groE* operon[78,79] by binding to an operator called CIRCE (for **c**ontrolling **i**nverted **r**epeat of **c**haperone **e**xpression).[80] It has been suggested that HrcA is present in two conformations, an active and an inactive one and the equilibrium between these two conformers is modulated by GroEL, which shifts this equilibrium toward the active conformation. This model is supported by three sets of experimental data: (1) Whereas an increase in the amount of GroEL reduced the basal level of the proteins encoded by the two operons, a decrease resulted in an increase. (2) In a bandshift assay, purified HrcA retarded more DNA in the presence of GroEL. (3) GroEL specifically binds to immobilized HrcA.[81,82] Based on these observations, the following model has been developed. Both, HrcA synthesized de novo and dissociated from its operator is present in the inactive conformation and interaction with GroEL converts it in its active conformation. After a heat shock, GroEL is titrated by nonnative proteins, leaving HrcA inactive thus leading to the induction of the *dnaK* and *groE* operons. The more nonnative proteins have been removed, the more GroEL will become available to take care of HrcA resulting in a gradual turn-off of the heat shock response.

The third example is the HspR-DnaK system of *Streptomyces coelicolor*. Here, the *dnaK* operon consists of the four genes *dnaK*, *grpE*, *dnaJ* and *hspR*, where *hspR* codes for a repressor protein of its own operon (and some other genes) binding to an operator designated HAIR (for **H**spR-**a**ssociated **i**nverted **r**epeat).[83] Here, the activity of the HspR protein is modulated by the DnaK chaperone.[84] This conclusion is based on four different observations: (1) In a band shift assay, HspR is active only in the presence of DnaK and this activity does not need neither DnaJ nor GrpE. (2) Addition of anti-DnaK monoclonal antibodies to the retarded complex produced a supershift, proving that DnaK is part of the DNA-binding complex. (3) HspR copurified with DnaK in column chromatography.

(4) Induction of the DnaK operon is partially decreased in the presence of overproduced DnaK. Based on these results, it has been suggested that DnaK acts as a transcriptional corepressor by directly binding to HspR at its operator site and by activating HspR or keeping it in its active form. As suggested for HrcA and GroEL, the appearance of nonnative proteins after a heat shock will titrate DnaK leading to derepression of the operon.[84]

So far, only one system has been described where a protease acts as a thermosensor. This protease, DegS, is anchored in the inner membrane of *E. coli* cells facing the periplasmic space. It consists of an N-terminal transmembrane domain followed by a central protease domain and a C-terminal PDZ domain.[85] PDZ domains are present in a large number of proteins and are known to recognize specific C-terminal polypeptide sequences.[86] In the case of DegS, the PDZ domain recognizes C-terminal peptides with the Y-X-F motif, common to a number of outer membrane porins (e.g., OmpC). It is assumed that the PDZ domain inhibits the protease domain most probably through direct contact between both domains. Upon appearance of denatured porins exposing their C-terminal tails, the PDZ domain is released from the protease domain and interacts with the Y-X-F motif. Denatured proteins are produced by a severe heat shock or by overproduction of a porin. The free protease domain now attacks the anti-sigma factor RseA. RseA consists of three functional domains, a periplasmic domain, a transmembrane domain and a cytoplasmic domain which sequesters the alternative sigma factor σE.[87,88] The DegS protease efficiently cleaves within in the periplasmic domain of RseA[89] and the remaining part of RseA is subsequently further degraded.[90,91] These proteolytic events destabilize the cytoplasmic domain of RseA, releasing σE to activate transcription of the genes of the σE regulon.[85] Removal of the denatured porins from the periplasm most probably leads to binding of the PDZ domain to the proteolytic domain of DegS resulting in to a shut-off of the heat shock response.

Protein Thermosensors and the LTR

Three different proteins have been reported to be active at low, but not at high temperatures. Example one is the VirA protein encoded by the Ti-plasmid of the soil bacterium *Agrobacterium tumefaciens*. VirA is the sensor kinase of a two-component signal transduction system, which phosphorylates the response regulator VirG which in turn activates a set of *vir* genes. These *vir* genes are involved in the processing and transfer of the T-DNA from the Ti-plasmid into susceptible plant cells.[92] Expression of the virulent genes is specifically inhibited at temperatures above 32°C. At temperature of 32°C and higher, VirA undergoes a reversible inactivation preventing both autophosphorylation and the subsequent transfer of the phosphate to VirG.[93] Why transfer of the T-DNA is inhibited at high temperatures? Since several plant proteins are involved in steps subsequent to T-DNA transfer, one or more of these proteins might be inactive at high temperatures blocking successful integration of the T-DNA into the plant genome.

A second example is the transcriptional activator NifA of *Klebsiella pneumoniae*. In diazotrophic bacteria, the *nif* operons are transcribed by the alternative sigma factor σ54 in conjunction with the transcriptional activator NIFA.[94] NifA binds to upstream activation sequences (UAS) that are located approximately 100 bp upstream of the *nif* promoters and catalyzes isomerization of closed complexes between Eσ54 and the promoters to produce open complexes. Activation occurs only at temperatures below 37°C and it has been suggested that the failure of NifA to bind to its UAS elements at 37°C is due to the fact that the helix-turn-helix motifs in different subunits are not correctly oriented with

respect to one another at 37°C.[95] Later, it was shown that the N-terminal domain plays an important role in the temperature sensitivity of the protein.[96]

The third example is the response regulator DegU of *Listeria monocytogenes*. In this bacterial species flagella-based motility is regulated in response to the growth temperature with the permissive temperature being 30°C and below.[97,98] The reason for not becoming flagellated at high temperatures relies on the *flaA* gene coding for the flagellin FlaA that is not expressed under these conditions.[99] Regulation of transcription of flagellar genes relies on three different proteins among them DegU, a response regulator. Since this protein is present at ambient temperatures and phosphorylation is not impaired,[100] its activity has to be modulated in response to the growth temperature. Either DegU is a temperature-sensitive protein being active at low and inactive at high temperatures, or the activity of DegU is regulated by another protein in a temperature-dependent way. Why synthesis of flagella should be prevented at 37°C, the temperature of the mammalian host? Downregulation of *flaA* expression during in vivo infection with *L. monocytogenes* may serve as an adaptive mechanism to avoid host recognition and activation of the host innate immune response.[101,102]

Protein Thermosensors and the CSR

Two major problems arise from exposing a cell to a sudden decrease in temperature.[103] First, membrane fluidity decreases, which affects many vital membrane and membrane-associated functions. Second, DNA and RNA topology will be stagnated causing halts in transcription and translation. Furthermore, warm-blooded pathogens leaving its host may need to shut off the expression of virulence gene expression. Therefore, one of the essential processes in the cold-shock response is the adaptation of the membrane to the new temperature. After a temperature-downshift, the physical properties of the cytoplasmic membrane change by undergoing a phase transition from its normal liquid-crystalline phase to a more rigid gel-like phase. In *B. subtilis*, adaptation occurs through two different mechanisms, where one involves desaturation of fatty acid moieties of the membrane. This is accomplished by enzyme fatty acid desaturase, which converts already existing fatty acid moieties into Δ5-unsaturated fatty acids, resulting in higher membrane fluidity.[104] Transcription of the desaturase gene *des* is cold-induced and regulated by the two-component system DesK and DesR.[105] The DesK histidine kinase consists of an N-terminal sensor domain composed of four helical transmembrane domains connected by a C-terminal cytoplasmic domain. Upon sensing the low temperature, the plasticity of the central four-helix bundle domain influences the catalytic activity of the DesK protein, either by modifying the mobility of the ATP-binding domains for autokinase activity or by modulating binding of its response regulator DesR.[106] The phosphorylated DesR binds to a DNA segment upstream of the promoter of the *des* gene and activates its transcription.[107] Upon return of the membrane to the fluid state, DesK becomes a phosphatase, dephosphorylates DesR, which leads to the shut-off of *des* gene activation.

EVOLUTION OF THERMOSENSORS

Based on the suggestion that our DNA world has been preceded by an RNA world, mRNA thermometers can be assumed to have evolved first. In their simplest form, mRNA thermosensors just need a simple hairpin structure, which sequesters the SD-sequence

at one temperature and allow access to the ribosomes at another temperature. More sophisticated mRNA thermosensors use more complex secondary structures exemplified by those coding for the heat shock sigma factor σ32 of *E. coli*[40] and those encoding small heat shock proteins in *Rhizobiae*.[45] Here, the additional secondary structures may influence the stability of that sequestering the Shine-Dalgarno sequence. It is important to stress that these mRNA thermosensors allow regulation of just one single gene at the level of translation. Theoretically, mRNA thermosensors could also regulate more than one gene provided translational coupling defined as the interdependence of translation efficiency of neighbouring genes encoded by the same polycistronic mRNA.[108] To regulate more than one gene by a mRNA thermosensor, they code for a transcriptional regulator, either an alternative sigma factor or a transcriptional activator.

DNA thermosensors are based on promoter occlusion. Here, bending of the DNA in the promoter region in conjunction with a silencing protein such as H-NS prevents binding of the RNA polymerase at low temperatures. High temperatures reduce the bending and destroy the whole architecture thus allowing access of the RNA polymerase to the promoter. Protein thermosensors also depend on conformational changes, where the low temperature favours the active, DNA-binding activity and high temperatures the inactive conformation of the protein. So far, only two protein thermosensors have been described the RheA and the TlpA repressor.[69,109] It is astonishing that not more protein thermosensors have evolved since a single point mutation can be sufficient to convert a stable into a temperature-sensitive repressor as exemplified by the cIts857 repressor of phage λ.[110]

The complex thermosensors represent the most sophisticated systems evolved so far. They depend on a molecular chaperone or a protease where both are able to sense denatured proteins. In their absence, they keep a positive regulatory protein inactive (DnaK—σ32) or a negative one active (GroE—HrcA, DnaK—HspR, DegS—RseA) and are titrated by the sudden appearance of nonnative polypeptide chains. The last example is the two-functional HtrA protein, where a switch from a molecular chaperone to a protease is dictated by the temperature.[111]

CONCLUSION

1. Temperature sensing is based on conformational changes of three different biomolecules: DNA, RNA and proteins.
2. Three different principles affect DNA as thermosensor: DNA supercoiling, promoter curvature and nucleoid-associated proteins.
3. RNA termosensors are either based on trapping the Shine-Dalgarno sequence in a secondary structure being destabilized at increasing temperature (*cis*-acting RNA thermometer) or through binding of a small RNA (*trans*-acting RNA thermometer).
4. During the cold shock response, mRNAs acquire secondary structures impairing translation, which is counteracted by specific cold shock proteins.
5. Protein-based thermosensors involve either temperature-dependent change in the conformation of the protein itself or in assembly of protein complexes.
6. Protein thermosensors include transcriptional and translational regulators, molecular chaperones and proteases.

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CHAPTER 2

MOLECULAR PLANT VOLATILE COMMUNICATION

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Abstract: Plants produce a wide array of volatile organic compounds (VOCs) which have multiple functions as internal plant hormones (e.g., ethylene, methyl jasmonate and methyl salicylate), in communication with conspecific and heterospecific plants and in communication with organisms of second (herbivores and pollinators) and third (enemies of herbivores) trophic levels. Species specific VOCs normally repel polyphagous herbivores and those specialised on other plant species, but may attract specialist herbivores and their natural enemies, which use VOCs as host location cues. Attraction of predators and parasitoids by VOCs is considered an evolved indirect defence, whereby plants are able to indirectly reduce biotic stress caused by damaging herbivores. In this chapter we review these interactions where VOCs are known to play a crucial role. We then discuss the importance of volatile communication in self and nonself detection. VOCs are suggested to appear in soil ecosystems where distinction of own roots from neighbours roots is essential to optimise root growth, but limited evidence of above-ground plant self-recognition is available.

INTRODUCTION

Plants are literally rooted to the ground and therefore unable to change location. Consequently, they are easy targets to organisms that wish to feed on them. Plants have evolved a vast array of defensive features that effectively reduce the number of their enemies.[1] However, defences are rarely flawless, meaning that plants cannot exist as static, non-interactive organisms. Instead they can benefit through exchanging information with other organisms. In order to communicate without physical contact, plants require a 'language' and volatile organic compounds (VOCs) are the 'words' in the plants 'vocabulary'. The quantities and relative proportions of VOCs in the bouquet

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emitted by plants allow the plant to send complex signals, which using the linguistic analogy could be described as 'sentences'.

Plants produce a huge diversity of different chemicals, which include an array of VOCs emitted by flowers, foliage, bark, roots and specialised structures.[2] Many of these chemicals play roles in structuring relationships that plants have with a plethora of arthropods. These relationships can be beneficial or deleterious to the plant. Scientific advances in the field of plant-plant communication have led to VOCs being assigned an important role in transmitting signals from a damaged plant to a healthy neighbour. Moreover, signals from a herbivore-damaged part of a plant can be transmitted to a distant part of the same plant via VOCs.

We currently have a fairly robust knowledge of the processes and metabolic pathways involved in the production of many VOCs,[3,4] but we have an extremely limited understanding of how plants can detect these signals. Even less is known about how plants may differentiate signals from conspecifics representing the same or different genotypes. In this chapter we will provide a short review of plant communication via VOCs, detail current knowledge on the detection of self and nonself in plants and complete the chapter with suggestions of future directions for this fascinating research field.

ROLES OF VOLATILE ORGANIC COMPOUNDS

Plant secondary chemistry is defined by Schoonhoven et al[1] as 'plant compounds that are not universally found in higher plants, but are restricted to certain plant taxa at much higher concentrations than in others and have no (apparent) role in primary metabolism'. Plant volatiles represent 1% of known plant secondary metabolites and to date, 1700 plant volatiles from over 90 plant families have been isolated.[3] Plants emit volatiles constitutively; it is known that constitutive isoprene and monoterpene production in chloroplasts is related to protection against heat stress,[5] and some constitutive VOCs can directly affect the physiology and behaviour of herbivores through their toxic, repellent and deterrent properties.[5-7] For generalist herbivores VOCs can be repellent signals, but species specific volatile signals released by a plant individual reveal the plant identity and if perceived by specialist herbivore species will increase feeding damage and reduce the plant's fitness.[6]

A number of different stresses induce plants to emit a broad range of volatiles in a temporally, qualitatively and quantitatively complex pattern.[7] Such stresses include abiotic factors including drought, heat stress and ozone,[5] and biotic stressors such as pathogens,[8] and herbivore feeding.[9,10] Feeding by herbivorous invertebrates is known to have profound and variable effects on the volatile bouquets emitted by a multitude of plant species in a range of taxa. It is these induced volatiles that are most active in mediating the numerous signalling processes involving plants.

When herbivores begin to feed, plants have two types of volatile response. The first response is the rapid emission of stored compounds, which are released when plant tissue is damaged. The second response is the de novo synthesis of compounds, which are not stored, but emitted as they are produced.[11] The compounds released by these two mechanisms may have some overlap with constitutively emitted volatiles, amounts of which are often increased by herbivore feeding.[7] Other compounds are completely exclusive to herbivore damaged plants. For instance, *Phaseolus lunatus* only emit the monoterpenes α-pinene and limonene when intact, but after 48 hours of feeding by spider

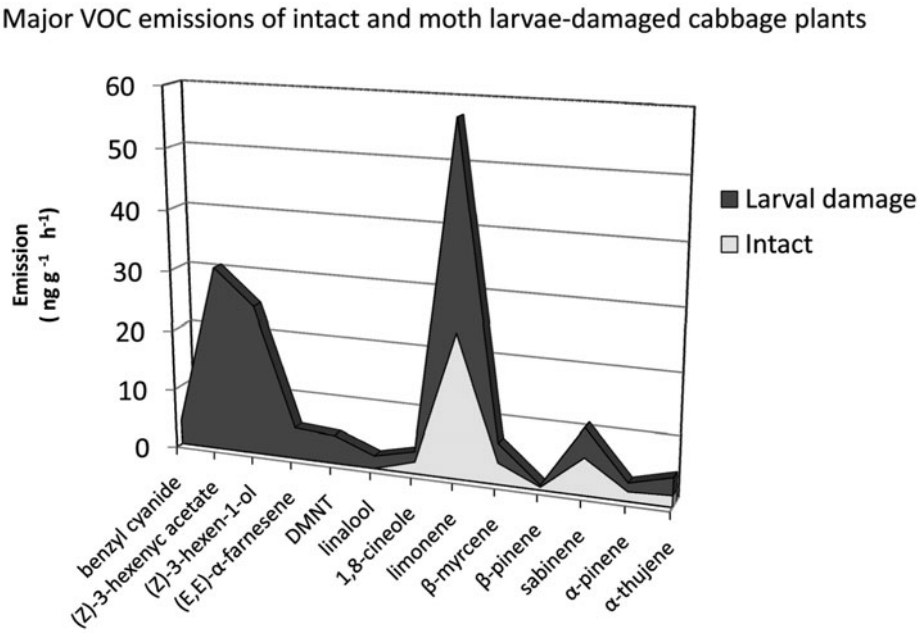

Figure 1. Example of VOC emission profiles of healthy intact cabbage plants and Diamond Back Moth (*Plutella xylostella*) larvae-damaged cabbage plants.

mites seven other VOCs are emitted.[12] Intact *Brassica oleracea* plants constitutively emit at least seven monoterpenes, but after 48 hours of feeding by diamond back moth larvae three more terpenes and three other compounds are emitted (Fig. 1).

These herbivore-inducible compounds or their relative ratios in the scent released by damaged plants are used by the natural enemies of plant feeding insects to locate their host and this has been shown in laboratory,[13] semi-natural[14] and natural conditions.[15] The quantity of VOCs released by damaged plants is much larger than the amount of VOCs released by the actual herbivores, for example herbivorous mites and insects[1] or by their faeces (Fig. 2).[16] It can be concluded that the specific VOC signals released by plants after damage by a herbivore are important signals that improve the fitness of the plant by eliciting behavioural responses in herbivore natural enemies and thus increasing the predation rate leading to reduced plant damage. This plant response has often been referred to as a 'cry for help', due to natural enemies of herbivores using these volatile signals as cues in the process of prey or host foraging. However, it could be suggested that the receiver of the signal, may interpret it as a 'cry',[17] while the complex nature of the signal could be deemed a far more eloquent monologue. Certain compounds seem to provide particularly reliable indication of herbivore feeding, such as the acyclic homoterpenes (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and 4,8,12-trimethyl-1,3(*E*),7(*E*),11-tridecatetraene (TMTT). These compounds are emitted by plants in different quantities and ratios depending on the herbivore, which determines the attractiveness of the emitted volatile blend to different species of foraging predators.[18] The composition of emissions is often plant specific and herbivore specific to the species level—and even to the level of larval feeding stage.[19]

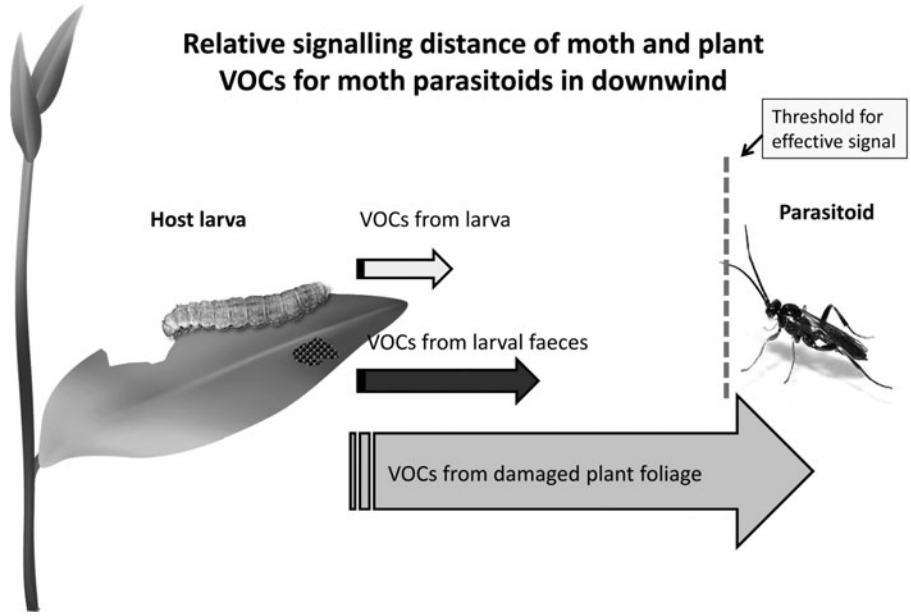

Figure 2. Schematic illustration of interactions between herbivores and their natural enemies (parasitoids) communicated via plant volatile molecules. Plant volatiles induced by herbivore feeding are emitted in higher quantities and have better communication value than direct emissions from herbivorous larvae or larvae faeces. It has been shown that parasitoids can learn to detect plant emissions related to herbivore damage.

PLANT-PLANT SIGNALLING

Above-Ground

The Ecology of Plant-Plant Signalling

As herbivore-induced volatiles are reliable indicators of herbivore presence, plants stand to gain a benefit if they can detect these volatiles and modify their defences accordingly. Plant-plant communication was first reported in 1983,[20] and has since been a topic of considerable debate. Much of the debate has centred on the ecological relevance of a process that had been demonstrated to occur in the laboratory,[20,21] but not observed in nature. However, in more recent years a body of evidence has accumulated to suggest plant-plant communication in field conditions. This evidence includes interspecific communication,[22-24] intraspecific communication [25-27] and within plant communication.[28]

Sagebrush, *Artemisia tridentata*, has been the subject of numerous studies conducted under field conditions. Interspecific communication was observed,[22-24] with wild tobacco plants shown to experience less foliar damage when exposed to clipped sagebrush neighbours than plants exposed to unclipped sagebrush. This communication was also shown to occur with sagebrush damaged by herbivores.[23] In both cases the distance over which this communication occurred was 10 cm.[23] Intraspecific communication has also been demonstrated in sagebrush,[25,23] whereby undamaged sagebrush with clipped

sagebrush neighbours received significantly less damage than sagebrush with unclipped neighbours. This communication occurred at distances of up to 60 cm from the clipped plants.[25] Methyl jasmonate is constitutively emitted by sagebrush, but upon damage the isomeric composition of emissions is altered, with overall emissions increased and emissions of the cis isomer proportionally increased.[29] Consequently, methyl jasmonate was predicted to be an important signal mediating interplant communication.[29] However, application of methyl jasmonate in concentrations representing the amounts naturally released by sagebrush did not elicit nicotine responses in open-grown plants.[30] Herbivore resistance in tobacco plants was recently shown to be primed,[31] see below.

The Chemistry of Plant-Plant Signalling

A large number of chemical compounds have been implicated in signalling to herbivores, predators and parasitoids, but we will focus on reviewing the compounds involved in signalling between and within plants. Typically many of the volatiles effective in plant to plant signalling are the compounds synthesised de novo upon herbivore attack.

To date several compounds (Fig. 3) have been reported to function as between and within plant signals, these include the green leaf volatiles (*E*)-2-hexenal,[32-34]

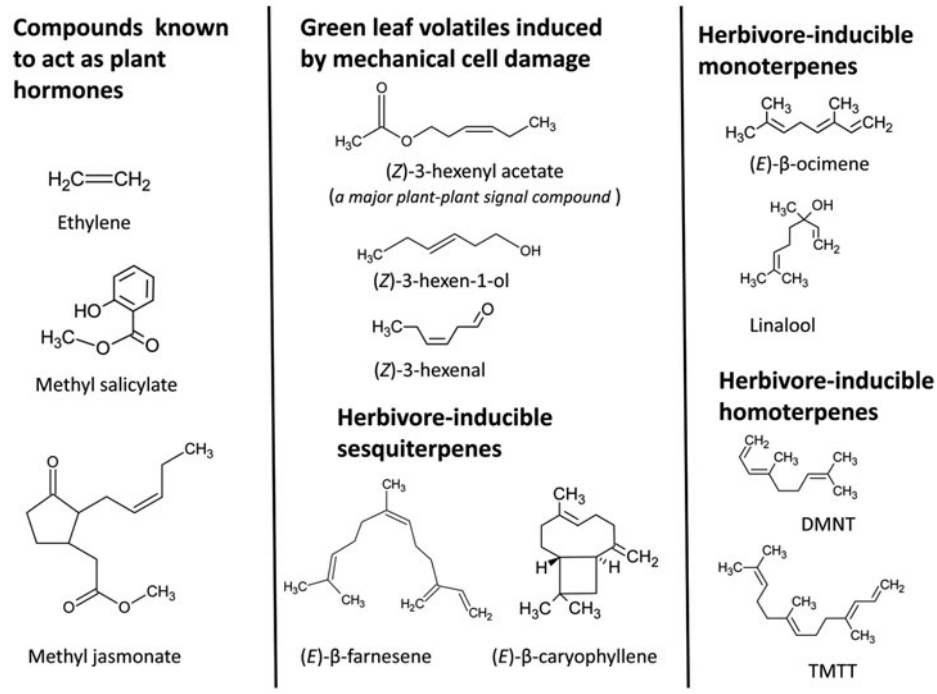

Figure 3. Chemical structures of selected plant volatile compounds, which are known to have function in intraplant, intraspecies and interspecies communication. Emissions of most of these compounds are induced by herbivore damage. (*E*)-β-caryophyllene (shadowed) is the only inducible volatile compound which is shown to be active in inter specific below ground communication. DMNT = (*E*)-4,8-dimethyl-1,3,7-nonatriene, TMTT = (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

(*Z*)-3-hexen-1-ol[35] and cis-3-hexenyl acetate,[36-38] the terpenes myrcene and blended ocimene volatiles ((*E*)-β-ocimene, (*Z*)-β-ocimene and allo-ocimene)[39] and the phytohormones methyl jasmonate,[21] methyl salicylate[40] and ethylene.[41]

Green-leaf volatiles include a range of C6 compounds including aldehydes, alcohols and esters. Formed via the lipoxygenase pathway, these compounds are emitted rapidly upon disturbance of the plant, by mechanical damage as well as herbivore feeding.[42] These compounds are therefore indicative of any mechanical damage and could provide early signals to receiving plants. However, they do not have the same reliability as emissions such as DMNT and TMTT, emissions of which are highly correlated with herbivore damage.[18]

Terpenoids are the largest group of secondary compounds, consisting of approximately 40,000 compounds,[43] including at least 1,000 monoterpenes and 6,500 sesquiterpenes.[1] All terpenoids originate from isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP), which are derived via two alternative pathways. In the cytosol, IPP is synthesised via the mevalonic acid (MVA) pathway, while in plastids it is synthesised via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, see Arimura et al[44] and Dudareva et al[3] for reviews. Some terpenoids are constituents of essential oils and resins and are constitutively produced and stored in specialised structures, such as glandular trichomes or resin ducts. Upon damage by herbivores these structures are broken and the compounds are released. The de novo biosynthesis of terpenoids can be induced locally and systemically by herbivore feeding. Terpenoids as a group are therefore, able to provide rapid, but also herbivore-damage related signals to receiving plants.

Methyl jasmonate is a volatile derivative of jasmonic acid, which is an integral component of plant defence responses to insect feeding. Application of methyl jasmonate to tomato plant leaves has been shown to increase production of proteinase inhibitors under laboratory conditions.[21]

Methyl salicylate is synthesised from salicylic acid, it is a phenolic compound and plays an important role in plant defence. It is released in significant amounts from plants in response to aphid feeding damage and is emitted by tobacco in response to tobacco mosaic virus infection. Tobacco plants exposed to methyl salicylate have been shown to have increased resistance to tobacco mosaic virus.[40]

Plant-plant signalling in maize was shown to be mediated by the green leaf volatile (*Z*)-3-hexen-1-ol, with ethylene synergising the effect. Plants exposed to (*Z*)-3-hexen-1-ol increased emission of several compounds associated with herbivore feeding by 2.5-fold. Treating plants with ethylene increased the effect to 5.1- to 6.6-fold.[35] Ethylene also plays an important role in shade avoidance in tobacco.[45,46] Wild-type tobacco leaves normally stop growing as they get close to neighbouring plants; however, a mutant variety of tobacco that does not produce ethylene does not reduce growth and results in overlapping leaves, reduced shade avoidance and possible loss of energy. This indicates that plant to plant communication mediated by ethylene occurs under laboratory conditions.[47]

The fate of VOCs in the atmosphere is particularly relevant to plant to plant signalling. Plants cannot move great distances towards an odour source and therefore rely on sufficient quantities of any signalling cues being transported through the atmosphere and to the plant. The atmospheric life times of VOCs are therefore relevant in determining how effective they will be in mediating communication. Many of the inducible VOCs including, monoterpenes, GLVs and sesquiterpenes have atmospheric life times of only a few minutes, a few hours or less than 24 hours.[48,49] Other VOCs, which are considered less reactive with atmospheric oxidants, have extended atmospheric life times of longer

than 24 hours.[48] High reactivity and short atmospheric life times significantly reduce the signalling distance of most reactive compounds.

Priming

Priming in terms of plant defence is where plants ready their defences in response to a signal or previous challenge so that they can respond with increased rigour should they be subsequently challenged by herbivores or pathogens. The priming of plants as a product of plant-plant communication via volatile organic compounds is a recent discovery, but work in this field has already gathered enough momentum to yield at least two reviews.[37,50] Although, research of priming in plant-plant interactions is still in the early stages, the phenomenon of priming in plant-pathogen interactions was recognised many years ago, with understanding of this phenomenon progressing substantially in recent years.[51-53]

The first study to clearly demonstrate priming via airborne signals from a herbivore-damaged plant to an undamaged neighbour was conducted by Engelberth et al[54] with corn plants. The authors showed that exposing plants to three different green leaf volatiles primed plants to emit inducible terpenoids and accumulate jasmonic acid with increased rigour following challenge with a wound and added caterpillar regurgitant extract. This is a protocol used for mimicking herbivory, whereby enzymes present in the regurgitant are responsible for causing significant differences in volatile emissions caused by caterpillar feeding and mechanical damage alone.[13] Interestingly, jasmonic acid accumulation was not primed to increase in response to mechanical damage alone. Other plant defence responses have also been shown to be primed. In Lima bean, the production of extra-floral nectar is increased by exposure to VOCs emitted by mechanically damaged conspecifics.[55] This exposure also primes receiver plants to increase extrafloral nectar more rapidly in response to both mechanical damage[55] and spider-mite feeding.[56] Therefore, it would seem that this priming is more general than the example of jasmonic acid in corn, which is not increased by mechanical damage alone. Other primed defence responses include accelerated production of trypsin-proteinase inhibitors in tobacco exposed to volatiles from damaged sagebrush.[31]

Analyses of gene expression have complemented these records of primed defence. Changes in transcription patterns of defence-related genes following exposure to volatile compounds have been described in several studies.[37,57-60] This suggests that signals have been detected by receiver plants, even though changes in phenotype are not observed.

In the future it is possible that we could purposefully prime crop plants to increase their resistance to herbivores or pathogens. The use of transgenic 'beacon' plants that are engineered to continually produce and release quantities of priming compounds has been suggested as a potential mechanism for increasing resistance.[61]

Within Plant Signalling

Within-plant signalling by VOCs is a potentially relevant discovery with regards to furthering our understanding of self and nonself recognition in plants. Heil and Silva Bueno[28] showed that herbivore-damaged Lima bean tendrils release a VOC signal that results in an undamaged tendril of the same plant increasing extrafloral nectar secretion. We know from previous studies that undamaged neighbouring plants increase their EFN secretion in response to a VOC signal, but the knowledge that damage induced VOCs function to transmit a signal within the same plant suggests that this could be the main

or the primary function. This lends credence to the expression 'eavesdropping plants'[62] with regard to plant-plant signalling. This term was first coined to describe plants that receive a VOC signal intended for a different recipient, with the intended recipient suggested to be the natural enemies of herbivores. However, the accumulating evidence for within-plant communication in different plant species suggests that the 'intended recipient' is likely to be the emitting plant itself. As well as in Lima bean, within-plant communication has been demonstrated in a tree, hybrid poplar,[63] and two woody shrubs, sagebrush[25] and blueberry.[64] In all these species, branches have either reduced or absent vascular connections, which means that regulation of a systemic response to herbivore attack is not possible via internal signals. Therefore, external volatile cues provide a means to negate these constraints.

Interestingly, in both Lima bean and hybrid poplar[37] the green-leaf volatile cis-3-hexenyl acetate has been shown to play a vital role in within-plant communication. This compound is also released by blueberry[64] and sagebrush[31] and is emitted within five minutes of the start of herbivore-feeding[44] and therefore a good candidate for providing a fast signal from damaged to undamaged parts of a plant. However, the commonness of cis-3-hexenyl acetate and the fact that it is released in response to mechanical damage as well as herbivore feeding, suggest that it is rather a general signal, detectable by multiple species and inducible by multiple stimuli.

Below-Ground Signalling

Roots of non-aquatic plants usually spend their lives below ground, but they are the site of synthesis of plant secondary metabolites such as alkaloids, which have been shown to be produced in the roots and transported via the xylem and into the leaves.[48] In the rhizosphere, free air and aerial communication is limited to soil pores as most of the root surfaces and soil particles are covered by a water film. Therefore, below-ground chemical communication is strongly based on nonvolatile hydrophilic plant root exudates, which are used to compete with invading root systems of neighbouring plants for space, water and mineral nutrients, but also with other soil-borne organisms, including herbivorous animals, bacteria and fungi.[65] Particularly in wet soils allelopathic effects between plant roots are mediated predominantly by phenolic compounds,[66] including e.g., catechins and various phenolic acids.[65] There is also evidence that these root exudates could be responsible for internal root communication by self-inhibition. Falik et al[67] were able to show that development of lateral roots of *Pisum sativum* towards an obstacle were reduced, when the lateral root first faced an obstacle, other lateral roots then withered. However, this avoidance growth pattern was suppressed in the presence of potassium permanganate or activated carbon which adsorbs active compounds of root exudates. The result indicates a significant role of root exudates in plant self-signalling to promote obstacle avoidance by other lateral roots of the same plant. External self-inhibition of root growth towards obstacles could increase plant performance by directing resource allocation in the root system to more profitable directions in the rhizosphere.[67]

Our knowledge of volatile communication in the rhizosphere is limited. Potential and reported volatile interactions above and below ground are summarised in Figure 4. Some volatile compounds such as the sesquiterpene (*E*)-β-caryophyllene are induced in plant roots by abiotic stresses like heat stress[68] and by biotic stress caused by insect feeding damage.[69] In sesquiterpene-rich plant species such as *Copaifera officinalis* several sesquiterpenes were found from roots, but two-thirds of the amount was (*E*)-β-caryophyllene,[70] which indicates a

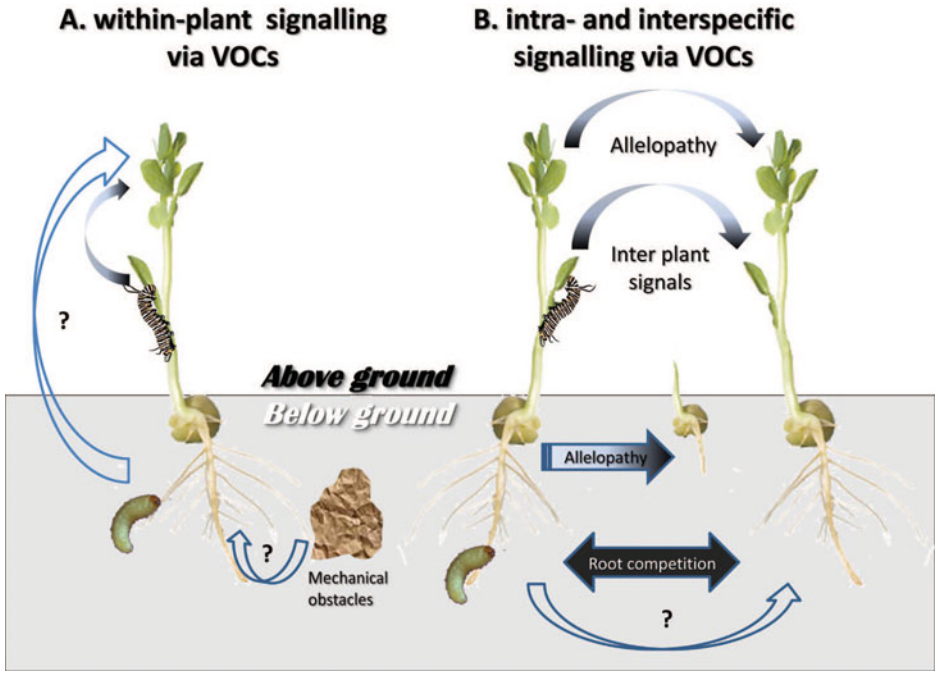

Figure 4. Example of participation of volatile compounds in above ground and below ground plant to plant signalling. A) Within-plant signalling via VOCs and (B) intra- and interspecific signalling via VOCs. Curved arrows with gradient colour describe reported signalling and nonfilled curved arrows indicate putative signalling routes.

root-specific role for this volatile compound. Maize root worm larvae (*Diabrotica virgifera virgifera*) feeding on maize roots induced (*E*)-β-caryophyllene production in the roots and attracted entomoparasitic soil nematodes to orientate toward damaged plant roots in tests with a sand-filled olfactometer.[69] It was shown that (*E*)-β-caryophyllene evaporated and moved in moist sandy soil rapidly, 90% of experimentally released compound was recovered through a 5 cm thick sand layer.[69] This is an indication that volatile signal compounds released from root systems can rapidly reach neighbouring roots and has the potential to transmit information between self and nonself root systems in soil. Interestingly, root exudates from aphid infested broad bean plants have been shown to make non-infested conspecific neighbouring plants more attractive to foraging parasitoids.[71] This interesting demonstration of positive plant-plant communication through the rhizosphere is in contrast with the many allelopathic effects of root exudates.

SELF AND NONSELF RECOGNITION IN PLANTS

Vascular plants can be unitary organisms appearing in individual units such as humans or other animals. Many plants, however, are modular organisms which look-like separate individuals, but are somehow connected like e.g., grasses which are often

connected through their root system and represent the same genotype.[72] In perennial and woody plants the situation may become more complicated. European aspen (*Populus tremula*) grows root extensions which develop new shoots, asexually produced modules, which are called ramets or clones. Clones can physically remain connected through roots to their sexually reproduced parent tree, called a genet. They could also become disconnected from parent trees and start to function independently, which means that "a tree" could have three phenotypes: The parent tree, physically connected clones and physically disconnected clones. In a forest ecosystem these genetically identical individuals compete for light, water and nutrients with half-siblings i.e., sexually reproduced seedlings of the parent tree and clones and seedlings of other conspecific and heterospecific trees and other plants.

Self and Nonself Recognition Belowground

Self and nonself recognition in plants has mainly been studied belowground, with focus on interactions between roots of plants competing for resources. To date, information to confirm self and nonself recognition involves comparing the root growth of plants grown in proximity to plants of differing levels of relatedness. Several studies have shown that when roots encounter nonself root growth there is a different growth response to encountering its own roots.[73] Root growth experiments with the clonal perennial grass *Buchloe dactyloides* showed that individuals have shorter root growth when confronted with self than nonself competing roots.[74] Gruntman and Novoplansky[74] conducted experiments with 'twin' plants originating from the same plant node and showed that while plants were able to recognise self and modify their root growth reflectively, they did not recognise separated plants as self after a prolonged period of separation. The authors concluded that in this circumstance self and nonself discrimination is mediated by physiological co-ordination among roots that develop on the same plant rather than allogenetic recognition. Falik et al[75] used a similar method to study discrimination between self and nonself in *Pisum sativum*, they also observed greater root growth when plants were grown in the same pot as nonself plants than when grown in the same pots as separated twins. Although the authors could not rule out allogenetic recognition, they also hypothesised that a physiological co-ordination among roots was the most likely reason for their results. These studies give firm support to the idea that recognition of nonself competing plants results in an increase in root production. However, there are some methodological issues that have complicated this field, with Hess and de Kroon[76] providing an enlightening account of the need to consider resource competition aspects in future experiments. They showed that in most previous studies the over-production of roots correlated with increased soil volume and nutrient availability for plants growing in competition than for plants growing alone.

Interestingly, in the annual plant *Cakile edentula*[77] individuals sharing a pot with a group of 'strangers' allocated more resources to root growth than plants sharing a pot with siblings. This indicated that kin recognition may occur as a result of root based communication. This shows that whereas some plants lose the ability to recognise genetically identical twin plants following a period of separation, effectively no longer recognising self, others are able to recognise kin. The authors suggested a different mechanism to that used in self/nonself recognition due to genetically identical individuals sometimes being determined as nonself.[74,75]

Self and Nonself Recognition Aboveground

The only study to have satisfactorily addressed the phenomenon of self and nonself recognition in plants above ground was recently conducted by Karban and Shiojiri[78] using sagebrush as a model plant system. The ecological relevance of plant-plant communication in sagebrush has been documented in a series of elegant experiments by Karban and colleagues carried out over a number of years. The sensitivity of different plants to signals released by sagebrush is variable, for example tobacco growing at up to 10 cm[22,23] from sagebrush neighbours receives a tangible benefit by responding to cues from the damaged intraspecific neighbour, whereas undamaged sagebrush plants are able to gain a benefit at distances up to 60 cm from a damaged conspecific neighbour.[25] The recent study has gone further and produced genetically identical sagebrush clonal cuttings to demonstrate that when a sagebrush plant is defoliated by clipping, a genetically identical neighbour will receive 42% less damage than genetically different neighbours. This is a landmark discovery in understanding to what extent and via what mechanism plants are able to distinguish self from nonself.

It has been shown that within-plant signalling in woody plants between different branches or adjacent leaves with little or no vascular connection can be based on volatile signals released from wounded leaves to prime the defence in receiving foliage.[63,37] These observations suggests that plants would benefit by recognising signals representing their own genotype.

Nonself Recognition by Parasitic Plants

One particularly interesting example of plants responding to nonself volatiles has been reported in the parasitic plant *Cuscuta pentagona*[79] or dodder. *C. pentagona*, an obligate parasite with little photosynthetic capacity, was shown to use volatile compounds to orientate toward host plants.[79] *C. pentagona* oriented toward its preferred host plant tomato (*Lycopersicon esculentum*) with significantly greater frequency than to wheat (*Triticum aestivum*) a nonhost. Three volatile compounds emitted by tomato, the terpenes β-phellandrene, β-myrcene and α-pinene, were significantly oriented toward when tested in isolation, while (*Z*)-3-hexenyl acetate emitted by wheat had a repellent effect. In order to respond to these volatile compounds the host-foraging parasitic plant needs to in some way detect or perceive them. The mechanism for this is still unknown and should be the focus of further research in this field.

CONCLUSION AND FUTURE DIRECTIONS

At the moment the most intriguing question in plant to plant communication by volatile compounds regards the mechanism; how plants perceive the signal molecules and how the potential VOC receptors function in plants. Moderate ozone concentrations (80ppb) have recently been shown to significantly reduce the distance over which plant-to-plant communication occurs, with oxidation of the signalling compounds indicated as the mechanism. Obviously the receiver plants are not able to sense the reduced concentrations of signalling compounds.[80]

In antennal sensilla of an insect there are general odour binding proteins and more compound-specific receptor molecules.[81] In plants, several salicylic acid (SA) binding proteins have been described e.g. from tobacco.[82] SA-binding proteins have methyl salicylate (MeSA) esterase activity, which is required to release the active defense phytohormone SA from MeSA.[83] Recently, several members of the AtMES gene family, which is functionally homologous to SA binding proteins, have been described in Arabidopsis.[83] Proteins produced by this gene family have potential for MeSA hydrolysis, which is essential to activate SA when MeSA serves as a long-distance signal for systemic acquired resistance (SAR) in Arabidopsis and tobacco.

After detection of volatile signal molecules by receiver cells, a signalling cascade will be activated in that plant part. Receiver cells could be located in another organ or another module of the emitter plant or in longer distance signalling receiver cells could be in conspecific or heterospecific neighbouring plants. According to our current understanding, compounds known to transmit information between plants are rather common in the plant kingdom and are released by many plant species. Thus the recognition may be based on the ratio of specific signalling compounds perceived concomitantly, as reported for insect antennae,[6] where different type sensors can be found. Thus, receiver plants should have different mechanisms or sensors for sensing e.g., GLVs and monoterpenes than the SA binding proteins used for MeSA sensing. Recently we have found[84] that plants are able to adsorb volatiles from neighbouring plants and re-release these molecules back to the atmosphere. This suggests that the plant epidermis may have importance in storing VOC molecules for signal perception and possibly enrich the concentration of received compounds for more accurate identification by potential sensor molecules. Furthermore, our observation could also suggest that plants can possibly use VOCs from neighbouring plants to camouflage themselves from their specialist herbivores by letting VOC molecules condensate on their leaf epidermis and then releasing misleading compounds for detection by the antennae of their herbivores. When the mechanism of signal perception for specific volatile compounds has been elucidated, hypersensitive genetically engineered crop plant varieties can be developed. VOCs released from plants first attacked by pest insects can probably elicit better pest protection in neighbouring plants by long-distance systemic acquired resistance. With this type of "primed" crop plant cultivar, defence can be elicited by a farmer as a part of a plant protection strategy. It requires the artificial release of the volatile compounds needed for the plant to be primed as a part of other plant protection actions in the field. To conclude, our knowledge of phytogenic organic compounds is still limited and their role in intraspecific communication between plant individuals and in interspecific communication is not fully elucidated. Better understanding of the VOC receptors and their functions in plants will improve our possibilities to assess the ecological significance of above-ground and below-ground molecular communication in plant communities.

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CHAPTER 3

PRIMARY PROCESSES IN SENSORY CELLS: Current Advances

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Abstract: In the course of evolution, the strong and unremitting selective pressure on sensory performance has driven the acuity of sensory organs to its physical limits. As a consequence, the study of primary sensory processes illustrates impressively how far a physiological function can be improved, if the survival of a species depends on it. Sensory cells that detect single-photons, single molecules, mechanical motions on a nanometer scale, or incredibly small fluctuations of electromagnetic fields have fascinated physiologists for a long time. It is a great challenge to understand the primary sensory processes on a molecular level. This chapter points out some important recent developments in the search for primary processes in sensory cells that mediate touch perception, hearing, vision, taste, olfaction, as well as the analysis of light polarization and the orientation in the Earth's magnetic field. The data are screened for common transduction strategies and common transduction molecules, an aspect that may be helpful for researchers in the field.

INTRODUCTION

Sensory cells provide the central nervous system with vital information about the body and its environment. Each sensory cell detects specific stimuli using highly specialized structures which operate as sensors for adequate stimuli. Thus, the posture of the body, its supply with nutrients and oxygen, the state of the cardiovascular and digestive systems, as well as the body temperature and ion concentrations are constantly monitored by a set of sensory cells. Moreover, information about objects in the environment, their shape, colour, chemical composition, their distance and movement are collected and conveyed

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to the central nervous system. This steady and complex flow of coded information is then integrated and used to generate sensible behaviour.

Sensory cells display a multitude of remarkable adaptations towards their tasks. The adequate stimulus is detected by a sensor which must be both selective and sensitive. To detect weak stimuli that transfer only little energy to the sensory cell, primary signals have to be amplified. And an output signal must be generated that can be interpreted by the brain. These primary processes constitute an efficient and characteristic transduction cascade in each type of sensory cell. In the evolution of animals, sensory acuity is continuously sharpened under intense selective pressure, and the transduction cascades are the prime targets of this process. The result is a set of cells with astonishing performance: Photoreceptors that detect single photons, mechanoreceptors that respond to movements on a nanometer scale, and chemoreceptors that report the detection of single molecules. Furthermore, the perception of electromagnetic radiation by many animal species amazes physiologists, and the research for the transduction mechanisms that mediate the analysis of infrared radiation, electrical fields or magnetic navigation cues is among the most exciting fields in sensory physiology. This chapter tries to provide a brief overview of current work on sensory transduction mechanisms. I focus on just one or a few research topics in each of the sensory modalities, and I try to point out the significance of recent findings for the scientific concepts of sensory function. The depth of knowledge and the accuracy of mechanistic models varies considerably between well-studied cells like photoreceptors and more enigmatic cells like the touch receptors in the human skin. However, as common transduction features begin to appear, new experimental approaches become available which are based on the observation that various sensory cells use similar or homologous proteins for transduction. Thus, the examination of transduction channels or amplification mechanisms in one type of sensory cell may help to advance studies of transduction in a different modality. The present chapter is designed to promote such effects.

MECHANORECEPTORS—TUGGING AT ENIGMATIC CHANNELS

Touching an animal usually triggers rapid and robust motorresponses, ranging from twitching of the skin to violent startle responses. Oddly enough, this basic and omnipresent sense appears to be one of the most difficult to study on the level of its transduction mechanism. It is quite clear that mechanical stimuli of various sorts can trigger opening of ion channels and cause depolarization in practically every cell.[1-3] However, sensory cells which are specialized for the detection of mechanical stimuli (mechanoreceptors) can use elaborate protein complexes to transduce adequate mechanical stimuli, and to report a sensory signal to the central nervous system. How intricate the structure of such a transducer can be was revealed by an extensive genetic screen of *Caenorhabditis elegans* mutants which took more than 25 years and brought to light a set of proteins that co-assemble to form a mechanotransduction complex.[4-6] This multi-protein system works by pulling transduction channels open when the worm's cuticula moves (Fig. 1). In the touch receptor neurons, transduction channels are tethered to the cuticula on the extracellular side of the plasma membrane and, possibly, to the cytoskeleton on the intracellular side. The channel itself is composed of two subunits, MEC-4 and MEC-10.[7,8] MEC-4 and MEC-10 belong to the degenerin/ENaC family of cation channels[9-11] and form the channel pore, while MEC-6 and MEC-2 are auxiliary subunits necessary for proper function of the MEC-4/MEC-10 channel.[8,12,13] MEC-2 belongs to the large group of

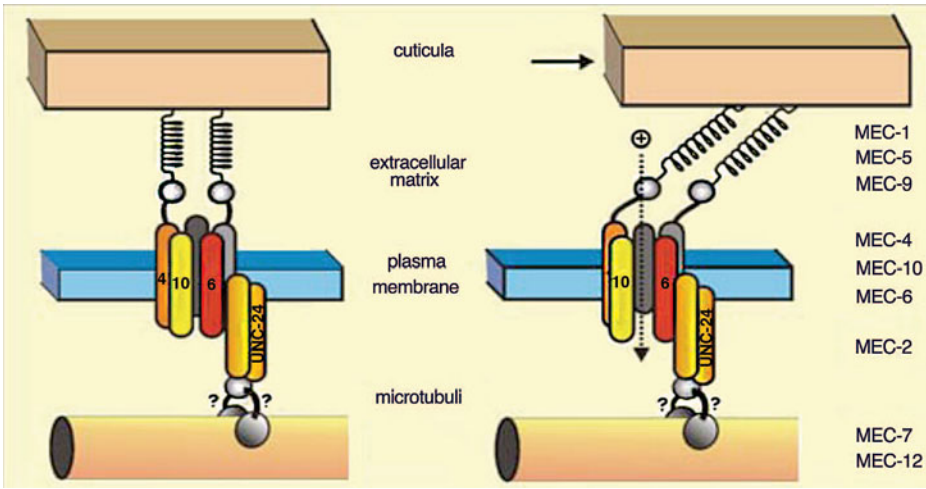

Figure 1. The mechanosensitive protein complex of *C. elegans*. *Left:* Nine different MEC proteins co-assemble to form an ion channel in the plasma membrane of a mechanosensory neuron. The channel is formed by MEC-4, MEC-6, and MEC-10. Other MEC proteins tether the channel to the cuticula and to the cytoskeleton. *Right:* When the cuticula is shifted by gentle touch, the channel is pulled open, and cation influx generates a receptor potential.

prohibitin homology (PHB-) domain proteins.[14] MEC-2 is a cholesterol-binding protein and associates with the channel through its PHB domain, apparently within a cholesterol-rich lipid-raft like membrane environment.[15,16] A similar protein, UNC-24, is also associated with the transduction complex, but its role in channel regulation is less well understood. The touch receptor neurons possess rather thick microtubules consisting of 15 instead of the usual 11 protofilaments.[17,18] MEC-7 and MEC-12 are necessary for the function of the transduction complex, but it is not clear how the channel subunits connect to the microtubuli, if indeed such a cytoskeletal tethering of the transduction complex exists. The extracellular anchoring of the complex is well characterized.[19,20] It involves a number of extracellular matrix proteins, at least three of which are associated with the transduction complex. MEC-5 is a unique collagen secreted by the epidermal cells of *C. elegans*, while MEC-1- and MEC-9 are matrix proteins with multiple protein-interaction domains. These proteins attach the transduction complex to the cuticula and may, therefore, play a critical role in mechanotransduction. Moreover, they are necessary to concentrate the MEC-4/MEC-10 channels in characteristical punctate clusters in the membrane of touch receptors. Thus, the model in Figure 1 illustrates the possible co-ordination of nine different MEC proteins to form a functional mechanotransducer. More proteins may contribute to this complex, and the list of potentially relevant proteins currently extends to MEC-18.

Mechanoreceptors in vertebrates are much less well understood, and there is hope that the *C. elegans* touch receptor neuron will serve as a blueprint for a corresponding model composed of homologous vertebrate proteins. This approach has only just begun, but there are already promising results. A member of the PHB domain protein family, stomatin-like protein 3 (SLP3), turned out to be necessary for normal touch sensation in mice.[21] The related protein stomatin is needed for sensory function in rapidly adapting D-hair mechanoreceptors.[22] These findings suggest that SLP3 and stomatin play a similar role

in vertebrate mechanotransduction as MEC-2 plays in the *C. elegans* touch receptor. Although it is too early to speculate, the notion that vertebrate mechanotransducers are protein complexes with tethered transduction channels appears to be a reasonable working hypothesis. Future work may lead to the identification of multiple transduction components and to gene ablation experiments with unambiguous phenotypes. Up to now, the search for mechanotransduction channels has only produced conflicting results,[23,24] and the molecular identity of the channels remains elusive. An important question is whether low-threshold touch receptors and high-threshold nociceptors use the same gating principle to generate mechanoreceptor potentials.[25] A recent report on the effects on pain behaviour of a spider toxin that blocks stretch-activated cation channels (GsMTx4)[26] suggested an involvement of transduction channels gated by membrane stretch. It is conceivable—although entirely speculative—that the detection of gentle touch relies on multi-protein transduction complexes in vertebrate touch receptor neurons, while nociceptors respond to their much stronger stimuli with simple stretch-sensitive channels. It is also possible that the *C. elegans*-type transduction complex and the stretch-sensitive channel do not represent mutually exclusive gating principles. There may be various intermediate structures with transduction channels attached to the cytoskeleton or to proteins in the membrane or the extracellular matrix. The challenge is to identify the channel protein itself, which can probably only be done by genetic means, because the proteins cannot be isolated from the fine sensory endings of mechanoreceptors. Once this is achieved, auxiliary subunits and associated proteins can be identified more easily.

A fascinating example of vertebrate mechanosensory transduction by tethered channels is the generation of receptor potentials in the hair cells of the inner ear. These exquisitely sensitive cells detect movements on a nanometer scale by their apical hair bundles and transmit the sensory signal to afferent neurons with high efficiency. Based on groundbreaking electrophysiological studies[27-29] and the discovery of protein filaments connecting the sterocilia within a hair bundle[30,31] a working hypothesis was formulated that explained hair-cell function in terms of a tethered transduction channel.[32-34] An impressive array of excellent biophysical investigations was since carried out to scrutinize and improve this hypothesis.[35-40] Today, the tethered-channel hypothesis is well established, and much of the current work is focussed on identifying the molecular components of the transduction complex.

In the organ of Corti, the sensory inner hair cells (IHCs) extend their sensory stereocilia into a thin layer of endolymph between the top of the sensory epithelium and the tectorial membrane (Fig. 2A). Lateral movements of this fluid layer deflect the stereocilia when the Corti organ responds to sound with local vibrations. The hair bundle consists of 50-300 stereocilia which move together because various protein filaments connect each stereocilium to its neighbours. In fact, all stereocilia of a bundle move in unison so that the entire hair bundle acts as a functional unit.[41] The tip of each stereocilium is connected to the lateral membrane of its larger neighbour by a protein filament called tip link (Fig. 2B). When the endolymph tilts the stereocilia along the tip links' axis, the two attachment points of each tip link move slightly apart, stretching the protein filament. Two cell adhesion proteins, cadherin 23[42] and protocadherin 15,[43] co-assemble to form the ~150 nm long tip-link filament.[44-48] Since both the setereocilia and the tip links appear to be rather stiff structures with low elasticity, each displacement of the hair bundle causes an immediate mechanical force to act on the two attachment points where it opens transduction channels. The resulting depolarisation leads to activation of ribbon synapses at the basal pole of the hair cell which transmit the signal with remarkable fidelity to multiple afferent neurons.[49,50]

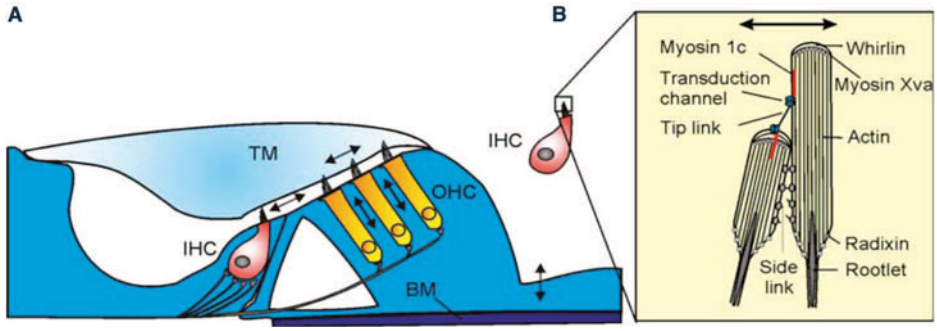

Figure 2. Hair cells in the organ of Corti. A) Schematical cross-section of the organ of Corti with the tectorial membrane (TM) covering the stereocilia of sensory inner hair cells (IHC) and electromotile outer hair cells (OHC). Sound induces local vibrations of the basilar membrane (BM) and a lateral displacement of hair-cell stereocilia (arrows). B) Enlarged view of the stereocilia of an inner hair cell. The transduction channels of the longer stereocilium are dynamically anchored to actin filaments through myosin 1c. A tip-link filament pulls the transduction channels open when the stereocilia move in the plane indicated by the arrow.

To ensure optimal operating conditions in all physiological situations, the hair cell actively adjusts the tension of the tip link using so-called adaptation motors.[51,52] These motors are thought to be myosin 1c molecules which are attached to the tip-link attachment points and can crawl along the actin filaments inside the stereocilia, thereby stretching the tip link (Fig. 2B).[53,54] This process consumes ATP and preserves the optimal tension of the tip link. When the hair bundle is displaced, the transduction channels open and admit K^+ and Ca^{2+} influx into the lumen of the stereocilia. Because the interaction of myosin 1c with actin filaments is disturbed by Ca^{2+},[55] the adaption motors let go, and the entire transduction complex slides down the plasma membrane, releasing tip-link tension and allowing the channels to close. Once Ca^{2+} is extruded from the stereocilia, the adaption motors can re-establish the optimal tension.

Much effort has been invested into the search for the molecular identity of the transduction channels that sit at one or both ends of the tip link. For each candidate channel protein, the expression at the tip of the stereocilia must be demonstrated, and it must be shown that the channel is gated by hair-bundle displacements. Moreover, ablation of the candidate gene must cause cochlear and vestibular dysfunction. The first promising candidate was the ion channel TRPN1. Gene silencing experiments caused the expected phenotypes in zebrafish.[56] However, TRPN1 was found to be expressed in the kinocilia of lower vertebrates, and not in the stereocilia,[57] and the *trpn1* gene it is not present in avian and mammalian genomes. A number of other proteins have been investigated as possible candidates for the hair cell transduction channel, including TRPA1, TRPML3, TRPV4, and TMHS.[36,40,58] But the channel has not been identified to date, and the search for the tethered channels of the inner ear remains one of the most urgent challenges in sensory physiology.

The primary transduction process in hair cells requires a complex dynamic environment to function properly. To generate neuronal signals upon detecting a very faint sound (the detection threshold in humans is ~10^{-16} Watt/cm^2), and to discriminate frequencies over a range of three orders of magnitude (20 Hz-20 kHz), the Corti organ must amplify the primary signal. Research in cochlear amplification has seen exciting

advances in recent years. I will only briefly outline this topic here, as it is more an auxiliary than a primary process. Amplification of the primary signal—which are local vibrations of the Corti organ due to resonance—is performed by the outer hair cells (OHCs) of the Corti organ.[37,59-61] OHCs possess the unusual (perhaps unique) property of electromotility. They contract upon depolarization, and they elongate upon hyperpolarization. This motorresponse to changes in membrane voltage is extremely rapid. An OHC can go through tens of thousands of contraction/elongation cycles per second and can thus follow the vibration frequency caused by resonance at any particular spot along the cochlear. Importantly, the stereocilia of OHCs are embedded in the tectorial membrane (Fig. 2A) and, thus, constitute a mechanical link between the sensory epithelium and the tectorial membrane. As the OHCs oscillate, they "shake" the entire structure at the point of resonance and amplify the local movement of endolymph that stimulates the sensory IHCs. In mammals, electromotility is mediated by the protein prestin, a membrane protein that changes its volume under the control of the membrane voltage.[62-64] The protein swells by voltage-dependent uptake of chloride ions, a process that is extremely fast. Electromotility is thus orders of magnitude faster than any motion based on ATP-consuming motor proteins. The OHCs amplify the primary signal by a factor of 100-1000 and hence allow the IHCs to operate at the levels of sound pressure that constitute our normal auditory environment.

PHOTOTRANSDUCTION—DYNAMIC SCAFFOLDS

Since more than five decades, a constant stream of excellent papers has been published on signal transduction in photoreceptors of vertebrates and invertebrates. Today, phototransduction is arguable the best understood of all sensory transduction cascades, and a number of excellent reviews describe the most recent advances.[65-71] In this chapter, I will concentrate on one particular aspect of phototransduction: The spatial organization of the transduction cascade and the temporal redistribution of transduction components. Research on this topic is critical to the understanding of primary processes in all sensory cells, and the advances in organellar proteomics is expected to promote this field considerably.[72-74] Proteomic studies provide a specific protein inventory of a sensory organelle which can be used to examine protein-protein interactions and their significance for sensory transduction.[75] *Drosophila* photoreceptors were the first sensory cells where such interactions were shown to exist on a large scale. In the compound eye, the microvilli of the rhabdomere contain the scaffold protein INAD (for the terminology of *Drosophila* photoreceptor gene products see ref. 70). INAD contains five PDZ domains[76,77] which serve as interfaces for the interaction with multiple proteins of the signal transduction cascade. INAD polymers constitute a scaffold which binds together the transduction proteins in a supramolecular complex, termed a signalplex.[78] Within the signalplex, diffusion distances are short and transduction is fast (Fig. 3). Upon illumination, the absorption of a photon converts rhodopsin to metarhodopsin which activates phospholipase Cβ via the $G\alpha_q$ subunit of a GTP-binding protein. The resulting release of diacylglycerol (DAG) opens the transduction channels TRP and TRPL which depolarize the photoreceptor through cation (Na^+, Ca^{2+}) influx.[79] The active second messenger for TRP and TRPL appears to be DAG itself or one of its metabolites, most likely a polyunsaturated fatty acid.[80,81] The Ca^{2+} signal induced by the TRP/TRPL channels plays a pivotal role in the termination of the light response. A Ca^{2+}-dependent protein kinase C phosphorylates TRP channels as well

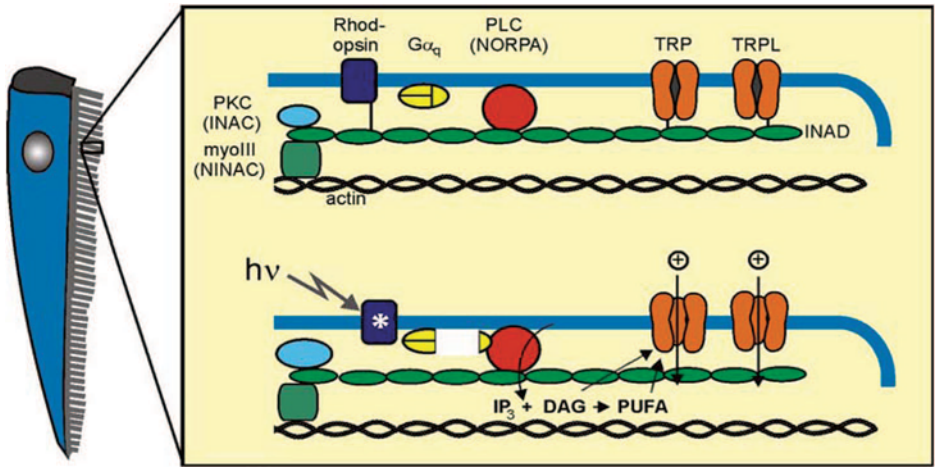

Figure 3. The signalplex of fly photoreceptors. Schematical view of primary processes in a microvillus from a fly rhabdomer. A polymer of INAD scaffold proteins co-ordinates the transduction proteins rhodopsin, $G\alpha_q$, phospholipase C (PLC), protein kinase C (PKC), *transient receptor potential* channel (TRP), and TRP-like channel (TRPL). Illumination leads to release of inositol-1,4,5-trisphosphate (IP_3), diacylglycerol (DAG), and polyunsaturated fatty acids (PUFA), which open the TRP/TRPL channels.

as myosin III (NINAC),[82] and both processes contribute to response termination. In fact, fast signal termination depends on the interaction of PKC with the INAD scaffold,[83] and this currently represents the only proven effect of protein co-ordination on transduction kinetics in this cell. It is still an open question to what extent the signalplex contributes to the extremely rapid light response in flies, the fastest G-protein mediated process known. An exciting development in this field is the finding that the INAD scaffold is not a fixed structure but that its interactions with other proteins can be regulated by light. Using X-ray crystallography,[84] found that the fifth PDZ domain of INAD can exist in two different forms. In one form the domain binds other proteins. In the other form an intramolecular disulfide bond prevents binding. Interestingly, PDZ5 toggles between the two conformations under the influence of light in such a way that illumination can switch off protein binding to PDZ5. This light effect on scaffolding appears to improve the temporal resolution of vision in flies and indicates a new approach to studying the photoreceptor signalplex. Dynamic effects on protein co-ordination may be important for the function of the phototransduction system.[85-87]

In vertebrate photoreceptors, studies of supramolecular transduction complexes were mainly focussed on the rim regions of the outer segment where the edges of the discs are in close proximity (<10 nm) to the plasma membrane (Fig. 4). In this rim region, the role of scaffold protein is thought to be played by the protein GARP (glutamic acid-rich protein). Having itself no intrinsic structure,[88] GARP binds to a number of phototransduction proteins and helps to assemble them into a molecular complex.[89] Interestingly, GARP comes both in soluble form (GARP1, GARP2) and as a membrane-bound appendage to the photoreceptor transduction channel, the cGMP-gated channel in the plasma membrane. As such, it can function as a molecular glue between the metabotropic transduction in the disc membrane, and the effector proteins in the plasma membrane that generate light-dependent electrical and Ca^{2+} signals. Indeed,

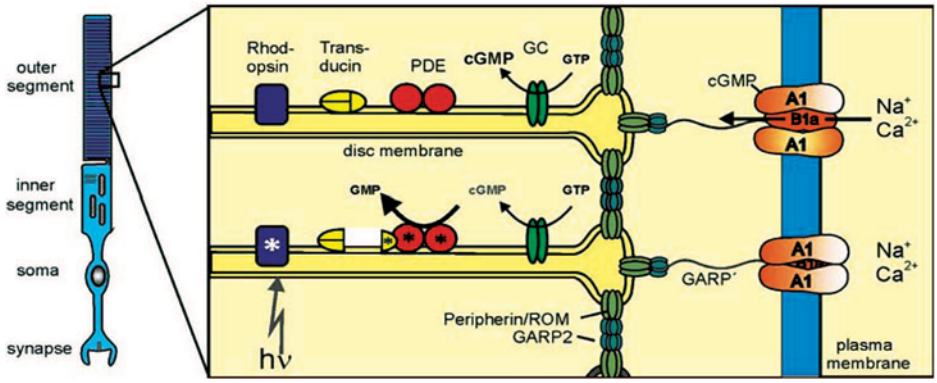

Figure 4. The rim region of the vertebrate photoreceptor outer segment. cGMP-gated transduction channels in the plasma membrane (with their subunits CNGA1 and CNGB1a) are connected to the intracellular disc membrane via a GARP' domain (GARP = glutamic acid-rich protein) which forms the C-terminus of each CNGB1a subunit. Soluble GARP and peripherin connect discs and transduction channels in the rim region. cGMP, cyclic guanosine monophosphate; GC, guanylyl cyclase; GTP, guanosine triphosphate; PDE, phosphodiesterase; ROM, rod outer segment membrane protein. Illumination leads to a decline of the cGMP concentration and closure of cGMP-gated cation channels.

in vitro binding experiments have shown that GARP co-ordinates phosphodiesterase, guanylyl cyclase, the retinal ATP-binding cassette transporter, and peripherin 2 on the disc side with the cGMP-gated channel, and the Na^+/Ca^{2+}-K^+ exchanger on the side of the plasma membrane.[89-91] It is not yet known whether this spatial organization serves structural or functional purposes—or both. But it points to the notion that the rim region of vertebrate photoreceptors shows a similarly high degree of molecular co-ordination as the signalplex in *Drosophila* photoreceptors. The speed and efficiency of sensory signal generation and termination may require such spatial order and may comprise more, as yet unidentified proteins. A recent addition to the molecular complex in the rim region is a retinal ryanodine receptor, related to the large Ca^{2+} channel protein known to mediate electromechanic coupling in skeletal muscle.[92] This protein is loacted in the edge of the disc and may contribute to the regulation of Ca^{2+} signals within the microdomain of the rim region. Ca^{2+} signals drive revovery and adaptation in photoreceptors[93] and the retinal ryanodin receptor may be involved in this process.

Reversible attachment to a transduction scaffold may constitute an effective mechanism for sensory adaptation. Proteins may be tethered to the signalplex for high sensitivity and may be removed from the complex to reduce sensitivity. This concept arises from observations of light-induced protein translocation in photoreceptors.[94] In both *Drosophila* and in mouse rod photoreceptors, key members of the phototransduction cascade are shuttled to and from the transduction site in a light-dependent way. The heterotrimeric GTP-binding protein transducin in vertebrates sticks to the disc membrane in the dark by a farnesyl group on its γ subunit and an acyl group on its α subunit. This double-anchor effectively attaches the inactive trimer to the discs. During photoactivation of rhodopsin, however, transducin dissociates into the $\beta\gamma$ dimer and the active $G\alpha$-GTP, both of which have increased solubility because each has only one membrane anchor. This "photo-solubilization" of transducin releases much of the protein from the disc during intense or prolonged illumination. The soluble protein is able to diffuse to the inner

segment where it is sequestered by a still unknown mechanism,[95,96] until it returns again to the outer segment in the dark. A similar translocation of $G\alpha_q$ is thought to contribute to light-adaptation in the fly photoreceptor.[97] In both cells, G proteins and arrestin—a key protein for response termination—were shown to travel in opposite directions.[98,99] Attenuation of the light response is brought about by sequestration of transducin in the inner segment and accumulation of arrestin in the outer segment.[94] Such translocation of proteins between the signaling complex and a nonphotosensitive compartment is not restricted to soluble proteins. The TRPL channel of fly photoreceptor undergoes a light-dependent, reversible translocation between rhabdomers and cell body,[100,101] a process that may involve endocytosis and intracellular transport by motor proteins. Whatever the exact molecular mechanisms of protein translocation are, the data available today clearly show that signaling complexes in photoreceptors can be subject to light-dependent restructuring. Such dynamic regulation of protein networks may have profound effects on transduction efficiency—not only in vision. Once again, the photoreceptor may serve as a model cell for the exploration of new principles in sensory transduction.

TASTE TRANSDUCTION—GUSTATORY GENETICS

Taste transduction research has rapidly advanced in recent years through genetic examination of the taste system.[102-104] In mammals, two families of metabotropic taste receptors, T1R and T2R (gene symbols *Tas1r* and *Tas2r*) are expressed in the chemosensory microvilli at the apical pole of taste receptor cells. T1R-expressing cells probe food for attractive stimuli (sweet and umami), while T2R cells mediate the aversive bitter taste. Sweet taste can be elicited by a range of mono- and polysaccharides but also by various amino acids, peptides and proteins as well as by artificial sweeteners and by certain salts.[104] The umami taste quality is caused by detection of certain L-amino acids, most distinctly by sodium glutamate which generates a pleasant meaty taste. The T1R protein family mediates sweet and umami detection by differential combination of its three members, T1R1, T1R2, and T1R3 (Fig. 5). Dimers of T1R2 and T1R3 (and possibly T1R3 homodimers) operate as sweet sensors, while T1R1/T1R3 dimers are umami-selective.[105,106] The receptors for bitter stimuli, the T2R family, are activated by various toxic and nontoxic substances mainly present in plants. It is generally believed that bitter taste serves to detect harmful substances and to prevent the animal from swallowing noxious material. T2R receptors differ from the T1R family in that they have short N-termini and do not form dimers (Fig. 5). They are coded by a gene family of 36 functional genes in mice and 25 genes in humans. Importantly, each bitter cell expresses multiple T2Rs and is, therefore, responsive to a wide range of bitter substances.[107,109,110,209] Both T1Rs and T2Rs couple the initial taste signal to activation of a phospholipase C (PLCβ2).[111] This process is mediated by the GTP-binding protein gustducin[112] and leads to the release of IP_3 and Ca^{2+}. The common transduction channel of this pathway is TRPM5, a cation channel gated by Ca^{2+}.[111,113,114] While the prominent role of the T1Rs and T2Rs in taste transduction, together with its transduction cascade that targets TRPM5, is now firmly established, evidence obtained in behavioural assays points to additional processes involved in sweet and umami transduction. Residual sweet and umami responses were found with mice after genetic ablation of either T1R3 receptors;[106,115,116] gustducin,[117] or TRPM5,[118] suggesting that a TRPM5-independent, yet unidentified transduction pathway exists in taste cell. Also, there appears to be cross-talk between sweet and bitter taste, which may result from direct effects of bitter compounds

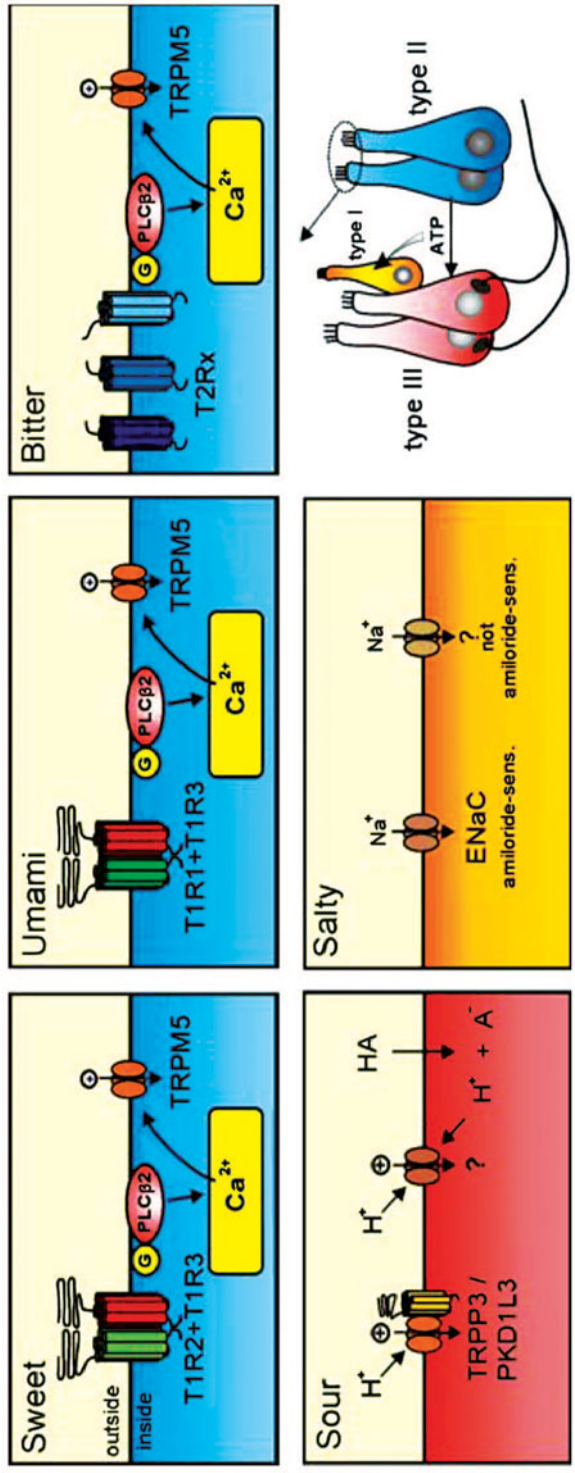

Figure 5. Primary processes in taste cells. Transduction models for the chemosensory membrane of taste cells. The metabotropic receptor families T1R and T2R mediate sensitivity to sweet, umami, and bitter by activating phospholipase β2 (PLCβ2) through a GTP-binding protein (G). The resulting release of Ca^{2+} from intracellular stores opens transient receptor potential M5 channels (TRPM5) which generate a depolarizing receptor potential. Sour taste is mediated by a combination of transient receptor potential P3 channels (TRPP3) and polycystic-kidney-desease-like ion channels (PKD1L3). Additional pH effects on other ion channels, as well as proton uptake into taste cells have also been reported. Salt taste may—in part—be mediated by Na^+-permeable epithelial sodium channels (ENaC). Sweet, umami, and bitter taste resides in Type II cells (blue) which release ATP as paracrine transmitter. Sour taste is located in the presynaptic Type III cells (red), and salt taste is mediated by Type I cells (yellow) which also remove ATP from the taste bud.

on the TRPM5 channel.[119] Finally, substantial species differences can be expected in taste transduction as different animals need different food.[120] Carnivorous *Felidae*, for example, (cats, tigers, cheetahs) were shown to have a *Tas1r2* pseudogene, so that the T1R2 protein is not expressed and the T1R2/T1R3 dimer cannot be formed.[121] This may explain the cats' indifference to sweet stimuli and reflect the lack of selective pressure on the maintenance of sweet taste in the evolution of these animals.

The search for transduction mechanisms of sour-sensitive taste cells has been hampered by the fact that the adequate stimulus, protons, affects virtually every protein with amino acid residues that can bind H^+. Thus, pH effects can be measured with most channels, transporters and proteins involved in signal transduction, and it is difficult to prove that a pH effect on an individual protein is related to the physiological proton sensor of a sour-selective taste cell. This conundrum is made worse by the fact that protons can reach the basolateral membrane of taste cells through the paracellular pathway, and that protonated acids can cross the plasma membrane and cause intracellular acidification. The actions of pH changes are, therefore, essentially unspecific and not localized. Physiological data, however, show that there is a subpopulation of taste cells equipped with a specific pH sensitvity. Such a property could arise from specific expression of proton-gated ion channels and/or by a reduced cytosolic pH buffer capacity. Indeed, intracellular acidification and stimulus-related Ca^{2+} signals could be demonstrated in a subpopulation of taste cells upon extracellular pH changes.[122] The most convincing set of evidence for a specific H^+ sensor in sour-specific taste cells comes from studies of an ion channel from the TRP family, TRPP3 (synonyms: TRPP3 = PKD2L1; TRPP = polycystin family).[123] This protein is expressed in a subset of taste cells that are not sensitive to other taste qualities and, in conjunction with the related protein PKD1L3, confers acid sensitivity when expressed in the cell line HEK 293.[124-126] When TRPP3-expressing taste cells are removed by genetic manipulation, animals do no longer respond to sour stimuli while the other taste qualities are intact.[103] While many details of the sour transduction process are not yet clear, these data strongly suggest that TRPP3 is part of the sour taste receptor.[127]

Salt detection is thought to work through cation ion channels which conduct Na^+ or K^+ from the surface of the tongue into salt-sensitive cells. In parallel, Cl^- ions are thought to take the paracellular route across the taste epithelium.[104] A candidate for Na^+ taste is the amiloride-sensitive epithelial Na^+ channel ENaC whose three subunits α, β and γ are expressed in some taste receptor cells.[128] Both amiloride-sensitive and amiloride-insensitive components of salt taste were identified by electrophysiology and in behavioural experiments.[129,130] Amiloride-sensitive, highly Na^+-selective channels are present in some taste cells.[131] However, the definite answer to the question whether ENaC mediates salt taste must probably await the generation of a taste-cell specific conditional knockout mouse, as global deletions of any of the three ENaC subunits results in perinatal lethality.[132] Thus, the transduction mechanism of salt taste is presently not well understood.

An important point for the examination of primary processes in taste transduction is to identify the right cell types within a taste bud. Evidence from a number of morphological and physiological studies supports the view that only a subset of cells in the taste bud expresses taste receptors and transduction proteins. Other cells have different functions including synaptic transmission or glia-like supportive function.[104] At present the data provide a scenario in which taste receptor cells (Type II cells; Fig. 5) respond to tastants with the release of ATP, probably through pannexin 1 hemichannels.[133,134] ATP appears to act as paracrine transmitter on Type III cells which express $P2X_2$ and $P2X_3$ purinergic receptors and form synapses with afferent neurons.[133] Finally, Type I cells may limit

wide-spread diffusion of ATP by an ecto-ATPase,[135] thus serving a glia-like function in the taste bud. This working hypothesis of a paracrine transmission system illustrates the complexity of signaling inside a taste bud. Many observations have yet to be integrated into this model. For example, amiloride-sensitive currents are restricted to Type I cells[136] suggesting that these glia-like cells are also responsible for salt taste. Sour taste, in contrast, was localized to the Type III cells of the taste bud.[137,138] Substantial differences in morphology and expression patterns have been demonstrated between taste buds of rats and mice[120] and between different taste buds on the same tongue.[139,140] Moreover, psychophysical effects of peripheral neuromodulators[141] have to be examined as they may regulate sensory signal processing in the taste bud.

OLFACTORY TRANSDUCTION—COPING WITH FUZZY RECEPTORS

The olfactory system of vertebrates is designed to detect an unlimited number of odorants.[142] To do this, the system exposes a set of roughly 400 (humans) to 1000 (dogs, rodents) olfactory receptor proteins to the air inside the nasal cavity. The receptors are encoded by a large family of intron-less genes,[143] scattered all over the genome (Fig. 6),

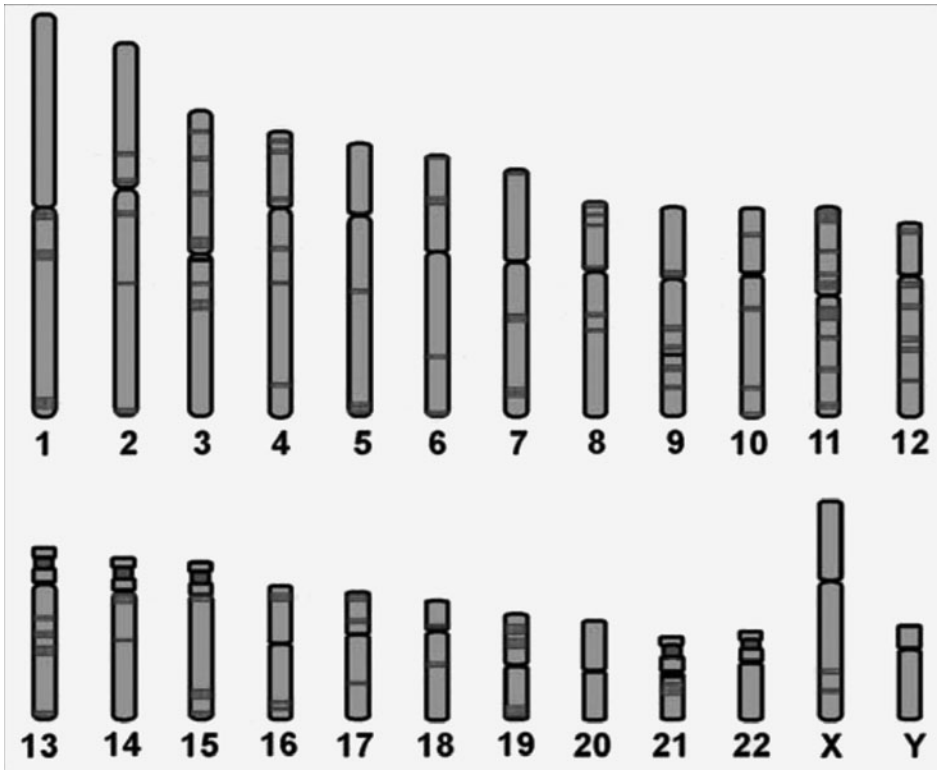

Figure 6. Odorant-receptor genes on human chromosomes. The dark (red) bands indicate gene clusters that contain groups of odorant receptor genes. Such clusters are present on almost all chromosomes. A color version of this image is available at www.landesbioscience.com/curie

and are expressed in olfactory receptor neurons (ORNs). The receptor proteins do not only determine to which compounds the ORN will respond. They also help to target the ORN axon to its appropriate connection point in the brain. Consequently, all axons of a ORN population that expresses the same receptor converge onto the same projection neurons in the olfactory bulb.[144] Most authors favour the notion that only a single olfactory receptor gene is expressed in each individual ORN.[145,146] Intense research efforts are currently focused on the question how a single receptor gene is chosen from the large receptor gene repertoire of the olfactory system. Only one allel of each olfactory receptor is transcribed in a mature ORN,[147,148] and the selected gene product appears to suppress the transcription of all other olfactory receptor genes.[149,150] Various minigenes, enhancer elements and homeodomain transcription factors have been shown to promote expression of receptor genes within a gene cluster, and they seem to play key roles in controlling the expression program.[151-153] Importantly, an ORN tolerates the simultaneous expression of two different olfactory receptors only if one of them is coded by a pseudogene and is, hence, not functional.[154] Transcription of the first receptor gene apparently exerts an effective feedback repression upon all other receptor genes. Alternatively, expression of multiple receptors may prevent the ORN from developing into a mature sensory cell. Despite remarkable progress in recent years, the precise genetic basis of the one cell-one receptor phenomenon is not fully understood.

Although the family of olfactory receptor genes is large, 1000 receptors appears to be a small repertoire of sensors compared to the multitude of possible odorants to which a limiting number cannot be rationally assigned. This means that each receptor-type must be able to bind a large number of different odorants. In other words, the olfactory receptors must work with relatively low odorant selectivity. This was first demonstrated by single unit recordings from ORNs, challenged with a diverse panel of odorants.[155] Even in experiments with only 20 test odorants, most ORNs responded to more than 10, illustrating that the activity of a single ORN, or a single ORN population expressing the same odorant receptor, does not provide the brain with conclusive information on odor identity. In fact, this information is extracted from the spatial and temporal activity pattern of *all* ORNs.[156-159] Such pattern analysis does not require highly selective sensors. It operates efficiently with a set of broadly tuned sensors which, collectively, produce unique signal patterns for each stimulus. In the olfactory system, the set of 400-1000 low-selectivity receptors accommodates an unlimited range of stimuli and still generates unique neuronal activity patterns for each of them. However, working with low-selectivity receptors brings fundamental problems for the task of signal transduction. Most odorants bind to the receptors with low affinity and activate the receptors only briefly.[160] Estimated the mean dwell time of an odorant bound to its receptor to be less than 1 ms. Such a brief contact is hardly sufficient to trigger the transduction cascade by activating the G-Protein G_{olf} (Fig. 7). Accordingly, the synthesis of the second messenger cAMP by adenylyl cyclase Type III (AC III, Fig. 7) proceeds at a low rate. Measurements from amphibian ORNs showed that the maximal rate of cAMP synthesis in an ORN is about 200,000 cAMP molecules per second.[161] This maximal rate is similar to the number of cGMP molecules hydrolyzed upon absorption of a single photon in a rod photoreceptor (250,000 molecules per photon).[163]

Thus, in contrast to phototransduction, the metabotropic transduction step on olfactory transduction operates with low efficiency. This point is supported by the

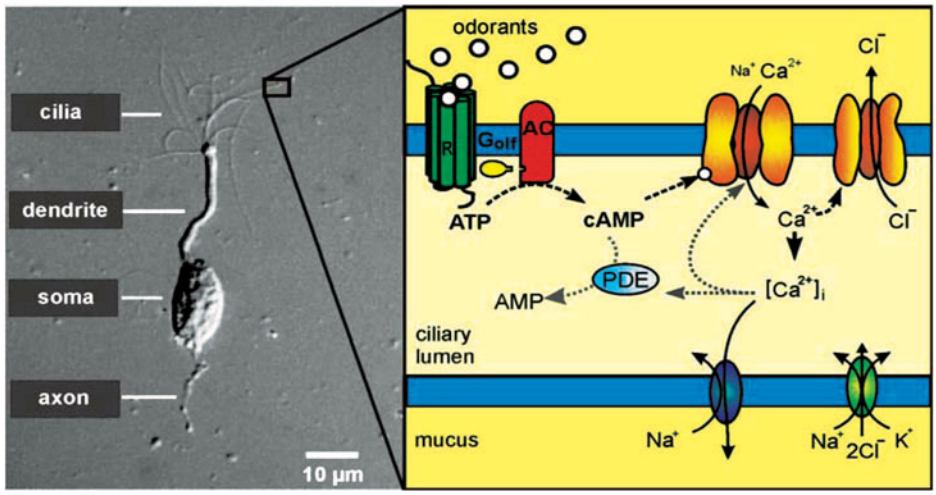

Figure 7. Primary processes in olfactory sensory cilia. Left: Micrograph of an isolated olfactory receptor cell showing the chemosensory cilia at the ending of the neuron's dendrite.[162] Right: Current transduction model. AC: adenylyl cyclase Type III; ATP: adenosine triphosphate; $[Ca^{2+}]_i$: intracellular calcium concentration; cAMP: cyclic adenosine monophosphate; G_{olf}: olfactory GTP-binding protein; PDE: phosphodiesterase. The two transduction channels, cAMP-gated cation channels and Ca^{2+}-gated chloride channels, are indicated in the upper membrane. The ion transporters that extrude Ca^{2+} and accumulate Cl^- are depicted at the bottom membrane of the cilium.

consistent observation that odorant concentrations used for physiological experimentation with ORNs have to be in the range of 1-100 µM to detect cAMP synthesis or to record cAMP-dependent receptor currents.[164-166] In fact, metabotropic transduction in ORNs appears to work without any molar amplification: Micromolar concentrations of odorants are needed to generate micromolar concentrations of cAMP in ORNs. The absence of effective metabotropic amplification results directly from the use of low-selectivity receptors which, in turn, is required for a system open to an unlimited range of odorants. How then, can the olfactory system work as the highly sensitive detection system with amazing powers of odor discrimination?

Odorants can be detected at extremely low concentrations, much lower than the 1-100 µM used in physiological experiments on isolated cells. It is difficult to compare results obtained from single ORNs with the performance of the olfactory system in vivo for at least three reasons: (1) ORNs show an extremely high degree of convergence, as roughly 2000 ORNs are connected with a single mitral cell in the olfactory bulb. It is conceivable that such a large ensemble of afferent neurons causes excitation in a mitral cell even if each individual ORN is only slightly activated. Thus, temporal summation of multiple weak signals may contribute to olfactory sensitivity. (2) The sensory membrane of ORNs is embedded in a mucus layer that, in terrestrial animals, contains high concentrations of odorant-binding proteins.[167-170] These small, soluble proteins belong to the lipocalin-family, proteins that can shuttle hydrophobic molecules through body fluids and across cell membranes. In the olfactory mucus, these binding proteins display odor-specificity[171] and can interact with odorant receptor proteins.[172]

The precise role of these proteins in olfaction is not understood, but they are expected to influence the interaction between odorants and their receptors. (3) ORNs possess an unusual signal amplification mechanism that boosts the odor-induced depolarisation and may be critical for responses to weak stimuli. This mechanism utilizes the Ca^{2+} influx through cAMP-gated transduction channels in the chemosensory membrane (Fig. 7). Ca^{2+} opens chloride channels which conduct a depolarizing efflux of chloride ions.[173] The anion influx strongly amplifies the receptor potential and depolarizes the ORNs sufficiently for excitation.[174-176] To support this excitatory chloride current, ORNs accumulate chloride and support an elevated intracellular chloride concentration.[177,178] Current research efforts in this field focus on the molecular identification of the calcium-dependent chloride channels[179-184] and on the mechanisms of chloride homeostasis that support this signal amplification.[185-187]

While ORNs have to operate with low selectivity, pheromone receptors in the vomeronasal organ display a high degree of specificity and sensitivity for the chemical compounds that orchestrate reproductive behaviour among the members of a species. Consequently, the primary processes are fundamentally different between these two sensory modalities. The prototypical pheromone receptors of the silk moth *Bombyx mori* basically respond to single pheromone-binding events, although the exact nature of this process and, in particular, the role of pheromone-binding proteins, is still not fully understood.[188] But mammalian pheromone detectors are highly sensitive as well. Studies of pheromone receptors in the mouse vomeronasal organ (VNO) revealed detection thresholds near 10^{-11} M for the neuronal response.[189] VNO neurons employ two distinct sets of pheromone receptors, the V1R and V2R families, each of which comprises 100-200 different receptors.[190-192] The V1R family recognizes small urinary molecules that act as pheromones in mammals. Each V1R neuron seems to express only a single member of the V1R receptor family and, consequently, diplays high pheromone specificity. A separate population of VNO neurons expresses V2R genes. These cells respond to urinary peptides, in particular to major histocompatibility complex (MHC) class 1 peptides[193] and help conspecific animals to gain information related to the immune system of their mates. The transduction cascade used by both V1R and V2R neurons is also different from that operating in ORNs. Phospholipase C is believed to be the target enzyme, releasing IP_3, Ca^{2+}, DAG and polyunsaturated fatty acids (PUFA) as second messengers upon pheromone stimulation.[194] Robust evidence is available for a central role of the protein TRPC2 as transduction channel.[195] TRPC2 is expressed in the chemosensory microvilli of VNO neurons,[196] the channel is gated by DAG,[197] and TRPC2 knock-out mice lose the ability to distinguish between male and female conspecifics.[198-200] Nevertheless, some aspects of pheromone-driven behaviour remain intact in the $TRPC2^{-/-}$ mice, in particular the detection of MHC 1 peptides.[201] This finding suggests that a different population of VNO neurons exists which does not use TRPC2 as transduction channel.

Intense examination of the VNO and the olfactory epithelium currently challenges the traditional view that the two systems are dedicated exclusively to two discrete functions, namely pheromone control and olfaction.[202] It becomes clear that both systems contain various different populations of neurons, each with a specific purpose and a specific molecular equipment. The characterization of these chemosensory cells and their sensory function is an exciting task for sensory physiologists.[203-205]

EVALUATING ELECTROMAGNETIC FIELDS—MORE PRIMARY PROCESSES

In addition to analyzing the intensity and wavelength of visible light, animals can extract vital information from the degree of light polarization, from infrared radiation, as well as from electrical fields and from the Earth's magnetic field. Exciting recent developments have yielded insights into some amazing primary processes that mediate these tasks. I will briefly review progress in polarization vision and magnetoreception. For the topics of electroreception and infrared perception, I refer the reader to a set of excellent reviews recently published in this journal.[206-208]

Many animals are able to detect light polarization and to obtain complex information through this sensory channel. Nonpolarized sunlight reaches the Earth and is polarized when it is scattered or reflected by all kinds of materials within the atmosphere, on the terrestrial surface, or in water. The atmosphere creates a stereotypical pattern of celestial light polarization which can be used for navigational purposes.[209-213] Moreover, reflective surfaces like water or glass, as well as scales, elythrae and other shiny animal surface structures, polarize light, an effect that can be used to search water, identify prey, and break other animals' camouflage.[214,215] The primary transduction process of polarization vision is based on photoreceptors with pronounced absorption anisotropy (dichroism). Such photoreceptors show a preferred response of their photosensitive organelles to polarized light with a certain electric vector (e-vector). The rhabdomeric photoreceptors of insects and cephalopods harbour their rhodopsin in microvilli, membrane tubes of ~50 nm diameter. Apparently, the rotational freedom of rhodopsin is limited in the microvillar membrane, such that the orientation of the retinal molecules is mainly parallel to the long axis of the microvillus. Polarized light is, therefore, best absorbed when its e-vector is aligned along the microvilli. Furthermore, all microvilli in a polarization-sensitive photoreceptor are aligned in parallel[216,217] so that the entire rhabdomer displays the same dichroism as each of its microvilli. In contrast to the rhabdomeric photoreceptors, the ciliary photoreceptors of vertebrates seem not to be very useful for polarization vision. It is generally held that rhodopsin molecules rotate freely within the disc membranes, without any preferred orientation. Light traveling axially through the outer segment thus hits retinal molecules that point into all possible orientations of the membrane plane, and no dichroism can occur. While most terrestrial vertebrates are polarization-blind, many fish species have been shown to be polarization-sensitive. Anchovies have tilted the discs in their cone photoreceptors by ~90° so that the incident light enters each disc from the side[218] and, presumably, hits retinal molecules that are more or less aligned with the disc membranes and preferably absorb light polarized in the membrane plane. This appears to be a solution to generate dichroic ciliary photoreceptors, but other strategies may also exist.[219,220] A new aspect of polarization vision is the recent discovery that marine mantis shrimps (stomatopod crustaceans)[221] are able to detect circularly polarized light, and even to distinguish left-handed from right-handed circularly polarized light.[222] Circularly polarized light arises when linearly polarized light travels through a birefringent material. Such material has different refractive indices for the x- and the y-components of the e-vector, retarding one component with respect to the other.[223] The resulting phase shift between the two components makes the e-vector rotate along the axis of the flight path, as the light travels through space. Importantly, only a material that retards one component by a quarter of the wavelength λ (a $\lambda/4$ retarder) generates circularly polarized light. If

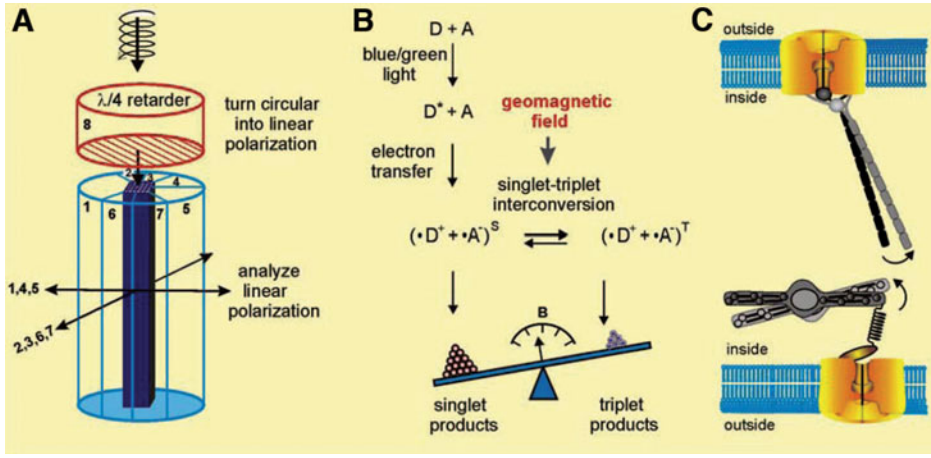

Figure 8. Primary processes in polarization vision and magnetoreception. A) Schematic representation of circular-polarization vision in the mantis shrimp. Photoreceptor 8 is positioned in the light path that enters an ommatidium. The cell converts circularly polarized light into linearly polarized light. The remaining 7 photoreceptors analyze the polarization plane.[221] B) The radical pair model of magnetoreception. Two domains of a cryptochrome molecule act as electron donor (D) and acceptor (A), respectively. Upon light absorption, the donor reaches the excited state D* and, subsequently, transfers an electron to the acceptor, giving rise to a radical pair in a spin-correlated singlet state $(\cdot D^+ + \cdot A^-)^S$. The yield of interconversions between the singlet and triplet states is affected by the geomagnetic field. The magnetosensory cell monitors the balance between singlet and triplet products as a measure of the magnetic field intensity B.[235] C) The magnetite hypothesis of magnetoreception. Top: A chain of single-domain magnetite particles is connected to the gating mechanism of an ion channel. When the animal changes its position within the Earth's magnetic field, the chain is displaced and opens the channel. Bottom: Small superparamagnetic magnetite particles are organized in plaques within the dendrite of a magnetosensitive neuron. These plaques consist of magnetite clusters (spheres) together with nonmagnetic maghemite chains (black lines) around an iron-coated vesicle (center). The entire structure is thought to change its shape with its position in the geomagnetic field and to drive the gating mechanism of an ion channels through an elastic connection.[251]

such light is again guided through a λ/4 retarder, the x- and y-components are shifted back into phase, and linearly polarized light results. It turned out that mantis shrimps possess caudal appendages which reflect patterns of circularly polarized light, and that the animals can be trained to distinguish between right-handed and left-handed circular polarization. Chiou and colleagues discovered that one of the eight photoreceptors in certain ommatidia acts as λ/4 retarder, turning circularly polarized light into linearly polarized light which is then analyzed by the remaining seven photoreceptors (Fig. 8A). The intricate architecture of the stomatopod eye provides the animal with two distinct channels of polarization vision and thus imparts additional visual qualities to the perception the environment.

Light polarization is not an exotic phenomenon to most of us, as we are used to polarizing filters on our cameras and our sunglasses, and the perception of light polarization appears to be just an additional aspect of vision. Magnetoreception, however, is a different matter. It is utterly amazing to observe the navigational skills of migratory animals and their use of magnetic cues. While human travellers need to be equipped with the Global Positioning System (GPS), a good map, a compass, and some geographic knowledge to find their way, animals apparently "see" or "feel" the geomagnetic field

and know how to use the magneto-sensory perception to travel over long distances. Two questions have to be addressed for navigation: Where am I? And which direction leads to my destination? Interestingly, animals seem to use different sensory strategies to obtain these informations, involving different primary processes.[224-228] Animals can exploit at least three parameters of the magnetic field: The inclination of the magnetic field relative to the Earth's surface, and the direction to magnetic north, parameters that we obtain from an inclination compass and a declination compass, respectively. Moreover, animals perceive the local intensity of the geomagnetic field, a parameter that we determine using a magnetometer. In the search for the primary processes that transduce these parameters into neuronal signals, two models are currently favoured, the radical pair model and the magnetite hypothesis. The radical pair model is based on the observations that certain modes of magnetoreception are light-dependent,[229] and that chemical free-radical reactions can be influenced by magnetic fields of ≤50 μT, the intensity of the geomagnetic field.[230] The candidate biomolecule for such a light-induced, magneto-sensitive free-radical reaction is cryptochrome, a photopigment that is present in the retina of migratory birds[231] and was found to be necessary for magnetoreception in *Drosophila.*[232] Cryptochrome absorbs blue light and forms long-lived radical pairs.[233] The light-dependence of magnetoreception in birds has given rise to the notion that the impact of the magnetic field on cryptochrome photochemistry may represent a primary sensory process.[228,234-236] The idea is that cryptochrome forms a singlet radical pair upon illumination, and that the kinetics of singlet/triplet interconversion is affected by the geomagnetic field (Fig. 8B). The balance between singlet products and triplet products depends to some extent on the orientation of a cryptochrome-containing cell in the geomagnetic field. If a cryptochrome-containing cell is able to compare the amount of chemical products resulting from singlet pairs to that orginating from triplet pairs, it can determine the yield of spin interconversion. Ritz proposed that a visual representation of the magnetic field can result from an ordered distribution of cryptochrome-containing cells in the retina.[235] While the radical pair model has not been established in all details, it represents a valuable hypothesis for directional magnetoreception in birds—it provides the molecular concept for a visual compass in the birds' eyes.[227]

But a compass alone does not bring you home if you do not know where you are. Thus, positional information is needed for navigation, information of the kind that we derive from comparing GPS readings with a map. Behavioural studies have revealed that migrating animals (birds, sea turtles, spiny lobsters) indeed possess positional information, and that this information is derived from the geomagnetic field.[237] The inclination and the local intensity of the magnetic field supply useful positional information for large areas of the globe. For example, each location in the atlantic ocean has a unique combination of inclination and intensity, as the lines of equal field inclination (isoclinics) are oriented roughly east-to-west, while the lines of equal field intensity (isodynamics) run roughly north-to-south. Isoclinics and isodynamics thus form a grid on a magnetic map, just like latitudes and longitudes do on a geographic map. There is strong evidence that migratory animals can follow both isoclinics and isodynamics and, therefore, must have the ability to gain and process positional information.[238,239] The primary processes underlying positional magnetoreception are thought to be distinct from the ones described by the radical pair model. The magnetite hypothesis was originally based on the microbiology of magnetotactic bacteria. These microorganisms contain strings of magnetite (Fe_3O_4) particles, each of which has a size of 30-120 nm, and is a stable, single-domain magnetic dipole.[240,241] The strings

restrain thermal movements of the individual particles, so that their magnetic moments add up, and the entire string tends to align with the geomagnetic field, just like a compass needle. Animal physiologists have long speculated that magnetite particles may transduce geomagnetic signals in migratory animals.[242-244] Strings of permanently magnetic particles may be connected to the gating mechanism of an ion channel so that the magnetic field can trigger channel opening and generate a receptor potential (Fig. 8C). Indeed, single domain magnetite particles were discovered in fish,[245] and indirect evidence points to a role of single domain magnetite in magnetoreception by mole rats and bats.[246,247] Curiously, the magnetite particles in various animals with robust magnetoreception are very small and are not aligned in orderly chains (e.g., homing pidgeon).[248] These particles have no stable magnetic moment, but they can assume a magnetic polarization in an applied field. An important recent finding is that such superparamagnetic material can, in principle, serve as a sensor in magnetosensory cells. Clusters of these particles change their shape when they are moved within a magnetic field (Fig. 8C). And the resulting forces are sufficient to gate ion channels.[249-251] Thus, cryptochrome and magnetite may be the transducing molecules in directional and positional magnetoreception. This concept will be scrutinized and extended in the coming years, with the still distant goal to understand magneto-electrical transduction in sensory neurons.

CONCLUSION

The data collected in this chapter illustrate several prominent similarities between primary processes of different sensory modalities: (1) *Stimulus detection:* G-protein coupled receptors (GPCRs) detect a wide spectrum of chemical and visual stimuli. At least five families of GPCRs mediate chemosensory qualities and a number of rhodopsin varieties cover the visual and ultraviolet spectra. Mechanodetectors directly couple movement to the opening of transduction channels. (2) *Transduction channels:* Most transduction channels belong to one of three protein superfamilies, die TRPs, the CNGs, and the degenerins. These are mostly nonselective cation channels, which are Ca^{2+}-permeable and show little voltage dependence. Transduction channels are often components of a supramolecular protein complex that regulates channel activity. (3) *Transduction complex:* A large set of proteins may co-assemble to form a transduction complex. The considerable plasticity of a transduction complex may underly adaptation, sensitization, response kinetics, and noise reduction. *(*4) *Amplification:* Primary receptor potentials may be amplified by prolonged activation of metabotropic receptors, by large electrochemical gradients for the receptor current, or by secondary currents that are conducted by distinct sets of ion channels. These common principles may also apply to primary processes in sensory cells where transduction mechanisms are not yet understood.

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CHAPTER 4

CHEMICAL COMMUNICATION IN INSECTS:
The Peripheral Odour Coding System of *Drosophila Melanogaster*

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Abstract: Animals use their chemosensory systems to detect and discriminate among chemical cues in the environment. Remarkable progress has recently been made in our knowledge of the molecular and cellular basis of chemosensory perception in insects, based largely on studies in the vinegar fly *Drosophila melanogaster*. This progress has been possible due to the identification of gene families for olfactory receptors, the use of electro-physiological recording techniques on sensory neurons, the manifold of genetic manipulations that are available in this species and insights from several insect model systems. The superfamilies of olfactory receptor proteins, the *Or* genes and the more recently discovered *IR* genes, represent the essential elements in olfactory coding, endowing olfactory receptor neurons with their abilities to respond to specific sets of odorants or pheromones. General odorants activate receptors in a combinatorial fashion, but some receptors are narrowly tuned to pheromones or to carbon dioxide. Surprisingly, olfactory receptors in insects are biochemically quite different to those in mammals and do not appear to signal via classical G protein pathways but rather via ionotropic mechanisms. Here we review the past decade of intensive research since the discovery of the first insect olfactory receptors in 1999, focusing on the molecules and cells that underly peripheral olfactory perception in *Drosophila*.

INTRODUCTION

As humans we tend to experience the world largely through our visual and auditory systems. However, for most animals the world is chemical and chemical

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senses are among the most basic tools evolved for locating resources and avoiding danger. Chemoperception is generally divided into the senses of smell (olfaction) and taste (gustation). The sensing of chemicals is a fascinating neural coding problem because of the vast number of possible chemicals in the environment, that are not only numerous, but diverse and complex. How does an organism detect and discriminate amongst hundreds or thousands of chemical stimuli that are vital for behaviours such as feeding, sex, defence or communication? How is the neural coding of chemical stimuli achieved and what are the mechanisms of signal transduction that ultimately result in the appropriate behavioural outputs?

In animals olfaction is mediated by the interaction of volatile ligands with a set of specialized membrane proteins known as odorant receptors (Ors). The genomics revolution has facilitated the discovery of large gene families of these Ors. In mammals such as mice and dogs there are upward of 1000 *Or* genes[1,2] while zebrafish have 143 *Or* genes.[3] The genetic model insect, the vinegar fly *Drosophila melanogaster*, has far fewer olfactory receptor genes, with only 60 *Or* genes encoding 62 receptors[4] as well as a few olfactory members of other chemosensory receptor families encoded by the *Gr* and *IR* genes. As model systems, insects offer distinct advantages over mammals for studying the chemical senses. Their chemosensory systems are similar in design but numerically simpler and mechanisms of stimulus-response relations can be quantified by studying simple innate behaviors. Electrophysiological recording techniques allow response properties of single chemosensory neurons to be correlated with behavior, something that still cannot be done in mammals. Though insects cannot tell us what they experience, behavioural conditioning experiments can teach us how their brains interpret chemical stimuli.[5,6]

Among insects, in recent years the vinegar fly *Drosophila melanogaster* has risen to prominence as a premier model for studies of olfactory receptor function. *Drosophila* offers the advantages of many powerful molecular genetic approaches to study olfactory system function and development. As well as the genome sequence of *Drosophila melanogaster*, genomes of another 11 *Drosophila* species have been completed, providing an excellent resource for comparative genomics and evolutionary studies. A unique in vivo assay system has been developed for olfactory receptor function, the so called "empty neuron" system.[7] This system allows any given *Or* (including *Ors* from other invertebrate species) to be transgenically expressed in *Drosophila* and its odour response profile to be determined. In addition *Drosophila Ors* have proven amenable to successful functional expression in heterologous cells in culture,[8,9] something that has proven very difficult to achieve with mammalian receptors.

IN INSECTS OLFACTORY RECEPTOR NEURONS ARE HOUSED IN OLFACTORY SENSILLA

The peripheral olfactory system of adult *Drosophila melanogaster* comprises two bilaterally symmetrical pairs of organs, the third antennal segments and the maxillary palps, the surface of which are covered by sensory hairs called olfactory sensilla. Odour molecules are thought to pass through pores in the external cuticle of the sensilla and into the underlying aqueous sensilla lymph, where they are transported to the plasma membrane of olfactory receptor neuron (ORN) dendrites (Fig. 1) and activate olfactory receptors and trigger action potential generation.

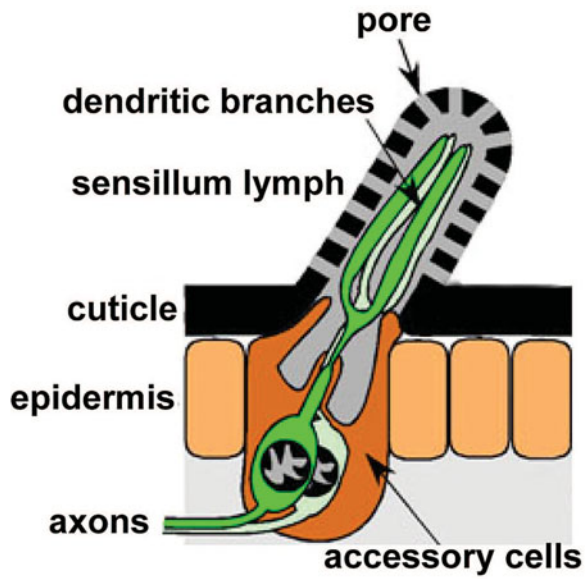

Figure 1. *Drosophila* olfactory sensillum. Cartoon of a single olfactory sensillum. Two to four olfactory receptor neurons (two in this example) are housed within a sensillum. Their cell bodies are at the base of the sensillum underneath the cuticle and are surrounded by accessory cells. Dendrites extend into the shaft of the sensillum and are bathed in sensillum lymph secreted by the accessory cells. Odorants enter through pores in the sensillum wall, traverse the lymph, then bind to olfactory receptor proteins in the plasma membrane of the dendrites, generating a change in receptor potential and ultimately an action potential.

The axons of ORNs converge onto ~49 functional processing units called glomeruli in the antennal lobe of the brain, the equivalent of the olfactory bulb in vertebrates. Glomeruli are the first synaptic relay centre of olfactory information from ORNs and are spherical bundles composed of synapses between terminating axons of ORNs, local interneurons and projection neurons. Glomerular organisation (the projection of ORN axons to discrete, condensed synaptic glomeruli in the brain) is remarkably conserved in essentially all invertebrates and vertebrates that have a differentiated olfactory system.[10] In *Drosophila* the number of ORNs and glomeruli is an order of magnitude less than in the mouse, with ~1300 ORNs converging onto ~50 glomeruli in *Drosophila*, compared with ~2 million ORNs and ~2000 glomeruli in mice.[10]

As in other insects there are different morphological types of olfactory sensilla in *Drosophila*. On the antenna there are three major morphological types of olfactory sensilla which differ in size, shape and cuticular structure; club-shaped basiconic sensilla, spine-shaped trichoid sensilla and small cone-shaped coeloconic sensilla. There is also one minor type, the intermediate sensilla. The maxillary palps bear only basiconic sensilla. Combined, the olfactory sensilla on the antenna total ~419 in males and ~457 in females.[11] Males possess about 30% more trichoid sensilla but 20% fewer basiconic sensilla than females,[12] resulting in a relatively even number of ~1200 afferent neurons from the antenna in both sexes. There are 60 basiconic sensilla on the palps, containing 120 ORNs. Most olfactory sensilla have a single cuticular wall that is a multi-porous structure to allow entry of odours. Each sensillum contains three accessory cells and one to four ORNs.

What are the roles of the different sensillum types? Behavioural experiments with strong hypomorphic alleles of the *lozenge* (*lz*) gene (a proneural gene involved in development), that lack most of their basiconic sensilla, showed that this sensillum type is not needed during courtship,[13] but is required for locomotor responses to food odours.[14] Conversely, trichoid sensilla respond to odours involved in mate recognition (odour extracts from males and virgin females) as well as the anti-aphrodisiac pheromone-like compound *cis*-vaccenyl acetate.[15-17] *Cis*-vaccenyl acetate has been suggested to inhibit male courtship towards fertilised females[18] and to function as an aggregation pheromone.[19] Accordingly, a sexual dimorphism is seen for this sensillum type[13] with small shifts in abundance. In moths, pheromone receptor neurons that are narrowly tuned to compounds like *cis*-vaccenyl acetate are always located in trichoid sensilla.[20]

Coeloconic sensilla are architecturally distinct in that they have two walls instead of one, with longitudinal grooves allowing odours to pass through, and they are the smallest type of sensilla.[11,21] These double walled coeloconic sensilla have a fourth accessory cell surrounding the two or three ORNs they contain. Intriguingly, the presence of this sensilla type is highly conserved over millions of years of insect evolution and is possibly found in all insect orders,[22] suggesting a critical function in chemosensory coding. In keeping with this they have been found to detect some universal odours, small amines and humidity.[21]

Despite these known functional differences, it is not clear how sensillum structure affects olfactory receptor neuron function and thus why these different sensillum types exist. We do know that sensillum types differ in the composition of proteins contained in the aqueous lymph surrounding the ORN dendrites. These proteins are involved in various perireceptor events, thought to be important for maintaining the sensitivity of odour detection systems, including aiding the activation and adaptation of ORNs. Important roles for these proteins may include acquisition and solubilisation of odour ligands in order to shuttle the hydrophobic odour molecules across the aqueous lymph or rapid inactivation of the odour stimulus after detection. One family of proteins thought to be involved in one or possibly all of these perireceptor roles are odorant binding proteins (OBPs). OBPs are a large family of small, highly abundant proteins secreted into the lymph by the accessory cells. They are differentially expressed in subsets of olfactory sensilla[23-25] and therefore could contribute to the sensitivity or selectivity of different sensilla types.

THE CELLULAR BASIS OF OLFACTORY CODING: FUNCTIONAL CLASSES OF OLFACTORY RECEPTOR NEURONS

The chemical information of odours is encoded neurologically by the firing patterns of ORNs. As a neuron fires, odour quality (chemical type), intensity (concentration) and dynamics (fluctuations in response time) are encoded simultaneously and are fundamentally interlinked.[26,27] One of the major advantages of studying insect olfaction is that physiological measurements can be recorded in vivo from either populations of ORNs or from individual ORNs to study peripheral olfactory perception and odour coding.

Electroantennograms (EAGs) and electropalpograms (EPGs) are electrophysiological techniques that allow the study of ORN physiology at a gross level.[28] EAG (and EPG) amplitudes for individual odorants puffed over the fly are summed receptor potentials of many ORNs, reflecting the number of ORNs responding and the location of the electrode with respect to structural properties of the antenna. This method can be used to detect odour specific changes in peripheral neurons of olfactory mutants when compared to

appropriate controls, but in most cases cannot directly measure individual ORN activation and is therefore not suitable for studying all fundamental aspects of odour coding.

The highly specialised technique of single unit electrophysiology takes precise recordings from a single sensillum using an electrode placed in contact with the lymph surrounding the dendrites of the sensillum. The relative amplitudes of action potential spikes fired by different ORNs contained in the sensillum can be used to reliably separate individual ORN responses indicating the number of neurons present and enabling analysis of their activity separately.[26,27] Therefore this technique can be used to measure the odour response spectra of individual ORNs, which is a major advantage compared to other organisms.

Recordings of ORN action potentials in response to odours has revealed that they are comprised of a limited number of discrete functional classes. Individual classes of ORNs respond to different sets of odours, but also exhibit a diverse array of response properties to different odours. Although most neurons display excitatory responses, some neurons have a high spontaneous rate of firing and are inhibited by certain odours. ORNs also have various modes of odour specific onset and termination kinetics[15,26,27] with a range of abrupt or prolonged responses. The different response spectra of the ORN types, along with their diverse response dynamics, provide the cellular basis for an olfactory code. Response spectra of *Drosophila* ORNs suggest that, like mammals, insects encode odours in a combinatorial way i.e., a single ORN responds to multiple odorants and a single odorant stimulates multiple ORN classes.

A complete analysis of the maxillary palp demonstrated that the 120 neurons in 60 sensilla can be classified into six functional classes of neurons (Table 1), which respond to various subsets of 47 diagnostic odours tested.[26] These functional classes have a stereotypical pairing with two particular functional classes of ORN always housed in the same sensillum, resulting in three functional classes of sensilla pb1, 2 and 3. Functional classes of ORN are named to reflect this organisation, such that, for example, in the pb1 sensillum, pb1A (palpal basiconic sensillum class 1A), a functional class of neuron with a broad response to a number of odours, is always housed with pb1B, which has a strong and narrow response to 4-methylphenol.

On the antenna electrophysiological analysis has so far characterised 18 functional ORN classes in eight basiconic sensilla classes, ab1-8.[27,29] Another two sensillum classes have been distinguished from gene expression studies, discussed later. The ab1 sensillum contains four ORNs (ab1A-D) and the others each contain two ORNs (ab2-10A and B) (Table 1). Thus, including the palp, 26 functional classes of ORNs in basiconic sensilla have so far been identified. These functional classes vary in the breadth of their odour response spectrum, with some ORNs narrowly tuned to specific odours and others broadly tuned. For example ab3A responds to a variety of esters, alcohols, ketones and other odours of varying chain lengths while ab5A and pb1B respond to only one of 47 odours tested, pentyl acetate and 4-methyl phenol respectively, at relatively high doses.

Electrophysiological analysis of ORNs in trichoid sensilla, at1-4, shows that the ORNs housed in these sensilla (Table 1) respond to a very different subset of odours to basiconic sensilla. ORNs of trichoid sensilla respond to the odours of flies,[16,17] where all trichoid sensilla are responsive to male extracts, while one type also responds to virgin-female extracts. Four coeloconic sensillum types on the surface of the antenna, ac1-4, house 7 detectable ORN classes[21] however, only one has so far been shown to be olfactory with a broad response to odours. Some of the others have been shown to detect small amines and humidity.

Table 1. *Drosophila* olfactory sensillum and neuron classes and their matching receptors and ligands

Sensillum	ORN	Receptors	Best Known Ligand
Maxillary palp basiconics			
pb1	A	Or42a	Ethyl proprionate
	B	Or71a	4-Methylphenol
pb2	A	Or 85e, Or33c	Fenchone
	B	Or46a	4-Methylphenol
pb3	A	Or59c	-
	B	Or85d	2-Heptanone
Antennal basiconics			
ab1	A	Or42b	Ethyl acetate
	B	Or92a	3-hydroxy-2-butanone
	C	Gr21a, Gr63a	Carbon dioxide
	D	Or10a, Gr10a	Ethylbenzoate
ab2	A	Or59b	Methyl acetate
	B	Or85a, Or33b	Ethyl-3-hydroxybutanoate
ab3	A	Or22a (Or22b)	Ethyl hexanoate
	B	Or85b	6-methyl-5-hepten-2-one
ab4	A	Or7a	E2-hexenal
	B	Or56a, Or33a	-
ab5	A	Or82a	Geranyl acetate
	B	Or47a	Pentyl acetate
ab6	A	Or13a	1-octen-3-ol
	B	Or49b	2-methylphenol
ab7	A	Or98a	3-octanol
	B	Or67c	Ethyl lactate
ab8	A	Or43b	Ethyl butanoate
	B	Or9a	2,3-butanediol
ab9	A	Or69aA (Or69aB)	-
	B	Or67b	-
ab10	A	Or49a, Or85f	Acetophenone
	B	Or67a	2-Phenylethanol
Antennal trichoids			
at1	A	Or67d	Cis-vaccenyl acetate
at2	A	Or23a	1-pentanol
	B	Or83c	-
at3	A	Or2a	Iso-pentyl acetate
	B	Or19a (Or19b)	1-octen-3-ol
	C	Or43a	Cyclohexanol
at4	A	Or47b	Fly extract
	B	Or65a, Or65b, Or65c	Fly extract
	C	Or88a	Fly extract
Antennal coeloconics			
ac1	A	IR92a, IR76b	Ammonia
	B	IR31a*	Water
	C	IR75d*	-

continued on next page

Table 1. Continued

Sensillum	ORN	Receptors	Best Known Ligand
ac2	A	IR75a*	1,4-diaminobutane
	B	IR75d*	Water
	C	IR76b*	-
ac3	A	IR75a, IR75b (IR75c)	Propanal
	B	Or35a, IR76b	Z3-hexanol
ac4	A	IR84a	Phenylacetaldehyde
	B	IR75d*	-
	C	IR84a*	-

ORN—olfactory receptor neuron name; Or—olfactory receptor; Gr—gustatory receptor; IR—ionotropic-like receptor. Note when two receptors are expressed in one ORN in most cases it is not known if both are functional. Receptors shown in parentheses are possibly expressed in these neurons but are too similar in sequence to the other gene in that neuron to be distinguished from it using RNA in situ hybridisation. Asterixes next to receptors indicates it is unknown exactly which neuron in that sensillum expresses that receptor. For example in ac4 it is known the A neuron expresses IR84a, but not which of the two other neurons expresses IR75d and which IR84a. Table assembled with the assistance of Dr Marien de Bruyne and using data from references 21,26,27,43,44,50,83.

Results of this characterization of response types in *Drosophila* are comparable to similar studies on other insects. For instance, overlapping response spectra in multiple classes of ORNs have been described in cockroaches[30,31] and several ORN classes with highly specialized responses to pheromone components or plant odorants are known from moths.[20,32]

MOLECULAR BASIS OF ODOUR DETECTION: THE *OR* GENE FAMILY

The different response spectra of ORN classes, along with their diverse response dynamics, provide the cellular basis for an olfactory code. Underlying these responses are distinct groups of genes encoding odorant receptor proteins. The first discovered and most well-characterised are the *Or* (odorant receptor) genes, these underly the responses of most of the ORN classes.

In 1991 Buck and Axel[33] discovered a large gene family encoding odorant receptors in vertebrates and following this many research groups attempted to find them in insects. It was only with the sequencing of the genome of *Drosophila melanogaster* that candidate receptor proteins mediating olfaction and gustation were identified.[34-37] Key to this success was the development of an algorithm to identify genes encoding seven-transmembrane receptor proteins.[38] Members of the first family described were called odorant receptor (*Or*) genes because they showed expression in antennae or palps. The *Drosophila Or* family comprises 60 genes encoding 62 proteins (two genes are alternatively spliced) with a unified naming system based on their chromosomal location.[4,39] Like odorant receptors from other organisms they have seven hydrophobic domains and were thus initially believed to be G protein-coupled receptors (GPCRs) (but more on this below). Predicted Or proteins are extremely divergent in amino acid sequence from each other and from all other known proteins, including Or proteins from other organisms. Nevertheless, the presence of several conserved intron locations suggests they arose from a common ancestor.[4]

Since their discovery in 1999, much evidence has been accumulated that the *Or* genes endow the ORNs in which they are expressed with all their response properties to odours. Most of the *Drosophila Or* genes are expressed in small subsets of 3-50 ORNs in the antennae or maxillary palps. RNA in situ hybridisation experiments revealed 32 *Or* genes to be expressed in the antenna and seven in the maxillary palps[34,36,37] with individual genes expressed in specific subsets of 3 to 50 ORNs. Only one or a small number of *Or* genes is expressed in each ORN with the RNA expressed in the cell body and the protein being transported to the ORN dendrites.[7,29] Many, but not all, of the remaining *Ors* are expressed in larval ORNs.[40]

Antibodies against two Or proteins, Or22a and Or43b, label the dendrites of a subset of ORNs, as expected for odorant receptors.[7,29] In addition, functional evidence has now been obtained for a number of *Or* genes. Over-expressing *Or43a* both in vivo in the *Drosophila* antenna and in vitro in the heterologous system of *Xenopus laevis* oocytes, led to increased responses to a particular set of aromatic compounds.[41,42] Subsequent genetic analysis of two different null mutations removing the genes *Or22a* and *Or43b* respectively, showed that removal of a single *Or* gene leads to the loss of responsiveness in a single functional classes of ORN,[7,29] providing convincing evidence that *Ors* are responsible for ORN response profiles and properties. These and later studies showed that in most cases one *Or* is expressed in a given ORN (with a few exceptions where two *Ors* are expressed in particular ORN classes) and is responsible for all its properties—the odour response spectrum, the spontaneous firing rate, the response dynamics and the signalling mode (excitation or inhibition).

Mapping *Or* Genes to ORNs and to Ligands

The *Or22a* null mutant has led to the establishment of an important in vivo experimental system that can be used to functionally characterise Ors and in many cases to map them to their neuron classes. In this mutant the ab3A neurons are still present and display spontaneous activity, but are insensitive to odour stimulation. Specific Ors can be expressed in this "empty neuron" and their odorant response profiles determined using the single unit electro-physiological recording technique.[7,43,44] To achieve this, the regulatory sequences of *Or22a* are used to drive expression of other receptors in the subpopulation of ab3A ORNs using the binary GAL4-UAS system.[45] The *Or22a* promoter drives the yeast transcriptional activator GAL4 (*Or22a*-GAL4) which in turn drives expression of an odorant receptor under control of a UAS sequence (UAS-*OrX*). When different *Or* genes are expressed in this way in many cases they confer onto the empty neuron an odour response spectrum matching that of a previously defined ORN functional class. For example, expression of *Or47a* in the empty ab3A neuron identified it as the receptor for the ab5B neuron.[7] Of 32 *Ors* expressed in antenna, 31 were tested in this manner and 24 generated odorant responses each with a distinct response spectrum.[43] Of these 24, 13 gave response spectra that closely resembled the profile of an identified ORN, thus using this approach many individual *Ors* were mapped to particular ORN classes and, importantly, to their ligands (Table 1).

The development of this initial Or-ORN map demonstrated that in most cases there is one *Or* expressed in one ORN functional class (but with some exceptions, discussed below). Expression of many Ors in the empty neuron demonstrated that the *Or* expressed in a neuron is responsible for encoding not only the odour quality (what odour it is) but also the odour intensity (the spike frequency; with more receptors activated at higher odorant concentrations providing a molecular basis for intensity coding), the dynamics

(abrupt or prolonged response or termination) as well as signalling mode (whether is it excitatory or inhibitory), plus the intrinsic spontaneous firing rates of each ORN class.[43] Interestingly these experiments also show that most Ors do not appear to require neuron specific or sensillum specific perireceptor molecules (such as OBPs) in order to confer the specific odour response spectrum.[7,43]

In an attempt to complete the de-orphaning of all *Or* genes by mapping them to their neuron classes, the map obtained from the empty neuron experiments (20 *Ors* in 24 ORN classes) was combined with detailed *Or* expression data. Reporter lines for many *Or* genes were used in combination with endogenous *Or* RNA in situ expression patterns to perform double label experiments.[46,47] In such experiments, when two *Ors* are expressed in two cells within the same sensillum, staining will appear as pairs of adjacent cells. Using these reporter lines the two groups of investigators were able to map the location of an *Or* of unknown ORN class by comparison to an *Or* of known ORN class. The unknown *Or* could be identified as being expressed in the adjacent ORN of the same sensillum, in an ORN in a different sensillum or in some cases in the same ORN. For example, reporter gene expression of *Or9a* is seen in cells adjacent to cells labelled by an RNA in situ probe for *Or43b.* As *Or43b* is known to be expressed in ab8A this enables the placement of *Or9a* into ab8B.[46]

Using these methods 45 *Ors* have been mapped to 38 distinct ORN classes in adults (Table 1 and for further review see ref. 48). Of the remaining 17 *Ors*, ~10-11 are expressed in the larval olfactory system and the remaining six either showed no expression or were ectopically expressed. Some ORN classes apparently did not express *Ors* and interestingly three gustatory receptors (*Grs*) were found to be expressed in antennal ORNs.[46,49] This analysis distinguished nearly the whole completed Or to ORN map for the basiconic and trichoid sensilla.

Interestingly, the mapping revealed that a number of classes of ORN express more than one receptor (not including the widely expressed coreceptor Or83b, see below). However, in four cases the co-expressed *Or* genes are closely linked in the genome and highly related to each other, suggesting that they arose through relatively recent gene duplications. These pairs of co-expressed receptors are likely to detect the same odorants and may not thus represent a meaningful exception to the one neuron-one receptor principle. In addition, in a number of cases only one of the two co-expressed Ors has been shown to be functional. For example, both *Or22a* and *Or22b* are believed to be expressed in the ab3A neuron. The abovementioned *Or22a* mutant that results in the loss of the odour response in neurons of functional class ab3A in fact has a deletion of both the *Or22a* and *Or22b* genes. However, rescue experiments with *Or22a* and *Or22b* demonstrated that *Or22a* appears to account for the full odour response spectrum of the ab3A neuron.[7] *Or22b* is highly similar to *Or22a* (78%) and appears to have arisen through a recent duplication event resulting in co-expression in the ab3A antennal neuron but a nonfunctional status.

However, there are some examples of co-expression of Ors with different functional properties in the same neuron. One example is the co-expression of *Or33c* and *Or85e* in the pb2A neuron. *Or33c* and *Or85e* are located on different chromosomes and show only 16% amino acid identity, however both are expressed in the pb2A neuron, with the axons of these neurons projecting to a single glomerulus in the antennal lobe. Functional electrophysiological analysis in the empty neuron system shows that while both receptors are odour responsive, Or85e confers most of the odour response of the pb2A neuron.[50] A strong ligand for Or33c has not been identified suggesting the possibility that Or33c is narrowly tuned to a specific ligand.

Other examples suggest that perhaps co-expression of receptors could modulate ligand response profiles. *Or47a* and *Or85a* are both co-expressed in their respective ORNs with *Or33b*.[47] Or47a and Or85a when individually expressed in the empty neuron system respond to more odours than the native neurons which co-express Or33b/Or47a and Or33b/Or85a. Or33b expressed alone in the empty neuron system responds weakly and with inhibition to most odours. Thus Or33b could function to temper the relatively broad tuning of Or47a and Or85a.[47] Co-expression patterns appear to be behaviourally significant as *Or33c* and *Or85e* are also co-expressed in *D. pseudoobscura* indicating that the co-expression of these two genes has been conserved for >45 million years of evolution.[50]

Or83b is Required for Localization of Other *Or* Proteins and as a Coreceptor

All members bar one of the *Or* gene family appear to encode ligand-binding olfactory receptors. However, one member of the family, *Or83b*, is unusual as it is expressed in all palpal and a large proportion of antennal ORNs in adults, as well as in larval ORNs.[51] Furthermore, unlike other *Or* genes, it exhibits a high level of conservation across four different insect orders.[52-54] These two features make it unlikely that *Or83b* can contribute to the specificity of odour responses in ORNs. The *A. gambiae* (mosquito) and *H. virescens* (moth) orthologues share 78% and 68% amino acid identity respectively with *Drosophila* Or83b and also exhibit expression in large numbers of ORNs. The overall predicted structure of Or83b is similar to other Ors but with a particularly large loop region between transmembrane domains four and five.

Or83b null mutants have been used to show that it is required for the correct localisation of two Ors (Or22a and Or43b) to the dendritic membrane of ORNs.[51] It seems likely that all Ors are mislocalised, as *Or83b* mutant flies show no EAG responses and have disrupted olfactory driven behaviours to a number of odours. The mutant phenotype in *Drosophila* can be rescued by expression of *Or83b* homologs from the mosquito (*A. gambiae*), moth (*H. zea*), or med fly (*C. capitata*) confirming an evolutionarily conserved function.[55]

It is now clear that each ligand-binding *Or* is expressed in a different subset of ORNs and is co-expressed with the noncanonical *Or83b*, with this co-expression being essential for odorant receptor function in vivo.[51,56] Or83b acts as a chaperone-like membrane protein that helps target the regular Ors to the dendritic membrane.[51] It has also been shown to form heterodimers with other Ors and increases in odour sensitivity are seen in heterologous assays when regular Ors are co-expressed with Or83b.[8] These findings could simply reflect an increase in the amount of the Or protein that is correctly localised to the plasma membrane in the presence of Or83b, or alternatively Or83b may have a second role such that the functional odorant receptor is actually an OrX-Or83b complex. This will be discussed further below.

Or Genes in Other Insects

That olfactory perception in insects is generally mediated by the *Or* genes has been confirmed by the identification of families of *Or* genes in other insect species. The advent of multiple genome sequencing projects in various insect species has proven particularly valuable to the field of olfaction for identifying and investigating the large and highly divergent families of Or proteins. These genes have diverged rapidly and thus identification of *Or* genes from a species nearly always requires the availability of a genome sequence. Families of *Ors* have been identified in a number of insects, including

the malaria vector mosquito *Anopheles gambiae*;[52,57] the silk moth, *Bombyx mori*;[58] and the honey bee *Apis mellifera*.[59]

A clear indication of how fast these genes are evolving can be seen by comparing Ors from two Dipterans, *D. melanogaster* and the mosquito *Anopheles gambiae,* that are estimated to have diverged ~100 million years ago (MYA). *A. gambiae* has only four Ors out of 79 with even slight homology to *Drosophila* Ors. For most Or families, a high level of divergence is found within as well as across species. In *Heliothis virescens* for example, 21 Ors have been identified which are highly divergent from each other (8-15% homology) but with common sequence motifs allowing assignment to the same family.[60]

Ors are not only rapidly evolving but are highly specialised, with Ors from moths and honeybees showing various species-specific lineage expansions, presumably enabling adaptation to specific odour environments. Interestingly, the honeybee has had a very large expansion of Or proteins and in total has 170 *Or* genes. The expansion of *Or* subfamily lineages observed in the honeybee presumably facilitates their exquisite olfactory behaviour and might indicate adaptations to specific odour environments, such as recognising diverse floral odours or complex pheromone blends that enable co-ordination of caste-specific tasks within the social colony.[59] In honeybees the large expansion of Ors is accompanied by a much larger number of olfactory glomeruli, faithful to the one Or-ORN-glomerulus rule.

For less genetically tractable insects such as mosquitoes, moths and bees, receptors have been functionally characterised either by ectopic expression in the *D. melanogaster* empty neuron assay or by heterologous expression in various cell assays. Empty neuron experiments showed that the *AgOr1* receptor from *A. gambiae* (a major malaria vector) responds strongly to 4-methylphenol, a chemical present in human sweat.[61] *AgOr1* is expressed only in females who feed on blood (males do not) and this receptor is downregulated after a bloodmeal.[57] In a large scale study 72 *AgOrs* were expressed in the empty neuron system and 50 were functional.[62] Of these some were narrowly tuned to volatiles produced by humans and may be central to the process by which the mosquito locates and identifies its human hosts. These results suggest transgenic functional studies are highly reliable and biologically relevant and interestingly that the peripheral signalling environments are compatible for Ors from different insects. Heterologous expression of various insect Ors in *Xenopus laevis* oocytes[58,63] and HEK293 cells[8] has also been very successful for studying Or-ligand relationships.

A Subset of *Ors* are Involved in Pheromone Detection

Some members of the *Or* family have been co-opted for a role in pheromone detection, as opposed to general odours. The first genes encoding pheromone receptors were identified by searching for moth *Or* genes that were expressed specifically in male but not female antennae. In the silkmoth *Bombyx mori* two male-specific *Or* genes were identified, *BmOR1* and *BmOR3* and using heterologous expression studies in both cell culture and in the *Drosophila* empty neuron assay these were shown to function as receptors for the two female-produced pheromone components bombykol and bombykal.[58,63] In the moth *Heliothis virescens* three *Or* genes, *HR13*, *HR15* and *HR16* were also found to be male-specific and respond to female-produced pheromone compounds in heterologous expression assays.[60] Thus it appears that the long sought after receptors for moth volatile pheromones are part of the *Or* superfamily.

In *Drosophila Or67b* is required for the response of the T1 sensillum to the aggression and contact sex pheromone *cis* vaccenyl acetate (cVA).[64] Interestingly additional factors have been found to be required for pheromone detection by an Or protein compared to detection of general odorants. The pheromone-binding protein LUSH appears to be a critical component of the cVA detection mechanism,[65] unlike other odorant binding proteins which so far have not been shown to be required for odour detection (see later). In addition the SNMP protein, which is a two transmembrane domain CD36-related protein, is required for cVA detection, mutants lacking this protein cannot detect cVA.[66] When the *H. virescens HR13* receptor is mis-expressed in *Drosophila* it can only function if SNMP is also expressed in the same neurons, whereas function of a conventional *Or, Or22a*, does not require SNMP for detection of its short chain ester ligands. This suggests that SNMP may play a general role in assisting the binding of pheromone molecules to receptors. Interestingly mammalian CD36 binds fatty acids and both cVA and the pheromone detected by HR13, (*Z*)-11-hexadecenal, are fatty-acid derived molecules with long hydrocarbon tails.

The *Or* Proteins Appear to be Directly Ligand-Gated Ion Channels

What type of proteins are the Ors? When they were discovered in 1999, *Drosophila* Ors were independently predicted by three groups to have seven transmembrane helices. As most such proteins are G protein-coupled receptors (GPCRs) and the mammalian Ors are GPCRs, the fly Ors were proposed to be part of this superfamily of proteins.[34,36,37] However, their structural topology is in fact difficult to predict due to the fact that they are extremely divergent in amino acid sequence from each other (~20% average identity at the amino acid level) and from all other known proteins, including Or proteins from vertebrates and *C. elegans*. Membrane topology prediction programs predict anywhere from 3 to 8 transmembrane domains for different family members.[67] In addition, many genetic studies have addressed the role of candidate GPCR-activated signal transduction pathways in *Drosophila* olfaction via knocking down or overexpressing proteins involved in canonical GPCR signaling, but with no compelling evidence for an essential role being obtained. This contrasts sharply with studies of mouse or *C. elegans* olfaction where knocking out such components completely eliminates olfactory responses.

If insect Ors do utilise G proteins, there are two major candidate signal transduction cascades which they may activate, the inositol phospholipid (IP_3) signalling pathway and the cAMP signalling pathway. These are G protein-activated signal transduction cascades used by many sensory systems to transduce ligand detection into electrophysiological activity of the receptor neuron. Vertebrate ORs primarily utilise the cAMP pathway, although there is some evidence for a role of the IP_3 pathway as well (for review see ref. 68). Of the two pathways, there is more evidence that the IP_3 pathway is involved in insect olfactory signal transduction, but this evidence is not conclusive. For example, *Drosophila norpA* mutants, which lack the phospholipase C that is an essential component of phototransduction (an IP_3 signalling cascade), exhibit reduced (but not eliminated) olfactory responses of the maxillary palp, however the antennal responses are unaffected.[69] The *Drosophila Gα49B* gene, which encodes a Gqα that activates phospholipase C in the visual system, has been shown to be expressed in ORNs[70] and flies expressing an RNAi construct for this gene exhibit olfactory behavioural defects to some, but not all, tested odorants.[71] Finally a rapid and transient increase in IP_3 has been observed in response to

pheromones and odorants in cultured ORNs from various insect species and this increase can be suppressed by pertussis toxin, which inactivates some G proteins.[72]

Thus, as mentioned above, conclusive evidence for an essential role for G protein signaling has not been obtained and the combination of this with some pivotal recent studies have indicated that the *Drosophila* Ors are not in fact GPCRs. Firstly, over the past several years several important studies have shown that while *Drosophila* Ors do appear to have seven transmembrane domains like GPCRs, they have the opposite membrane topology to that of GPCRs, with an intracellular N terminal domain and extracellular C terminal domain. Benton et al[56] first showed using several biochemical methods that Or83b has an N-terminus that is intracellular. Lundin et al[73] then extended this finding and used glycosylation sites as topological markers to show that Or83b expressed in *Drosophila* rough microsomes has seven transmembrane domains, an intracellular N-terminus and an extracellular C-terminus.

These findings were then extended to the ligand-binding members of the Or family. Benton et al[56] showed that the N-terminal domain of Or9a was intracellular when it was expressed fused to a single transmembrane domain in S2 cells and using a different approach, an in vivo YFP protein-fragment complementation assay, showed that the N-terminal domain of Or43a is also intracellular. A detailed topology study of Or22a was performed by expressing tagged versions of the receptor in S2 cells and testing for an intracellular vs extracellular location of the tag.[67] This study supported the orientation of Or22a being the direct inverse of that of a classical GPCR, such that the N-terminus faces the cytoplasm and the C-terminus is extracellular, with seven transmembrane domains interconnected by three extracellular and three intracellular loops.

This distinct topology raised the question of whether *Drosophila* Ors do in fact signal via heterotrimeric G proteins and several pivotal recent studies have shown that the Ors do not depend on G protein-activated pathways for signalling and in fact appear to encode directly odour-gated ion channels. Using a combination of a ligand-binding Or and Or83b expressed in various heterologous cell culture systems, Sato et al[74] and Smart et al[67] showed that odour-induced currents are unimpaired upon application of pharmacological inhibitors of G protein signalling. Sato et al[74] further obtained direct evidence that the Or/Or83b complex itself possesses ligand-activated channel activity through recordings of odour-evoked currents in excised membrane patches expressing these receptors. Wicher et al[75] also describe an odour-dependent, rapidly activating ionotropic current in Or/Or83b-expressing heterologous cells, but also show that after the initial rapid ionotropic response there is a slower and longer lasting current that appears G protein-dependent. It is possible that G protein signalling modulates Or function, as a recent study showed that though odour-evoked neuronal responses are observed in neurons lacking a chemosensory G alpha subunit they are reduced in intensity.[76]

The above studies suggest that the functional Or is either a heteromeric complex of a ligand-binding Or protein and Or83b, which together form a ligand-gated ion channel, or alternatively Or83b forms a channel that physically associates with a ligand-binding Or and is activated directly by odour binding to the ligand-binding Or. Most ion channel subunits have an even number of transmembrane domains with intracellular N-and C-termini, although ligand-gated channels such as channelrhodopsin 2 from the green alga *Chlamydomonas reinhardtii* and the metabotropic glutamate receptor, are seven transmembrane domain proteins.[77,78] However, unlike the *Drosophila* Ors, both these receptor-channels have a standard GPCR topology with an extracellular N terminus. Thus the insect Or proteins appear to be quite unique.

It therefore appears that the molecular basis of insect olfaction is quite different to that of mammals and also that of *C. elegans* and that different groups of organisms have evolved quite different ways of achieving the peripheral coding of olfactory information. The insect odorant receptor proteins appear to define a completely new, insect-specific, receptor family, opening the door to the development of novel chemicals that could modify insect behaviour without affecting humans and other mammals. Why might insects have evolved a completely different olfactory signaling mechanism to that of mammals? At present we can only speculate, but possibly directly ligand-gated ion channels provide a faster signaling mode that might provide a selective advantage in insects that need to make very fast decisions during flight behaviour.

OTHER FAMILIES OF CHEMOSENSORY RECEPTORS IN *DROSOPHILA*

The *Gr* Genes Function in Multiple Chemosensory Modalities

A second family of chemosensory receptors in *Drosophila*, encoded by the *Gr* (gustatory receptor) genes, has been so named by virtue of their expression predominantly in gustatory neurons.[35,49,79] This family consists of 60 genes that encode 68 proteins via alternative splicing. The *Grs* are even more divergent from each other than the *Or* genes, with most of them sharing as little as 8% identity.[4] Phylogenetic analysis suggests that the *Or* and *Gr* genes comprise an ancient superfamily of chemoreceptors, with the *Or* family being a single highly expanded lineage within the superfamily.[4] The expression patterns of the *Gr* genes have proven difficult to examine, however using RNA in situ hybridization and reporter gene constructs 11 genes have been shown to be expressed in subsets of either adult taste neurons, adult olfactory neurons, or larval taste and olfactory neurons.[49,79] Thus the *Gr* gene family seems to encompass both olfactory and taste receptors.

Whilst some members of the *Gr* family have now been shown to be taste receptors and function in contact chemoperception, at least three *Gr* genes, *Gr10a/b*, *Gr21a* and *Gr63a*, are expressed in *Drosophila* antennae,[49] suggesting a role in olfaction (Table 1). Of these, *Gr21a* and *Gr63a* have been shown to be required for the perception of CO_2.[80,81] The two genes are co-expressed in the ab1C neurons, which are highly specialized for CO_2 detection and drive an innate avoidance behaviour.

Interestingly Or83b is not co-expressed in the ab1C neurons with these two *Gr* genes and is also not co-expressed with other *Gr* members nor required for the function of any of the *Gr* genes. Although it has not yet been shown, if *Gr* genes also encode directly ligand-gated ion channels, they either do so without such a coreceptor, or this coreceptor remains to be identified. Similarly to olfaction there is no compelling evidence for G protein-activated signaling pathways in *Drosophila* taste perception. In addition, a fascinating *Gr* gene has been functionally characterised in *C. elegans*, which has three *Gr* homologues. One of these, the *LITE-1* gene, mediates UV light response in worms and drives an escape behaviour.[82] *C. elegans* lack other known light-transducing proteins and a *Gr* gene appears to have been co-opted as a novel molecular solution for UV light detection. Most interestingly the *LITE-1*-driven behaviour has been shown to not require G protein-activated signalling pathways as it is unaffected in mutants lacking these pathways. Thus, as for the fly *Ors*, the *LITE-1* signalling pathway does not require classical G protein signalling and it seems highly likely the fly *Grs* also do not and instead use an ionotropic mechanism.

The *IR* Gene Family is Expressed in Neurons of Coeloconic Sensilla

The *Or* genes are expressed in neurons of the basiconic and trichoid sensilla, but (except for one member, *Or35a*) not in neurons of the coeloconic sensilla. The *Gr* genes are also not expressed in these sensilla. Thus some chemosensory receptors were obviously missing and indeed recently a newly identified family of genes was found to be expressed in coeloconic neurons and appear to encode a second family of novel receptors for odorants.[83] This family, called the *IR* family, was also identified using a bioinformatic approach in which the authors screened for insect-specific genes expressed in ORNs. Among these they found representatives of a family of 61 genes that they named the ionotropic receptors (IRs), which although very divergent from each other (10-70% amino acid identity), show some similarity in structure to ionotropic glutamate receptors.

Of the 61 genes, 15 were found to be expressed in ORNs by RNA in situ hybridization.[83] The remainder were either not expressed in adult tissues or expressed at levels too low to detect. This expression was not observed in *atonal* mutants that lack coeloconic sensilla, indicating that the *IR* genes are expressed in ORNs in this sensillum type. Double and triple labeling studies were used to show that the IR-expressing neurons are organised in four distinct clusters of 2-3 neurons and in combination with single unit recording experiments these clusters were able to be linked to the four different known functional classes of coeloconic sensilla (Table 1). An antibody to IR25a was generated and immunostaining showed that the IR25a protein localized to both the distal tip of the dendrite as well as the cell body of ORNs, consistent with a role in odour detection. Finally functional evidence for a role in olfaction was obtained by misexpressing two members of the family in neurons in which they are not normally expressed and observing that this conferred non native odorant specificities. For example, misexpression of *IR84a*, normally found in the ac4 sensillum, in the ac3B neuron using the *Or35a*-GAL4 driver conferred a strong response to phenylacetaldehyde not seen in controls.

As mentioned above, the IRs encode proteins that are related to ionotropic glutamate receptors. They have the same predicted structure, with a bipartite extracellular ligand-binding domain, three transmembrane domains and an ion channel pore region between TM1 and TM2. The pore region is the most conserved with ionotropic glutamate receptors which suggests the IR proteins retain ion conducting properties. However, residues in the ligand-binding domain known to be important for glutamate-binding are not conserved in most members suggesting they do not bind glutamate. This family thus appears to represent a second divergent family of ionotropic olfactory receptors in *Drosophila*.

OBPs ARE SECRETED PROTEINS THAT MAY MODIFY LIGAND-RECEPTOR INTERACTIONS

Most odorant molecules are hydrophobic, yet the extracellular environment in which odorant receptors operate is aqueous, ORN dendrites are bathed in a lymph through which odorants must pass to reach the receptor. The sensillum lymph contains high concentrations of another family of proteins, the odorant binding proteins (OBPs). OBPs are small proteins secreted into the sensillum lymph by accessory cells. The first members were identified in pheromone processing sensilla of male silkmoths and dubbed pheromone binding proteins (PBP).[84,85] It has been hypothesized that OBPs help to shuttle

highly hydrophobic odorants across the aqueous sensillum lymph. In support of this theory, specific binding of pheromone components has been demonstrated,[86] and OBPs are very abundant in subsets of olfactory sensilla. In moths, the expression of specific PBPs or other OBPs is correlated with different sensillum types, which detect different pheromone components or plant odours.[85]

There are 51 *obp*-like sequences in the *Drosophila* genome, none of which are closely related to moth PBPs.[87,88] Like *Ors* and *Grs* they are differentially expressed, however their expression patterns do not correlate in an obvious way with functional classes of sensory neurons. For instance, *obp28a* is expressed in most basiconic sensilla of the antenna, in taste sensilla on the legs and in the larval olfactory organ,[23,24] whereas *obp76a* is expressed only in trichoid sensilla, a subset of which also co-express *obp83a* and *obp83b*.[89,90] *Obp19d* is expressed in coeloconic sensilla, but also in epithelial cells of antennae and labellum.[24] Thus, *obp* genes are expressed in olfactory and gustatory sensilla or the epithelia surrounding them.

Despite their clear association with chemosensory tissues, heterologous expression studies of *Drosophila Or* genes suggests that OBPs do not generally play an essential role in determining binding specificity of chemical stimuli.[42,43] However, pheromone perception may be an exception to this, as discussed above the odorant binding protein LUSH is required in vivo for cVA detection by the *Or67b* receptor. More OBP mutants need to be studied in order to clarify their roles in vivo. For example it is possible that in vivo they are required to solubilise odorants in the sensillum lymph and this requirement is bypassed in heterologous functional studies of the receptors. There may also be some redundancy in OBP function. Alternatively if OBPs are not generally required for odour detection they have also been proposed to play roles in removing deleterious compounds from the lymph, or in the deactivation of odours following receptor activation (reviewed in ref. 91).

CONCLUSION AND FUTURE DIRECTIONS

Remarkable progress has been made in the last decade in our fundamental understanding of the principles of the cellular and molecular basis of odour detection in *Drosophila*. A number of extremely diverse families of olfactory receptor genes have been found, that most surprisingly utilize entirely different signaling mechanisms to receptors in other organisms. The sophisticated approaches for studying gene expression and function in *Drosophila* has led to extensive ligand information for many of the *Drosophila* Ors, making its receptor repertoire by far the best characterized of any organism in this regard. However, relatively little is known regarding the structure of *Drosophila* Ors and the recent experiments suggesting they encode directly ligand-gated ion channels need to be confirmed in *Drosophila* olfactory neurons. In addition, the identification of the Or family has led to the ability to genetically label and trace subsets of neurons in the brain and has spawned another entire area of investigation not discussed here, namely the processing of olfactory signaling in the antennal lobe and higher order brain centres and is leading to key understanding of behavioural neuronal circuits.

Future areas of intensive investigation include identifying how olfactory receptors bind odorant molecules. In addition many groups are currently expanding this understanding to other insects and to the evolution of olfactory perception, as well as linking this underlying molecular and cellular information to differences in insect behaviour and ecology.

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CHAPTER 5

EUSOCIAL EVOLUTION AND THE RECOGNITION SYSTEMS IN SOCIAL INSECTS

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Abstract: Eusocial species, animals which live in colonies with a reproductive division of labor, typically have closed societies, in which colony members are allowed entry and nonmembers, including animals of the same species, are excluded. This implies an ability to discriminate colony members ("self ") from nonmembers ("nonself "). We draw analogies between this type of discrimination and MHC-mediated cellular recognition in vertebrates. Recognition of membership in eusocial colonies is typically mediated by differences in the surface chemistry between members and nonmembers and we review studies which support this hypothesis. In rare instances, visual signals mediate recognition. We highlight the need for better understanding of which surface compounds actually mediate recognition and for further work on how differences between colony members and nonmembers are perceived.

INTRODUCTION

In the eusocial animals recognition of group membership is an essential component of evolutionary success.[1] In this chapter we introduce the defining characteristics of eusocial species and their colonies. We then develop an analogy between recognition of group membership and other types of "self" versus "nonself" recognition. We develop some key theoretical issues, including phenotype matching, neutral substitution in the evolution of signal diversity and response thresholds. Recognition of group membership has been well studied in ants, honeybees, wasps and to a certain extent in termites; we review examples in each of these types of eusocial insects.

A eusocial species is one in which colonies are formed by family groups.[2] Some of the young in the colony have permanently diminished reproductive capacities and devote

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their lives caring for their sibs, defending the colony, collecting food for the colony and constructing the nest in which the colony lives. This reproductive division of labor results in a reproductive caste, the queen (and sometimes a king) and a nonreproductive worker caste within the colony.[2] The workers, in some instances, are further subdivided into specialized groups that perform specific tasks, like colony defense.

Many of the commonly mentioned examples of eusocial species are members of the insect order Hymenoptera.[1,3-5] These include honeybees (*Apis*), bumblebees (*Bombus*), ants (family Formicidae), all of which are eusocial and social wasps, such as paper wasps (*Polistes*) and yellowjackets and hornets (family Vespidae, genera *Dolichovespula*, *Vespula* and *Vespa*). Termites, the insect order Blattodea (formally Isoptera), are also all eusocial. In addition to these species, which are all frequently encountered in the temperate zones, the stingless bees (genera *Trigona* and *Melipona* and their relatives) and numerous species of eusocial wasps in the vespid tribe Epiponini are found in the tropical zones. Eusocial insect colonies are remarkable for their coordination of labor among colony members and, in some cases, for their very aggressive and effective mechanisms of colony defense.

In recent years eusociality has been discovered in a variety of other types of animals.[6] Perhaps most notable is the naked mole rat (family Bathyergidae, genus *Heterocephalus*). These small mammals, which occur in the southern part of Africa, have societies that are remarkably analogous to those of eusocial Hymenoptera and termites.[7-9] Colonies of naked mole rats live in complex tunnel systems and typically have 50-100 individuals. Eusocial shrimp (genus *Synalpheus*, in the Decapoda) live within marine sponges.[10] Thrips (family Thysanoptera) and Aphids (families Hornaphididae and Pemphigidae), are both plant-feeding insects which contain species that nest in galls in the plant tissue and produce a defensive caste, soldiers, that defend the gall at the expense of individual reproductive capacity.[11-13]

A defining characteristic of eusocial colonies is their closed membership.[14-16] Like a multicellular organism, membership in the colony is "self" and nonmembers, even if they are of the same species, are treated as "nonself". Separation of "self" from "nonself" allows eusocial colonies to prevent invasion by parasites and predators.[15] This is a nearly perfect analogy to the function of the immune system in multicellular organisms and it is worth noting that in many vertebrates odors correlated with variation in the major histocompatibility loci (MHC) facilitate social recognition processes.[17]

Colony closure can center at entrances to the nest or may extend to territorial boundaries that are distant from the nest. In the ecotypes of the western honeybee, *Apis mellifera*, found in Europe and most of North America, guard bees at the nest entrance examine incoming insects and exclude bees from other colonies as well as other species.[18] The major cost of admitting nonnestmates is the risk of having honey-stores robbed and weak colonies, which cannot effectively defend themselves, may be decimated by intraspecific robbing.[19] Western honeybee colonies are sometimes clustered in nature because acceptable nesting habitats—hollow trees or small caves—occur in close proximity to each other. Intraspecific nest defense in the western honeybee occurs only at the nest entrance. In other species aggressive interactions may also occur at flowers.[3] Other ecotypes of *Apis mellifera* may defend a much larger perimeter against potential vertebrate predators, but this extended defended area does not result in territorial aggression against other bees.[20]

In contrast, harvester ants (genus *Pogonomyrmex*) aggressively defend not just their nest, but also an extended area around the nest in which they forage.[21] This exclusion limits competition for food and results in colonies being evenly distributed across the habitat. Similarly, many species of the tropical stingless bees are aggressive at flowers in their colony's foraging range; this also results in an even distribution of colonies within the

environment. The evolutionary trade-offs that result, in some cases, in defense of the nest only and in other cases in defense of a feeding territory are not well understood. In both types of defensive systems, discrimination of colony members ("self") from noncolony members ("nonself") is a critical behavioral element.

There are exceptions to colonial closure among eusocial organisms and these exceptional cases merit some discussion here. In some instances, colonies of a eusocial species are isolated from other colonies of the same species, or have no significant problems with social parasites or problems with robbing and probably as a consequence, show little expression of aggressive behavior to nonnestmates. *Apis cerana*, the eastern honeybee, fits this pattern.[22] Some ant species are adapted for colonization of disturbed habitats and rapid colony expansion so that a single large colony occupies a large habitat patch. In these species termed unicolonial—all ants in a population belong to the same large colony.[23] Unicolonial ants are often polygynous (colonies have many queens) and polydomous (a single colony occupies many nests); in these species exclusion of nonnestmates is often not expressed. *Formica podzolica*, an ant common in subalpine habitats in North America, is a good example of this social lifestyle.[24] Some invasive ant species, such as the Argentine ant, *Linepithema humile*, adopt a similar strategy of unicoloniality.[25]

In sum, eusocial colonies can conveniently be viewed as multiorganism assemblages that are analogous to multicellular animals. While this superorganism analogy has limitations, it provides an excellent frame of reference for thinking about the evolution of closed societies and the importance of recognizing "self" and "nonself" in social interactions. In the next section we extend this argument to a discussion of how recognition phenotypes are constructed and perceived.

RECOGNITION THEORY AND PHENOTYPE MATCHING

The closure of eusocial colonies relies on two mechanisms. First, there must be phenotypic features that differentiate among colonies.[15,19] Second, animals within a colony must be able to use this phenotypic information to discriminate members from nonmembers and must be able to act in ways that exclude nonmembers from the colony.[20]

Phenotypic variation among colonies could occur in any conceivable signaling modality. Chemical cues,[15] visual characteristics,[26] or audible signals seem, from a human point of view, to be the most plausible, but we should not lose sight of the fact that animals can use unexpected and therefore surprising means of communication. Having made this point, the overwhelming preponderance of evidence from insects suggests that chemical cues, perceived either as volatiles or by contact chemoreception, are the recognition phenotype for the vast majority of eusocial insects.[15] Most eusocial insects use hydrocarbons from the cuticle in phenotypic matching for nestmate recognition. In a few eusocial wasps, white or yellow markings in the cuticle, called maculations, vary among individuals and are used as visual recognition phenotypes.[26]

For small colonies of eusocial animals, individual distinctiveness of the phenotypes of colony members is possible and colony members may recognize each as discrete individuals.[26,27] For larger colonies the sheer number of animals and the likelihood that any pair of colony members will meet infrequently during their life argues against individually distinct phenotypes.[19] In these species the most efficient way to accomplish recognition of colony membership is for the members to all carry the same phenotype.

This could come by merging of individual phenotypes due to workers rubbing together within the nest, to the function of a gland that establishes a common (or gestalt) odor, to shared nesting materials, or to the secretion of a unique labeling mixture by the queen. All of the mechanisms have been demonstrated and no single rule dictates how the shared phenotype is established in eusocial colonies.[28-30]

Animals that need to make discriminations, such as entrance guards, can then learn the phenotype of their colony and use that information in excluding nonnestmates, even if they are contacting individuals for the first time. This mechanism is termed phenotype matching and is likely the most generalizable rule in social recognition in eusocial animals (Fig. 1).[19] Through phenotype matching, colony members gain a template that identifies "self" and then compare that template with the cue profile of animals they encounter.

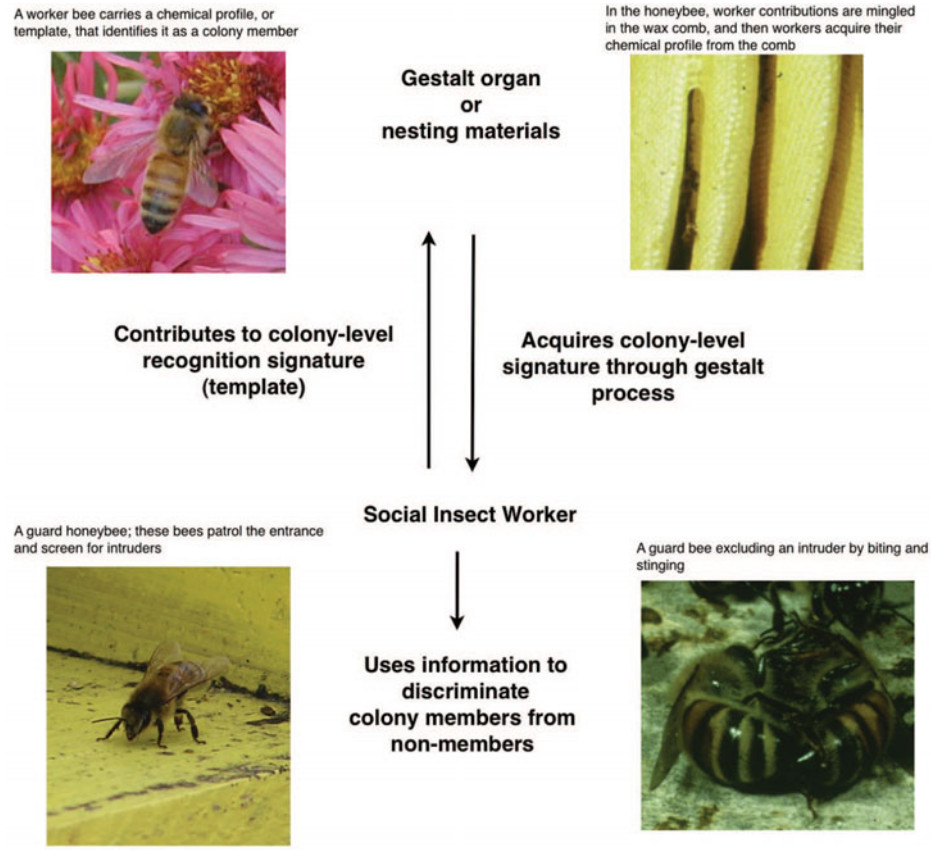

Figure 1. A schematic diagram of the process by which colony-level, or gestalt, recognition cues are gained by social insect workers. Colony workers contribute cuticular compounds to either nesting material, as is the case in honeybees and *Polistes* wasps, or acquire cuticular compounds from colony members in a gland, as occurs in *Camponotus* ants. This information is then used as a template in phenotype matching to distinguish colony members from nonmembers. The photographs illustrate each of these phases: Upper right, combwax, which is constructed by honeybees, is the intermediary that provides uniform cuticular chemistry among colony members, lower right, a guard bee aggressively excludes a nonnestmate, lower left, a guard bee on alert at a colony entrance, upper left, a foraging bee which must regain entrance to its colony.

Phenotype matching is also used in social discriminations in mammals and is particularly well studied in rodents.

Returning to the chemical cues used for discriminations, in nearly all cases hydrocarbons secreted to the outer cuticle of the insect form the basis for the recognition phenotype.[31] These hydrocarbons probably first evolved as cuticular waterproofing and were later co-opted for social recognition. Cuticular hydrocarbons are known to serve as social signals in some other types of insects, such as the sex pheromone of houseflies, which is Z-9-tricosene.[32] The following sections present our knowledge of recognition chemistry in eusocial animals in more detail.

NEUTRAL SUBSTITUTION AND PHENOTYPIC VARIATION

The MHC loci code for hypervariable phenotypes, which give vertebrates the flexibility to respond to novel parasites and pathogens. This phenotypic variability provides a perfect backdrop for recognition of kin or individuals, as it provides the basis for individually unique external phenotypes. Breed and Buchwald[19] argued that when the functional requirements for a phenotype in one context are met, then "neutral substitution" of aspects of that phenotype can enhance the use of those characteristics in social recognition. For example, the morphology of the human face is constrained by the need for a functioning jaw, open airways through the nares and appropriately aligned eyes. Facial phenotypes, though, are hypervariable within these constraints, facilitating recognition. Noses that are wide, narrow, long, pug, or flat all serve equally well in breathing, so substitution among these shapes is neutral with respect to function but provides variability that can help to distinguish individuals.

In insects, cuticular hydrocarbons probably evolved as waterproofing and later in evolution were co-opted as social signals. Effective waterproofing requires hydrophobic compounds that will not crystallize at ambient temperatures. Many hydrocarbons of various carbon chain lengths can serve this waterproofing function. In addition, of theses hydrocarbons, alkanes, alkenes and methylalkanes, which are found on insect cuticles are all effective. This means that cuticular hydrocarbon phenotype can vary substantially—facilitating social recognition—without impairing the insect's water balance. In honeybees, fatty acids strengthen comb wax. A number of fatty acids serve equally well to enhance the mechanical properties of the wax, but can be neutrally substituted to generate variable recognition phenotypes.[33]

THRESHOLD MODELS FOR EXPRESSION OF DISCRIMINATIONS

In 1989 Reeve[34] investigated recognition with a unique approach. He wanted to learn what factors affect nestmate recognition in a way to maximize an organism's inclusive fitness. Reeve investigated what constitutes an optimal acceptance threshold for the guards of a nest. This model assumes that nestmates and nonnestmates have overlapping recognition cues which make it likely that recognition errors will occur. Guards that are too strict with their acceptance threshold may inadvertently reject true nestmates while those that are too lenient may incorrectly allow nonnestmates into the nest. With this thinking, Reeve introduced the idea that the optimal acceptance threshold would be one that varies with the cost of accepting a nonnestmate, benefits of accepting nestmates and the frequency in which nestmates and nonnestmates are encountered.

In 2000 Downs and Ratnieks[35] applied Reeve's theoretical model to honeybees in the field. They found that the acceptance threshold is dependent on ecological conditions and may shift. For example, as nectar conditions improved honeybee guards were less selective about who to allow into the nest because there was not a great cost associated with allowing an intruder into the nest. Ecological changes influence the behaviors of guards at both an individual and colony level. By rapidly increasing the number of nonnestmate intruders encountered by a guard Couvillon et al[36] showed that changes in the acceptance threshold of both individual guards and the colony could occur within 15 minutes. For individual guards mean acceptance of nestmates and nonnestmates declined. At the colony level the mean number of guards at the entrance increased.[36]

The most common context in which the response threshold model may apply is seasonal variation in defensiveness. Defensiveness should be highest under conditions of intense competition, or when food stores within the colony are relatively large and should be lower when competition is less fierce. In an ant, *Plagiolepis pygmea*, Thurina and Aron[37] found that aggressiveness among colonies varied seasonally, peaking in the spring, when intercolonial competition for food may be at its highest. However, Kudo and Zucchi[38] found that in a eusocial wasp, *Polybia paulista*, expression of nestmate recognition remained constant through the year, even though seasonally shifting competition had caused the authors to predict that they would find shifting acceptance thresholds. This is an area in which further studies will define the phenotypic flexibility of animals in the expression of social discriminations.

ANT CHEMISTRY

Most ants behave cooperatively with nestmates and exclude alien conspecifics from their nests. Typically this occurs when individuals from the same nest share a recognition cue to form a common nest odor. The collective profile of the cues are considered a template which can be learned by colony members as the shared odor of the nest. The learned template is then compared to the detected hydrocarbon profile in social discriminations. If the detected hydrocarbons are different from the nest template, then visiting insects will be considered intruders and be prevented from entering the nest.[39] The use of a learned template is sometimes referred to as phenotypic matching, which we discussed above. During phenotypic matching individuals are identified as familiar and unfamiliar by having formed a template of kin (or nestmates) by learning the phenotypes from familiar individuals (or nestmates).[40,41]

Studies in ants have shown that the postpharyngeal gland (PPG) is important in forming nestmate recognition cues.[31,42,43] After extracting hydrocarbons from the PPG of ants Sorokoer et al[44] found that hydrocarbon composition in the PPG is species specific and these hydrocarbons are similar to those found on the cuticle.[44] These finding are important because the maintenance of the colony odor requires the continuous production of recognition cues which are provided by the PPG.[44,45] The PPG is involved in the active exchange of cuticular hydrocarbons via allogrooming or trophallaxis.[42,44]

Cuticular hydrocarbons are thought to play an important role in nestmate recognition. Cuticular hydrocarbons consists of n-alkanes, alkenes and methylalkanes and can range in carbon chain length from about 21 to greater than 40 carbons.[46] Hydrocarbons tend to be highly species specific and intraspecific compounds typically vary in relative proportions that can be colony specific. Because of the low volatility of cuticular hydrocarbons, acquisition

of the common nest odor typically comes from the exchange of cuticular hydrocarbons via allogrooming or trophallaxis.[42] Recent studies, however, have found that mixed species of ants living in close proximity, but not able to touch, can become familiarized with the neighboring species hydrocarbon signal.[47] This suggests that in addition to tactile olfactory cues, volatile cues may also affect nestmate recognition template formation.

Studies on ants have examined the effects of the three types of cuticular hydrocarbons on behavior and, although still controversial, some patterns have emerged. The profiles shown during gas chromatography may not be identical to those perceived by the insect.[48,49] Dani et al[48] found that in honeybees changes in the alkene pattern and not the n-alkane pattern affects nestmate recognition. Martin et al[49] determined that nestmate recognition signals in the ant *Formica exsecta* come from Z-9 alkene signatures even though there are other compounds present on the cuticle. Further investigation into this phenomenon by Martin and Drijfhout[50] suggest that the n-alkane component of the hydrocarbon profile is independent of the nestmate signal and is strongly influenced by worker task. These finding suggest that in some species of *Formica* ant the Z-9 alkene signature is strongly influenced by genetic factors while the n-alkane signature is influenced by environmental factors. In the ant *Formica japonica*, an ant with a relatively more simple hydrocarbon profile, both differences in the alkene and n-alkane signatures are necessary to elicit and aggressive response.[51] Greene and Gordon[52] also found that in *Linepithema humile* behavioral nestmate recognition responses only occurs when there are mixtures of hydrocarbon structural classes. In other words, the response may be due to the structural complexity of the signal. Table 1 presents a brief summary of cue chemistry in some social insects.

Table 1. An overview of the chemistry of recognition in selected social insect species

Insect Family	Genus	Cue Compounds	References
Bees, Superfamily Apoidea	Sweat bees, *Lasioglossum*	Macrocyclic lactones	19
	Honey bees, *Apis mellifera*	Free fatty acids, alkenes	19,48,71,73
	Stingless bees, *Trigona fulventris*	Free fatty acids, alkenes	19
Wasps, Family Vespidae	Yellowjackets and Hornets, *Dolichovespula, Vespula, Vespa*	Methylalkanes, alkenes	15,82
	Paperwasps, *Polistes*	Methylalkanes, alkenes, Fatty acid ester (some evidence)	15,75,76,77,79, 80,81
Ants, Family Formicidae	Wood ants and their relatives, genus *Formica*	Alkenes	15,49,50,51
	Carpenter ants, genus *Camponotus*	Methylalkanes, alkenes	15,31
	Bulldog ants, genus *Myrmecia*	Methylalkanes, alkenes	19
	Argentine ant, genus *Linepithema*	Methylalkanes, alkenes	52,64, 66,67,68,69,70

Although it seems that eusocial insects do not use entire cuticular hydrocarbon profiles as recognition cues, variation in the cuticular chemistry must still be unique in order for recognition cues to be informative. Because nestmate recognition cues are colony specific there must be more cue phenotypes in the population than there are nests. Breed and Buchwald[19] predict that phenotypic cue profile diversity will mainly depend on the number of compounds in the cue profile and to a lesser extent depend on fine olfactory distinctions between profiles. They argue that evaluators with a template profiles composed of 13-16 compounds can discriminated high and low concentrations of compounds. Additionally, evaluators with fewer compounds in their template profile (8-10) can discriminate compound concentrations at a finer scale. Although cue diversity is an important factor attributed to nestmate recognition, within cue diversity, we must investigate acceptance thresholds with a focus on slight differences among cues.

Recognition chemistry has been investigated in many genera of ants. In the next two sections we focus on two particularly well-studied ant systems, *Formica*, which includes the wood ants and the invasive Argentine ant, *Linepithema humile.*

FORMICA ANTS

Formica is a large, widely distributed ant genus, some members of which have been intensively studied. *Formica* ants are important members of nearly all temperate terrestrial communities. One of the intriguing aspects of *Formica*, as a genus, is variation among species in colony structure. Some species, such as *Formica argentea*,[53,54] have colonies with a single or multiple queens; these colonies occupy a single, discrete, nest and have relatively limited foraging territories around the nest. Many ecologically important species of *Formica*, such as *Formica podzolica*,[24,53,54] *Formica* exsecta[49] and *Formica aquilonia*[55] are unicolonial, as described above, with multiple queens and nests within a supercolony and ecological dominance of a large area by a single extended colony.

Although eusocial insects are generally aggressive towards nonnestmates, there is still variation among how aggressive a particular species, nest, or individual should be to maximize the benefits of recognition while reducing the costs of fighting. Many studies investigate intraspecific aggressiveness. However, deconstructing interspecific aggression can lead to interesting behavioral findings that may be difficult to tease apart. Oftentimes variation in aggression is due to the context in which social insects, or animals in general, encounter another individual. Tanner and Adler[56] investigated different factors that affect levels of aggressiveness in several species of *Formica* ants. They found that compared to ants in neutral territory, ants within their own territory tended to be more competitive towards nonnestmates demonstrating that aggression levels in ants can be context dependent.[56] Additionally, as resource value increases so does aggressiveness.[56] Behavior can be affected by the behavior of their competitors; this is shown in intraspecific interactions, but Tanner and Adler[56] have also clearly found this in an interspecific context. Many factors can affect aggressive behavior and in some species it is more context dependent.[56,57]

When social animals encounter competitors they use group size to evaluate how aggressive they will be toward who they encounter. In social insects studies have shown that group size can affect whether an individual will enter a competition, with individuals from a larger group being more willing to enter a competition.[58] Little is known about how social insects collect information about group size. This is important because these

decisions about entering an interaction must be quick thus insects must communicate this information in an efficient manner. Tanner[58] found that direct contact with nestmate cuticular hydrocarbons can elicit aggressive behavior towards competitors suggesting that this is the cue some ants use to asses group number. Interestingly, it took about 25 minutes of nestmate hydrocarbon exposure to elicit and aggressive response to competition suggesting it takes a period of assimilation for ants to process group size. However, once this information was assessed the ants continued to be aggressive for 25 minutes after exposure.[58] This suggests that the ants remember the information about group size for at least this long.

ARGENTINE ANTS

Invasive species are a concern to ecologists due to their potential to disturb native habitats. In social insects, the Argentine ant, *Linepithema humile*, is a case of great interest. The altered social structure of *L. humile* in its introduced range (for example, in California) contributes to its success as an invasive species.[25] In its introduced range Argentine ants are unicolonial forming large supercolonies that lack territorial boundaries.[59] Although nests are separated by physical space, individuals that are part of these supercolonies are tolerated when moving between nests.[59] In its native range *L. humile* mainly form smaller distinct colonies that show aggression towards ants from other colonies.[60,61] In a more recent study Pederson et al[62] found that in its native range *L. humile* can also be unicolonial. However, unicolonial colonies in the native range are several orders of magnitude smaller than those in the introduced range.[62] By examining differences between the Argentine ants in its native and introduced range scientists are trying to ascertain factors that have caused its altered social structure.

Several hypotheses have been proposed about how Argentine ants switched from a multicolonial social structure to a unicolonial one. Tsutusi et al[61] attribute multicoloniality to reduced genetic diversity. The "genetic cleansing" hypothesis proposes that unicoloniality in Argentine ants arose by selection against less common recognition alleles.[63] Another hypothesis suggests that selection against individuals from genetically diverse groups has contributed to unicoloniallity in the introduced population.[64] These hypotheses are based on the idea that ants in the native range are multicolonial while those in the introduced range are unicolonial. However, Pederson et al[62] found that unicoloniallity exists in the native range of *L. humile* as well. Although unicoloniallity exists in both native and introduced ranges the levels of chemical and genetic diversity are much lower in introduced versus native range suggesting that although they may be unicolonial these colonies are in fact different.[65] Despite the large amount of work that has been done on these questions, the matter of why these colonies are different is far from resolved.

In the Argentine ant cuticular hydrocarbons can cause intraspecific aggression.[66] Vasquez et al[67] found that unrelated *L. humile* colonies that share similar cuticular hydrocarbons will readily fuse. This suggests that plasticity in cuticular hydrocarbon profiles maintain the fusion of unrelated *L. humile.*[67] Tsutsui et al[61] suggests that recognition cues in *L. humile* are heritable due to the genetic similarity between individuals. Conversely, studies have shown that cuticular hydrocarbons derived from prey can affect the recognition system in Argentine ants.[68,69] However, the affect of these environmental cues varies among introduced populations based on genetic diversity where recognition cues are more genetically based in populations with greater

genetic diversity while environmentally based cues are more important populations with reduced genetic diversity.[70]

THE WESTERN HONEYBEE, *APIS MELLIFERA*

In addition to cuticular hydrocarbons, in honeybees, comb wax is important to nestmate recognition.[71] Cuticular fatty acids found in bees are not only key compounds in nestmate recognition but also have a structural role in beeswax.[19] Like cuticular hydrocarbons in ants and wasps, all individuals in a honeybee colonies have fatty acids but they differ in relative proportion.[33] The primary components of bees wax are variable proportions of n-alkanes, wax esters and free fatty acids.[72] All of the fatty acids found in the comb wax, except steric acid, provide a cue for nestmate recognition.[71] These fatty acids include saturated: palmitic acid and tetracosanoic acid and unsaturated: palmitoleic acid, oleic acid, linoleic acid and linolenic acid.[71] In addition to varying in chemical composition comb wax varies in mechanical properties depending on the ecology of a particular species of bee.[73,74] The inherently interesting connection between the mechanical and behavioral importance of fatty acids in honeybee ecology lead to interesting questions about how natural selection has acted upon these compounds.

Within a species of bee there must be enough variation in wax composition to ensure the phenotypic diversity of recognition cues. However, because of the importance of maintaining the mechanical integrity of combwax to bee ecology, the differences in wax composition must not compromise the mechanical properties of combwax. Buchwald et al[33] found that phenotypic variation in the relative proportion of fatty acid composition of combwax has little impact on the mechanical properties. More specifically, variation in the majority of the unsaturated fatty acids did not affect the mechanical properties of comb wax.[33] Interestingly, the relative proportions of these unsaturated fatty acids between nests varies suggesting that changes in unsaturated fatty acids lead to phenotypic cue diversity without compromising nest mechanical properties.[33] These findings suggest that in other social insects, who use cuticular hydrocarbons as recognition cues, a similar type of selection has occurred. Although it has never been tested, there is likely enough variation in the composition of hydrocarbon type within the profile to provide recognition cue diversity but this variation most likely does not affect the integrity of the waterproofing qualities of the exoskeleton.

SOCIAL WASPS

Like ants, several species of eusocial wasps exhibit colony-specific cuticular hydrocarbon profiles comprised of n-alkanes, methylalkanes and alkenes.[75-78] Of these hydrocarbons, in some species, methylalkanes and alkenes seem to be most critical in inducing an aggressive response typically seen when a nonnestmate tries to enter a nest.[79] While in most species it is unclear which particular compounds elicit a nestmate recognition response, it is clear that combinations of these hydrocarbons are responsible for nestmate recognition.[79,80] In addition to cuticular hydrocarbons, some species of eusocial wasp use nest paper hydrocarbons for recognition.[81,82]

In paper wasps olfactory cues may not be the only factor important to communication, the variety of facial markings in *Polistes* lead investigators to examine the use of visual

cues in *Polistes*. Tibbetts[26] found that in at least one species of wasp, *Polistes fuscatus*, individuals can recognize a nestmate from a nonnestmate using facial patterns. This evidence indicates further investigation into such a phenomenon in other wasp species may lead to similar findings. Tibbetts[83] found that eight *Polistes ssp.* had variable enough facial markings that cue diversity from the facial marking of these species could provide enough phenotypic diversity for individual recognition. Further investigation into these species' recognition system could lead to similar finding.

TERMITES

The matter of how termites recognize a nestmate from a nonnestmate is still unresolved. Termites, although they have a different genetic structure than the Hymenoptera, also exhibit colony specific hydrocarbon profiles.[84,85] This suggests that cuticular hydrocarbons may be mainly responsible for nestmate recognition in termites. Studies that examine the link between cuticular hydrocarbons and aggression have had mixed results with some showing increased aggression to cuticular hydrocarbons[86] while others were not able to make this link.[87] These opposed finding have lead investigators to test different avenues for recognition besides cuticular hydrocarbons.

Exogenous environmental factors may play a role in termite recognition. Researchers have linked diet to increased interspecific aggression.[88] However, individuals from neighboring nests may have similar diets which may not lead large cue diversity for recognition. There is some evidence that intestinal bacteria play an important role in nestmate recognition.[89] Matsuura[89] found colony specific microbial communities in termite guts and that termites that had absorbed unfamiliar bacterial odor were recognized and nonnestmates. Matsuura[89] suggests that volatile cues from fecal bacteria may be responsible for nestmate recognition cues. Further investigation is required in order to elucidate nestmate recognition in termites.

EXPERIMENTAL APPROACHES TO RECOGNITION STUDIES

Studies examining cuticular hydrocarbons need to rely on a method of detecting and identifying hydrocarbons. Typically researchers extract hydrocarbons from the cuticle of the insect using and nonpolar solvent such as pentane or hexane. Extractions are then separated and identified using Gas Chromatography—Mass Spectrometry (GC—MS). Although this method is commonly used in these types of investigations, researchers must be careful to employ the proper temperature and column conditions as improper examination could lead to the underestimation of hydrocarbons present on the cuticle.[90] Once these compounds have been analyzed using GC—MS researchers typically uses multivariate statistics to look for colony-specific patterns in hydrocarbon profiles.[91] Although this approach seems to suggest that hydrocarbons are colony specific they rarely link behavioral evidence with chemical evidence leaving the link between hydrocarbons and nestmate recognition highly circumstantial.[71,91]

To determine nestmate recognition researchers typically perform aggression behavioral bioassays where they observe the behavior of interacting pairs or groups of individuals. In these bioassays those individuals perceived as nonnestmates typically act aggressively towards one another.[92] Although most researchers use aggression bioassays

for these studies, they tend to be highly varied in many aspects including but not limited to duration, number of individuals, detail of observations and ways data are collected.[92] Bioassays are critical to nestmate recognition but, because results can vary, researchers must be sure to choose the appropriate assay for the question they are trying to ask.[92] Behavioral bioassays begin to make the link between chemistry and behavior but more information is still needed to fully understand this phenomenon.

There is evidence that cuticular hydrocarbons elicit behavioral responses in social insects but the mechanism by which they perceive these odors is still weakly understood.[84] The use of electro-antennography (EAG) is a common technique used study the perception of volatile compounds in insects. However, because cuticular hydrocarbons are not very volatile at room temperature, this technique is rarely used to investigate social insects. Some investigators have been able to successfully link antennal responses with the presence of hydrocarbons in ants[93,94] and termites.[95] Future studies using this technique are needed to provide further insight into the mechanism by which odors are perceived.

CONCLUSION

Eusocial insects provide excellent models for studying discriminations of self versus nonself. Analogies are easily drawn between MHC mediated self-recognition in vertebrates and social recognition in insects. In both systems, hypervariable phenotypes provide the necessary information for self- and social recognition. In the vast majority of species, recognition in eusocial insects relies on cuticular hydrocarbons; neutral substitution among hydrocarbons can yield immense phenotypic variation for social signals.

The recent suggestion by Richard et al[96] that immune response, cuticular hydrocarbons and social recognition are linked in honeybees is intriguing and merits further study. If, indeed, immune function and social recognition are linked in eusocial insects, this would build an even stronger analogy with MHC mediated recognition systems. Comparative studies of the chemistry of social recognition will give further insight into the evolution of how social identity is signaled and perceived. This knowledge will also test the neutral substitution hypothesis and indicate whether neutral substitution should be accepted as the primary force in generating the variable phenotypes needed in social recognition.

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CHAPTER 6

ODOR AND PHEROMONE SENSING VIA CHEMORECEPTORS

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Abstract: Evolutionally, chemosensation is an ancient but yet enigmatic sense. All organisms ranging from the simplest unicellular form to the most advanced multicellular creature possess the capability to detect chemicals in the surroundings. Conversely, all living things emit some forms of smells, either as communicating signals or as by-products of metabolism. Many species (from worms, insects to mammals) rely on the olfactory systems which express a large number of chemoreceptors to locate food and mates and to avoid danger. Most chemoreceptors expressed in olfactory organs are G-protein coupled receptors (GPCRs) and can be classified into two major categories: odorant receptors (ORs) and pheromone receptors, which principally detect general odors and pheromones, respectively. In vertebrates, these two types of receptors are often expressed in two distinct apparatuses: The main olfactory epithelium (MOE) and the vomeronasal organ (VNO), respectively. Each olfactory sensory neuron (OSN) in the MOE typically expresses one type of OR from a large repertoire. General odors activate ORs and their host OSNs (ranging from narrowly- to broadly-tuned) in a combinatorial manner and the information is sent to the brain via the main olfactory system leading to perception of smells. In contrast, pheromones stimulate relatively narrowly-tuned receptors and their host VNO neurons and the information is sent to the brain via the accessory olfactory system leading to behavioral and endocrinological changes. Recent studies indicate that the functional separation between these two systems is blurred in some cases and there are more subsystems serving chemosensory roles. This chapter focuses on the molecular and cellular mechanisms underlying odor and pheromone sensing in rodents, the best characterized vertebrate models.

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INTRODUCTION

Detection and discrimination of various chemicals in the environment are critical for the survival of most organisms. These chemical cues are classified into two major categories: General odors, which signal potential food and environmental hazards and pheromones, which signal social or sexual status among individuals of the same species or conspecifics. Natural odors often comprise complex mixtures of odorants, which are volatile chemical compounds with molecular weights of less than 300 Daltons. Detection of these odorants by the olfactory system generally leads to perception of smells. In contrast, pheromones are substances released by one individual and can elicit stereotyped behaviors or endocrinological changes in the conspecific receivers.[1-3] Pheromones range from small organic molecules to large peptides and often do not lead to conscious perception of smells. However, the distinction between odorants and pheromones is sometimes ambiguous since some compounds can serve as both pheromones and odorants.

To detect numerous chemicals with diverse structures and to discriminate chemicals with subtle structural differences, many species have developed sophisticated chemosensory systems which express a large family of chemoreceptors. The first set of ORs was cloned from rat in a seminal study published nearly twenty years ago.[4] Since then, genomic analysis has led to a comprehensive list of chemoreceptors for a number of representative species. The worm *C. elegans* devotes more than 5% of its genes to encode 500-1000 chemoreceptors.[5] Insects (fruitfly, mosquito, honeybee, beetle and moth) express 80 to 400 chemoreceptors including both olfactory and gustatory receptors with substantial species differences.[3] In vertebrates, the odorant receptor gene family ranges from ~100 in fish to ~1500 in some mammals . In addition to the large number of chemoreceptors, the olfactory systems are also well-adapted at the molecular, cellular and circuit levels for detecting and discriminating odor molecules. This chapter reviews recent advances in our understanding of odor and pheromone sensing in vertebrates by focusing on rodents.

The rodent nose contains several distinct olfactory organs which express different chemoreceptors and connect to different parts of the brain. The two major olfactory apparatuses in the nasal cavity are the main olfactory epithelium (MOE) and vomeronasal organ (VNO), each of which contains several subsystems (Fig. 1A). Most sensory neurons in the MOE are ciliated OSNs, which express G-protein coupled ORs and utilize the cAMP cascade to transform chemical energy into electrical signals. However, some ciliated OSNs express unconventional receptors. For example, a subset of OSNs expresses trace amine associated receptors (TAARs) and responds to amines and urine.[6] Another subset of OSNs expresses guanylyl cyclase-type D (GC-D)[7,8] and detects ambient CO_2[9] and natriuretic peptides (uroguanylin and guanylin).[10] Additionally, the VNO is separated into the apical and basal compartments expressing two classes of vomeronasal receptors V1Rs and V2Rs, respectively.[11] Recent studies have identified formyl peptide receptor-like proteins as a new family of chemoreceptors expressed in a subset of VNO neurons.[12,13] Finally, two spatially segregated clusters of neurons form the septal organ of Masera[14] and the Grueneberg ganglion[15] (Fig. 1A). The septal organ predominantly expresses a small subset of ORs,[16,17] but most of the neurons respond broadly to general odorants and also to mechanical stimulation.[18,19] The Grueneberg ganglion cells also express chemoreceptors[20,21] and sense alarm pheromones[22] and cool ambient temperature.[23] Considerable progress has been made in the last few years in understanding the molecular and anatomical organization of the olfactory systems and linking chemoreceptors to neuronal activity and in some cases to behavioral significance.

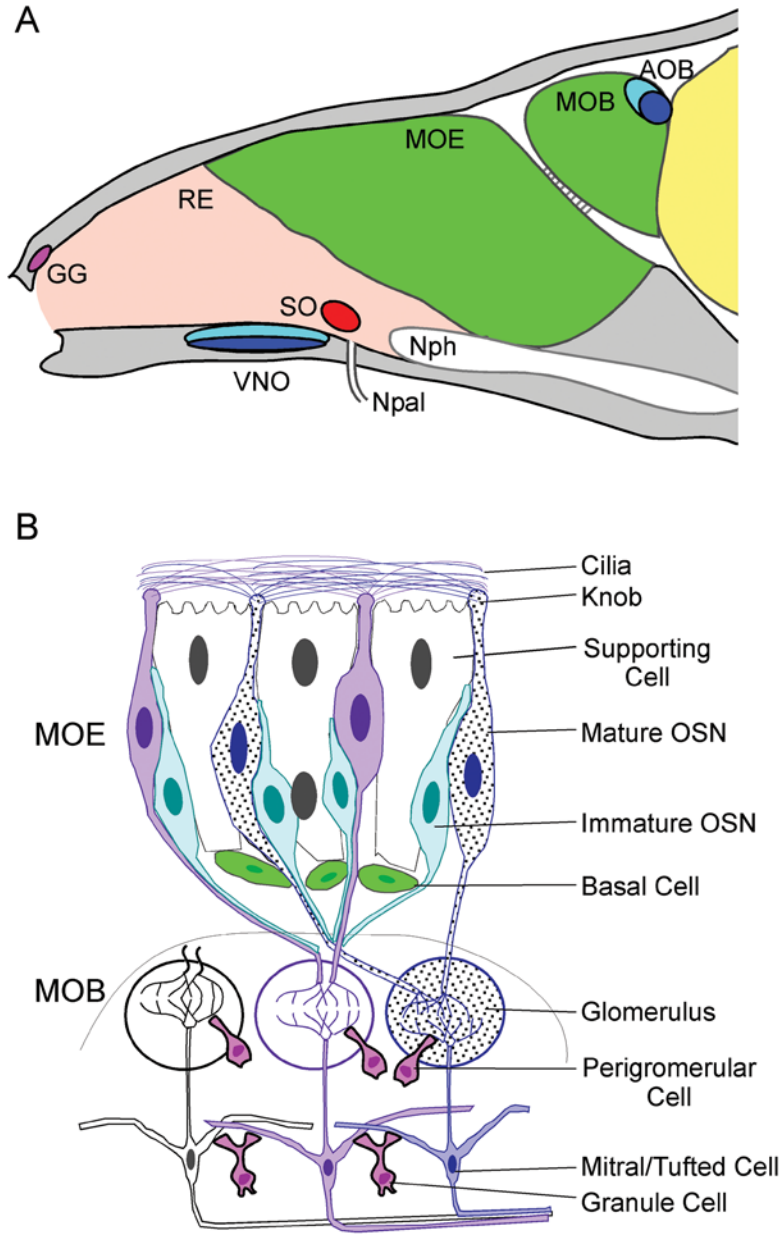

Figure 1. Organization of the mammalian olfactory system. A) Schematic illustration of the mid-sagittal view of the rodent nasal cavity. The olfactory sensory neurons in the main olfactory epithelium (MOE) project to the main olfactory bulb (MOB). The apical and basal sensory neurons in the vomeronasal organ (VNO) send axons to the anterior and posterior accessory olfactory bulb (AOB), respectively. The septal organ (SO) (surrounded by the respiratory epithelium, RE) projects to the ventroposterior MOB and the Grueneberg ganglion (GG) to the caudal MOB. Npal, Nasopalatine duct. Nph, nasopharynx. Modified from.[58] B) Cytoarchitecture of the MOE and MOB.

ODOR SENSING VIA THE MAIN OLFACTORY SYSTEM

Olfactory Sensory Neurons and G-Protein Coupled Odorant Receptors

The MOE is a pseudostratified neuroepithelium located in the posterior nasal cavity lining the cartilaginous turbinates and septum. The rodent MOE harbors 6-10 million ciliated OSNs among several other cell types (Fig. 1B). As the primary sensory neurons, OSNs are responsible for detecting odor molecules and transduce the chemical energies into electrical signals, which carry the information into the olfactory bulb. The glia-like supporting (or sustentacular) cells provide metabolic and physical support in the olfactory epithelium. The basal cells (a type of stem cells) undergo continuous division and differentiation to replace OSNs and supporting cells throughout life or after injury.[24,25]

Detection and discrimination of various odorants by OSNs critically depend on a large family of G-protein coupled, seven transmembrane ORs. The vertebrate chemoreceptor gene family has undergone molecular evolution involving gene duplication and pseudogenization. From fish (zebrafish and pufferfish) to amphibians (frog), the repertoire of the OR genes has expanded from ~100 to ~900, probably reflecting evolutional adaptation from aqueous to terrestrial environments.[26] In mammals (human, chimp, dog, mouse, rat and opossum), the number of OR genes ranges between 800 to 1500. For instance, the mouse and rat genome contains 1375 and 1576 OR genes, respectively, while the human genome contains 851 OR genes.[27] The fraction of pseudogenes increases from ~20% in rodents to 25-35% in nonhuman primates and to >50% in humans,[28-32] consistent with the fact that primates rely more on other senses. Interestingly polymorphism is evident in OR genes[33-35] and single-nucleotide polymorphisms may lead to individual differences in detecting specific odorants.[36,37]

Remarkably, each OSN typically expresses only one functional OR with few exceptions,[38-41] a process that involves negative feedback from the selected functional OR gene.[42-46] The mechanisms underlying OR choice in single OSNs are still elusive and currently under extensive investigation.[47-49]

Each OR is exclusively expressed in one of the few circumscribed bands in the MOE, which was originally divided into four zones.[50,51] More recent studies suggest that ORs are expressed in multiple, overlapping zones arranged along the dorsomedial (center) to ventrolateral (periphery) axis.[52,53] Such arrangement may match the OR types with the physicochemical features of the odorants (stability, volatility and water solubility) that each region tends to encounter during odor sampling.[54-56]

Olfactory Signal Transduction—The Canonical cAMP Cascade

Bipolar OSNs possess a thin axon and a thick dendrite with a swelling ending (called dendritic knob) bearing 10-20 cilia, which contain OR proteins and associated signal transduction machineries (Figs. 1B, 2A). Binding of odorant molecules to ORs activates a series of events, which eventually lead to generation of action potentials carrying the neural code into the brain.

Most ciliated OSNs utilize the canonical cAMP cascade for signal transduction. The ligand-bound OR activates an olfactory specific G protein (G_{olf}), which in turn activates the Adenylyl Cyclase-Type III (ACIII). The cyclase catalyzes the production of cAMP, a second messenger that directly opens a cyclic nucleotide-gated (CNG) channel. This nonselective cation channel allows Na^+ and Ca^{2+} to flow into the cell, which depolarizes

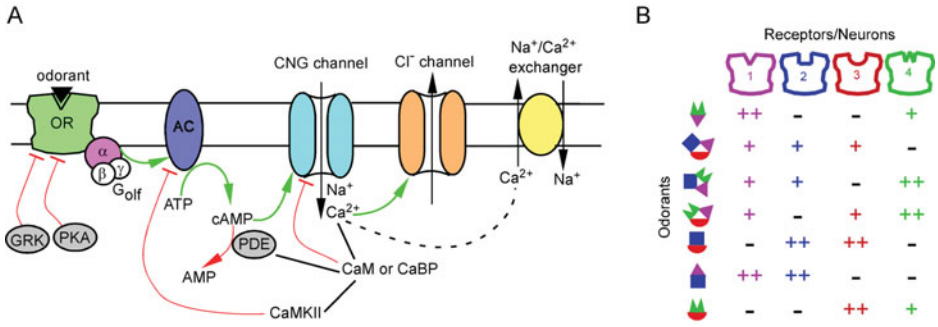

Figure 2. Odor sensing by olfactory sensory neurons (OSNs). A) The canonical cAMP pathway underlies signal transduction in most OSNs. Within the cilia of an OSN, binding of odor molecules to odorant receptors (R) triggers a cascade of enzymatic activity that leads to channel opening, thus transducing chemical energy into an electrical signal. G_{olf}, olfactory specific G protein. AC, adenylyl cyclase (Type III). CNG channel, cyclic nucleotide gated channel. CaM, calcium-calmodulin. CaBP, calcium-binding protein. CaMKII, calmodulin kninase II. PDE, phosphodiesterase. PKA, protein kinase A. GRK, G-protein coupled receptor kinase. NKCC1, Na^+-K^+-$2Cl^-$ cotransporter. Modified from.[58] B) The odor information is encoded by combinations of various receptors at the epithelial level. Representative receptors (1-4) are listed in the upper row and odorants in the left column. Plus signs indicate activation of a receptor by an odorant (more plus signs mean stronger responses) and minus signs indicate no response.

the cell membrane. An additional outward Ca^{2+} activated Cl^- current further amplifies the response.[57-60] Genetic ablation of G_{olf}, ACIII or the CNG channel dramatically reduces odorant-induced responses and causes smell dysfunction, strongly supporting the vital role played by the cAMP cascade.[61-63] A recent study suggests that anoctamin two forms the Ca^{2+}-activated Cl^- channel in OSNs because its transcripts are highly enriched in the cilia and when expressed in vitro, it forms functional Ca^{2+}-activated Cl^- channels that resemble those in OSNs.[64] Further studies on the response properties of OSNs with disrupted anocamin two function would help to make this finding conclusive.

As in all sensory systems, the acuity of olfactory perception depends on response termination and adaptation. The odorant-activated cAMP cascade is subjective to negative feedback regulation, which can occur at multiple sites (Fig. 2A). The best studied site is the CNG channel, which is inhibited by Ca^{2+}-calmodulin upon odorant stimulation.[65,66] Such inhibition is believed to play a dominant role in fast adaptation during repeated stimulation.[67,68] Surprisingly, a recent study indicates that selective deletion of the calmodulin binding domain in the CNG channel slows down the response termination but does not affect fast adaptation upon recurring stimulation.[69] In addition, the ACIII activity is inhibited by phosphoralytion via Ca^{2+} activated CaM-dependent protein kinase II (CaMKII), which potentially attenuates the olfactory signal,[70] especially during prolonged odor stimulation.[71] Moreover, two forms of phosphodiesterases (PDE) have been identified and speculated to contribute to response termination. PDE1C is highly enriched in the cilia of OSNs, while PDE4A is expressed in the entire OSNs except the cilia.[8,72-74] Contrary to the common belief, genetic ablation of PDE1C or PDE4A does not affect response termination. Instead, a double knockout impairs fast adaptation as revealed in odorant-induced EOG signals.[75] These and other feedback processes are likely activated under different odor stimulation paradigms (the duration ranges from a fraction of a second to minutes or hours) to ensure proper function of the sensory neurons under all conditions.

Odor Coding by Combinations of ORs and OSNs

How is a given odorant represented in the MOE which contains millions of OSNs expressing >1000 ORs? Both odor quality and intensity are encoded by combinations of specific ORs and their host OSNs (Fig. 2B). Although a single sensory neuron expresses only one OR-type, it can respond to multiple odorants with different sensitivity and concentration dependence. This is probably because a single receptor can broadly, yet selectively bind to multiple odorants via relatively weak hydrophobic and van der Waals interactions.[76] Conversely, a single odorant can be recognized by multiple receptors that detect different molecular features. A given odorant at a higher concentration elicits stronger responses in individual neurons and recruits more receptors/neurons, presumably with lower affinities to that odorant. As a result, both odor quality and intensity are encoded by combinations of multiple receptors/neurons.[77,78] A combinatorial strategy based on ~1000 receptors permits the olfactory system an almost unlimited capability of odor detection and discrimination.

How narrowly or broadly individual ORs are tuned to the large array of odorants? Two major approaches have been adopted to acquire such information. The first one is to test the responses of heterologously expressed ORs to a panel of odorants. Recent characterization of molecular chaperones and other factors involved in functional expression of OR proteins in heterologous systems has facilitated identification of chemical ligands for this large family of ORs.[79-83] The second approach is to test the responses of individual OSNs with known ORs. This can be achieved by introducing a known OR into OSNs,[84] by identifying the ORs after characterizing the response profiles of individual OSNs,[85,86] or by recording from OSNs with genetically-targeted ORs.[87,88] Up to date, the best-studied mammalian ORs respond preferably to a small number of structurally related compounds from a list of up to a few hundred, such as rat I7 to octanal,[89] M71 to acetophenone,[87] and mOR-EG to eugenol.[76] Interestingly, a mouse receptor SR1 (or MOR256-3), which is abundantly expressed in the septal organ and also in the MOE, has been shown to respond broadly to structurally distinct compounds with a high sensitivity and a wide dynamic range.[18] It is plausible that mammalian ORs and their host OSNs show diverse response profiles, ranging from highly selective to broadly responsive, similar to the ORs in the fruitfly *Drosophila melanogaster*.[90]

Central Processing of Odor Information

The OSN axons form the olfactory nerve bundles and project to the main olfactory bulb (MOB) (Fig. 1B). The axon terminals of OSNs make synaptic contacts with the dendritic arbors of second-order neurons in specialized structures called glomeruli, which are distributed as a layer under the surface of the bulb. Although the OSNs expressing a particular OR are scattered in a broad zone in the MOE, their axons typically coalesce onto two glomeruli in each bulb.[91] A single glomerulus is innervated by 25 to 50 projection neurons called mitral/tufted (M/T) cells, each of which extends a single primary dendrite into the glomerulus and receives excitatory inputs from OSNs. Consequently, a dispersed activity pattern elicited by an odorant in the MOE is transformed into a distinct spatial and temporal pattern in the glomerular units in the MOB.[92] The high convergence ratio (~5000 OSNs per glomerulus in rodents) may help to amplify weak signals, increase signal-to-noise ratio and expand the dynamic range in responding to odorants. Via reciprocal dendrodentric synapses, M/T cells also excite local interneurons

(heterologous periglomerular cells and granule cells) which in turn inhibit the same and other M/T cells allowing communication within and between glomerular units. How the bulbar circuits shape the odor representation is not fully understood, partly due to the technical difficulties in direct comparison between the input and output signals of M/T cells with known OR identity. The potential roles of lateral inhibition include sharpening the receptive field of M/T cells, providing a gain control of the input signals and/or synchronizing the output signals.[92]

The M/T cells in the MOB form the lateral olfactory tract and project to the primary olfactory cortex including the anterior olfactory nucleus, the olfactory tubercle, the piriform cortex and the entorhinal cortex.[93,94] Olfactory information is subsequently relayed to the higher cortex including the orbitofrontal cortex for cognitive processing. The M/T cells receiving inputs from the same receptor-type project diffusely to the olfactory cortex, which enables individual cortical neurons to serve as coincidence detectors for multiple M/T populations receiving different OR inputs.[95-97] Compared to the MOB, the odor representation in the piriform cortex is more sparse, presumably due to widespread inhibition mediated by local interneurons.[98,99] The sparse odor representation in the olfactory cortex may help the system to discriminate similar odorants.

The MOB is also connected with the limbic system, including the amygdala, presumably involved in the emotional aspects associated with odors. In addition, the main olfactory system also carries the chemosensory information to the hypothalamus, a key regulator in feeding, aggression and reproduction behaviors.[100,101] Several physiological and imaging studies have demonstrated that the MOE and the MOB respond to both general odorants and social cues.[102-107] The emerging picture is that the main olfactory system functions in conscious perception as well as in subconscious processing of olfactory cues. These findings suggest that organisms without a functional VNO (such as humans) can potentially use the main olfactory system for intraspecies pheromone communication.

PHEROMONE SENSING VIA THE VOMERONASAL ORGAN

G-Protein Coupled Vomeronasal Receptors and Signal Transduction

The rodent nose possesses a well-developed VNO, a bilateral blind-ending tube encased within bony capsules in the ventral nasal septum (Fig. 1A). Lining the medial wall of the VNO is the sensory neuroepithelium, which harbors microvillar Vomeronasal sensory neurons (VSNs) whose axons project to the accessory olfactory bulb (AOB). The accessory olfactory system clearly comprises at least two separated subsystems with different vomeronasal receptors (VRs) and central targets. The VSNs in the apical neuroepithelium express V1Rs with G_i proteins and project to the anterior portion of the AOB. In the mouse genome, there are 308 V1R sequences but only 187 of them have full-length open reading frames.[108] In humans, there are only five intact V1R genes which are expressed in the olfactory epithelium.[109,110] Similar to the singular OR expression pattern in the MOE, a single apical VSN expresses only one V1R-type.[11]

The VSNs in the basal compartment express V2Rs with G_o proteins and project to the posterior portion of the AOB.[11,111] In the mouse genome, there are 279 V2R genes but only 121 of them are intact.[108,112] Although both V1Rs and V2Rs belong to the GPCR superfamily, they share little sequence similarities with each other or with the ORs expressed in the MOE. Unlike ORs and V1Rs, V2Rs have a very large N-terminal

extracellular domain, a feature shared by metabotropic glutamate receptors.[11] A single basal VNO neuron appears to express only one V2R[113] with the exception that the V2R2 gene is co-expressed with other V2Rs throughout the basal layer.[114]

Signal transduction in both V1R and V2R expressing neurons depends on a Phospholipase C (PLC) mediated pathway. Via different G proteins, ligand-binding in the VNO neurons activates PLC, which results in production of phosphatidylinositol-3-phosphate (IP_3) and Diacylglycerol (DAG), followed by generation of arachidonic acid.[115,116] These processes eventually lead to the opening of a transient receptor potential channel, TRPC2, which is expressed in the microvilli of the VSNs.[115,117] The critical role that TRPC2 plays in pheromone detection in the VNO is further confirmed by targeted gene deletion. TRPC2 null mice exhibit reduced electrophysiological responses to urines and pheromones recorded in the VNO as well as impaired aggressive and mating behaviors.[118-120] However, detection of some compounds by the VNO is apparently independent of TRPC2-mediated transduction.[121]

Pheromone Coding by Specific VRs and Their Host VSNs

How are pheromone molecules represented in the VNO? Up to date, only a small number of pheromones have been identified in rodents and few of them have been linked to specific VRs and their host VSNs. Nevertheless, V1Rs are likely responsive to small organic molecules. Lacking a cluster of V1Rs leads to impaired responses to some volatile pheromones and decreased aggressiveness and sexual behavior in mutant mice.[122] In addition, one V1R member, V1Rb2, has been shown to respond to 2-heptanone, a component of urine that can extend the estrous cycle.[123] Furthermore, calcium imaging of the VNO slices demonstrates that the apical VSNs respond to various volatile pheromones.[124] Having a large N-terminal region, V2Rs and their host VSNs tend to respond to larger, nonvolatile peptides and proteins, including major urinary proteins (MUPs), major histocompatibility complex (MHC) peptides and exocrine gland-secreting peptides (ESPs).[3] MUPs are shown to activate the basal VSNs[125,126] and synthetic MUPs are sufficient to elicit aggressive behaviors in male mice.[126] MHC peptides also elicit calcium signals in the basal VSNs[127,128] and they may serve as the signals underlying mate recognition in the context of pregnancy block.[127] It should be noted that MHC peptides stimulate the MOE as well, even though the MOE does not express VRs.[103] ESP1 is a male-specific peptide that specifically stimulates the basal VSNs that express the receptor V2Rp5.[129,130] The VRs and their host VSNs respond to appropriate ligands with high sensitivity and specificity and a given ligand at a higher concentration does not recruit more receptors/neurons.[124] This is in sharp contrast with the combinatorial strategy used by the OSNs in the MOE (Fig. 2B), although it is plausible that populations of VSNs are used in combination to encode strain, gender and individual information.[128]

In addition to pheromones, some VNO neurons are responsive to volatile odorants, which may trigger instinctive behaviors.[131] This finding is further supported by a study using ACIII knockout mice, in which odor detection via the MOE is severely impaired.[132] The ACIII null mice still detect certain odorants, which elicit electrical responses in the VNO. It is unclear whether the volatile odorants can directly bind to V1Rs or V2Rs and consequently activate the same PCL-TRPC2 pathway. Identification of certain ORs in a subset of VNO neurons provides an alternative mechanism underlying detection of

volatile odorants by the VNO.[133] The OR-positive VNO neurons lack ACIII and G_{olf}, but instead express G_i and TRPC2 and project to the anterior part of the AOB. Taken together, current evidence indicates that certain compounds are processed in parallel by the main and accessory olfactory system, suggesting that these compounds may serve both as common odors leading to smell perception and as social cues to trigger innate behaviors.

Central Processing of Pheromone Information

The axons from the apical and basal VSNs project to the anterior and posterior part of the AOB, respectively. The VSNs expressing the same VR converge their axons onto several glomeruli and make synapses with the second-order neurons.[113,134] The projection neurons in the AOB directly send axons to the limbic system, including the medial amygdaloid nucleus, the posteromedial amygdaloid cortical nucleus, the bed nucleus of the stria teminalis and the nucleus of the accessory olfactory tract. These structures are in turn connected with several hypothalamic nuclei, such as the medial pre-optic area, the ventromedial hypothalamus and the premammilary and supraoptic nuclei, leading to behavioral and endocrinological responses.[2,135] In addition to processing social cues, recent studies indicate that the VNO readily detects small organic molecules including volatile odors that elicit responses in the MOE, suggesting a greater overlap of the stimulating ligands between the two systems in chemoreception.[2,136,137]

CONCLUSION AND FUTURE PROSPECTS

To increase the chance of survival, all organisms have evolved complicated chemosensory organs to detect and discriminate chemical cues in the environment. The mammalian nose is more complicated than previously appreciated with multiple physically segregated apparatuses including the MOE and VNO. Both MOE and VNO contain heterogeneous cell types with distinct chemoreceptors, transduction machineries and/or central targets. More subsystems may still emerge with new molecular markers and more detailed anatomical/functional analysis. The advantage of having multiple olfactory subsystems is manifold. Different chemoreceptors expressed in these subsystems can expand the overall detection capacity of the olfactory system for chemicals and other stimulations. In addition, critical information can be processed in parallel by multiple subsystems, which send signals to different brain regions for further integration and execution. The diversity and complexity of the chemosensory systems allow the organisms to accurately perceive their chemical surroundings and respond appropriately by adjusting their behaviors, emotions and hormones.

Despite the recent explosion of our knowledge on chemosensation, there are still many unanswered questions. Do mammals especially those without a functional VNO use pheromones for individual and species appraisal and recognition? If so, do the OSNs in the MOE serve dual functions as odorant and pheromone detectors? What are the molecular identities and behavioral effects of these so called pheromones? How are the odorant and pheromone information processed and integrated in the brain? The future goal of the field is to understand how the animals use sophisticated chemosensory systems in concert to perceive the chemical world and respond appropriately by linking the chemoreceptors and the sensory neurons to the neural circuits and to the behavioral outputs.

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CHAPTER 7

IDENTIFYING SELF- AND NONSELF-GENERATED SIGNALS:
Lessons from Electrosensory Systems

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Abstract: This chapter provides a short review of the mechanisms used by electroreceptive fish to discriminate self- from nonself-generated signals. Electroreception is used by animals to detect objects of electric impedance different from the water, to detect natural electrogenic sources and to communicate signals between conspecifics. Electroreceptive animals may generate electric fields either with the purpose of electrically illuminating the neighborhood or as an epiphenomenon of other functions. In addition, the presence of the fish body as a conductive object in a scene funnels the current flow and, consequently, animal movements also generate signals by changing the body shape or the spatial relationship of the body with the surrounding objects. Therefore, mechanisms for discrimination between self and externally generated signals are very important for constructing a coherent representation of the environment. Some mechanisms facilitate and stream the flow of signals carried by the self-generated electric field. Others are designed to reject unwanted interference coming from self-generated movements or even the self-generated electric field. Finally, more complex operations involving sensory motor integration are used for discriminating between self- and conspecific- generated communication signals. Despite the evolutionary distance between animals endowed with electric sense, mechanisms for self-identification reappear with few differences between

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species. This suggests that many of the possible strategies are present in vertebrates may be found in these fish. Therefore, we have much to learn about self recognition from the study of electroreception.

INTRODUCTION

Perception emanates from the dynamic process of interaction between animals and their environment. As a control system, the main role of the brain is to predict the next stage of the system under control.[1,2] Brains act as "reality emulators" to generate predictive images of the events to come. In the words of Llinas "Such an image may be considered a premotor template that serves as a planning platform for behavior or purposeful action".[2]

According to this view, to perceive is to evaluate and predict the consequences of self-generated actions or nonself-generated events and to discriminate between them. Self-generated actions generate sensory inputs to the organism's own sensory system. Some of these inputs may produce useful information for the individual but others may interfere with perception of externally generated sensory events and thus should be eliminated. The aim of this chapter is to review some neural mechanisms for separating self- and nonself-generated signals that flow together in the same sensory channel.

The formal study on how the sensory effects of self-generated actions are recognized by the individual was started by von Holst and Mittelstaedt.[3] They proposed a language to deal with this subject and a hypothesis (the re-afference principle) on how unwanted sensory effects of the organism's own actions could be eliminated during sensory processing. According to von Holst and Mittelstaedt self-generated sensory input is called *re-afference*. Re-afference may derive either from self-generated energy fields that serve as carriers for the sensory signals (for example exerting pressure in touch) or by animal movements that shape the pattern of energy stimulating the sensory surface (for example moving the eyes in vision). If a sensory system generates the sensory carrier, the system is called an active sensory system.

Afferent input has two components. One informs the individual about the external world (*exafference*) and the other results from self-generated actions (*re-afference*). The re-afference principle states that, in order to extract *exafference* from *afference*, *re-afference* must be cancelled. Nevertheless, the validity of the re-afference principle does not preclude the brain from using the opposite process (evaluating the motor commands from *re-afference*), when the data derived from self-generated sensory stimuli are necessary to implement a certain neural algorithm.

We focus this chapter on electroreception. This sensory modality is present in all major branches of vertebrates with the exception of Birds and Reptiles: Petromyzonts: Lampreys; Chondrichthys: Skates, rays and sharks; Teleostei: Catfish and electric fish; Amphibians: Salamanders, caecilians; and Mammals: Platypus). However, common ancestors for some of these taxa have not been identified as electroreceptive, thus it appears to have been re-invented several times.[5] To deal with self- and nonself-generated signals electroreceptive animals have evolved a variety of mechanisms organized at different levels (from subcellular to behavior) and it is possible that many of the possible strategies for sensory discrimination of such signals are present in these animals.[6]

ELECTRORECEPTION ILLUSTRATES ON VARIOUS FORMS OF RE-AFFERENCE

Electroreception is the ability to sense and to communicate using electricity as a sensory carrier. Electric fields stimulating the electrosensory mosaic may arise from real or virtual electric sources. In fact, as the impedance of objects is different from water, objects behave as virtual electric sources in the same way that the moon behaves as a virtual source of light when it reflects the sun. Thus, the electric sense is not only able to detect signals emitted by electrogenic objects but can also detect signals generated by the "reflections" or "shadows" produced by the presence of non-electrogenic objects.

Electroreception has two modes: Passive and active.[6-10] In passive electroreception, the signals are modulations of an externally generated electric field, whereas in active electroreception signals are modulations of a self-generated electric field.

Passive electroreception does not mean that self-generated actions do not modulate electrosensory signals. In fact, because the fish body is itself an object of different conductance from water it modulates the local electric field. Thus, the active orientation of the body in a scene funnels the electric field "illuminating" such a scene and as a consequence the fish's own presence modifies the stimulus pattern.[4,11] Additionally, the movement of the conductive body in magnetic fields (as the Earth field) induces electric currents that potentially stimulate electroreceptors. In this way, some fish (i.e., sharks) navigate using a biological compass.[4,9] Thus, passive electrolocation is a re-afferent sense. Pasively electrolocating fish must distinguish whether a change in the received signal is due to a change in the environment or a consequence of the fish's own movements. In addition when the same animal has both active and passive electroreception it has also to discriminate between external electrical stimuli and its own discharge (see below).

Active electrolocation is a sensory modality that appears when fish evolve electric organs (EO) for generating electric fields (EOD). Self-generated-fields have a dual function: (a) to carry self-generated electric images of the environment (so called active electroreception) and (b) to send communication signals to conspecifics (a form of passive electroreception).[8,10] The pattern of currents generated by the electric organ stimulate electroreceptors even in the abscence of objects, but such stimulation is modulated by the presence of objects.[4] Thus the pattern of self-generated elecrosensory stimuation constitutes a re-afference from which the exafference due to the presence of objects must be extracted. Similarly, when two fish communicate self- and conspecific-generated fields are summed at the periphery. Consequently, signals due to the fish's self-generated current and signals from other electric fish must be centrally discriminated.

Active electrolocating electric fish also generate re-afference with their movements. They orient and bend their bodies to funnel the self-generated currents. In this way, the body acts as a flash light reflector to electrically illuminate the objects of interest.[4,11] In addition, they have an electrosensory fovea at the perioral region.[13-18] Electrosensory foveas are characterized by a very high density of receptors, a variety of receptor types and a large central representation.[13,15,18,19] In the special case of *Gnathonemus petersii* this fovea is in a mobile appendix at the chin used to dig and explore for food at the bottom of water streams.[14]

In the distantly related taxa of African mormyriformes and American gymnotiformes evolution has converged on the following strategies for generating EODs: (a) brief electric pulses, irregularly emitted by a localized source (pulse mormyriforms); (b) a continuous sinewave like discharge generated by multiple sources having the same principal frequency component (wave mormyriforms and gymnotiforms); (c) brief electric pulses resulting

from the weighted sum of multiple sources generating specific temporal-waveforms along the EO (pulse gymnotiforms). These three strategies of active electrolocation are not only different on the motor side but are also different, in a corresponding manner, on the sensory side.

Last but not least, re-afference may be an unwanted epiphenomenon. Potentials generated by fish's own muscles and skin, or even the self-generated EOD are a source of "noise" for passive electroreception, this self-generated noise could perturb perception, if it is not eliminated by sensory processing.[20]

We can conclude that identification of self- (wanted and unwanted) and nonself-generated signals are very important tasks for passive and active electroreceptive animals. In the next sections we discuss several examples.

STRATEGIES FOR REJECTING UNWANTED SELF-GENERATED SIGNALS

Unwanted electrosensory signals are mainly suppressed at the first neural stage of sensory processing, by subtracting a plastic sensory expectation from the input signal.

Pulse Mormyriforms Eliminate Unwanted Re-Afference Using an Adaptive Corollary Discharge

One of the clearest examples of the use of a corollary discharge to avoid the effects of a self-generated action on a sensory pathway was found by Curtis Bell in the electrosensory lobe (ELL) of *G. petersii*.[21] A corollary discharge is an internal signal originated in motor commands that affects sensory processing. The ELLs are cerebellum-like initial stages of sensory processing structures that receive and process the information carried by electrosensory primary afferents.

The cortex of the ELL of pulse mormyriforms has three zones. The ampullary zone receives a combined input of exafferent and re-afferent signals coming from ampullary receptors sensing mainly low frequency, exafferent electric fields but also responding to the fish's own EOD.[22,23] These afferents fire spontaneously, increasing their firing rate when current flows inward and decreasing it when current flows outward.[22] Because of their extreme sensitivity, stimuli generated by weak external sources of electricity are masked by the EOD, which causes a damped oscillation of ampullary afferents firing rate.[23] Thus, the use of this pathway for exafferent signals would be precluded by saturation if the deleterious effects of the EOD were not eliminated. This suppression of self-generated interference is achieved by an adaptative sensory filter based on the anti-Hebbian plasticity of the electric organ corollay discharge.[21]

There are two main types of output or efferent cells in the ampullary zone of the ELL that convey the results of ELL processing to higher centers: Efferent cells that increase firing rate when currents flow out of the skin of the fish and efferent cells that increase firing rate when currents flow in the opposite direction.[23] The morphology of these cells is adapted to compare two main inputs. At their basal dendrites they receive the signal coming from the skin through primary afferents. At their large apical dendritic trees they receive also synaptic contacts from parallel fibers bringing information from superior centers (Fig. 1).[24] One of the most important descending signals is an electric organ corollary discharge coming from the command nucleus that triggers the EO activation.[25-27]

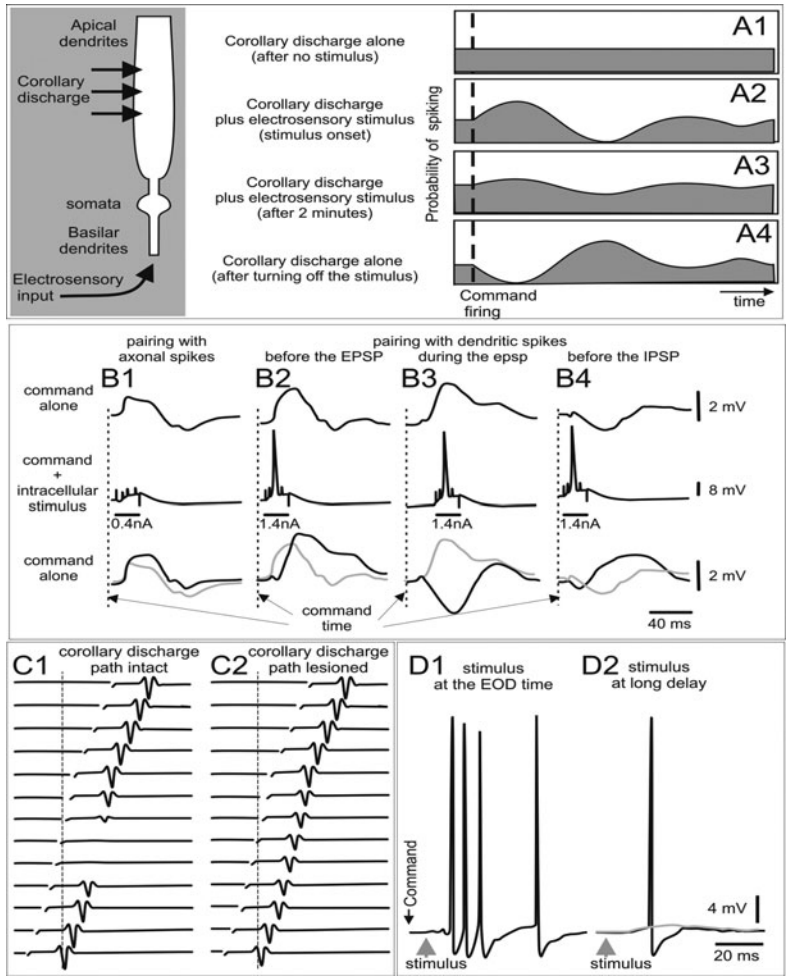

Figure 1. Pulse Mormyrids, the masters of the corollary discharge. A) The unwanted sensory effects of the EOD are cancelled by an adaptive corollary discharge. Output cells compare the electrosensory input with the plastic effects of a corollary discharge (inset). Probability of spike generation by an output cell of the ampullary zone of the ELL is plotted vs time after the command at four stages of the experiment (redrawn from ref. 21). A1) After a period without electrosensory stimulation on a curarized animal. A2) Just after turning on the stimulus at the time of the EOD. A3) After two minutes of continuous stimulation. A4) Just after turning off the stimulus. B) Cellular basis of the adaptive filter. B1) Pairing with a pulse that evoked only axonal spikes had little effect on the response to the corollary discharge. B2) Pairing at the same delay and duration but with a more intense pulse that evoked a dendritic spike resulted in reduction of early excitation and enhancement of later excitation. B3) Pairing with the same current pulse but at a longer delay resulted in a large hyperpolarizing response to the corollary discharge. B4) Pairing at the same delay and duration as in B2 resulted in an early IPSP followed by an EPSP (traces redrawn from ref. 28). C) Field potentials evoked by an electrosensory stimulus applied at different delays from the command at the mesencephalon. The dotted line indicates the time of the EOD. C1) In the intact fish a nonresponse window is observed when the stimulus is applied at the time of the EOD. C2) The lesion of the path from the command to the ELL resulted in an abolition of the non respose window (traces redrawn from ref. 57). Corollary discharge creates an AND gate. D1) output cells fire a train of spikes when the electrosensory stimuli is given at the time of the command and (D2) fire a single spike or fails to fire (see the gray EPSP) when the stimulus is applied at a long delay (traces redrawn from ref. 78).

The effect of this corollary discharge is to create a negative image of the expected afferent input to the efferent neurons of the ampullary zone.[21]

Curarized preparations allowed Bell to open the sensory-motor loop and also to close it at will using artificial stimuli triggered by the discharge of the command nucleus.[21] In these experimental conditions, it was possible to show that the corollary discharge is not fixed but depends on the pattern of ampullary input that occurred in the recent past. Figure 1 illustrates, schematically, the results of a typical experiment.[21] In absence of afferent stimulation the output cells of the ampullary zone show a flat post command firing probability (Fig. 1,A1). If after a silent period an artificial stimulus was applied to the center of the receptive field, at the exact timing of the EOD, the cells initially responded with a discharge pattern similar to that evoked in primary afferents (Fig. 1,A2). As time went by, this pattern vanished and post command spike histogram became flat again until the stimulus was suppressed (Fig. 1,A3). This reduction is due to the development of a new response to the command, opposite to the effect of the stimulus. The effect of the command alone can be seen in isolation by simply turning off the stimulus. At this moment, the experimental paradigm was exactly the same used at the beginning. However, the response, instead of consisting of a flat histogram, was a faithful negative image of the temporal and spatial pattern of sensory input that has been associated with the EOD motor command. It consisted of a damped oscillation completely opposed in phase to the afferent input (Fig. 1,A4). In the absence of a stimulus this pattern returned again to a flat pattern. However, the modified effects of the corollary discharge did not disappear if active rematching was prevented for 30 minutes by injecting local anesthetic into the command nucleus.[27]

This suggested the hypothesis that the effects of the corollary discharge are built and rebuilt to construct a mirror image input to the apical dendrites that opposes to the effects of primary afferent input to the basal dendrites. A change in synaptic weight opposed to the effect of another synapse synchronously active is called associative anti-Hebbian plasticity. The hypothesis of such plasticity was tested in intracellularly recorded single neurons in similar preparations.[28] By substituting the afferent synaptic input with an intracellular current pulse Bell et al[28] were able to demonstrate that the extracellularly observed plastic effect is due to anti-Hebbian synaptic plasticity at the synaptic contacts located at the apical tree of output cells. However, contrary to the initial expectations, pairing the corollary discharge with an intracellular current pulse that caused only a change in membrane potential or provoked axon spikes only did not result in any change of synaptic weight of the corollary discharge input (Fig. 1,B1). In fact, changes of the synaptic weight were observed only when the intracellular current pulse evoked a dendritic spike. After pairing with a dendritic spike, a compound inhibitory-post-synaptic-potential (IPSP) was observed at the time of the spike (Fig. 1,B3). This compound IPSP was flanked by small excitatory compound post synaptic potentials (EPSPs). Moreover, when the dendritic spike was evoked at delays before or after the expected self-generated input, pairing with the command caused the development of an EPSP preceded by a small IPSP (Figs. 1,B2 and 1,B4). This indicated a change in the weight of the apical synapses that opposed the effects of the sensory input. Furthermore, not only anti-Hebbian plasticity occurred at the time of the spike but also opposite changes occurred before and after the time of the spike. This finding was the first evidence for the phenomenon called associative time dependent synaptic plasticity later confirmed in in vivo and in vitro preparations of the ELL and other structures.[29-32]

Skates and Rays Eliminate Self-Generated Noise to Communicate

The ampullae of Lorenzini of sharks, skates and rays are the most sensitive electroreceptors known.[33,34] Their extreme sensitivity is adapted to detect very small electrical signals that are attenuated by the high conductivity of the sea water.[35]

One of the functions of the EOD emitted by rays and skates is to serve as a carrier of sexual calls. During the mating season harems of females lay on the bottom of the sea emitting EODs to attract males. Communication signals are also buried in a background noise, often much stronger than the communication signals, caused by the animal's own movement. The main source of this self-generated electric noise is the ventilatory movements of the receiving animal. While the firing rate of primary afferent fibers is correlated with the breathing movements, the output neurons of the first electrosensory relay, the dorsal octavolateral nucleus (DON) are silent. On the contrary, the firing rate of the afferent fibers barely reflects exafferent stimuli but the efferent neurons of the dorsal nucleus show a clear response in phase with such stimuli. This indicates the presence of cancellation mechanisms that selectively eliminate the re-afference while keeping the neurons near their firing threshold and responsive to exafference.[35]

It was initially proposed that lateral inhibitory interactions may select for signals showing local contrast.[36] In addition, cancellation takes some time to be fully effective, suggesting the presence of an adaptive filter like the one described above for mormyrids. In fact, the structure of the first relay and the characteristics of the phenomenon are much alike.[37] The DON is a cerebellum like structure in which principal effector cells are also bipolar. They receive the local input from primary afferents on their basal dendrites, while the apical dendrites are contacted by a set of parallel fibers.[38] These fibers carry information from several sources including motor commands, proprioceptive sensors, other sensory signals and feedback loops from higher stages of electrosensory processing.[35,37,38]

Computational models based on anti-Hebbian synaptic plasticity at the apical dendritic tree propose that the cancellation signal comprises independent but complimentary contributions from proprioceptive signals, motor commands and descending electrosensory feedback.[35,39] Experiments performed on curarized skates, recording DON's neurons responses to local sensory stimuli paired with passive fin movements, ventilatory motor commands or whole-body electrosensory stimuli confirm model predictions and the role of synaptic plasticity. As in mormyrids, intracellular excitatory current pulses paired with motor commands yielded a reduction of the sensory stimulus synaptic potential at the end of the pairing.[35]

Wave Gymnotids Eliminate Re-Afference Resulting from Body Movements

Tail and body bending is a common exploratory strategy used by electric fish when exploring the environment. Tail bending varies the electric "illumination" of the scene emphasizing or masking different saliencies of nearby objects.[4] However, a change in "illumination" has no information about the external world by itself. In addition, unwanted changes in illumination introduced by the fish's own movements may mask object features. Thus, removing baseline illumination and changes imposed by body movements reduces the computational cost while increasing accuracy.[4,44]

The main cells of the ELL of wave gymnotids (called pyramidal cells) are also comparators. They receive input from different types of parallel fibers on their apical dendrites and electrosensory signals from primary afferents on basal dendrites.[40-44] The apical input includes information from proprioceptive receptors sensitive to tail bending and also electrosensory feedback from higher centers. Primary afferents of wave fish strongly respond to the changes in stimulus strength caused by passive movements of the tail but pyramidal cells projecting to the torus semicircularis hardly notice this change.[44] This indicates that subtraction of the effects of movement is already done at the ELL.

Bastian applied a local stimulus with modulations in amplitude linked to the cycle of tail motion in a freely discharging fish[a] paralyzed by curare.[44] Responses to these electrical and mechanical combined patterns of stimulation were maximal at stimulus onset, gradually decaying with time, disappearing after two minutes. When the stimulus was turned off after the pairing, the tail bending alone (which previously caused little effect), now evoked a strong modulation of the response. As in the cases of mormyrids and rays, this phenomenon can be interpreted as the development of a negative image cancelling the effect of the mechano-electrical paired stimulus. Synaptic plasticity, backpropagation of dendritic spikes and regulation of firing dynamics of pyramidal cells by recurrent feedbacks, play complementary roles for the development of such "negative image".[45-50]

Pyramidal cells carrying the main output of the ELL have large apical dendritic trees. These apical dendrites are able to fire action potentials following somatic depolarization. In addition, when this spike invades again the somata the cell emits a burst.[49,50] Therefore, electrotonic coupling between the somata and dendrites regulates the intensity of the pyramidal cell's response.[49,50]

Besides the main output cells, there is another group of pyramidal neurons whose firing pattern follows the afferent input.[46,47] This is because they have a relatively short apical dendritic tree, thus receiving scarce feedback from higher stages of processing. The role of short apical tree cells is to drive a feedback loop that controls the gain of the ELL. These neurons project to the praeminential nuclei, feedback relays located in the transition between rombencephalon and mesencephalon.[51,52] GABAergic neurons driven by the output of the praeminentialis nuclei project back to the ELL making synaptic contacts on the initial shaft of apical dendrites of large apical tree pyramidal cells.[52] This inhibitory feedback loop provokes a relative decoupling of the apical and basilar inputs to large pyramidal cells preventing their bursting. Thus, by regulating the level of activation of recurrent GABAergic synapses fish exert an automatic control of the system's gain.[49,51,53] The slow development of the negative image suggests the presence of associative anti-Hebbian plasticity at the synaptic contacts between parallel fibers and apical dendrites. This was confirmed by in vivo and in vitro studies.[43,48,54]

Pulse Mormyrids also Block Self-Generated Signals with an Efference Copy

Pulse mormyrids use the simplest form of corollary discharge to block self-generated signals, in a path exclusively committed to communication.

This path originates from very sensitive receptors (called Knollenorgans) responding to rapid changes in transcutaneous voltages such as those caused by both self- and

[a] Apteronotus generates an EOD using purely neural mechanisms. Because of this, the EOD is not blocked by curare.

conspecific- EODs.[55] Knollenorgans are innervated by thick myelinated fibers discharging a single spike per EOD. These fibers make electric synaptic contacts with the cells of a compact cell group in ELL, the nucleus of ELL. In addition to these synaptic contacts, the cells of this nucleus also receive multiple GABAergic contacts from a mesencephalic relay of the corollary discharge.[56] These contacts provoke a strong inhibition completely blocking the signals resulting from the self activation of the Knollenorgans.[56] However, the signals evoked by the EOD of a conspecific, seldom fall within the same window as the self-generated EOD and therefore, they are rarely blocked by the efference copy. This temporal window can be evidenced at the nucleus extralateralis anterior of the mesencephalon, where the cells of the nucleus of ELL project (Fig. 1,C1). When the efference copy is disabled by cutting the path between the command nucleus and the ELL nuclei this window is abolished (Fig. 1,C2).[57]

Using this communication path, pulse mormyrids exchange a large repertoire of messages coded in the inter-EOD firing patterns.[58] The meaningfulness of these messages was recently confirmed by showing the presence of neurons specifically responding to some of these patterns.[59]

STRATEGIES FOR IMPROVING THE SIGNAL TO NOISE RATIO OF SELF-GENERATED SIGNALS

Re-afferent electrosensory signals generated by the distortion of the self-generated field by nearby objects have to be extracted from co-existing noise. Electric fish use two strategies for that purpose: (a) matching the tuning properties of the electroreceptors with the waveform of self-generated carriers and (b) gating the self-generated signal with a facilitatory corollary discharge.

Gymnotids Electroreceptors are Tuned to the Local Self-Generated Electric Fields

Gymnotid electric fish provide excellent examples on how matching the spectra of the self emitted carrier with the tuning band of the sensory receptors may maximize the information obtained and the signal-to-noise ratio.[4,60]

The EODs of wave gymnotids are sine-wave-like and can be characterized by their amplitude, phase and frequency. One type of electroreceptor in wave gymnotids follows the EOD cycles discharging with a probability dependent on amplitude. These receptors show narrow tuning curves with a maximum responsiveness at the same frequency as the EOD.[60] This tuning provides wave species with a private channel for active electroreception and communication. As explained below, as this single channel is used for both tasks other mechanisms have to be implemented to separate the two types of signals.

The EODs of pulse gymnotids exhibit complex waveforms resulting from the weighted contributions of different portions of the fish's body to the external field.[61] Consistently with a site specific local EOD pattern the responsiveness of tuberous electroreceptors is highly tuned to the local EOD waveform. This implies not only a narrow frequency band of response but also a phase pattern preference.[62-66]

Pulse gymnotids show an additional mechanism related to electroreceptor tuning. It has been proposed that in these fish electroreceptor tuning might also be involved in

separating active electrolocation from communication signals.[15] At the perioral region, where electroreceptor density is maximum, the local electric field is coherent (i.e., has a constant waveform), is collimated perpendicular to the skin and much stronger than elsewhere in the fish surface.[13,15] This field is mainly generated at the abdominal region and extends over a narrow fringe around the head in most species of the genus.[67] It can be considered the main carrier for active electrolocation since it "illuminates" objects near the region where the sensory mosaic has the best resolution.

In contrast the central and caudal regions of the EO contribute with higher frequency components to the EOD and are best represented in the far field. This is because of the large dipole arm and the higher internal resistance of the equivalent source. Due to their extended range, signals carried by the caudally generated field are more easily detected by conspecifics.[15] Consistently, the caudally generated components show the widest diversity among species and have seasonal variations linked to sex in the same species.[67-69] These observations support the hypothesis that the complexity of the EOD of pulse gymnotids is important for generating two signals carriers, one for active electro-location and the other for electro-communication.

Sensory Gating and Adaptive Corollary Discharges are Used for Streaming Self- Generated Signals

Mormyromast electroreceptors respond to the EOD of pulse mormyrids with a train of impulses. These trains code amplitude and waveform of the local stimuli by varying the latency of the first spike and number of spikes in the burst.[23,70-72] The evidence indicates that first spike latency is the critical signal. Primary afferents coming from Mormyromast electroreceptors project onto small "granule"neurons that receive an excitatory corollary discharge from the command nucleus. This corollary discharge consists of an EPSP locked in time to the command firing.[70-74] Early primary afferent spikes arrive to the ELL within the same time window as the corollary discharge EPSP. In addition, the ELL circuit shows lateral inhibition driven by the periphery of the receptive field and also by the corollary discharge.[75] This lateral inhibition occurs after the gating EPSP. Therefore, early spikes in the afferent train are facilitated by the excitatory corollary discharge and later spikes are blocked by the lateral inhibition. In this way, the grain neurons work as an AND-gate[b] selecting self-generated signals among all possible stimuli.[23] As a consequence of this gating mechanism some output cells fire a train of spikes when the electrosensory stimuli is given at the time of the command (Fig. 1,D1) and fire a single spike or fails to fire when the stimulus is applied at a long delay (Fig. 1,D2).

In addition to the gating corollary discharge, several neuron types of the mormyromast zone receive a second type of corollary discharge at their apical dendritic tree.[76-79] This corollary discharge is plastic and allows the fish to recognize novelties in the stream of sensory images.[76-79] As in the ampullary zone the role of dendritic spikes is crucial for building this expectation.[28,78-81] By means of time dependent anti-Hebbian plasticity mormyrid fish build expectation images and compare them with present input images. Cellular and subcellular synaptic mechanisms underlying this process have been further characterized in vitro.[29,82]

[b] ANDgate: logical operator that yields "true" only when both inputs are "true".

EMISSION PATTERNS, ACTIVE ELECTRORECEPTION AND ELECTRO-COMMUNICATION SIGNALS

Controlling the timing of the EOD is also a way of separating self- from conspecific-generated signals in species displaying different electromotor strategies.

Pulse Mormyrids Use Their Electromotor Patterns to Segregate Communication Messages

The EOD of pulse mormyrids is irregularly activated by a command nucleus that drives the electric organ and sends a corollary discharge to the sensory structures. By firing their organs 10-12 ms after the EOD of a conspecific they are able to place their EOD in an optimal time window for communication.[83,84] At that time the facilitatory effects of the gating corollary discharge at the ELL cortex and the inhibitory effects of the efferent copy at the nucleus of the ELL have already finished. This "echo response" is frequent in social interactions and may also have a meaning for sex communication. In fact, *Mormyrus rume* and *Pollymyrus isidori* show a mirror image behavior called preferred latency avoidance.[84] This behavior consists of the specific avoidance of an EOD at a fixed latency after the conspecific discharge. Lücker and Kramer found that while "echo responses" is more frequent in sexually mature males, "preferred latency avoidance" is characteristic of sexually mature females and speculated that sex recognition and mating disposition may be coded by these responses.[84]

Wave Fish Shift Pacemaker Frequency to Avoid Jamming

Wave mormyriforms and wave and pulse Gymnotiforms control their EOD in a regular way[8,85-87] Their EOs are commanded by pacemakers nuclei located at the ventral aspect of the medulla.[85,88] Two conspecifics discharging at similar rates will interfere with each other. In fact, the addition of two wave EODs firing at very close frequency (i.e., with a difference less than 6 Hz) may yield an ambiguous image consisting of a self-generated sine-wave-like carrier, locally modulated in amplitude and phase.[89] Since electroreceptors are tuned to a frequency band specific for the species EOD these local signals may either mean either the presence of nearby objects or the presence of a conspecific. To solve this ambiguity, wave fish shift their pacemaker frequency away from the frequency of interfering signals.[90-93] This behavior, which is widespread among wave fish, is called jamming avoidance response (JAR) of wave fish.

Several pieces of evidence indicate that this behavior does not depend on the presence of a corollary discharge.[85,91] First, JAR is elicited by purely artificial stimuli (one local mimicking the local effects of a conspecific EOD and the other global mimicking the self-generated EOD) even when the electromotor-electrosensory cycle is opened by blocking the EOD with curare. Second, the shift in pacemaker frequency is also elicited when the artificial stimuli evoking the JAR and the pacemaker firing are not phase locked and even when they differ in frequency.[85,91]

Two neurocomputational mechanisms have to be simultaneously active for implementing JAR: (a) fish must be able to measure the difference between the frequencies of the self-generated EOD and the interference signal and (b) fish must be able to shift the pacemaker frequency in the direction that increases the difference in frequency.

These mechanisms were brilliantly described by Walter Heiligenberg and his group.[91,92] The backbone hypothesis is that wave fish are able to extract the difference in frequency by combining the data obtained through the two types of tuberous electroreceptors. One type (T) of electroreceptors code the phase of the local wave and the other (P) code the amplitude. The sign of the frequency difference implies an opposite rotation of the signal in a phase plot of the momentary amplitude vs. the phase difference.[91,92] Evaluation of whether the difference in frequency is positive or negative requires: (a) a stimulus phase computation between different regions of the fish body; (b) a computation of the amplitude modulation of the stimulus signal; and (c) an integration mechanism for detecting whether the change in phase difference is positively or negative correlated with the local amplitude of the stimuli.[91]

It was known that T receptors project on a specific pauci-dendritic neuron type (spherical cells) that is receiving mainly the primary afferent input.[61,51,91] These cells in turn project to the layer VI of the torus semicircularis where Carr et al showed a Jeffreys' like circuit.[51,94,95] This kind of network allows the computation of local phase differences between different regions of the fish's body with high precision and accuracy.[c]

On the other hand, momentary amplitude has a double estimation. In the slow electrosensory path of the ELL the circuit is complex and (as explained above) receives the influence of recurrent loops from electrosensory and other sensory regions. This circuit has two main output cells. One type increases its firing probability with the increase in stimulus amplitude within its receptive field (E cell) and the other type decreases its firing probability with the increase in stimulus amplitude within its receptive field (I cells). The properties of these cells and the ELL circuit were described in detail by the groups of Bastian, Turner and Maler.[41,43,48-51,53,54,96]

At the mesencephalon (torus semicircularis and tectum opticum) there are specific cells that receive convergence between cells sensitive to phase difference and cells sensitive to amplitude. These cells project to the nucleus electrosensorius, a sensory motor integration link whose output influences the frequency of the EOD pacemaker via prepacemaker nuclei.[91] The net result of phase and amplitude computation at the nucleus electrosensorius is different for neurons receiving information from E and I cells of the ELL. "E-driven" cells would tend to increase pacemaker frequency when the interfering stimuli has lower frequency. "I-driven" cells would tend to decelerate the pacemaker when the interfering stimuli have higher frequency.[91] Thus, the prepacemaker structures receive complementary signals that tend to shift the pacemaker frequency away from that of the conspecific.

Pacemaker Accelerations in Pulse Fish: Communication or Jamming Avoidance?

The pacemaker of pulse gymnotids is a very regular oscillator, tonically accelerated by convergence of excitatory descending inputs from motor and sensory structures.[97,98] Punctual sensory events such as changes in the impedance of an object,[99] vibratory stimuli,[100] or motor commands[101] generally cause sharp accelerations followed by slow relaxations. The sum of multiple descending inputs exert a tonic but not constant level of excitation, in which acceleration peaks are observed. These peaks generate an asymmetric interval distribution skewed to the short interval side.

[c]Details of these mechanisms are beyond the scope of a general article, but can be found in references 52,85,91,92,95.

Pacemaker accelerations can also be elicited by artificial electrosensory stimuli applied either in coincidence or sweeping the pacemaker interval from one EOD to the next. Bullock described this effect as "phase sensitivity shortening of the interval".[102] In a series of articles, Westby[103,104] developed further this idea and described two main patterns of interaction between conspecifics: (a) Synchronization bouts, in which the frequency of both fish is the same for several seconds and the phase (defined as the interval between the EOD of one the fish and the EOD of the other) varies very slowly changing in direction; (b) JARs of pulse fish occurring when the frequencies are slightly different. In this case the phase of the slower discharging fish increases over time; consequently its EOD approaches the time of the next EOD of the faster discharging fish. When coincidence is imminent, either the faster fish or both fish accelerate shifting their phases, thus avoiding multiple coincidences.

Westby proposed a conceptual model to explain these phenomena postulating the existence of a "detection window" preceding the EOD.[104] In the JAR, when the EOD of a more slowly discharging neighbor precedes the self-generated EOD within the detection window, the fish becomes "aware" that a coincidence is imminent and accelerates to avoid a sequence of coincidences. In synchronization bouts, fish avoid coincidence by maintaining the phase difference in such a way that the conspecific's EOD falls away from the detection window.[103,104]

This conceptual model was implemented computationally by Capurro et al proving its feasibility.[105] However, the neural bases of these behaviors are still unknown. Two complementary explanations were proposed: Westby related the JAR with the changes in responsiveness of the central structures of the electrosensory system,[104] while Baker et al[106] proposed that the JAR depends on the adaptation of electroreceptors of the slow electrosensory pathway.

Recent research of our group has shown that there is a low responsiveness window of the fast electrosensory pathway in *Gymnotus omarorum*.[107] This phenomenon is caused by the path activation, does not depend on a corollary discharge and is mainly generated at the spherical cells of the ELL.[108] Spherical neurons are "onset cells" firing a single spike phase locked to the stimulus with very high precision. The axons of these cells are the input of a Jeffress'-like circuit at the mesencephalon, analogous to that described in wave fish.[91,94,95] The intrinsic properties of spherical cells are dominated by a low threshold K^+ conductance.[108] This low threshold conductance is partially open at rest, which causes a reduction of membrane potential variability and a low sensitivity to noise (Nogueira and Caputi, unpublished). Cell spiking fully activates this low threshold current which in turn causes a long refractory period that blocks further responses. Conductance dependence on time and voltage affects the firing precision of the cell after the spike. This suggests that firing precision and firing probability depends on the pattern of self and conspecific generated EODs.

In order to study how the descending control of pacemaker rate affects the interval between the EOD and a constant frequency stimulus (referred to as phase) we applied a series of pulses at the EOD rate at rest (Fig. 2). We found that even though the frequency of the artificial interfering EOD was kept constant along the trial, the phase relation of the interfering stimulus within the fish's EOD cycle depended on the type of behavior displayed by the fish. Synchronization bouts increase the probability that the EOD falls at the low responsiveness region of the inter-EOD interval. Paradoxically, JAR appears to generate a peak just before the EOD at the largest responsive region of the inter-EOD interval. Taking into account spherical cell intrinsic properties

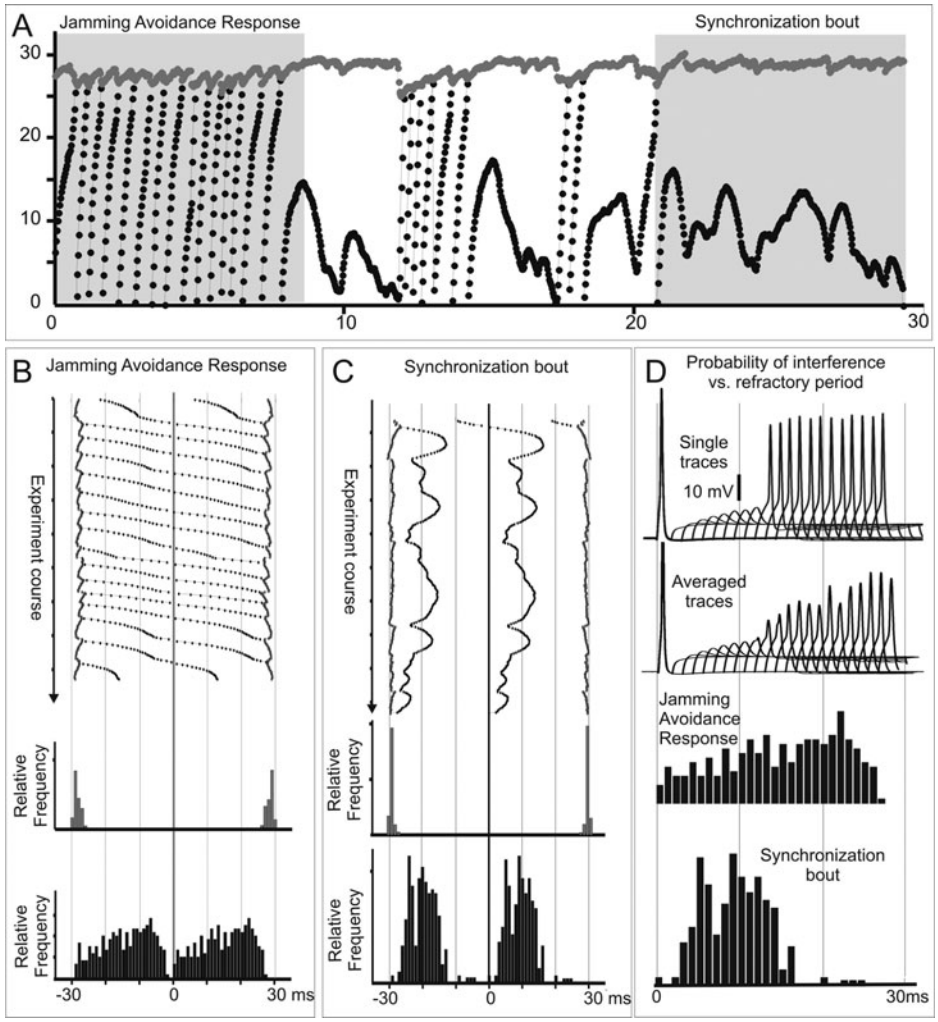

Figure 2. Pacemaker control. Jamming avoidance or Communication? A) Typical behaviors of a fish in the presence of a train of pulses at the same mean rate of the EOD represented as an intervalogram. Gray dots represent the consecutive inter-EOD intervals and black dots represent the phase of the interfering stimuli on each EOD interval. In a 30s period of recording we identified two different behaviors (a) JAR characterized by a swept of the interval by the interfering stimulus and short accelerations when the interfering pulse occurs on the initial wave components of the EOD and (b) a synchronization bout in which the phase is maintain bounded within the initial portion of the inter EOD interval. B,C) Peri-EOD raster and its corresponding histograms (middle self-generated EOD, bottom interfering pulse). D) Comparing the refractory period shown by single traces of spherical cells responses to paired pulse stimulation (top) or by the average of 10 responses at each inter-pulse delay that indicate that besides the drop in probability at short delays there is a progressive increase in latency variability (middle top) Note that while the classical jamming avoidance response locates the interfering stimuli at the region of the interval with best "visibility" (middle bottom histogram) the synchronization bout locates the stimuli at the region of the inter EOD interval with worst "visibility" (bottom histogram).

one can conclude that, while synchronization bouts avoid interference, the JAR behavior appears to have a double consequence: (a) increasein the probability of the conspecific EOD at the phase of the inter EOD interval where it is more "visible" and (b) avoid consecutive coincidences. These results suggest that the so called JAR is not an avoidance to jamming but a response to jamming when fish is looking for communication signals whereas the synchronization response may always minimize interference in active electrolocation.

CONCLUSION

The experimental and theoretical analyses of the electric sense provide illustrative examples of some sensory functions in which self- and externally- generated signals need to be identified and segregated.

Interestingly, mechanisms used by species that are distant in the evolutionary tree fall within common basins of convergence when nature has to implement such functions.

These mechanisms are multiple and involve a parallel evolution of motor and sensory structures at different levels of organization. From peripheral to central one may consider the following: (a) matching between carrier waveform and receptor dynamic range as observed in gymnotids; (b) gating signals by efference copies rejecting self-generated signals or corollary discharges facilitating self-generated signals, as observed in pulse mormyrids; (c) rejecting unwanted self-generated signals by adaptive filters, based in the combination of teaching pathways, recurrent loops, and/or corollary discharges, with synaptic plasticity; (d) complex processing combining image feature detection (as for example the correlation between amplitude and differential phase over the receptive surface) and motor actions (control of EOD rate or movements).

Finally, it has to be noted that these same mechanisms are present in other sensory systems throughout the animal kingdom. For example, the uses of corollary discharges for dealing with re-afference as well as the role of feedback loops are well known in vision, somatosensory and vestibular systems[1,2,109,110] Mechanisms identified in electrosensory systems can therefore serve as proof of principal or sources of hypotheses for other systems. These mechanisms can also serve as a source of inspiration for the design of robots and neurprosthetic devices, opening new horizons for human progress and health.

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CHAPTER 8

MAGNETORECEPTION

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Abstract: Animals can use the direction of the magnetic field as a compass and the intensity of the magnetic field as a component of the navigational 'map'. Two fundamentally different mechanisms of magnetoreception have been discussed: (1) light-dependent reactions in specialized photopigments lead to radical pairs, with the ratio singlet/triplet depending on the molecule's alignment with respect to the ambient magnetic field and (2) reactions involving small crystals of magnetite, a specific iron oxide of biogen origin. The first mechanism provides birds and possibly amphibians and insects with compass information; the second, which can theoretically provide animals with information on direction and intensity, appears to mediate intensity information in birds and compass information e.g., in mammals. Little is known about the magnetoreception mechanisms in other animals.

INTRODUCTION

The geomagnetic field is an omnipresent feature of the earth. Hence it is not surprising that many animals are able to perceive it and make use of it for orientation and navigation, among them mollusks, arthropods and members of all major groups of vertebrates. This may seem alien to us, as we ourselves cannot sense the geomagnetic field, at least not consciously (see ref. 1). However, we can also make use of it by technical means: A magnetic compass is a traditional instrument to locate directions—the magnetic field indicates even to us where north, south, east and west lie.

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THE GEOMAGNETIC FIELD AND ITS ROLE IN NAVIGATION

To fully understand magnetoreception, we must first consider what type of information the geomagnetic field can provide and—even more important—which type of information animals do actually use. The earth itself is a huge magnet, with its poles situated close to the rotational poles. The field lines leave the surface of the earth at the southern magnetic pole, run around the earth and re-enter at the northern magnetic pole. As a consequence, the magnetic field lines point upward on the southern hemisphere, run parallel to the earth's surface at the magnetic equator and point downward in the northern hemisphere, with inclination or dip changing continuously, showing a fairly

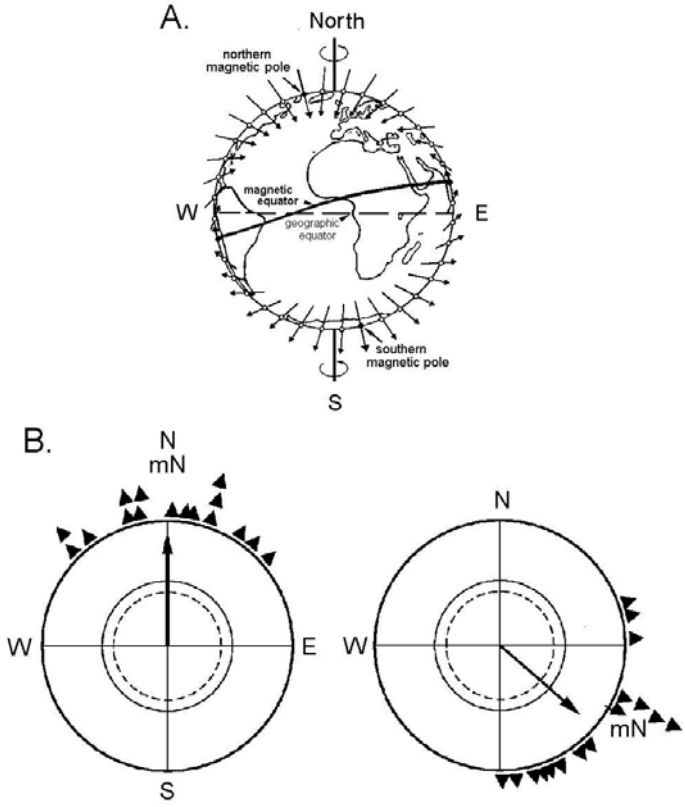

Figure 1. Magnetic field of the earth and demonstration of birds using the magnetic field as a compass. A) The geomagnetic field: Arrows indicate the local magnetic vectors with their lengths proportional to the intensity of the local field. The magnetic poles and the magnetic equator are marked. Reproduced with kind permission from Springer Science+Business Media: Wiltschko R, Wiltschko W. Magnetic Orientation in Animals. Berlin, Heidelberg, New York: Springer Verlag, 1995.[3] B) Orientation behavior of European Robins during spring migration, tested in the local geomagnetic field and in an experimental fields with magnetic North shifted by 120° to East-southeast. *mN*, magnetic North. The *triangles* at the periphery of the circle mark mean headings of individual birds, the *arrows* represent the grand mean vectors with their lengths proportional to the radius of the circle. The two *inner circles* are the 5% (dashed) and the 1% significance border of the Rayleigh test indicating difference from a random distribution. Reproduced with kind permission from Springer Science+Business Media: Wiltschko W, Wiltschko R. J Ornithol 2007; 148(Suppl 1):S61-S76.[19]

Table 1. Animals demonstrated to use a magnetic compass (Numbers in parentheses give the number of species where the respective type of compass is indicated; ??? means that it has not been tested)

Animal Group		Type of Compass?
Mollusks		
Snails	1 species	???
Arthropods		
Crustacean	5 species	Polarity compass (1)
Insects	9 species	Inclination compass? (1)
Vertebrates		
Cartilageous fish	1 species	???
Bony fish	4 species	Polarity compass? (1)
Amphibians	2 species	Inclination compass (1)
Reptiles	2 species	Inclination compass (2)
Birds	20 species	Inclination compass (9)
Mammals	5 species	Polarity compass (2)

regular gradient (Fig. 1A). With 60 to 65 μT (mikroTesla), the intensity or field strength is highest at the two poles, decreasing to 25 to 30 μT near the magnetic equator, thus forming gradients running from the poles to the equator on each hemisphere. This regular field can be locally distorted by material in the upper crust resulting in magnetic anomalies with increases or decreases in intensity; it is temporally altered by electromagnetic radiation originating in the sun causing daily variations and occasionally by magnetic storms (see ref. 2 for details). These changes, however, are mostly very small compared to the regular field.

The geomagnetic field thus represents a reliable, always available source of navigational information. This information can be of two kinds: The magnetic vector provides directional information for a compass, whereas total intensity (and/or inclination) provides information on something like 'magnetic latitude' and might be used as a component of the navigational 'map' indicating position. Animals have been shown to use both types of information.

A magnetic compass appears to be rather widespread among animals. It was first demonstrated in migratory birds, taking advantage of a spontaneous behavior, namely their urge to move into migratory direction during migration season. It is so strong that even captive birds head into the respective direction in their cages. Shifting magnetic north results in a corresponding shift in their headings (Fig. 1B), which clearly shows that they orient with the help of the magnetic field. Meanwhile, magnetic compass orientation has been demonstrated in a number of other animals; they include various birds species, fishes, amphibians, marine turtles, rodents, bats, crustaceans, insects and a mollusk species (Table 1). The behaviors involved range from spontaneous directional preferences, imprinted directions, building activities in social insects to directional training and other acquired directions (for review see refs. 3,4). Interestingly, not all animals have the same type of magnetic compass (see Table 1): Some, like mammals, have a 'polarity compass' that is based on similar principles as our technical compass, using the polarity of the magnetic field; others, like birds, have an 'inclination compass'

that ignores polarity and derives directions from the course of the field lines and their inclination in space. In other groups, the mechanism of the magnetic compass are unclear or have not yet been analyzed.

However, indicating directions is not the only role of the magnetic field. Animals can use magnetic intensity and/or inclination as a component of their navigational 'map', a mental representation of the spatial distribution of various geophysical factors that indicates position. The 'map' allows animals to determine the course to a desired goal. Evidence for this use of magnetic information is much rarer than that supporting compass use and the number of species involved is very much smaller. The best documented examples involve homing pigeons, marine turtles and spiny lobsters. Additionally, specific values of total intensity and/or inclination may serve as 'sign-posts', marking specific regions where animals must act in a specific way (see ref. 4 for review).

GENERAL CONSIDERATIONS ON MAGNETORECEPTION

Because of the different magnetic parameters used, magnetoreception is not a uniform phenomenon. We must expect animals to have specialized receptors for mediating magnetic information on directions and others for mediating intensity, just as man uses a compass for the former and a magnetometer for the latter task. Also, the two types of magnetic compass—polarity compass and inclination compass—imply that here, too, different mechanisms may be involved.

For a complete understanding of these 'magnetic senses', one needs to know (i) details on the primary processes mediating magnetic input, (ii) the location of the sensory organ, its structure and its connections to the central nervous system and (iii) the parts of the brain that are involved in processing magnetic information. Unfortunately, the knowledge on the physiological and neurobiological processes associated with magnetoreception is still rather limited and only very few species have been analyzed in some detail, with the various animal groups by far not equally represented. The literature on magnetoreception is a mosaic of findings with different aspects often studied in different species. Birds are by far the best studied group; some behavioral, neuroanatomical and electrophysiological evidence is available from most other vertebrate groups, but only little from arthropods.

A number of models for magnetoreception based on fundamentally different principles have been proposed, the three most prominent ones being (i) induction, (ii) interactions of chemical processes with the ambient magnetic field and (iii) processes involving magnetic material. Induction would be restricted to marine animals because it requires sea water as a surrounding medium with high conductivity. It is discussed for skates and rays with very sensitive electric organs: Their ampullary organs are sensitive enough to detect the differences in voltage induced when the fishes are heading in different directions (see ref. 5). However, direct evidence that this information is indeed used to derive compass orientation is still lacking.

The other two models are more general and would also serve terrestrial animals and those living in fresh water. They are supported by experimental evidence and, at least in birds, the position of the respective receptors is largely known.

MAGNETORECEPTION BASED ON RADICAL-PAIR PROCESSES AND ASSOCIATED FINDINGS

The Radical-Pair model, first proposed in the 1980s and presented in detail by Ritz and colleagues, postulates a 'chemical compass' based on direction-specific interactions of special photopigments with the ambient magnetic field.

The Model

In the initial step, the radical-pair model assumes that specialized photopigments absorb a photon and transfer an electron to an acceptor. Donor and acceptor form singlet radical pairs with antiparallel spin, which, by singlet-triplet interconversion, in part turn into triplet pairs with parallel spin (Fig. 2). The magnetic field alters the dynamics of the transition between spin states; as a consequence, the triplet yield depends on the alignment of the receptor-molecule in the ambient magnetic field (for details, see ref. 6)—it can thus convey information on magnetic directions. To obtain this information, animals must take advantage of the fact that triplet products are chemically different from singlet products and compare the triplet yields (or singlet yields) in different spatial directions. This requires orderly arrays of photopigments oriented in the various directions. Ritz and colleagues[6] suggested the eyes as location of the respective magnetoreceptors, because the required conditions could be met by their spherical shape and the arrangement of

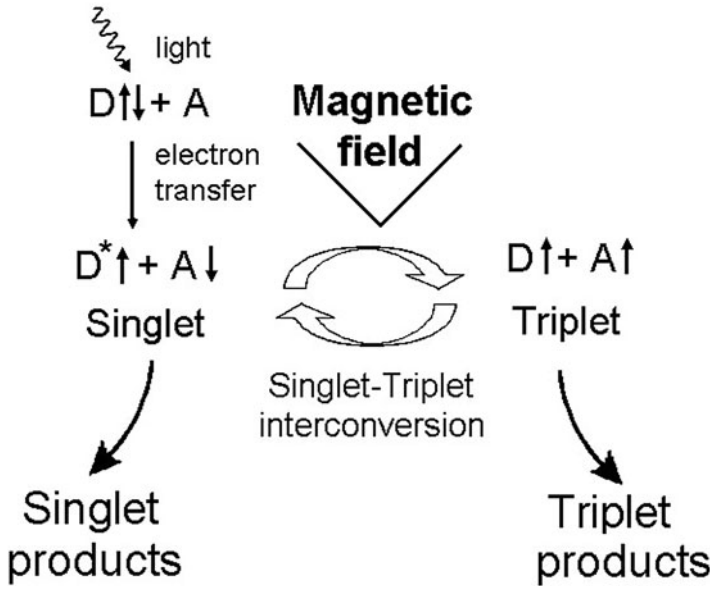

Figure 2. Schema of a radical pair mechanism: A donor absorbs a photon and, by electron transfer, a singlet radical pair is formed. Singlet-triplet interconversion leads to triplet pairs, with the triplet yield depending on the alignment of the molecules in the ambient magnetic field. Triplet products are chemically different from the singlet products and thus may play a role in magnetoreception (after ref. 6, modified).

receptors—radical-pair processes would generate characteristic patterns of activation across the retina. These patterns would be centrally symmetric to the axis of the field lines and could enable animals to detect the direction of the ambient field.

Since the radical-pair reactions are axial rather than polar, this mechanism would results in an inclination compass. Hence one would expect a magnetic compass based on radical-pair processes primarily in animals with this type of compass, namely birds, amphibians and marine turtles and insects (see Table 1). At the same time, the model allows specific predictions that can be experimentally tested: The initial photon absorption would make magnetoreception by a radical-pair mechanism light-dependent and if the relative singlet or triplet yield were crucial for magnetoreception, then interfering with the singlet-triplet interconversion should alter the output of the receptor markedly and thus disrupt magnetoreception.

Light-Dependency of Magnetic Compass Orientation

Experiments under different light conditions showed that light is indeed required for magnetic compass orientation in birds. First evidence came from behavioral experiments with young homing pigeons that rely on recording the direction of displacement with their magnetic compass: Displaced in total darkness, they were disoriented, just as young pigeons displaced in a distorted magnetic field had been.[7] Later tests with migratory birds under monochromatic lights of various wavelengths revealed a wavelength-dependency of the birds' magnetic compass: Orientation was possible only under light from the short-wavelengths end of the spectrum, from 373 nm UV to 565 nm green light. Under 590 nm yellow and under 635 and 645 nm red light, the birds were disoriented. This pattern seems to be common to passerine species, homing pigeons, *Columba livia f. domestica* and domestic chickens, *Gallus gallus*. Experiments with interference filters could narrow down the onset of disorientation in European robins, *Erithacus rubecula*, even further to between 561 and 568 nm. This rather abrupt transition from orientation to disorientation over just a few nm increase in wavelength is remarkable and suggests the interaction with another type of receptors (see ref. 8 for details and discussion).

The findings mentioned so far were obtained under rather low light levels at intensities found in nature more than half an hour after sunset or before sunrise. This seemed to be appropriate, because the passerine species tested were either nocturnal migrants or migrating during the twilight hours. When the intensity of the monochromatic light was increased, the response of birds changed: Passerine migrants no longer preferred their natural migratory direction, but instead showed axial preferences or showed odd unimodal tendencies that varied with the wavelength of light. Unimodal preferences and directions other than the migratory directions were also observed when yellow light was added to blue, turquoise and green light and in total darkness, with the specific directions preferred depending on the ambient light regime. These behaviors represent a fundamentally different type of responses that no longer originate in radical pair processes (see below). The observation that they occur under specific light conditions not found in nature, however, suggests interactions between the magnetoreception system and the visual system, although wavelength-dependency of magnetoreception shows no relationship to the peaks of the four color cones of birds. The interactions between the two systems probably take place at higher centers in the brain and are not yet completely understood (see ref. 8 for discussion).

Disorientation in the absence of visible light was also observed in a salamander species.[9] These salamanders likewise showed a wavelength-dependency for their magnetic compass, albeit one that differs markedly from that found in birds: Normal orientation was observed only in a rather narrow wavelength band at the short-wavelength end of the spectrum up to about 450 nm blue light; under wavelengths of 500 nm or longer, their headings were shifted by approximately 90° counterclockwise. A similar shift under long wavelength was also observed in tadpoles of bullfrogs. Keeping salamanders for a few days under long wavelengths made them show a mirror-image clockwise shift under 'white' light, but they now headed shoreward under long-wavelength light (see ref. 10 for review). Other manipulations led to an axial preference that roughly corresponded with the magnetic north-south axis under both, 'white' and long-wavelength light, with this response discussed as being possibly no longer controlled by a light dependent- mechanism.[11]

Marine turtles, on the other hand, proved well oriented in total darkness.[12] Hence birds and amphibians are so far the only vertebrates where a light-dependent magnetic compass mechanism is indicated, even if the spectral range where birds can obtain magnetic compass information includes the larger part of the visual spectrum until yellow, whereas it is restricted to the short-wavelength end, ending in the blue wavelengths in salamanders.

There are also indications for a light-dependent magnetic compass in insects. In the fruitfly *Drosophila*, the range of normal compass orientation likewise ended at wavelengths shorter than 500 nm; at longer wavelength, the flies showed a ca. 90° shift in direction. Beetles of the genus *Tenebrio*, disoriented in the dark, showed a similar wavelength-dependency. The responses under longer wavelengths in amphibians and insects likewise suggest an interaction of magnetoreception with parts of the visual system (see ref. 10 for review). How they are to be interpreted and whether they represent a parallel to those observed in birds is still unclear and requires future studies.

Radio-Frequency Fields as a Diagnostic Tool

A diagnostic test aimed at obtaining more direct evidence for a radical-pair mechanism possibly underlying the avian magnetic compass made use of the fact that the singlet-triplet interconversion can be significantly affected by radio-frequency oscillating fields in the MHz range.[6] The effect of these fields depends on their frequency and on their orientation with respect to the static background field. First critical tests were performed with migratory birds, using orientation in migratory direction as an indicator whether or not the reception of directional information from the magnetic field was undisturbed. European robins, tested under the influence of weak radio frequency fields were disoriented when these fields were presented at an angle to the geomagnetic field, whereas they were oriented when the same MHz field was presented parallel to the geomagnetic vector (Fig. 3). This clearly shows that the observed effect of radio-frequency field is a specific one.[13] Similar experiments with radio frequency fields showed that two sedentary bird species, the domestic chicken and the zebra finch, *Taeniopygia guttata*, also have a magnetic compass based on radical-pair processes (see ref. 8). The only group besides birds studied are insects, where radio frequency fields were found to alter the activity pattern induced by frequently changing magnetic fields in cockroaches of the genus *Periplaneta*.[14]

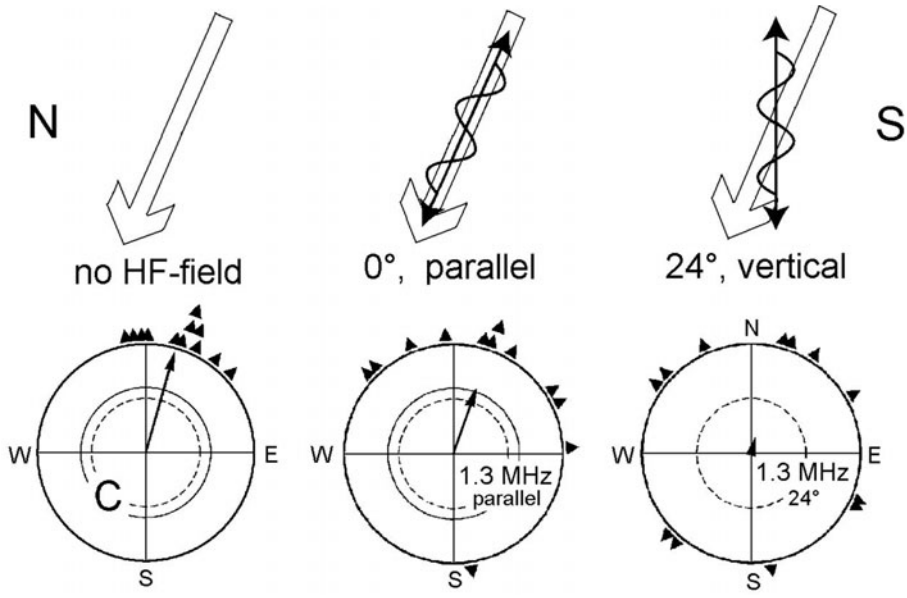

Figure 3. Orientation of European Robins in the geomagnetic field (Control, *C*) and in radio-frequency fields added to the geomagnetic field in two different orientation. The *upper part* of the diagram illustrated the orientation of radio-frequency field with respect to the geomagnetic field in the three test conditions; symbols in the circular diagrams as in Figure 2. Reproduced with kind permission from Springer Science+Business Media: Wiltschko W, Wiltschko R. J Ornithol 2007; 148(Suppl 1):S61-S76.[19]

In summary, a radical-pair mechanism underlying the magnetic compass has so far only been demonstrated in birds and is indicated in one species of insects. Light-dependency suggests such a mechanism also in amphibians; in marine turtles, although their compass is an inclination compass, it is unclear whether a radical-pair mechanism is involved.

The Receptor-Molecule

The question about the receptor-molecule forming the crucial radical pairs cannot yet be answered with absolute certainty, but evidence is accumulating for cryptochromes being the most likely candidates. The opsins, the pigments mediating visual information, cannot be involved because they do not form radical pairs; here, photon absorption leads to a change in configuration. Therefore Ritz and colleagues[6] suggested cryptochromes, another class of photopigments that possess the chemical properties crucial for the model, including the ability to form radical pairs. These photopigments were known from plants, but also occur in animals where they are involved in the internal clock (see ref. 15 for review). In vertebrates, they were found first in mammals, but also in chicken and passerine birds, where they occur in the retina, i.e., at a place where one would expect them if they were involved in magnetoreception (for reviewed in see ref. 16).

Cryptochromes are blue-light receptors, with a flavin and a pterin as photoactive cofactors. During photoreduction, they absorb short-wavelength light. Behavioral tests with migratory birds in radio-frequency fields of various frequency and intensities had

indicated a lifetime of the crucial radical pairs of 2-10 μs and revealed an extreme sensitivity to fields oscillating with the local Larmor frequency, the precession frequency of the electron: Here, a field of only 15 nT (roughly 1/3000 of local geomagnetic field) was sufficient to disrupt orientation, indicating a strong resonance. Theoretical calculations indicated that such a strong resonance is to be expected only in rather special radical pairs.[17] Cryptochromes possess the properties indicated for the receptor-molecule if one assumes that not photoreduction, but re-oxidation is the crucial step. This supports the idea that cryptochromes are involved in the radical-pair processes underlying the avian magnetic compass.

In the fruitfly *Drosophila*, experimental evidence directly indicates a crucial role of cryptochromes in the reception of magnetic directions: Wild-type flies showed spontaneous and trained responses to the magnetic field, whereas cryptochrome-deficient mutants showed neither response.[18] Altogether, the case for cryptochrome being the photoreceptor molecule that forms the radical pairs mediating magnetic information on directions in birds and insects is getting strong, although the evidence so far is mostly indirect.

Location of the Receptors and Neuronal Pathways

Theoretical considerations had favored the eyes as site of magnetoreception because of their almost spherical shape[6]—this prediction was also confirmed in birds, with the surprising finding that magnetoreception seems to be restricted to the right eye: Passerine migrants and chickens tested with their left eye covered were just as well oriented as binocular birds, whereas the same birds failed to show oriented behavior when their right eye was covered (see ref. 19). In salamanders, however, the receptors were found to be located in the pineal, the ancient third eye of vertebrates, which is directly sensitive to light in amphibians. Critical tests in which the skull above the pineal was covered with a color filter, but the eyes were open to the natural light clearly showed that the magnetic compass in salamanders depended solely on the spectral properties of the light reaching the pineal.[20]

Our knowledge on the neural pathways and the parts of the brain involved in processing magnetic compass information is still somewhat limited; the available evidence comes entirely from studies with birds. With the receptors located in the eye, magnetic input is mediated by the optic nerve and processed in parts of the visual system. Electrophysiological recordings from the nucleus of the basal optic root (nBOR) and from the *tectum opticum* revealed units that responded to changes in magnetic direction. Individual neurons in the nBOR as well as the *tectum* showed distinct peaks of response at particular alignments of the magnetic field. These varied between cells so that the input of a number of units would represent all directions in space. Processed collectively and integrated, they would thus provide a suitable basis for a compass as predicted by the radical pair model.[21]

The finding that magnetic input is mediated by the right eye indicates a strong lateralization of the magnetic compass that appears to be rather widespread among birds,[19] yet does not seem to include all species. Because of the very few connections between the two hemispheres of the brain, it means that magnetic information is processed almost exclusively by the left hemisphere of the brain. This is intriguing, as a number of morphological asymmetries have been described in the tectofugal system, a part of the visual system,[22] which, aside from the *tectum opticum*, comprises parts of the tectofugal system including the *nucleus rotundus,* where activation by magnetic stimuli was indicated by the glucose method of marking activity. But also parts of the thalamofugal pathway

are involved: Cluster N, a structure in the forebrain that represents a specialized part of the visual Wulst, was found to be connected with retinal ganglian cells. Differences in Zenk expression, indicating early gene activity, in birds kept in different magnetic fields and lesions furthermore suggested an involvement of cluster N in processing magnetic directional information.[23] Together, the few available findings suggest that magnetic compass information shares neuronal pathways with the visual system and is processed in centers associated with this system. Details of the neuronal processing and which other parts of the brain might also be involved remain to be determined.

MAGNETORECEPTION BASED ON MAGNETITE AND ASSOCIATED FINDINGS

Magnetite is a specific form of iron oxide Fe_3O_4 whose general properties depend on the size and shape of the particles. Spin interactions cause the spins of adjacent atoms to align, thus forming *domains* with all spins parallel. Large particles include multiple domains with their magnetic moments largely canceling each other; particles in the range between about 1.2 and 0.05 μm consist of a *single domain* and have a stable magnetic moment, acting as tiny permanent magnets. Even smaller particles are superparamagnetic (Fig. 4A); their magnetic moment fluctuates as a results of thermal agitation, but it can easily be aligned by an external magnetic field (see ref. 24 for details).

The Model

In the 1970s, certain bacteria were discovered to contain chains of single domain magnetite that act as small magnets, aligning the bacteria along the magnetic field lines.[25]

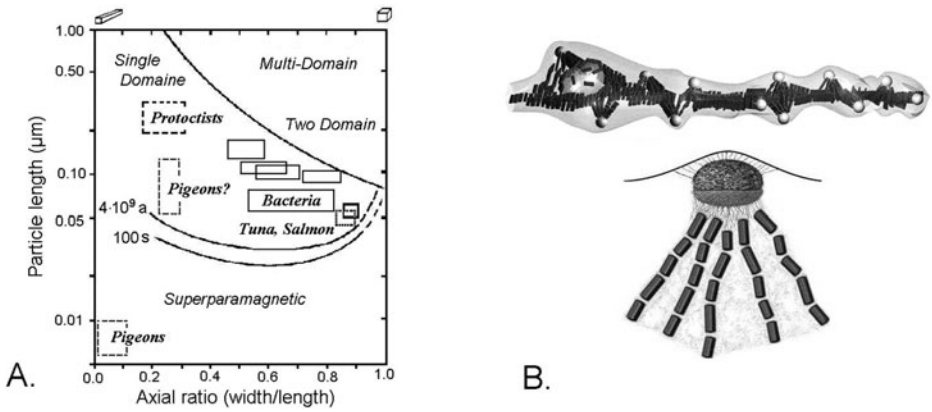

Figure 4. Properties of magnetite particles and reconstructions of a putative magnetoreceptor. A) Magnetic moments of magnetite crystals depending on size and shape: Domain stability field diagram with size of particles found in various living beings is indicated (after 40, modified). B) Schematic reconstructions of a structure in the skin of the upper beak of pigeons, based on ultra-thin section series. Above: The terminal region of a nerve containing a scaffold of platelets and numerous spherules of superparamagnetic magnetite particles; below: A spherule and the structures surrounding it. Reproduced from: Fleissner G et al. J Comp Neurol 2003; 458:350-360;[30] with permission from John Wiley and Sons.

These bacteria are anaerob or microaerophil; if they are stirred up from the bottom, this alignment will allow them to propel themselves along to field lines from the open, oxygen-rich water down to the sediments and thus into more favorable conditions. This 'orientation' phenomenon is fundamentally different from magnetic compass orientation in higher animals, because it is based on the magnetic *force* acting upon the magnetite particles: The alignments occurs without the bacteria and similar algae being active; dead bacteria align just in the same way. Higher animals, in contrast, must obtain *information* from the magnetic field to orient their movements, with the movements under their own active control.

Magnetic information mediated by tiny magnets was an attractive idea and the existence of magnetic material of biogen origin caused many authors to speculate about a potential role of magnetite in the orientation of animals. Based on theoretical considerations, the magnetite hypotheses consists of a variety of models on how magnetite particles might mediate magnetic information, some of them involving single domains others superparamagnetic particles or a combination of both (e.g., refs. 26,27). A uniform concept on how magnetite-based magnetoreceptors would mediate magnetic information does not yet exist. Model calculations showed that magnetite-based receptors could convey directional information or information on magnetic intensity, depending on their specific structure and on the amount of magnetite included (e.g., refs. 28,29).

Histological Findings and Neuronal Pathways

Magnetite has been discovered in a large number of species belonging to all major phyla, mostly by measuring the natural and induced remanence with highly sensitive magnetometers. In honey bees, *Apis mellifera*, magnetic material was described in the front part of the abdomen; in vertebrates, it appears to located mostly in the ethmoid region in front of the head.[24]

Histological studies indicating details about their arrangements are only available from fish and from birds. In salmonid fish, chains of single domain magnetite had been isolated from ethmoid tissue. Magnetite particles were found to be embedded in specific cells within the basal lamina of the olfactory lamellae in rainbow trout, *Oncorhynchus mykiss*.[27] Applying a strong external magnetic field could change the direction of their magnetization. In birds, two types of magnetite-containing structures have been described: Single domain particles were reported from the orbital and the nasal cavity, whereas clusters of very small crystals were described at six specific locations in the skin of the upper beak, identified by crystallographic means as superparamagnetic magnetite.[30] These clusters were located within nervous tissue and associated with a remarkable framework of platelets (Fig. 4B). These structures were first identified in pigeons, but recently also described in several passerine migrants and domestic chickens.[31] The magnetite-containing structures found in birds and fish do not seem to be identical, implying that the respective magnetite-based receptors might differ in their general characteristics.

The region of the head where magnetite particles were found in birds and fish is innervated by the *ramus ophthalmicus*, a branch of the *nervus trigeminus*. Electrophysiological recordings from this nerve and from the trigeminal ganglion in passerine birds showed units responding to magnetic stimuli,[32] and so did recordings from the corresponding nerve in rainbow trouts.[28] This indicates that in these two vertebrate groups, information from the magnetite-based receptors is mediated by the trigeminal system. Recent findings using markers to indicate neurobiological activity confirmed these findings, showing increased activity in neurons in the trigeminal brain stem complex after magnetic stimulation.[33]

In rodents, the *superior colliculus* was identified as a site of neural activity caused by magnetic stimulation.[34] The origin of this activity is unclear; an involvement of magnetite-based receptors indicated by the pulse effect (see below) is not unlikely.

A Strong, Short Magnetic Pulse Identifying Magnetite-Based Receptors

Authors reporting magnetite particles speculated about their possible functions as magnetoreceptors. The first behavioral tests designed to demonstrate an involvement of magnetite in magnetoreception used the response to a short, strong magnetic pulse as a diagnostic tool. The pulse would interfere with potential receptors by altering the magnetization of single domain magnetite crystals or by disrupting clusters of superparamagnetic magnetite.[35] This was expected to change the output of the respective receptors in a dramatic way and thus cause a lasting after-effect on orientation behavior. Since other reception mechanisms would not show any after-effect following pulse treatment, the observation that the pulse had an effect clearly indicates that magnetite particles are involved in the receptors controlling the observed behavior. A popular method was to apply a brief, strong magnetic pulse to the head of the test animal—the pulse had to be strong enough to remagnetize the magnetite particles but, at the same time, short enough to prevent these particles from rotating into the pulse direction and thus escape remagnetization.

In migratory birds, a pulse prior to the tests caused a marked 90° change in direction: Australian Silvereyes, *Zosterops lateralis*, preferred easterly headings or showed an axial preference of the east-west axis, regardless of whether they had been heading northward in autumn or southward in spring (Fig. 5, left and center). This effect of the pulse lasted for about 3 days; after that, the birds first became disoriented and later slowly resumed their original headings. Interestingly, the effect of pulsing was restricted to experienced migrants; young birds that had been captured immediately after fledging proved to be unaffected and continued in their normal migratory direction. The same pulse also caused experienced homing pigeons to deviate from the mean of untreated control birds, but here, the effect was much smaller, probably due to the fact that the pigeons were released in the field so that other, nonmagnetic cues were also available. Treating birds with pulses oriented differently with respect to their head let to deviations in different directions.

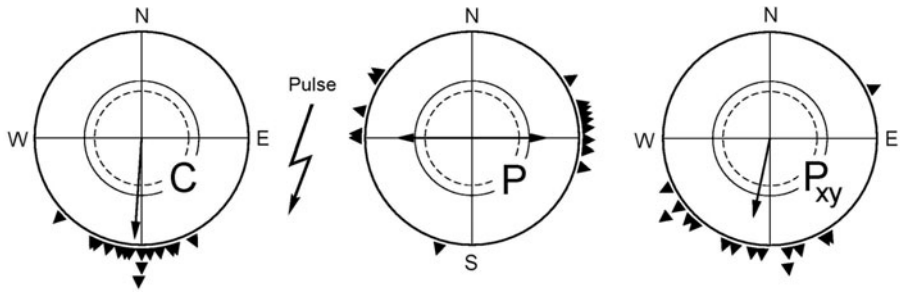

Figure 5. Effect of a short, strong magnetic pulse on the orientation behavior of Australian Silvereyes in Australian spring. *Left*: Orientation during the control phase before pulse treatment (C), *center* and *right* diagram: Orientation after pulse treatment. *Center*, without anesthesia (P); *right*, upper beak anesthetized with the local anaesthetic Xylocaine (P_{XY}). Symbols as in Figure 2. Reproduced from: Wiltschko W et al. Proc R Soc B 2009; 276:2227-2232;[39] with permission from The Royal Society.

This implies that the pulse did not deactivate the receptors altogether, but instead caused them to provide the birds altered information (for reviewed in see ref. 19).

Treating other vertebrates with the similar pulses also induced noticeable effects indicating an involvement of magnetite-based receptors. Zambian molerats, *Cryptomys anselli*, shifted the position of their nest from the south to east; testing the same animals repeatedly showed that this altered preference was stable for at least three months.[36] At the same time, the molerats were unaffected by radio-frequency fields, revealing the absence of a radical-pair mechanism. Big Brown Bats, *Eptesicus fuscus*, showed axial preferences after pulse treatment.[37] Hatchling loggerhead turtles, *Caretta caretta*, treated with five brief pulses applied in different directions were disoriented.[38]

The observed effect of the magnetic pulse also gives some hints on the type of magnetite particles—single domains or superparamagnetic particles—involved. Remagnetization of single domains particles should be just as stable and lasting as the original one, whereas clusters of superparamagnetic particles, when disrupted by a pulse, would rearrange themselves within a few days. In birds, where both types have been described, the short duration of the pulse effect—only about 3 days—speaks against single domains and points to the receptors in the skin of the beak containing the superparamagnetic particles as being the ones that affect the behavior. This is in agreement with the finding that local anesthesia of these receptors indeed renders the pulse ineffective,[39] with migrants continuing to head in their migratory direction (Fig. 5, right). In rodents, however, the long duration of the pulse effect[36] would be in accordance with single domains. The effect of the pulses on the behavior of loggerhead turtles cannot be easily interpreted in either way.

What Type of Information Do Magnetite-Based Receptors Convey?

Theoretically, magnetite-based receptors could provide information on magnetic directions as well as information on magnetic intensity.[28,29,40] However, we might expect different specializations of the receptors depending on which type of information they mediate, just as we ourselves use different instruments to measure the different variables of the magnetic field: a compass for directions and a magnetometer for intensity.

In birds whose compass is based on a radical pair mechanism, there are indications that the magnetite-based receptors provide information on magnetic intensity, used as a component of the navigational 'map'. Early electrophysiological studies produced responses to changes in intensity from the ophthalmic nerve. These responses showed a logarithmic characteristic; the minimum intensity difference tested were 200 nT, where the birds still showed a clear response. Similar recordings are reported from the trigeminal ganglion.[32] This is in agreement with the above-mentioned observation that the magnetic pulse failed to affect the behavior of a young, inexperienced birds—it suggests that the pulse interferes with an experience-based mechanism. This points to the position-finding system, where magnetic intensity could be used to indicate north-south displacements. Conditioning experiments likewise suggest a role in detecting magnetic intensity, which is also supported by the behavior of pigeons released in a strong, highly irregular magnetic anomaly. Deactivating the receptors in the beak with a local anesthetic or disrupting the ophthalmic nerve, on the other hand, did not disrupt compass orientation (see Fig. 6, right), indicating that the magnetite-based receptors are not involved in the avian magnetic compass.

Some findings, however, suggest that the receptors in the beak can also provide information that causes the birds to head in specific directions, albeit only under conditions that disrupt the normal magnetic compass. These directions are different from the migratory direction and do not show a seasonal change. The manifestation of these 'fixed direction' responses depends on the ambient light regime, but they obviously do not provide information that is helpful to the birds, e.g., in locating their migratory direction. Local anesthesia of the upper beak disrupts these 'fixed direction' responses, indicating the magnetite-based receptors as their origin. So far, they represent a phenomenon observed only under rather unnatural light conditions in the laboratory; their biological significance is unclear—they are discussed as possible phylogenetic relicts of an ancient magnetite-based compass mechanism that is replace by the radical-pair mechanism today (see ref. 8 for review).

Very little is known about magnetite-based receptors in other vertebrates. In fish, where single domain particles have been described within the olfactory lamella, electrophysiological recordings from the nerve innervating these structures produced responses to changes in intensity.[28] In marine turtles and bats, an involvement of magnetite-based receptors is likewise indicated by the response to a strong pulse[38] (see above), yet the nature of the response does not allow to distinguish between directional or intensity information. In Zambian molerats, on the other hand, the pulse changed the direction of nest-building,[36] which might be considered a compass response. If this is true, the magnetite-based receptors would convey compass information is this species.

CONCLUSION

The use of the magnetic field for orientation and navigation was already discussed in the 19th century,[41] but first evidence that animals can use the magnetic information was published in the 1960s. In recent years, the number of publications on various aspects of magnetoreception and processing of magnetic information greatly increased. It became clear that magnetoreception is not a uniform phenomenon: There is evidence for two different aspects of the geomagnetic field—direction and intensity—to be perceived and for at least two fundamentally different biophysical mechanisms mediating magnetic information.

Only in birds, the various pieces of the puzzle begin to form a consistent picture, even if many questions remain unanswered. The available data indicate the existence of two magnetoreceptor systems that mediate different types of information: A radical-pair mechanism in the right eye providing directional information and magnetite-based receptors in the upper beak recording differences in magnetic intensity—one might say: Birds have a compass in their eye and a magnetometer in their beak.

In other vertebrates, our knowledge is rather limited to certain aspects of magnetoreception. In marine turtles, the various uses of magnetic information are well documented, yet magnetoreception has not yet been analyzed. Primary processes of magnetoreception are indicated by behavioral data in salamanders, where the light-dependency suggests a radical pair mechanism in the pineal, and in mammals, where the pulse effect points to receptors based on magnetite. The position of the receptors and anatomical details about their structure as well as some of the neuronal pathways are known in fish, where electrophysiological recordings indicate that information on magnetic

intensity is mediated by the trigeminal system; yet details of the mechanism underlying the polarity compass are still unknown.

In view of the many open questions, we can only hope that the 'magnetic sense' continues to meet with great interest and that further research in the coming years will lead to a better understanding of how magnetic information is perceived and processed.

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CHAPTER 9

THE MOLECULAR BASIS OF MECHANOSENSORY TRANSDUCTION

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Abstract: Multiple senses, including hearing, touch and osmotic regulation, require the ability to convert force into an electrical signal: A process called mechanotransduction. Mechanotransduction occurs through specialized proteins that open an ion channel pore in response to a mechanical stimulus. Many of these proteins remain unidentified in vertebrates, but known mechanotransduction channels in lower organisms provide clues into their identity and mechanism. Bacteria, fruit flies and nematodes have all been used to elucidate the molecules necessary for force transduction. This chapter discusses many different mechanical senses and takes an evolutionary approach to review the proteins responsible for mechanotransduction in various biological kingdoms.

INTRODUCTION

Behind the development of all senses is an organism's need to sample the surrounding world and extract information relevant for survival. Our bodies are bombarded by thermal changes, light and chemicals, each of which has devoted encoding mechanisms; however, mechanisms that encode mechanical stimuli are perhaps the most commonly represented in the mammalian repertoire of senses.

Mechanosensation is necessary for mammals to perceive signals from the external world through touch and hearing, to monitor our internal states through sensing flow, osmotic pressure and blood pressure, and to perceive the relationship of our bodies to the external world through balance and proprioception. Though these senses seem quite disparate, they each encode a physical measurement of force. Advanced mechanosensory systems are

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not just present in mammals. All living things require some form of mechanosensation to survive- every cell responds to osmotic pressure and even single cell organisms react to touch. The ubiquitous presence of mechanotransduction suggests that it was one of the first senses to evolve. Although there is some evidence for convergent evolution, the majority of mechanisms for mechanotransduction are thought to have arisen divergently.[1] Microbial organisms developed the earliest mechanotransduction mechanisms to protect against cell rupture from osmotic shock. This basic mechanism is the evolutionary beginning of what sprouted into the vast array of mechanical senses present in more complex organisms.

Progress in elucidating fundamental mechanisms of mechanotransduction has come from exploring these processes in simple organisms. Despite their importance and prevalence, the mechanical senses remain the least understood in mammals. This chapter will discuss the development of mechanotransduction throughout the phylogenetic tree and the molecular mechanisms that underlie force sensing.

FORCE TRANSDUCTION

The process of transforming a stimulus into a cellular signal is called transduction. In the nervous system, sensory transduction culminates in change of the electrical potential of a neuron. This is accomplished by proteins in the membrane called ion channels, which are gated pores that allow the exchange of ions across the cell membrane. Sensory systems use specialized transduction molecules to convert stimulus energy into appropriate cellular signals. For example, the transduction molecule for vision (light) is rhodopsin, which changes conformation upon exposure to a photon. This begins a G-protein-coupled receptor signaling cascade that culminates in the gating of an ion channel. Although G-protein signaling is a commonly utilized mechanism for signal transduction, in mechanosensory systems, stimuli induce electrical activity by directly gating transduction channels.

Two primary models for the gating of mechanotransduction channels have been proposed (Fig. 1). The first model predicts that mechanotransduction channels are directly gated by force applied to the lipid membrane (Fig. 1A). This is the mechanism used by all known prokaryotic mechanosensitive ion channels. The second model posits that efficient channel gating requires a tether to the cytoskeleton or extracellular matrix (Fig. 1B). Force placed on this tether creates a tension that opens the ion channel. In the case of the mammalian hair cell, this mechanism seems to be most likely.

Although it is possible that gating occurs indirectly through a secondary messenger cascade, there is no experimental evidence supporting this in any known mechanotransduction channel. Corey and Hudspeth used vestibular hair cells to demonstrate that the time between mechanical stimulation and an electrical response is ~40 μs.[2] By contrast, the latency for phototransduction is tens of milliseconds due to signaling intermediates. The speed of transduction indicates that, at least for hair cells, force transduction molecules are force-gated ion channels.

PROKARYOTIC MECHANOTRANSDUCTION

One can imagine a primordial world full of water, solutes and the first life: A unicellular prokaryote. With the development of a plasma membrane encapsulating this first organism, there came an inherent delicacy. The semi-permeability of the plasma membrane is essential

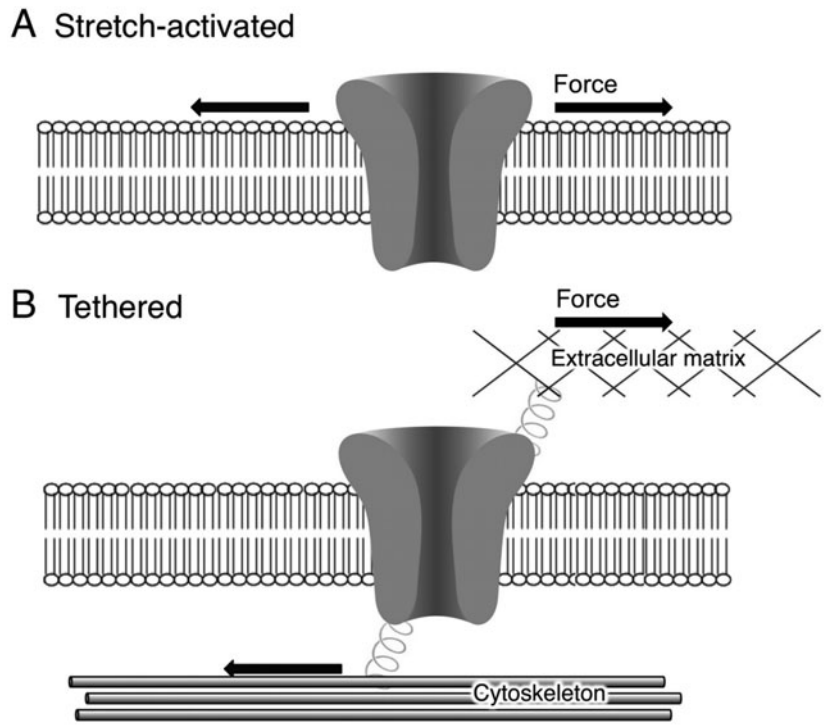

Figure 1. Two models of gating for mechanotransduction channels. A) Lipid bilayer tension is sufficient to cause channel opening, or B) mechanotransduction channels require a tether connected to either extracellular matrix and/or cytoskeletal elements.

for a cell's ability to sense and respond to its external environment, but requires tight control of the cell's internal environment and all of the solutes regulating its metabolic functions. The internal environment of this plasma membrane bag is dominated by water and thus is subject to the laws of osmosis. Water will flow through the plasma membrane into a cell from a hypotonic environment and out of a cell in a hypertonic one, so there must be a mechanism in place to preserve cell volume and membrane integrity. This is why osmotransduction is the most evolutionarily ancient mechanical sense and the primary mode of mechanotransduction in the prokaryotic kingdoms Bacteria and Archaea.

Bacteria

When exposed to an hypotonic external stimulus such as rain, bacteria require a mechanism to protect themselves from lysing. Osmotic pressure develops inside a cell when its external solution is diluted, causing the cell membrane to swell outward. In the 1950's, scientists observed that *E. coli* eject intracellular solutes to compensate for a hypotonic challenge. Nearly 50 years later, the osmosensitive channels MscL, MscS and MscM (mechanosensitive channels of large, small and mini conductance) were discovered to be the emergency release valves that mediate this response.[3,4] They are named for the size of their conductances, ~3 nS, ~1 nS and ~0.3 nS respectively, and require increasingly

more membrane tension to open with increasing channel conductance. The pores of these channels are mostly large and nonselective to allow passage of a wide variety of solutes. Initially, Msc channels were studied using the patch clamp technique, as applying suction to the membrane causes large current steps. The same results were subsequently obtained by diluting the external solution, which deforms the plasma membrane due to increased intracellular osmotic pressure.[5] Membrane deformation opens the Msc channel pore through which solutes are released and membrane tension is then relieved.[3]

Martinac and colleagues expressed MscL in liposomes to examine the gating mechanism and found that the channel was opened by changes in lipid tension alone. When lysophosphatidylcholine (LPC) was added symmetrically to liposomes reconstituted with MscL, the channel remained closed. However, with asymmetric addition of LPC, there is an asymmetric change in pressure across the bilayer that parallels the change in membrane curvature achieved with suction or cell swelling. This change in the lipid pressure profile was enough to open MscL in the absence of any suction, confirming the bilayer tension model in Figure 1.[6,7] The crystallization of MscL gave insight into the structural changes involved in the physical mechanism of pore opening: the subunits twist against one another and flatten when tension is applied to the membrane, opening like the iris of a camera.[3]

Mutational studies provide compelling evidence that these channels are playing a protective, osmoregulatory role in bacteria. Booth and colleagues created *E. coli* with dysfunctional mutant MscS or MscL, as well as a double mutant.[8] Each single mutant was able to compensate for increased turgor during a hypotonic challenge, but the double mutant *E. coli* lysed even with mild hypotonic shock. This showed that the smallest mechanotransduction channel, MscM, is not sufficient to protect bacteria from hypotonic environments.

The discovery of these channels in bacteria launched searches for similar genes in other organisms, the most related of which are prokaryotic members of the kingdom Archaea.

Archaea

Osmoregulatory channels allow bacteria to survive in a broad range of osmotic environments, but members of a separate prokaryotic domain, Archaea, have adapted to the most extreme habitats on earth. These unicellular organisms are known to live in hypersaline marine environments such as the dead sea (halophiles), deep sea volcanic vents (methanogens) and acidic hot springs (thermoacidophiles). Mechanotransduction channels have been discovered in all three types of Archaea. The genome of *Methanococcus jannaschii* was the first Archaeon to be sequenced and provided an opportunity to look for mechanosensitive channel homologs. When probing with the most highly conserved region of the MscL protein, transmembrane domain 1 (TM1), scientists identified a residue with 38.5% homology.[9] Conserved residues in the matching sequence were shown to be functionally important in bacterial MscL and this new archaeal protein was named MscMJ. Eventually two mechanosensitive channels, MscMJ and MscMJLR, were identified in this species. These channels biophysically correspond most closely with MscS and MscL of *E. coli*, respectively. Interestingly, these sequence probe studies led to the discovery of another homolog to an *E. coli* protein named YggB, whose function was unknown at the time. YggB was later identified as the bacterial mechanosensitive channel MscS.[8] The halophile *Haloferax volcanii* contains two distinct mechanosensitive channels, MscA1 and MscA2 and the thermoacidophile *Thermoplasma acidophilum* contains MscTA.[9]

Like bacterial mechanotransduction channels, all discovered Archaeal channels are gated through lipid bilayer force and open a large, mostly nonselective pore. These common channel properties fulfill the essential requirements for small, unicellular osmosensors, whose primary purpose is to rapidly compensate for osmotic changes in the environment. The sequence similarities of mechanosensitive channels in both Bacteria and Archaea point to a common MscL-like ancestor that likely arose before the two domains separated during evolution.

Eukaryotic Mechanotransduction

In-depth studies of MscL channels provided a promising starting point for finding mechanically sensitive homologues in eukaryotes. The function of mechanotransduction channels has expanded to immensely diverse systems within the eukaryotic kingdoms, but osmosensation is still prevalent and integral to eukaryotic functioning. In addition, eukaryotes can react to varied stimuli, including touch, sound and gravity. Plants contain channels similar to bacterial MscS, although no eukaryotic MscL-like channels have been found in the sequenced genome. These channels are proposed to confer osmoregulatory function and touch-sensitivity to plants.[10]

Plants

Plants have learned to thrive in myriad forms, but do so while being rooted in a single location, unable to escape from danger. To cope with this static lifestyle, they have developed defense mechanisms to protect themselves from assaults by wind, rain and predators. Plant shoots and roots have also developed the ability to detect and avoid barriers encountered during growth, a response called thigmomorphogenesis.

All plants respond to touch, but can do so in dramatically different ways. Specialized sensory cells allow some plants to react to touch on a very quick timescale with less than one second reaction time. Although for most plants this response is protective, some plants have co-opted this ability for predatory use. Touching a *Mimosa pudica* will result in rapid leaf folding, as will brushing the trigger hairs of a Venus Fly Trap. Even in the absence of specialized cells that result in fast touch responses, plants respond to touch by changing gene expression and growth patterns.[11]

The model plant *Arabidopsis thaliana* has ten MscS-like proteins (MSL1-10) with some sequence homology to bacterial and archaeal mechanotransduction channels. MSL 2 and 3 have been identified as necessary for proper organelle morphology and volume regulation, which are both dependent on osmotic sensation and control. To demonstrate the osmotransduction capacity of MSL3, Haswell and Meyerowitz expressed this channel in mutant *E. coli* that lacked all native osmoregulatory Msc channels. Normally these cells lyse in response to a hypotonic solution, but expression of plant MSL3 was able to rescue the bacterial cultures, demonstrating this channel is capable of functioning as an osmosensor.[12] MSL9 and MSL10 have also been identified as stretch-activated channels and if all of the expressed MSL channels are removed from the plant root protoplasts, they lose all mechanically induced electrical activity.[10]

Strangely, mutants lacking all of these genes are phenotypically indistinguishable from wild-type, therefore, our understanding of their physiological role is still limited. Although MSL conductances are considerably smaller than those of MscS channels in bacteria, they are among the largest channel conductances ever recorded in plants. The relatively high

conductances indicate these channels could function similarly to bacterial Msc channels, as this allows for a rapid membrane depolarization to correct for increased intracellular pressure.

Caenorhabditis elegans and the Molecular Basis for Touch

When considering the sense of touch, the first thought that arises is distinctly mammalian: The feel of another's skin or the texture of an object in hand. This view neglects the widespread presence and importance of touch sensation in other organisms. Plants were the first unlikely touch-sensitive organisms discussed, but the eukaryote that has helped us understand the most about touch is a worm, *C. elegans.*

This small soil nematode is an ideal candidate for elucidating the molecular mechanism of touch because the genetics and development of all 302 neurons is well understood. *C. elegans* lives underground and therefore must rely heavily on its mechanical senses to interact with its environment. The nematodes exhibit several stereotyped touch-response behaviors, many of which rely on different neurons that have been identified with laser ablation and genetic studies. The nose touch response, in which *C. elegans* reverses direction upon contacting an object in its path, is mediated by mechanosensory neurons ASH, FLP and OLQ. Two ion channels belonging to the transient receptor potential (TRP) family have been shown to be responsible for mediating this behavior: OSM-9 and OCR-2.[13,14] These same channels are also required for avoidance of hypertonic stimuli, which indicate a functional relationship between channels in Bacteria and Archaea.

Although first discovered in the fruit fly (*D. melanogaster*) retina, TRP channels have been widely implicated in mechanotransduction. They are broadly expressed throughout eukaryotic phyla and have provided numerous leads in the hunt to find mechanotransduction channels in various organisms. For example, yeast (*Saccharomyces cerevisiae*) requires TRPY1 to have proper fluid regulation responses to hypertonic stimuli.[15]

Another well understood touch response in *C. elegans* is the body touch response, in which the nematode changes directions when gently brushed with an eyelash along its body wall. Six specific neurons mediate this response and have distinct structural features that distinguish them from other body neurons. Gentle touch neurons are surrounded by a specialized extracellular matrix called the mantle and they are filled with unusual 15-protofilament microtubules.[16] These features suggest a tethered gating mechanism as they are unique to touch-sensitive neurons. Pioneering genetic screens by Chalfie and colleagues[17,18] identified 16 *mec* genes (mechanosensory abnormal) specifically required for the gentle touch response. Out of these 16 genes, two encode ion channel subunits (*mec-4* and *mec-10*). These channels are a part of the DEG/ENaC superfamily of ion channels, which includes many ion channels expressed in mammals.

Along with the TRP family, DEG/ENaC channels are now primary candidates for investigating mechanotransduction. Mutant animals with nonfunctioning *mec-4* and *10* have morphologically and developmentally normal touch neurons, but do not respond to mechanical stimuli. Nevertheless, when these two channel genes along with accessory subunits encoded by *mec-2* and *mec-6* are expressed heterologously in *xenopus* oocytes, they cannot be gated by mechanical stimuli.[19] The common hypothesis to explain this phenomenon is that proper function of the proteins requires expression of the other mec genes, many of which encode structural proteins that could help gate the channel or increase functional activity. For example, extracellular matrix components encoded by *mec-1, mec-5* and *mec-9* aide in the proper localization of the Mec complex.[20] Although some studies support a model in which several mec proteins provide structural support

that contributes to channel gating,[21] recent evidence shows otherwise. Specialized microtubules encoded by *mec-7* and *mec-12* are required for proper touch transduction, but do not interact with the mechanotransduction complex.

Defects in other mechanical senses in *C. elegans* have also pinpointed putative mechanosensory channels. Loss of *unc-8*, which encodes a Deg/ENaC subunit similar to MEC-4 and MEC-10, causes an unco-ordinated phenotype in worms, suggesting a possible role in proprioceptive co-ordination of muscle movement.[22]

Identifying important genes that encode molecules necessary for touch was key groundwork for furthering the study of mechanotransduction. The next area ripe with possibilities was a different model animal with powerful genetic accessibility and more complex mechanical senses than *C. elegans*.

Drosophila Melanogaster

Fruit flies may be pests in a home but they are a boon to scientists in the lab. They have been a valuable tool to parse out the functions of myriad genes, as they have a short generation time and numerous tools are available to manipulate their genome. Even as a simple model system, they have developed many mechanical senses that are essential for their survival. In fact, mechanosensory mutant flies are completely incapacitated and fail to thrive in a laboratory setting. Along with sensing touch, flies require hearing to detect mating songs, proprioception to co-ordinate six multi-jointed legs and strain gauges on their wings to control their flight path.

Externally, flies sense touch through Type I mechanosensory bristles and chordotonal organs that cover their body. Each mechanosensory organ is independently innervated by a single neuron that extends its ciliary structure into the bristle base (Fig. 2A). Bristles act as levers that transmit force when they are moved and, at their base, they may be able to detect movements as small as 1-2 nm.[23] When a bristle is bent, the proximal end compresses the dendritic shaft surrounding the cilia against the outer segment membrane, which contains tubular bundles (Fig. 2). Without these tubular bundles, mechanotransduction is lost, so the compression of the ciliary membrane against this structure is the putative gating stimulus.[24] Alternatively, this compression may be converted into a shear or tension force to gate mechanotransduction channels. Once the channels are opened, electrophysiological recordings reveal an inward K^+ current upon displacement of the bristle tip. As replacing the pipette solution with Na^+ does not affect the current, transduction molecules are nonselective cation channels.

Chordotonal organs are mechanosensory units that have no external process. They are attached to the cuticle and the neuron is secured basally and apically, thus acting essentially as a stretch organ. An array of chordotonal organs, named Johnston's organ, comprises the fly's antennal ear. Although bristles rely on compression to gate channels, shear forces pulling on a dendritic cap connected to neuronal cilia is the stimulus in chordotonal organs (Fig. 2A).

The first putative *D. melanogaster* mechanotransduction channel to be identified, NOMPC, is a member of the TRPN branch of the TRP channel family. Mutation in the *nompC* gene eliminates most of bristle's mechanotransduction current and the protein that it encodes is a fitting candidate for a mechanotransduction molecule. Despite seemingly different stimuli, the *nompC* mutants that showed no bristle potential also had greatly reduced receptor potentials for antennal sound responses,[25] which implicates *nompC* in chordotonal transduction and hearing.

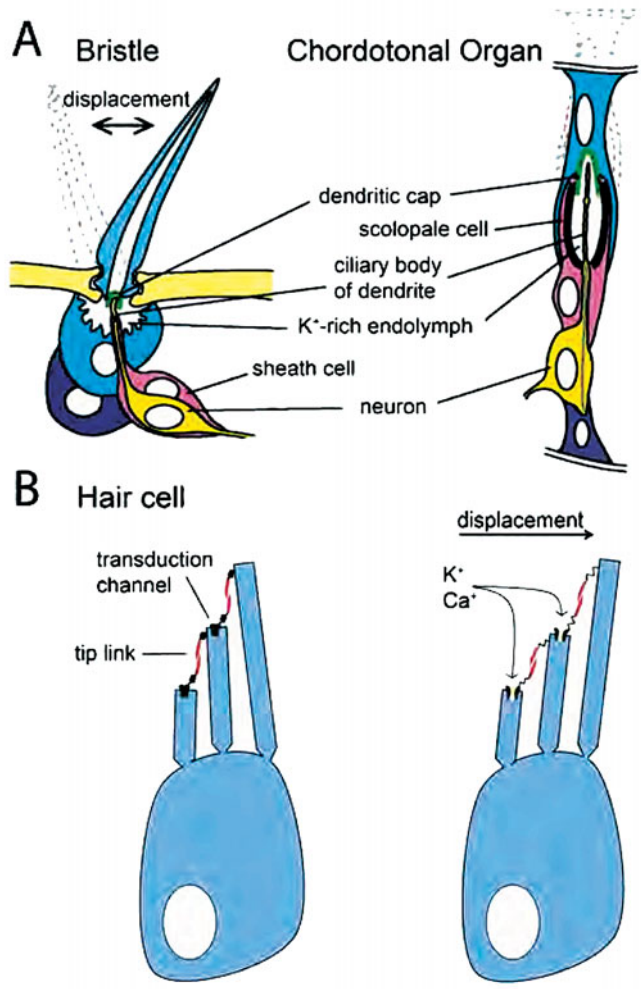

Figure 2. Mechanosensory cells. A) A depiction of two mechanosensory organs in *D. melanogaster* and their proposed mechanisms of activation upon position change (shown by dotted outlines) as a result of a mechanical stimulus. In both organs, the neuronal dendrite is attached to a dendritic cap that is affected by stimulus-induced movement, activating mechanotransduction channels. Adapted with permission from: Jarman AP et al. Studies of mechanosensation using the fly. Human Molec Genetics 2002; 11:1215-1218; ©2002 Oxford University Press. B) A vertebrate hair cell before (left) and after (right) displacement by endolymph movement. The shear force pushing stereocilia creates tension in a spring-like element (as yet unidentified) that is proposed to be in series with the tip link. Increased tension results in transduction-channel opening.

Although its subcellular location has not been confirmed in *D. melanogaster*, the *C. elegans* homologue TRP-4 localizes to ciliary outer segments of specific neurons that contain tubular bundles similar to those in fly bristle sensory organs, making it a mechanotransduction molecule candidate.[26] Researchers have also been interested in another potentially important feature of NOMPC channels: Out of the entire TRP superfamily, they contain the largest number (29) of ankyrin repeats.

Ankyrins are structural proteins that commonly mediate protein-protein interactions. Many models of mechanical gating include a spring-like component in series with a tether and the ankyrin helical structure makes it an attractive possibility for an intracellular gating spring.[27] These aspects together fit with the possibility that NOMPC is a mechanotransduction channel for fruit fly bristle sensation. A homolog of NOMPC, TRPN1, has also been shown to contribute to mechanotransduction in zebrafish hair cells.[28]

Other members of the TRP family, TRPV channels, also seem to be required for transduction in chordotonal organs. Mutations in *nanchung* (*nan*) and *inactive* (*iav*) result in deaf flies without sound-evoked potentials.[29,30] Both of these proteins are found specifically in the mechanosensory cilia of chordotonal organs. Moreover, each requires expression of the other for proper subcellular localization, suggesting that they form a heteromeric channel. This is comparable to the relationship of functional interdependence seen with closely related *C. elegans* channels OCR-2 and OSM-9.[14] The exact subcellular location of the IAV protein is notable, as it is restricted to the proximal end of the neuronal cilia, which would require it to be gated by membrane tension rather than direct pulling by the dendritic cap[29] (Fig. 2). The mechanosensitivity of these channels has been confirmed in heterologous expression systems, as both NAN and IAV are independently opened by hypotonic stimuli. The currents produced in this setting are very slow and thus do not reflect the true speed of transduction in vivo. It is possible that proper transduction only occurs with the presence of either both channels together or another accessory molecule.

The fly's primitive ear possesses intriguing parallels with the vertebrate ear. It consists of a sail-like flagellum called the arista connected to the funiculus. When moved by mechanical force, the arista rotates the third antennal segment relative to the second, thus deforming the cuticle between the joint of the two segments, where the sensory units attach.[24] The mammalian cochlea is structurally and functionally quite different from the fly antennal auditory organ and this has led to a common belief that the two arose separately during evolution. Surprisingly, genetic evidence has now revealed that many key genes required for proper development of the antennal auditory organ are also required for normal development or function of the vertebrate ear.[31] The proneural gene *atonal* is essential for differentiation of all chordotonal organs. Mammalian knockouts of the atonal homolog gene (*atoh1*) lack both the mechanosensory hair cells of the ear[32] and mechanosensitive Merkel cells of the skin.[31,33] Additionally, the vertebrate cochlea has the ability to amplify and augment sounds so that they respond nonlinearly to acoustic energy. The mechanism varies in mammals and tetrapods, but is accomplished by contraction of the hair cell itself or through movement of apical hair-cell bundles. Gopfert and Robert found that auditory neurons in the fly show a remarkably similar nonlinear tuning, thus mirroring both the actuating and transducing roles of the vertebrate cochlea.[34]

This small organ has provided much insight into mechanisms of hearing, as well as other mechanical senses. Aside from hearing courtship songs and other relevant stimuli, the Johnston's organ is also responsible for *Drosophila*'s ability to sense gravity and wind.[35,36] The third antennal subunit is deformed by gravity irrespective of head orientation, thereby providing the "gyroscope" function needed for proper flight.[35]

In addition to ciliated mechanosensory neurons, nonciliated neurons that innervate the fly's body wall have been proposed to function as proprioceptors and nociceptors, or pain neurons. Tracey and colleagues found that larvae have very different behavioral responses to a harsh touch or a painfully hot (>38°) probe than they do to a gentle touch. The distinct writhing escape response indicates that there is a pain-specific sensory pathway and three *painless* mutants lacked this response.[37] All three *painless* lines had mutations in a TRPA

channel subunit, which was later found to be required for thermal nociception in adult flies and avoidance of isothiocyanates.[38] The closely related channel TRPA1 is found in mammals, where it is also essential for isothiocyanate sensitivity. Thus, this polymodal channel senses mechanical, thermal and chemical stimuli. This pathway is completely distinct from chordotonal transduction, as *atonal* mutants (which lack chordotonal organs) retain the writhing response and *painless* mutants have no deficit in chordotonal transduction.

These mechanosensory neurons in *Drosophila* have been immensely powerful in teaching us about the mechanisms underlying various representations of mechanical senses. These sensory abilities only become more fascinating, complex and varied when investigating higher branches of the phylogenetic tree.

VERTEBRATE MECHANOTRANSDUCTION

Of the many specialized mechanosensory systems found across phyla, vertebrates have developed the most elaborate. The following section will focus primarily on mammalian mechanosensory systems.

Several attributes of mammalian mechanotransduction channels make them difficult to identify. Mechanosensory cells or receptor endings are few in number and are not readily separated from surrounding cells. This is most evident in the cochlea, which only has about 15,000 hair cells, making biochemical assays difficult. Additionally, each hair cell is estimated to only have 50-100 transduction channels. Lastly, it is possible that mammalian mechanotransduction channels are multiprotein complexes, unlike experimentally tractable bacterial channels that are encoded by single genes.

These hurdles in uncovering mammalian mechanotransduction channels are slowly being overcome with new technology and creative scientific minds, but the molecular mechanisms behind mammalian touch reception still remain a mystery.

The Somatosensory System—Touch, Nociception and Proprioception

The skin acts as our first line of defense, sensing both damaging and pleasant stimuli. Sensory neurons innervating the skin are responsible for conveying thermal, chemical and mechanical information. It is clear that some of these stimuli activate separate transduction mechanisms, whereas other proteins can be activated by more than one stimulus. TRPV1 (transient receptor potential vanilloid 1), for instance, is activated both by heat and chemicals.

The skin is densely innervated by mechanosensory nerve endings, such as lanceolate endings around hair follicles, Pacinian corpuscles, Meissner's corpuscles and Merkel cell-neurite complexes. As yet, the molecules that confer force sensitivity to any of these sensory endings are unknown.

One promising avenue to identify candidate transduction molecules is to analyze mammalian homologs of *C. elegans Mec* genes. For example, knockout mice lacking a MEC-2 related protein, stomatin-like protein 3 (SLP3), show deficits in discriminating texture and in electrophysiological recordings from touch-sensitive afferent nerves. This indicates SLP3 is essential for mechanotransduction in some cutaneous sensory afferents.[39]

Similarly, several studies implicate DEG/ENaC ion channels in mammalian somatosensory mechanotransduction. Mice lacking acid-sensing ion channels (ASIC) 2 and 3 resulted in either no touch deficits,[40] or moderate ones;[41,42] however, the mechanical threshold for these touch-sensitive neurons are not changed. A dominant-negative mutant

of ASIC3 actually increased the sensitivity of fibers to mechanical and chemical stimuli.[43] These data suggest that mammalian ASIC channels shape the output of touch-sensitive afferents, rather than serve as mechanotransduction channels in these neurons.

Another useful approach for analyzing mechanotransduction mechanisms is to study sensory neurons in simplified, in vitro preparations. The cell bodies of somatosensory neurons cluster in dorsal root ganglia (DRG) and trigeminal ganglia (TG), which can be easily dissociated and cultured so that mechanically activated responses can be monitored with electrophysiology or calcium imaging. Dissociated DRG neurons possess a number of mechanically activated currents that may reflect bona fide mechanotransduction mechanisms. For example, subsets of DRG neurons can be activated by radial stretch,[44] hypotonic stimuli,[45] suction[46] and direct touch.[47] Intriguingly, Lewin and colleagues recently reported that some mechanically evoked currents in DRG neurons require extracellular tethers.[48]

Another promising model system for analyzing touch transduction mechanisms lies in a specialized light-touch receptor called the Merkel cell-neurite complex. These touch receptors cluster close to the skin's surface and each cluster is innervated by a single sensory afferent that branches multiple times, sending a single neurite to each Merkel cell. Merkel cells are thought to be required for essential tasks that rely upon fine tactile discrimination. The sensory afferent innervating the Merkel cell-neurite complex has a unique electrophysiological signature and abolishing these cells removes this neural light-touch signal.[49] Touch researchers have pioneered methods to culture and enrich Merkel cells so that their mechanotransduction mechanisms can be directly assessed. In culture, Merkel cells are activated by hypotonic-evoked cell swelling and their response involves amiloride-insensitive, calcium-permeable ion channels.[50,51] To begin the search for the underlying mechanotransduction channels, Lumpkin and colleagues investigated the gene expression profile of Merkel cells. Interestingly, several TRP channels are expressed in Merkel cells, which provide a starting point for investigating putative mechanotransduction channels.[52]

Along with the sense of touch, somatosensory neurons allow us to determine where our limbs are in space, by conveying joint angle, muscle tension and muscle length. These proprioceptive afferents include muscle spindle organs, which are responsible for conveying muscle length information. At the end of the muscle in the tendon are Golgi tendon organs, which interweave with collagen fibrils to convey information about muscle tension. Both of these afferent types provide feedback to the brain, allowing animals to protect their muscles from overstretching and to form a neural representation of their body position. Ion channels involved in these mechanisms are not known.

The Acousticolateralis System—Hearing and Balance

The cochlea, an intricate organ responsible for sound-detection in mammals, is one of the most specialized and sensitive mechanisms developed to detect mechanical force. It sits encased in the skull, with a coil of three fluid-filled chambers. The center chamber contains a fluid called endolymph, which has a high concentration of potassium. Distributed along the partition of these chambers sits the Organ of Corti, which contains mechanically sensitive hair cells. Pressure from a sound wave deforms the tympanic membrane (eardrum), vibrating the small bones of the middle ear. The vibrations are transmitted to the oval window, which pushes waves of pressure into the fluid filled cochlea. Endolymph movements cause displacement of hair bundles, clusters of specialized microvilli called stereocilia that are the site of mechanotransduction.

Stereocilia are actin-filled processes arranged in a height-dependent manner, with rows cascading down from a single microtubule-filled kinocilium. Each level of stereocilia is connected to the one above it by a spring protein strand called the tip link, which is proposed to be in series with the gating spring that opens mechanotransduction channels when hair bundles move (Fig. 2B). Although this intricate structure of the hair bundle is well understood, many of the molecular components responsible for mechanotransduction remain a mystery, however; protein components of the tip link were recently shown to be cadherein 23 and protocadherin 15.[53]

Hair cells of the cochlea share common morphology and mechanisms of action with hair cells of the vestibular system. The utricle, saccule and semicircular canals allow for the detection of gravity and provide feedback for balance. Due to common morphology and function, it is possible that all hair cells in the inner ear will employ mechanotransduction channels encoded by the same genes.

Mammalian Stretch-Sensitive Ion Channels

Although hair cells are proposed employ tethered transduction channels, stretch-sensitive ion channels have been found widely in mammalian cell types. In most cases the molecular identities of these channels is unclear; however, one class shares a notable structural feature with prokaryotic channels. MscL, MscS and MscMJ share a cluster of charged residues in their C-terminus and removal of this sequence from MscL abolishes the channel's mechanosensitivity.[54,55] A very similar sequence was found in the c-terminus of the mammalian mechanically gated potassium channel TREK-1.[9] TREK-1 is a stretch-sensitive two-pore domain potassium channel that loses mechanosensitivity with loss of its C-terminus.[56] Evidence from knockout animals suggests that TREK-1 may be important for tuning the mechanosensitivity of nociceptors, as deletion of TREK-1 causes allodynia, an increase in sensitivity to nonpainful mechanical stimuli. Intriguingly, mice lacking TREK-1 show decreased sensitivity to mechanical and thermal hyperalgesia, which indicates this channel plays a role in sensitizing nociceptors.[57] Two related potassium channels TREK-2 and TRAAK, are also stretch-sensitive.[58]

Other senses in mammals that might require stretch-sensitive channels include baroreception, bladder osmosensation and bladder voiding. The TRP channel TRPV4 contributes to osmosensation in mammals, as mice lacking this channel are not able to regulate their osmotic equilibrium as efficiently as wild-type mice.[59] Additionally, TRPV4 contributes to mechanically evoked visceral pain.[60] This channel was also found to transduce mechanical shear stress in an ex vivo carotid artery preparation.[61] In TRPV4 knockout artery tissue, there was an absence of this stress response, indicating this channel could contribute to baroreception.

CONCLUSION

The myriad mechanical senses of mammals are excellent examples of the power of evolution to shape complex sensory responses. We communicate, navigate through space and control our internal states by detecting forces inside and around us. The mystery of these abilities is slowly being uncovered, but mechanotransduction remains a ripe area for discovery.

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CHAPTER 10

THE EVOLUTION OF VERTEBRATE COLOR VISION

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Abstract: Color vision is conventionally defined as the ability of animals to reliably discriminate among objects and lights based solely on differences in their spectral properties. Although the nature of color vision varies widely in different animals, a large majority of all vertebrate species possess some color vision and that fact attests to the adaptive importance this capacity holds as a tool for analyzing the environment. In recent years dramatic advances have been made in our understanding of the nature of vertebrate color vision and of the evolution of the biological mechanisms underlying this capacity. In this chapter I review and comment on these advances.

INTRODUCTION

The fossil record suggests that photosynthetic cyanobacteria (blue-green algae) appeared at least 2.8 billion years ago,[1] and there are recent claims that organisms having the capacity to harvest light and convert its energy to alternative uses existed considerably earlier than that.[2] Since those ancient times photosensitivity has been inextricably linked to the evolution of life.[3] Vision, a complex process through which animals perceive the qualities of objects, is one of the most remarkable ways in which the process of evolution has exploited a photosensitivity capacity. Vision is initiated by the activation of photopigments, photosensitive molecules typically sequestered in densely-packed mosaics of receptor cells found in specialized structures called eyes. Much has been learned about the nature and evolution of visual photopigments in recent years. In this chapter we consider how new information about photopigments along with a developing appreciation of the linkages between photopigments and visual performance have opened a window

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to understanding the evolution of one of the most important and highly advantageous features of vertebrate sight—color vision.

DEFINING COLOR VISION

For members of a species like ours, possessors of a keen color sense, the nature of color may seem self-evident because we automatically appreciate conditions under which color is an inextricable feature of our experience—when color is an inferred property of objects and light sources—and when it is not. Despite this outward simplicity, there have long been contentious debates about the locus of color featuring alternate arguments as to whether color inheres in objects or in viewers.[4] A prevailing sentiment among visual scientists is that color is a computed inference, one derived by an active nervous system based on an analysis of the distribution pattern of light reaching the eye and evaluated in the context of previous experience. This general position was first clearly articulated by Isaac Newton when he famously pointed out that it is misled to think of light itself as being colored by noting, "for the Rays to speak properly are not coloured, in them there is nothing else but a certain Power or Disposition to Stir up a Sensation of this or that Colour".[5]

Formal examinations of color vision necessarily require technical definitions. A standard operational definition is that color vision is the capacity that allows one to discriminate between objects or lights presenting different wavelength compositions to the eye irrespective of the relative intensities of the two.[6,7] Key to this definition is the idea that while an individual absent a color vision capacity, or one possessing the capacity but operating under environmental conditions that are unfavorable for color vision, may in fact be able to make such discriminations at certain intensity relationships of the two stimuli (typically because they appear to differ along the perceptual dimensions of brightness or lightness), they will inevitably fail for other combinations. Operating within this definitional constraint makes it possible to study color vision using stringent behavioral criteria without concern as to the nature of the quality of the color experience and this strategy permits one to equally well objectively study color vision in man, monkey, mouse, or moth. It may be noted that it has recently been cogently argued that, quite beyond these basic considerations, the definition should be expanded to additionally require that an animal must also be shown capable of making color discriminations in the context of the discrimination of objects.[8]

How must biological systems be organized to support color vision? It has long been appreciated that, at minimum, two things are required: The animal must (1) possess multiple tuned photic sensors with each sensor type having a different spectral sensitivity and (2) have the neural machinery to foster a comparison of the patterns of activations of the separate sensors.[9] Although other organizations can be imagined that could fulfill these requirements, all vertebrates employ an arrangement that shares two common elements: (1) the sensors are two or more types of photopigment resident in photoreceptors giving them spectral absorption properties that are unique to each type and (2) there are downstream nerve cells whose inputs are configured such that they can contrast the magnitude of the signals generated in the various types of photopigment. Such an organization is illustrated in Figure 1 for a case involving three types of sensors and two classes of comparator elements.

It has proven to be very useful to characterize color vision according to its dimensionality. The responses of photopigments are univariant, i.e., the signals they can deliver reflect only the total quantal harvest, not the spectral distribution of incident light. A consequence of

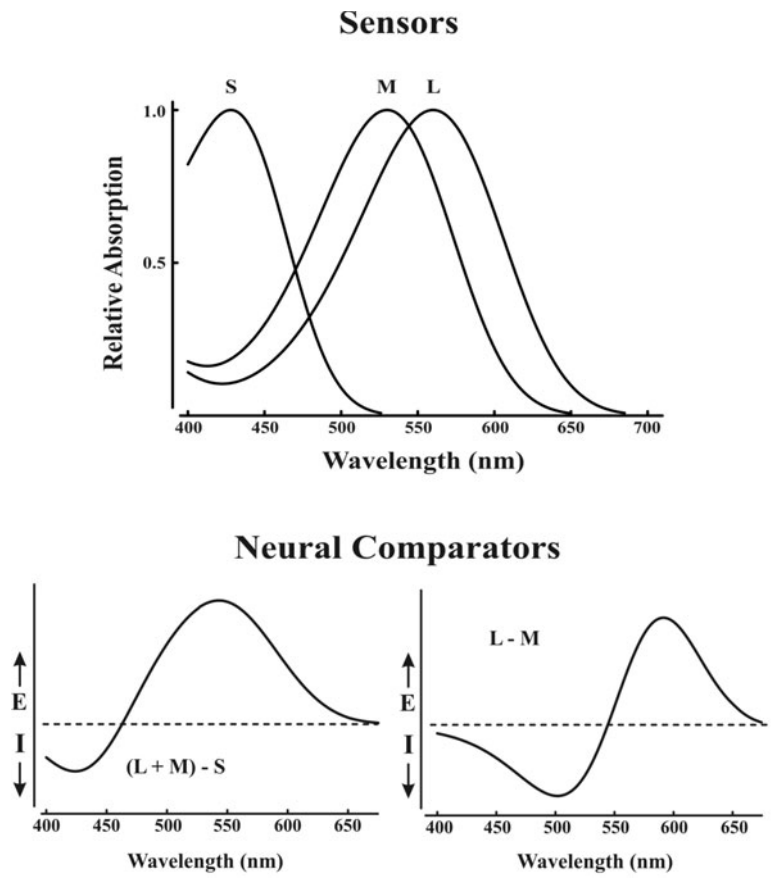

Figure 1. Two biological mechanisms required to support color vision are tuned spectral sensors and neural comparators. The tuned sensors of vertebrate color vision are two or more types of photopigments. Illustrated at the top are the absorption curves for sensors found in the human eye. These are three types of cone photopigment, conventionally designated as S, M and L. The outputs from these three sensors are combined by excitatory/inhibitory interactions in two classes of neural comparator units (bottom). There response patterns represent the subtractive combinations of signals derived from the cone types the nature of the combinations being indicated. [E = excitation; I = inhibition] In general, each type of comparator element is capable of supplying the information required to support one dimension of color vision.

this, in line with the above definition, is that an animal having only a single photopigment necessarily lacks color vision (they are called monochromatic). A comparison of the signals derived from activation of two classes of photopigment can yield a single dimension of color vision, a condition referred to as dichromatic color vision. If an additional comparison is made between two other photopigments (or combinations thereof) a second dimension of color vision emerges and such individuals are said to have trichromatic color vision (Fig. 1). Dimensionality of color vision can escalate beyond this—for instance, adding a fourth class of pigment to the array shown in Figure 1 (along with an additional neural comparator element) adds another dimension to the emergent color vision (it becomes tetrachromatic). In general, each added dimension significantly expands the animals' abilities

to make more subtle color discriminations; for example, one theoretical estimate suggests that a shift from dichromatic to trichromatic color vision may escalate the total number of surface colors that an animal can discriminate from around 10,000 to over 1 million.[10]

THE UTILITY OF COLOR VISION

The evolution of color vision requires the emergence of the requisite biological machinery, i.e., the appropriate photic sensors and neural comparators. In addition, of course, the new capacity must provide the organism with some survival advantage. Over the years there have been extensive discussions of the advantages that might be linked to the presence of a capacity for color vision.[11-13] The most general conclusion is that color vision serves to significantly enhance the visibility of objects in the animal's visual world. There are a number of different aspects encompassed in this use of the word "visibility". As a simple illustration of these, consider the example of a hungry monkey foraging through a tropical forest. From the monkey's perspective a distant tree laden with fruit may be perceived as standing out in a sea of other fruitless trees. Why is that? Physically, the fruit will almost certainly have a different total surface reflectance than the foliage that surrounds it and thus it may be perceived as being lighter or darker than the foliage depending on direction of the difference in reflectance. Brightness differences such as these may serve to help make discriminations, but such cues can be ambiguous since they can vary enormously dependent on illumination conditions (e.g., the presence of clouds or sunlight) and viewing circumstances (e.g., viewing the tree through an intervening screen of foliage). An animal with color vision however can additionally exploit the fact that the fruit also has different spectral reflectance properties than the foliage and that difference can be used to produce a percept of color variation. These spectral difference cues are relatively consistent irrespective of illumination/viewing conditions and thus can provide much more reliable information. In short, one thing color vision does is to enhance object detection. As the monkey approaches the tree, the actual nature of the color differences among the fruits may also come into play, as for instance toward the goal of discriminating between fully ripe and partially ripe fruits. This second aspect of the use of color vision corresponds roughly to what may be called object discrimination. Quite beyond these basics properties of detection and discrimination, primates (and probably many other vertebrates) also use color as a means of object categorization, a process that an organism may develop as result of experience. This capacity allows color to convey a particular significance and examples of its utility abound; for example, the use of skin coloration as indicators of sexual receptivity or health. The actual utility of color vision is obviously species and circumstance dependent but in general, as these examples have suggested, the addition of a color capacity serves to substantially enhance the ability of an animal to successfully cope with its environment thus promoting its evolution.

OPSIN GENES AND PHOTOPIGMENTS

Visual pigment molecules consist of a G-protein-linked transmembrane protein, an opsin, that is covalently bound to a chromophore (either 11-*cis* retinal or 11-*cis*-3, 4-dehydroretinal) by means of a Schiff base linkage. Photon capture isomerizes the chromophore from an 11-*cis* to an all-*trans* form and that conformational change in the

protein is converted, via a multi-stage phototransduction cascade,[14] to a signal that can be communicated to other neurons positioned along the visual pathway.[15]

The spectral tuning of photopigments is controlled by variations in the amino acid sequences of the opsins. Consequently, since they were first cloned some twenty-five years ago,[16] opsin genes have been the focus of intensive study with the result that at least 500 opsin genes obtained from ~180 vertebrate species have now been characterized.[17] Phylogenetic analysis indicates that opsin genes can be divided into five paralogous groups. The photopigments specified by these genes are spectrally positioned such that they have their peaks of maximum absorbance (λ_{max}) in different portions of the spectrum. One family (Rh1) yields pigments expressed in rod photoreceptors, while the other four (LWS, Rh2, SWS2 and SWS1) specify pigments found in cones. Although there are scattered exceptions, rods generally underlie dim light (scotopic) vision while signals from cones support daylight (photopic) vision, including color vision. The total λ_{max} ranges for pigments drawn from each of the five groups for cases where 11-*cis* retinal is the chromophore is indicated in Figure 2 (top). Taken as a group, such vertebrate photopigments may have λ_{max} values that can vary from ~360 nm to 565 nm.

Although their length is somewhat variable across species, opsins are comprised typically as a transmembrane polypeptide chain of around 350 amino acids. Of these, residues at only a relative handful of positions have been found to be critical for influencing the spectral positioning of the photopigment. For example, a total of nine amino acid replacements can account for all of the λ_{max} variations in Rh1-specified photopigments[17] while five amino acid substitutions suffice to capture the full range of variations detected in LWS photopigments.[18] The relative conservatism of spectral tuning of opsins has two important consequences: (1) photopigments are quantized in the sense that their spectral positions occur at stepped locations across the spectrum,[19] and (2) sequence analysis of novel opsin genes can now be used to provide strong inferences about the spectral absorption properties of the pigments they specify.

Although there is a tight linkage between opsin structure and the absorption properties of photopigments, the spectral sensitivity of photoreceptors can be modified significantly by several other features of visual system organization. Two of these merit mention. First, differences in the inherent structure of the two chromophores used to construct vertebrate photopigments cause shifts in the spectral absorption properties of the photopigment such that the absorption spectrum of a pigment comprised of a given opsin bound to the 3-dehyroretinal chromophore is shifted toward the long wavelengths relative to that for the same opsin now linked to the 11-*cis* retinal chromophore.[20] The magnitude of this effect can be substantial; for example, the longest λ_{max} value for a retinal-based pigment is at ~565 nm while λ_{max} value for the corresponding 3-dehydroretinal pigments extends out to ~615 nm. In general, again with some scattered exceptions, the 3-dehydroretinal chromophore is characteristic of the photopigments of freshwater species of fish, amphibians and reptiles while 11-*cis* retinal is commonly found in photopigments for those species that inhabit marine and terrestrial environments and, importantly, it is routinely found in all of the photopigments of birds and mammals.[21]

A second modifier of the spectral sensitivity of photoreceptors is the presence of ocular filters. Such filters serve to condition the light incident on the photoreceptors and they come in a wide variety of forms including pigmentation sequestered in the cornea, the lens, the retina and in the tissue lining the eye behind the photoreceptors.[22] Most of these devices act as long-pass filters preferentially absorbing short-wavelength light. For their potential to impact color vision the most important of these filters are the

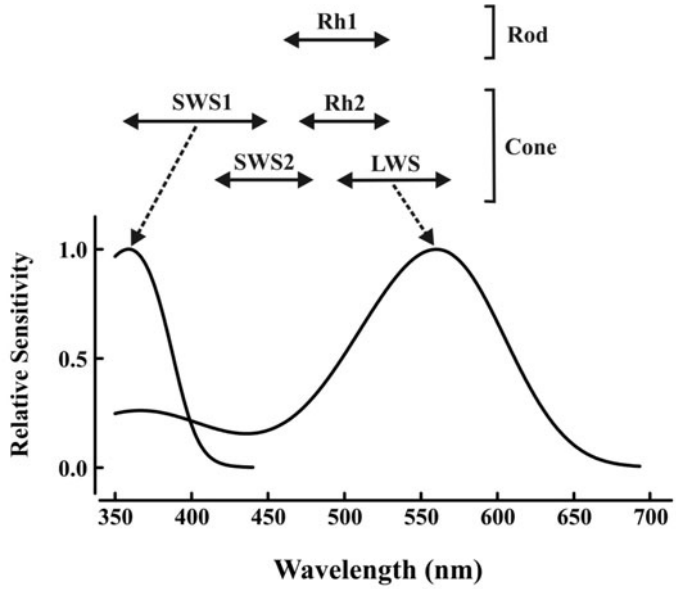

Figure 2. Opsin genes from five families specify all vertebrate photopigments with one family providing rod pigments while genes from the other four families yield cone pigments. Sequence variations in the opsins spectrally tune the photopigments so that the spectral locations of maximum absorbance fall within the range of the double arrow lines shown at the top of the figure. The ancestor of all eutherian mammals is believed to have had two types of cone pigment drawn respectively from the SWS1 and LWS gene families with the absorbance properties sketched at the bottom of the figure.

so-called oil droplets found in the inner segments of cone photoreceptors in many birds, reptiles and some amphibians. The photoreceptors in the retinas of such species can be paired with any of several types of such oil droplets that vary in the spectral location of their long-wavelength cutoff. The effect of these oil droplets is to narrow the absorption spectrum of the resident photopigment, thus lowering its overall sensitivity to light and shifting its maximum sensitivity toward the longer wavelengths. On theoretical grounds it has been argued that the presence of oil droplets in such species may serve to enhance their ability to discriminate among colors.[23] Direct evidence on that possibility is still scarce, although early experiments comparing color vision in conspecific birds that did or did not have colored oil droplets do suggest that color vision of the latter may be somewhat degraded.[12]

LINKING OPSIN GENES AND PHOTOPIGMENTS TO COLOR VISION: A CAUTION

In extant species the interrelationships between opsin genes, photopigments and color vision can be evaluated directly by measuring all three and in the relatively small number of cases where that has been accomplished the three link in the manner just described. That success has fostered the view that studies of the phylogeny of cone opsin genes can be used to provide strong insights into the color vision enjoyed by ancestral

vertebrates, enough so that it has now become routine for vision scientists to talk about where in vertebrate evolution various types of color vision emerged or disappeared. In the discussion below I will largely follow that tradition. Even while doing so, however, it is important to be aware that while change in opsin gene complements may provide strong suggestion of color vision change they are not, by themselves, entirely compelling. For one thing, whereas the presence of at least two cone pigments is a necessary condition for the emergence of color vision, it is not sufficient by itself since, as noted above, a means of comparing photon capture in receptors containing the different cone types is also required. At the present there are no consistent genetic markers that can be used to trace the phylogeny of such comparator mechanisms. In addition, opsin genes are not in themselves informative about the retinal distribution of the various cone types or of their overall numbers. Both of these features are critical for supporting efficient extraction of color information. In sum, although tracing the phylogeny of opsin genes has provided a window through which the evolution of color vision may be viewed, one should keep in mind the fact that the view so derived still lacks some critical details.

THE BEGINNINGS OF VERTEBRATE COLOR VISION

Photoreceptors are believed to have appeared prior to the divergence of protostomes and deuterostomes, an event that occurred some 600 MYA.[24] To gain insight into the prospects for color vision in the early vertebrates, scientists have relied on comparative studies. Of particular interest are examinations of contemporary agnathans (jawless fish), primitive creatures that stand as relatively unchanged representatives of lineages that diverged from all other vertebrates at a point early in vertebrate evolution. There are two groups of such animals—hagfishes and lampreys. Hagfish eyes are small and buried beneath a skin overlay, perhaps serving not to support vision but rather to function as some sort of a circadian organ.[24] Interestingly, at metamorphosis the lamprey eye changes from one similar to that of a hagfish to one that appears to be a quite typical image-forming vertebrate eye. Although there are significant variations in the arrangements found in various contemporary lampreys, as well some uncertainties as to the distinctions between rods and cones in these animals,[21] in one well-studied species of southern-hemisphere lamprey (*Geotria australis*) genes representative of all five of the opsin gene families (Fig. 2) have been detected.[25] Since this lineage is believed to have diverged from that leading to jawed vertebrates in excess of 500 mya this would imply that the pigment basis for an elaborate color vision system was already present in very early vertebrates.[26]

What can one infer about color vision in early vertebrates from the presence of multiple types of cone pigment? First, it is worth noting that the presence of multiple cone pigments could, at least theoretically, provide visual advantage entirely in the absence of any mechanisms for producing color vision. That's because the absorption bandwidths of photopigments are limited (note Fig. 1) and, consequently, simply to expand the total extent of the spectral sample necessarily requires the addition of new photopigments with displaced λ_{max} values. Thus it is conceivable that the early opsin gene duplications that led to new photopigment types may have evolved, at least initially, entirely in the absence of means for extracting color information.

Assuming color vision did emerge in early vertebrates coincident with the presence of multiple types of cone photopigment what role(s) may it have played? Early vertebrates are believed to have lived in brightly-illuminated shallow-water environments. Maximov[27]

noted that light passing through surface ripples in shallow water produces significant luminance flicker that could serve to interfere with accurate visual detection of looming predators. He suggested that a neural comparison of signals from two or more classes of spectral receptors (as in the arrangement sketched in Fig. 1) is one possible mechanism that can be employed to diminish the visual noise inherent in such flicker and that utility alone might have supported the evolution of color vision. Whether vertebrate color vision initially evolved for such a purpose is not known, but at any rate it seems quite certain that the potential for color vision was present in our earliest vertebrate ancestors. Maintenance of a color vision capacity over the long span of time since then provides strong testament to its enduring utility.

TYPES OF CHANGE IN THE EVOLUTION OF VERTEBRATE COLOR VISION

If the inferences drawn from the study of opsin genes outlined above are correct, the earliest vertebrate representatives possessed four classes of cone pigment that could have supported a rather elaborate color vision sense—potentially, a tetrachromacy. The development of phylogenies for cone opsin genes, viewed in conjunction with an expanded understanding of color vision among contemporary vertebrates, suggest several types of change that may occur during the evolution of vertebrate color vision. Here we identify and comment on these potential changes.

The four classes of cone pigments of the early vertebrates are believed to have originated in their ancestors, probably as a result of whole genome duplications.[28] Although apparently some species of fishes may constitute exceptions to this generalization,[29] most contemporary vertebrates express no more than four classes of cone pigment suggesting that over the long history of vertebrate evolution there has been no further expansion beyond those found in the earliest vertebrates. If, as noted above, adding successive dimensions of color vision can expand the possibilities for discriminating color one may wonder why that number has not been increased even further. There are at least two factors that may explain why no vertebrate retina seems to need more than four classes of cone pigment. First, based on theoretical calculations it appears that three or four types of cone photopigment, appropriately spaced along the spectrum, suffice to efficiently sample the full spectrum of light available to support the vision of most veretebrates.[30,31] If that conclusion is correct, no advantage would accrue from adding more types of photopigment than were available in ancestral vertebrates. Second, a broad range of evidence shows that nervous systems are very energetically expensive (for example, in the blowfly a full 8% of its total resting metabolic rate is consumed by the retina[32]) and so the emergence of excess capacity of signal processing likely incurs severe adaptive penalties.[33] Color vision may well qualify in this way because the generation of a new dimension of color vision requires not only the maintenance of an additional class of pigment but also the presence of many neural comparator elements to permit the extraction of new color information and, very likely, also a significant expansion of the central nervous system to support the abilities resulting from the exploitation of this added information. Given these costs, it may well be that selective pressures operate to limit the expansion of color processing capabilities.

It should be noted that if there are adaptive factors that limit the evolution of the number of vertebrate cone pigments so as to not exceed four, even that arrangement still

allows very high sensitivity to spectral variation; for example, in some parts of the visible spectrum trichromatic humans have been shown to be capable of reliably distinguishing wavelength differences amounting to well less than 1 nm![34] Although few contemporary species seem to have gone beyond the four cone pigments already present in the early vertebrates, many contemporary lineages feature fewer than four types of cone pigment. In some cases the mechanisms that have led to a decrease in the number of pigment types have been well analyzed, as will be described below.

The most common type of change to occur during cone pigment evolution involves shifts in the spectral absorption properties of the pigment. This has frequently been accomplished by residue changes in the opsin. For example, it has been inferred that the ancestral SWS1 pigment peaked in the ultraviolet, at about 360 nm[35] (Fig. 2, bottom left), but in many eutherian mammals this pigment has been peak shifted toward the longer wavelengths, often resulting in a change in the λ_{max} of the pigment by as much as 60-70 nm. In some well-studied cases that shift is explained as resulting from the change of a single amino acid change, a substitution of Tyr for Phe.[36] Quite a number of other examples of similar kinds of changes have also been documented.[17] Although in many cases the mechanics of such changes in spectral absorption properties of cones can be understood, there are virtually no instances in which the functional utility associated with that change has been confidently identified.

Most vertebrate retinas contain a mixture of rod and cone photoreceptors. Whether cones or rods evolved first was topic often debated in the past, but the clear weight of contemporary opinion is that cones were the primordial vertebrate photoreceptor with rods only appearing later in support of the specialized function of vision under low light levels.[37] As noted above, rod opsins are specified by genes from the Rh1 family and it is believed that this family of genes emerged by a duplication of ancestral Rh2 cone opsin genes.[38] The fact that rod receptors are the more derived makes sense in light of the fact that they are very highly specialized cells being configured so as to be capable of transducing and signaling the energy captured from a single photon and then become functionally saturated when they are irradiated with only very modest amounts of light.[39] Among the vertebrates there are enormous species variations in the relative representation of rods and cones; for example, rods comprise 99% of all photoreceptors in the rat[40] while making up fewer than 10% of the receptor complement of the tree shew.[41] These variations in rod/cone ratio roughly track the photic specialization of species, ranging from nocturnal to diurnal respectively.

Although rods do not play a primary role in determining the nature of color vision, the relative representation of rods and cones can be important in the sense that retinas that have relatively sparse populations of cones appear in animals in which the salience of color as a cue for guided behavior is relatively reduced. Changes in the rod/cone mixture can occur quite rapidly during evolution. One example of such change has been documented for the owl monkey (*Aotus*), the only nocturnal anthropoid. This primate has a heavily rod-dominated retina yet it evolved from fully diurnal ancestors quite recently, probably no more than about 15 mya.[42] Recent work provides some insights into how such rapid changes in the rod/cone mix might occur. One such possibility derives from the observation that the temporal ordering of neurogenesis of the various cell types in the mammalian retina is quite consistent across taxa and there is evidence that simply shifting the timing of the cell cycles will produce large changes in the rod/cone ratio as well as several other features usually associated with differences between diurnal and nocturnal eyes.[43,44]

OPSIN EVOLUTION IN VERTEBRATES: THE BIG PICTURE

With the above general principles and caveats in mind, we now turn to some of the ideas that have emerged regarding the evolution of color vision in species representing the major vertebrate groups. The principal information available for this task comes from analyses of the evolution of opsins. This general topic has been the subject of many recent reviews which the reader may consult to gain entry to what has by now become a large literature.[9,21,45-50]

The five opsin gene families arose through a series of gene duplications. Although many details of the history of those events remain murky, the general outline of the process is reasonably clear. A phylogeny of the five families of opsin genes illustrated for a few contemporary representative species from each of the major vertebrate groups is given in Figure 3. The gene duplications that initially gave rise to the five groups were ancient events that occurred early in the history of vertebrates (as noted above, the four families of cone opsin genes are believed to have emerged by at least 540 mya while the family of rod opsin

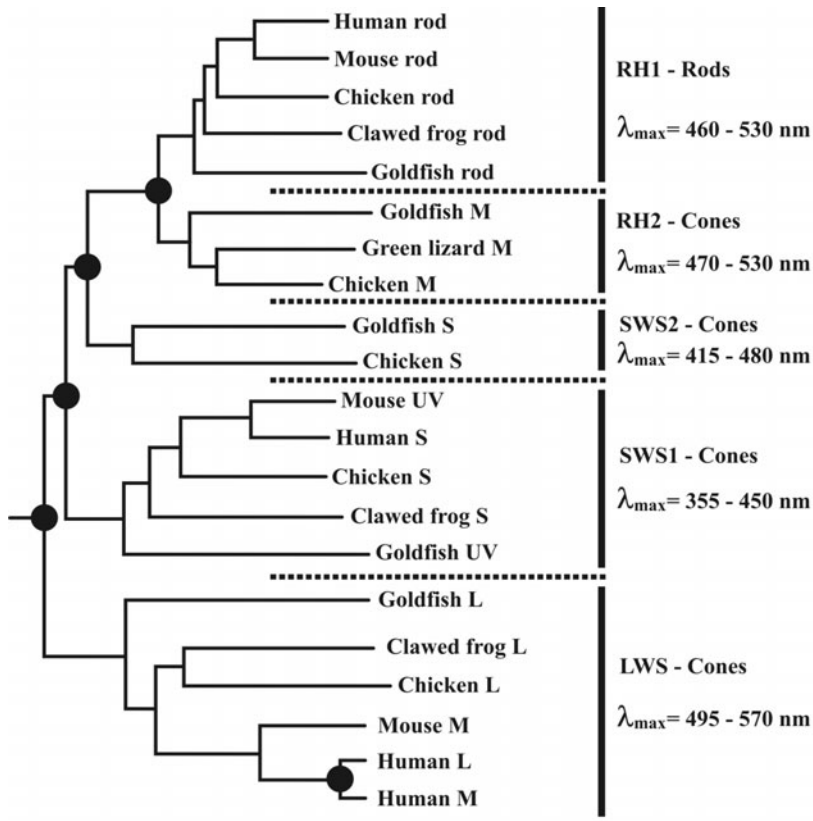

Figure 3. Phylogeny of the five vertebrate opsin gene families. Shown are a few representative species drawn from the major vertebrate groups.[21] The solid circles indicate where gene duplications are believed to have occurred, the four at the left of the figure all having been accomplished at times that were early in vertebrate history. Further details are provided in the text.

genes (Rh1) probably appeared shortly thereafter). An additional opsin gene duplication occurred in the primate lineage much more recently. Most modern diurnal reptiles, birds and shallow-water teleost fishes express at least one gene from each of the four cone opsin families (as illustrated in Fig. 3). At the same time, representatives from one or more of these gene families have been lost in primitive fishes, amphibians and mammals.[21] As an example of such evolutionary changes we now turn to consider in more detail the story of the evolution of mammalian cone opsins and its implications for color vision.

EVOLUTION OF MAMMALIAN CONE OPSINS AND COLOR VISION

Mammals emerged during the early Jurassic, somewhere around 200 MYA. In line with the account given about, the progenitors of these early mammals very likely had cone pigment representation drawn from each of the four cone opsin gene families (Fig. 4). Most authorities agree that the early history of mammals was characterized by a prolonged period of time during which these animals were principally nocturnal.[51] Perhaps because of that association, all mammals have retained representation from the Rh1 opsin gene family and thus share in common the presence of rod pigments. Over this same period a variety of changes have occurred in the complements of the cone opsin genes. First, there appears to be no representation of viable Rh2 genes in any contemporary mammals implying that this gene family was lost at a point prior to any significant divergences in mammalian evolution. At the same time, all contemporary mammals retain representation of genes belonging to the LWS cone opsin gene family. Contemporary monotremes (such as the platypus and the echidna) retain SWS2 derived opsins and have a pseudogene from the SWS1 family.[52,53] SWS2 genes are absent from both marsupials and eutherian mammals, but each of these groups have SWS1 genes (Fig. 4).[48]

Estimates of the spectral properties of the cone pigment are available for some contemporary monotremes and marsupials. Inferences drawn from gene structure indicate that monotremes have two types of cone pigment with respective λ_{max} of 451 and 550 nm.[52] This arrangement would allow for dichromatic color vision, but whether that capacity has been realized remains to be determined. The cone pigments of contemporary marsupials

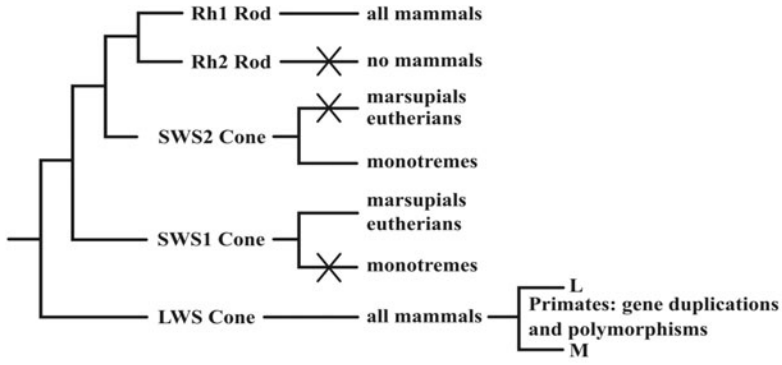

Figure 4. A schematic to illustrate the pattern of pigment loss and retention believed to have occurred during the evolution of mammals. The Xs indicate the loss of pigment types due to gene loss. The pattern shown here is elaborated in the text.

are more varied. The tammar wallaby (*Macropus*) has two types of cone pigment that have been shown to support a dichromatic color vision capacity.[54,55] By contrast, some other marsupial species appear to have three types of cones and behavioral tests suggest they can use these to support a trichromatic color vision capacity.[56,57] Curiously, however, it appears that the third class of cone found in the retinas of these animals may not contain a cone pigment, but rather expresses a rod pigment in a cone photoeceptor.[58] In sum, we do not yet have a completely clear picture of the distribution and nature of color vision among the monotremes and marsupials.

Eutherians comprise the vast majority (~95%) of all contemporary mammals. All of these species share in common the fact that their cone pigments are specified by only two of the cone opsin gene families—SWS1 and LWS (Fig. 4). Examination of cone opsin gene phylogenies suggests that ancestral eutherians had two types of cone pigment with respective peak values of ~360 nm and 560 nm (Fig. 2, bottom) and thus they had the photopigment basis for dichromatic color vision.[59,60] Most contemporary eutherians have retained this basic pattern deriving, respectively, a single-type of cone pigment from the SWS1 and LWS gene families. This pattern holds for all of the common domestic animals (dog, cat, cattle, pig, sheep, etc.) and many others as well so that this arrangement encompasses, in total, at least some species drawn from each of thirteen orders.[50] Overlaid on this commonality are considerable species variations in the spectral positioning of the pigments derived from the SWS1 and LWS gene families so that they cover the spectral ranges shown in Figure 2 and, as noted, much variation in the relative mixtures of retinal rods and cones. Both of these features have important implications for color vision, issues that have been considered in detail elsewhere.[61,50]

Although most eutherians derive a short-wavelength sensitive photopigment from genes of the SWS1 family, this association is not universal. About fifteen years ago it was discovered that two species of nocturnal primates fail to express a short-wavelength sensitive pigment.[62,63] Since this loss leaves them with only a single-type of cone pigment, they must by definition also lack a capacity for color vision. An examination of the opsin genes in these animals revealed that the absence of S cones resulted from the presence of mutatational changes in these genes that result in their being unable to express viable pigment, i.e., they have become pseudogenes.[64] Since the original discovery of such loss a number of other species have been found to similarly lack viable S cones, the list now includes various rodents, primates, cetaceans and carnivores.[50,65] Such losses are particularly striking among marine mammals in that every species of cetacean (whales) and pinniped (seals, etc.) is without S cones.[66,67] In each of these cases the loss of this cone class can be traced to mutational changes in the S-cone opsin genes.

The transition of functional genes to pseudogene status is usually said to occur when the function(s) the gene supports becomes dispensable.[68] The eutherian species that have lost S cones in this fashion represent a highly diverse lot being often only very distantly related and sharing little in the way of dominant lifestyles. The one common element that does unite most of these animals is that they are predominantly nocturnal. Nocturnal species are most often behaviorally active under conditions where the ambient light level is too low to support much in the way of color vision. Since the principal contribution of S cones is to support a dimension of color vision, it is possible to argue that the loss of S cones would only minimally impact nocturnal visual capacities and perhaps that is reason why such species have frequently abandoned their S cones. On the other hand, that argument may well be too facile and there is at least some suggestion that other reasons will eventually be found to underlie the loss S cones and color vision in some

eutherian mammals.[69] For the present we can do no better than conclude that we still lack a completely satisfactory explanation for this gene-driven loss of a cone-pigment type and with it the behavioral functions it supports.

PRIMATES—EXCEPTIONS AMONG MAMMALS

So far as we now know all contemporary eutherian mammals have either maintained the basic two-cone-pigment arrangement and have dichromatic color vision or, like those species just described, have lost a potential short-wavelength pigment through mutational changes to the SWS1 opsin gene and thus lack color vision. The primates present striking exceptions to these patterns.

Although the topic is one of ongoing debate, it appears that the last common ancestor of modern primates dates to ~77 mya.[70] Since that time a series of lineage divergences have occurred to yield the ~350 species of extant primates. For our purposes these animals can be divided into three major groups: catarrhines (humans, apes, Old World monkeys), platyrrhines (New World monkeys) and the more primitive strepsirrhines (lemurs, bush babies, etc.), the latter having diverged from the other two groups very early in primate evolution.[71] It is generally believed that early primates were nocturnal and probably therefore had the two-cone pigment arrangement common to most eutherian mammals. Several significant changes in opsin gene/cone pigment arrangements subsequently occurred in primates that served to render them much more visually competent under daylight illumination. These alterations included some interesting expansions in their color vision capacities.

Mammalian LWS genes map to the X-chromosome while SWS1 genes are autosomal (chromosome 5).[72] Among the mammals, catarrhine primates are exceptional in having more than one LWS-derived gene on the X-chromosome. That difference resulted from a duplication of the LWS gene that occurred at a point close to the base of the catarrhine radiation, perhaps 30-40 mya.[73] The two genes emerging from this duplication are positioned in a head-to-tail tandem array on the q-arm of X-chromosome. The added LWS gene specifies a second-type of cone pigment whose peak maximum absorbance is shifted further toward shorter wavelengths than was the pigment specified by the progenitor gene. Together, the two are generally referred to as M and L pigments and they have respective λ_{max} values of about 530 nm and 562 nm; these two are illustrated in Figure 1. The gene duplication event thus provided catarrhine primates with a third-type of cone pigment and allowed them to achieve an additional dimension of color vision—they became trichromatic. Although there are some minor species differences,[74,75] all of the contemporary catarrhines have effectively identical trichromatic color vision.

The evolution of cone opsin genes and color vision in New World primates is strikingly different. With only two known exceptions, all of the monkeys in this group display color vision polymorphisms such that some members of a species have trichromatic color vision while other individuals are dichromatic and, beyond that, there are significant variations in the nature of color vision found within each of these broad categories.[76] Key to understanding the nature of this polymorphism was the observation that whereas female platyrrhine monkeys may have either dichromatic or trichromatic color vision all males are dichromatic.[77] This fact predicted the presence of an X-chromosome opsin gene polymorphism and that turns out to be correct.[78,79] Specifically, these New World monkey species usually have polymorphic X-chromosome opsin genes (typically, three) each of which specifies a cone pigment with λ_{max} somewhere in the range of 535 nm to 562 nm.

Since males have only a single X-chromosome this limits them to having a total of two cone pigments, one based on an SWS1 cone pigment and the other one of the three polymorphic X-chromosome specified pigments yielding a total of three different forms of dichromatic color vision. Females who are homozygous at the X-chromosome opsin gene locus similarly have a total of two cones pigments and dichromatic color vision. However, random X-chromosome inactivation, a process that occurs during early embryonic development, provides heterozygous females with two spectrally separate M/L pigments that get segregated into different cone types and this allows them to achieve trichromatic color vision. This polymorphic arrangement yields three types of trichromatic color vision among females and there are thus a total of six distinct forms of color vision within the species. Since most New World monkeys live in social groups, this pattern of cone pigment inheritance provides an most intriguing problem: How do conspecific animals solve a lifetime of shared visual problems while employing strikingly different sets of visual capacities?

Within the general polymorphic context shared by almost all platyrrhine monkeys some additional important variations have been discovered. First, although many species share in common the three-allele arrangement described above, other species feature only two X-chromosome opsin gene alleles.[80] This difference has visual implications as those species with two alleles have reduced variations in their color vision, both quantitatively and qualitatively. Second, even species that share the same number of alleles may not share the same sets of M/L pigments. Third, the cone pigment/color vision arrangement found in two genera of platyrrhine monkeys departs from the polymorphic theme. One of these is the howler monkey (*Alouatta*) which features an arrangement similar to that described for the catarrhines, i.e., two separate X-chromosome genes specifying spectrally discrete M and L cone pigments. This allows them to also achieve species-wide trichromacy.[81] One important implication of this arrangement is that an event similar to the X-chromosome opsin gene duplication that occurred early in catarrhine evolution must have occurred independently and at a much later date in a single platyrrhine lineage.[82] Finally, we have already noted that some nocturnal primates have lost a functional SWS1 gene and thus have been reduced to monochromacy. This occurred only once among the platyrrhine monkeys—in the only anthropoid genus that is nocturnal, the owl monkey (Aotus).[62,64]

Photopigments, opsin genes and color vision have been much less intensively investigated in the strepsirrhines, those animals making up the generally much more primitive third branch of the primate family. Three different arrangements have been discovered thus far, each of which is effectively the same as one of the several color vision arrangements already described.[74] Some of the nocturnal strepsirrhines, the bush baby (*Otolemur*) being one example, have a mutated and nonfunctional S-opsin gene and a single X-chromosome LWS gene yielding only a single class of cone pigment and monochromatic color vision.[64] Others follow a pattern most like that characterizing many nonprimate mammals—a single-type of S-cone pigment and a single-type of LWS-cone pigment. A common example of this occurs in the ring-tailed lemur (*Eulemur*).[83] A third variant is similar to the polymorphic opsin gene/color vision arrangement just described as occurring commonly in platyrrhines. Such animals, the Sifaka (*Propithecus*) being one example,[84,85] have not yet been subject to very detailed behavioral studies of color vision but this gene/pigment arrangement implies a within-species mixture of dichromatic and trichromatic individuals similar to that seen in the platyrrhine monkeys. How all these various cone pigment/color vision arrangements evolved in the strepsirrhines remains very much a topic for future study.

CONCLUSION

Eyes are present in some 95% of all contemporary animals.[86] These organs vary strikingly in their configurations, but they share in common a first-stage mechanism for light reception—a light-sensitive transmembrane protein, an opsin, containing a retinal chromophore. The enormous increase in our understanding of opsins and of the genes that specify them that has emerged over the past 20 years has in turn revolutionized our understanding of how vision evolved. In this chapter we have seen how the presence of multiple types of opsins in an eye, in conjunction with the appropriate neural machinery, has been exploited to yield color vision in vertebrates. Multiple types of opsins appeared very early in vertebrate history and it is probable that event triggered the emergence of color vision. The recent development and refinement of opsin gene phylogenies has revealed a vertebrate history that is replete with gains, losses and transformations of photopigments and these have been accompanied by corresponding changes in color vision. Among the mammals, primates stand out for having added significant new color capacities such that many primate species and individuals have gained a new dimension of color vision and, as a result, have also gained a much more elaborate and useful color world.

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CHAPTER 11

TRANSFORMING THE VESTIBULAR SYSTEM ONE MOLECULE AT A TIME:

The Molecular and Developmental Basis of Vertebrate Auditory Evolution

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Abstract: We review the molecular basis of auditory development and evolution. We propose that the auditory periphery (basilar papilla\organ of Corti) evolved by transforming a newly created and redundant vestibular (gravistatic) endorgan into a sensory epithelium that could respond to sound instead of gravity. Evolution altered this new epithelia's mechanoreceptive properties through changes of hair cells, positioned the epithelium in a unique position near perilymphatic space to extract sound moving between the round and the oval window, and transformed its otolith covering into a tympanic membrane. Another important step in the evolution of an auditory system was the evolution of a unique set of "auditory neurons" that apparently evolved from vestibular neurons. Evolution of mammalian auditory (spiral ganglion) neurons coincides with GATA3 being a transcription factor found selectively in the auditory afferents. For the auditory information to be processed, the CNS required a dedicated center for auditory processing, the auditory nuclei. It is not known whether the auditory nucleus is ontogenetically related to the vestibular or electroreceptive nuclei, two sensory systems found in aquatic but not in amniotic vertebrates, or a de-novo formation of the rhombic lip in line with other novel hindbrain structures such as pontine nuclei. Like other novel hindbrain structures, the auditory nuclei express exclusively the bHLH gene *Atoh1*, and loss of *Atoh1* results in loss of most of this nucleus in mice. Only after the basilar papilla\organ of Corti evolved could efferent neurons begin to modulate their activity. These auditory efferents most likely evolved from vestibular efferent neurons already present. The most simplistic interpretation of available data suggest that the ear, sensory neurons, auditory nucleus, and efferent neurons have been transformed by altering the developmental genetic modules necessary for their development into a novel direction conducive for sound extraction, conduction, and processing.

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INTRODUCTION

Evolution has shaped the myriad of life forms that we see on the planet today. Changes over time in morphological structure are a result of changes in gene expression and action during development whereas the maintenance of such developmental programs is the result of selection. Alterations in morphology may relate to modifications of pre-existing structures (hymandibular bone to stapes) or can be regarded as 'new,' forming without any obvious precursor such as limbs, jaws, or the vertebrate ear. A clear definition of what novel tissues are will ultimately be dependent upon our ability to asses at the molecular level the developmental pathways that give rise to these structures given that most developmentally relevant transcription factors are highly conserved.[1] Molecular origin and their cellular specification events may predate the morphological appearance of a particular structure making it nearly impossible to determine exactly when a novelty first appears in evolution: (1) Is it when the ortholog of an important transcription factor for a morphologic novelty is first detected? (2) Is it when the cellular specification cascade is in place that guides the main known aspects of development of the morphological novelty? (3) Is it when all details of the developmental cascade are in place but have not yet achieved unique co-expression to develop the morphologic specialty? A good example of this problem can be found in the formation of major germ layers during development. Coelenterates have been shown to lack a morphologically defined mesoderm layer and are referred to as diploblasts or two-layered animals. However, more recent molecular evidence suggests that this layer is also specified in this group, but is less obvious.[2]

As partially outlined above, there are two basic mechanisms in which a morphological novelty can evolve: (1) An already present structure is no longer needed in its function resulting in loss of selective pressures upon it. (2) A structure multiplies or enlarges creating an additional new structure without a functional constraint. An old structure developing a new identity and role has been extensively researched in comparative studies dealing with the evolution of the middle ear. Reichert[3] (1837) and Gaupp[4] (1898) originally suggested that the hyomandibular bone, used by anamniotic vertebrates as a support of the jaws, has been adopted to become a portion of the middle ear ossicle(s) in tetrapods. This was subsequently validated and it was shown that the hyomandibular bone was independently freed three times to become a newly devised structure.[5,6] In ratfish it is thought that the hyomandibular bone regressed to its previous function as a support for the first gill arch.[7,8] In most extant lungfish the hyomandibular bone is reduced in size and serves an unknown, if any, function.[7,8] This regression is most likely the result of the hyomandibular bone becoming a near functionless, vestigial structure like that of the hind-limb in cetaceans.[9] In modern tetrapods the hyomandibular bone became situated between the tympanic membrane and the oval window. In this location the hyomandibular bone is able to transmit vibration of the tympanic membrane to the inner ear. These modifications are the result of developmental genetic changes in the neural crest cells that form the anlage of the hyomandibular bone. For this to occur there had to be a loss of ancestral functional constraints in the utility of the hyomandibular bone. The addition of a bone structure to facilitate this transmission of sound induced tympanic membrane movement eliminated the impedance mismatch that occurs with hearing in an air environment, only needed after the water to land transition was completed and airborn sound was mostly reflected at the oval window before entering the fluid filled inner ear.

Multiplication and diversification is most often associated with gene evolution. A gene can become duplicated through action of a transposase, copy number variation, or genome duplication. When an entire gene including the coding sequence and cis-regulatory elements are duplicated the two copies become redundant with one another. The DNA enocoding this new gene can either degenerate (become a pseudogene) due to lack of selective pressure, both genes can be retained and become alternately regulated with each gene taking on part of the role of the original, or the new gene can become altered and serve a new function. The same processes can influence a novel structure within the animal formed though interactions of multiple genes.

One of the outstanding achievements of evolution has been the development of senses to perceive our environment, the specialized sense organs and central pathways related to a sensory modality, and its integration with the rest of the information flow in the central nervous system to guide appropriate responses in terms of motor outputs. Hearing is an elegant example of how this can occur. We will first briefly discuss the early hypothesis surrounding evolution of the ear. This will be followed by outlining each step of ear evolution toward hearing starting with the inner ear and ending in the hindbrain. Conceptually, evolution had to generate developmental programs that can gave rise to a vertebrate auditory system resulting in the formation of (1) an appropriate endorgan and assignment of sensory cells, (2) dedicated neurons to transmit the hearing information to the hindbrain, (3) formation of dedicated processing nuclei in the central nervous system, and (4) an efferent system to modulate the hair cells and afferent neurons. We will focus here on many of the emerging developmental studies that shed light on molecular changes that could lead to such a complex modality as hearing.

OVERVIEW OF EVOLUTION OF THE EAR

The evolution of the ear is challenging to understand given the complex three-dimensional system requiring a carefully choreographed emergence of several different cell types that evolved in a stepwise fashion with each step being functional (Fig. 1). Evolution of the ear has also been perplexing because only vertebrates have a true ear, but several invertebrates have evolved ear-like structures built on different basic principles.[10-12] There are two main camps of thought as to how the vertebrate ear evolved. One idea, the octavo-lateralis hypothesis, contends that a pre-existing lateral-line-like organ through evolution invaginated to create the ear.[13] Later, this hypothesis was slightly amended and van Bergeijk[14] advocated that the lateral line and the ear develop from a shared mass of ectodermal tissue, the acoustic-lateral placode. This idea also stated that both systems have the same type of sensory cells, the hair cells, and both systems are innervated in a similar manner that project to the same central nuclei. A modern offshoot to this idea is that all placodes share a common preplacodal domain. Thus all placodally derived tissue, including highly diverse organs such as the lens and ear, share a common developmental origin and a common molecular mechanism to initiate their formation.[15] However, it is unclear whether all placodal domains share common detailed developmental programs.[16] It has been seen in many amphibians that there is a developmental time difference with the inner ear placode developing much earlier than the lateral line placode.[17] In addition, the lateral line has been lost in terrestrial vertebrates indicating that these placodal regions are not tightly coupled with one another.

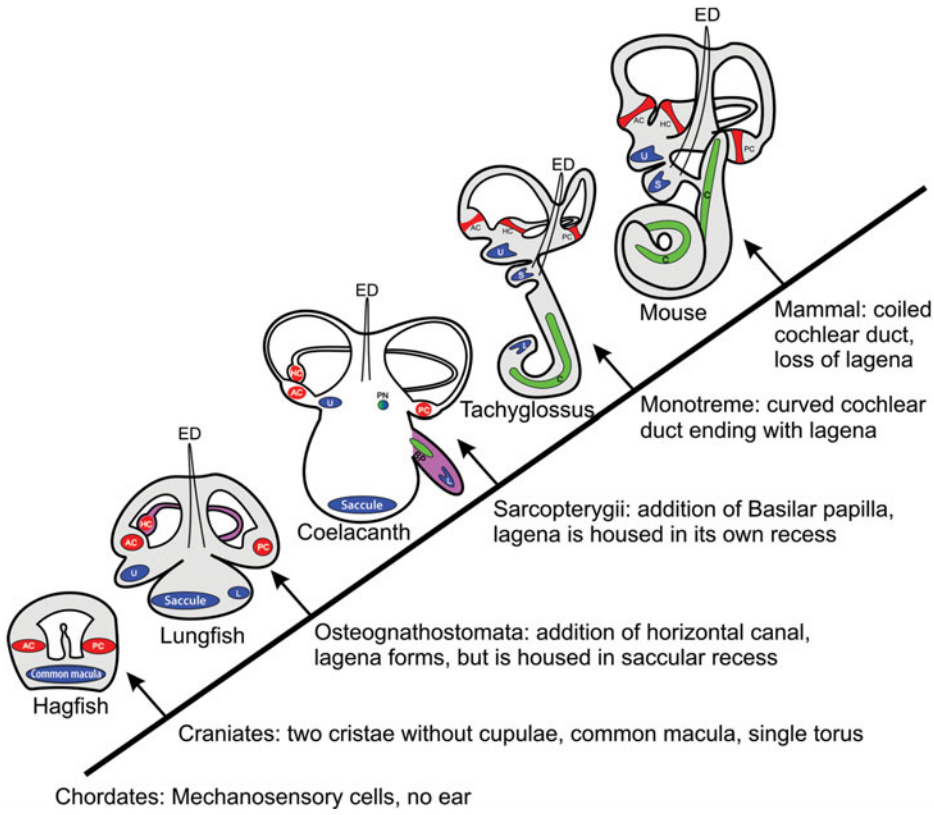

Figure 1. Morphological evolution of the ear. Gravistatic organs are depicted in blue, angular acceleration in red, and sound pressure in green. There is an apparent increase in complexity of the ear moving from left to right. It is assumed that the chordate outgroup had mechanosensory cells but no ear. The hagfish ear shows a single torus and three sensory patches, including one gravistatic and two for detecting angular acceleration. The sensory cristae lack a cupula indicating that this may be the primitive state. Evolution results in the addition of endorgans through developmental segregation, with 9 endorgans being the most found to date in any given vertebrate ear. Highlighted here is the addition of a third endorgan in the lungfish to detect an additional plane of angular acceleration (HC) as compared with hagfish, and the concurrent appearance of a lagenar recess with the bailar papilla in the coelacanth. With the increase in sensory epithelia there is also the morphological increase in complexity resulting in three semicircular canals and three recesses housing the saccular, lagenar, and utricular macula. AC, anterior crista; PC, posterior crista; HC, horizontal crista; U, utricle; L, Lagena; BP, basilar papilla; PN, papilla neglecta. Red, canal crista to detect angular acceleration; Blue otolith bearing endorgans to detect gravistatic and linear movement; Green, sensory epithelia for hearing; Violet, indicating first appearance of select structures.

The second idea on ear evolution is known as the statocyst hypothesis. This idea implies that hearing organs across phyla are morphologically conserved and that the function of hearing is retained in various forms of statocysts predating the vertebrate lineage. Wever argued that several invertebrates including co-elenterates, ctenophores, echinoderms, and crustaceans all have small sacs containing sensory cells with cilia in contact with calcareous materials, which he likened to the vertebrate ear. This was given

further evidence by molecular comparison with tunicate atrial chambers which contain sensory receptors and supporting cells, but are used as hydropressure sensors.[18] These atrial chambers express *Pax258* the homolog of mammalian *Pax2*. PAX2 and 8 have been shown to be necessary for the development of both the chicken and mammalian ear.[19-21] Thus, PAX258 may play a role in mechanoreceptor development across phyla in much the same way as PAX6 is necessary for eye development across phyla.[22] The underlying theme is that the hair cell and ear have had an intertwined evolution since the start as compared with the octovo-lateralis hypothesis which states that the hair cell evolved first followed by the morphological evolution of the ear. A third idea by Fritzsch and Beisel[23] proposes as in the octovo-latarealis hypothesis that the mechanosensory receptor developed first and evolved into the modern hair cell independent from the evolution of the ear. The difference lies in that this theory does not require the prior evolution of a lateral line system. This hypothesis has gained ground in the finding that all hair cell mechanosensory transducers rely on a single gene Atonal and its orthologs for development as well as a highly conserved set of microRNAs that are crucial for hair cell development.[24] Aggregating such hair cells into a placodal area next to the forming brain could have been the starting point for both ear and lateral line evolution.[12]

Once a placodally derived ear was formed further evolution was apparently based on multiplication of existing sensory patches followed by progressive segregation and specialization of sensory epithelia embedded in an increasingly complex three-dimensional network of tubes and recesses (Fig. 1). The least complicated extant ear known is that of the hagfish. The hagfish ear has only three sensory epithelia, two crista and one macula communis. These three epithelia are set within a single tube. The largest number of endorgans known in vertebrates is that of gymnophionan amphibians which have nine endorgans: three semicircular canal cristae, a utricle, a saccule, a lagena, a basilar papilla, a neglected papilla and an amphibian papilla.[25] Most likely all nine of these epithelia are dedicated to detecting specific aspects of mechanical stimulation, and each unique structure allows transduction of a previously unexplored property of the mechanical energy that reaches the ear.[26] It has long been thought that the evolution of these multiple sensory epithelia came about through splitting of a single sensory anlage,[27] and developmental evidence showing experimental fusion of one or more sensory epithelia into a single one support this notion.[28]

An example of multiplication and respecification of vestibular epithelia is the formation of a third sensory patch for detecting angular acceleration, known in all jawed vertebrates as the horizontal canal crista. As stated before, hagfish have only two canal cristae, a feature that is thought to be primitive and is shared with lampreys. A single homeobox gene, *Otx1*, has been indicated as being necessary for the development of this epithelia. When OTX1 is absent in mice there is an absence of the horizontal canal. Lampreys lack *Otx* expression in the ear,[29] leading some to believe that the entire horizontal canal system depends on *Otx1*.[30] Close examination revealed that some displaced patches of hair cells remain in the *Otx1* null ear[31] leading to the thought that this remaining hair cell patch is reminiscent of the lamprey dorsal papilla.[26] If this were true, it would indicate a two-step evolution of the horizontal canal system. First, a multiplication of hair cells and a segregation of the dorsal sensory patch would occur. Followed by the evolution of a distinct horizontal canal, of which *Otx* plays a role. The finding that another gene, *Foxg1*, is necessary for the normal development of the horizontal canal cristae clearly is in line with this suggestion.[32]

AUDITORY SENSORY EPITHELIUM IS A TRANSFORMED VESTIBULAR SENSORY EPITHELIUM

The cochlea of mammals or basilar papilla of other sarcopterygian vertebrates evolved from vestibular organs.[33,34] More specifically it is likely that the cochlea, the mammalian auditory organ, evolved through embryonic transformation of parts of the saccule.[35] This has been shown developmentally by several markers indicating the progressive segregation of the mouse cochlea from the saccule during development such as *Lfng* and *Bdnf*.[36-38] Similar developmental segregations of various sensory epithelia have been noticed in amphibians, and resulted in the proposition that splitting of sensory epithelia is a general mechanism to form new endorgans within the ear that are originally functionally uncommitted and allow for specification in the course of evolution.[26] After the initial segregation of the lagena from the saccule the two epithelia continued to be closely associated with each other, sharing in several species the same otoconial mass. It is only in those species where the lagena is situated at the end of its own recess does the basilar papilla appear. It has been suggested following the formation of a new lagenar recess, the sacuular/lagenar sensory epithelia anlage extended into this recess to provide it with the lagenar sensory epithelium.[39] This extension of presensory epithelia into the lagenar recess enabled the development of a new endorgan, which subsequently segregated from both the saccular and lagenar sensory epithelia. It is unclear how the other properties needed for the extraction of sound could have evolved including association with the perilymphatic space.

After the cochlea\basilar papilla segregated during development and became situated in its own recess the evolutionary transformation of auditory hair cells from a vestibular to an auditory receptor required changes in molecules to govern the emergence of novel properties. Some of the genes necessary for the segregation of saccular from cochlear epithelia in the mouse are *Lmx1a, Wnt5a,* and *Mycn*. In the absence of each of these genes there is a fusion of the sensory epithelia such that both saccule and cochlear hair cells remain in continuity in a single variable shaped recess. In the absence of *Mycn* there remains the stereotypical one row of inner and three rows of outer hair cells in the cochlear portion of the epithelia, except at the apex where there is a breakdown of this segregation pattern.[40] *Lmx1a* mutants also show a fusion of the saccule and cochlear epithelia. The cochlea, which is delineated from the saccule by the expression of *Gata3*, shows a vestibular like arrangement of hair cells and supporting cells in the base, but a more cochlear like organization in the apex.[28] *Wnt5a* null mice also show a fusion of the utricle, saccule, and cochlea. There is an alteration of the number of rows of outer hair cells, but inner hair cells and outer hair cells are distinguishable from each other.[41]

One of the pathways that has been suggested in separation of these two cell types is Wnt signaling which has been shown to be necessary for the development of the vestibular hair cell phenotype. When constitutively active β-catenin (a key player of Wnt signaling) was expressed in the developing auditory sensory patch, hair cells and supporting cells in that region developed characteristics consistent with a vestibular phenotype. Similar results were found in chicken with the overexpression of WNT3A. Thus the auditory sensory epithelium of tetrapods may have down-regulated the Wnt pathway mediated activation and shed some of its vestibular molecular burden, allowing for a new phenotype and the emergence of new genetic networks. Wnt signaling may also be involved in the transformation of an otoconia-based structure covering the hair

cells to a tectorial membrane. Many proteins necessary for the formation and integrity of both types of matrices are shared between the two structures. However, minute alterations, perhaps mediated by the absence of Wnt induced differentiation of the sensory epithelium, could be involved in turning the otoconia covering into a tectorial membrane. Otoconia being the major necessary component for the saccule to receive transduction by force of gravity, or other linear acceleration, and allowing a switch to reception of auditory stimuli.

One of the unsettled questions in hair cell development is that of polarity. Hair cells of the ear become polarized with respect to their stereocillia and this is essential for the direction of force that they respond to. In the utricle the hair cells are oriented toward the striola where as in the saccule there is a 180 degree shift and the hair cells are oriented away from the striola. In the cochlea instead of having opposing polarities all hair cells are oriented toward the abneuronal portion of the cochlear duct. However, the polarization is different in various basilar papillae. The molecular mechanism resulting in these distinctions is not currently known.

While all hair cells, including both vestibular and cochlear, require the bHLH transcription factor ATOH1 very few distinguishing markers are known for individual hair cell types. FGF8 is known to be necessary for mouse and chick otic induction.[42] During later development the inner hair cell row of cochlear hair cells is known to express FGF8. Its deletion or overexpression does not influence hair cell number but rather other cell types of the organ of Corti, the inner and outer pillar cells.[43] Nevertheless, Fgf8 is a good marker of inner hair cells, but also of a subset of hair cells in gravistatic organs but not the canal cristae, reinforcing the conclusion that the cochlear sensory epithelium evolved from gravistatic receptors. One FGF receptor FGFR1 has been shown to have specific effects on the auditory epithelia of mice.[44] With the full null and conditional deletion with the ear specific *Foxg1cre* there is a loss of hair cells specifically within the organ of Corti while the vestibular system remained normal.

As stated previously for the saccule to give rise to the cochlea there has to be an increase in cellular proliferation. Although also expressed in the vestibular epithelia deletion of *Cdkn1b(P27$^{kip1)}$* results in a slightly increased number of cell cycles and increased hair cells and supporting cells within the organ of Corti. This preferential effect indicates that CDKN1B must play a unique role in the cell cycle of the cochlea.

AUDITORY NEURONS ARE DERIVED FROM VESTIBULAR NEURONS

Just as auditory sensory epithelia are ontogenetically related to the saccular epithelia so too are the nerve fibers that innervate the cochlea. Auditory and vestibular neurons are derived from the same embryonic cellular source, the otic placode. To become distinct the newly derived auditory neurons must reach auditory rather than vestibular epithelia at the periphery, and acquire the ability to project to auditory rather than vestibular nuclei in the central nervous system. Both types of neurons show dependence on NEUROG1, and the null mouse shows a complete loss of both vestibular and auditory afferent neurons. Both auditory and vestibular neurons also require NEUROD1 for survival and proper migration. However, there is a preferential loss of auditory neurons compared to vestibular neurons in the *Neurod1* null mouse. To serve their function auditory neurons must contact the auditory epithelia instead of the vestibular epithelia, and they must project to the auditory, rather than the vestibular

nuclei in the CNS. One gene shown to be uniquely expressed in the auditory neurons is *Gata3*. GATA3 has been shown to modulate the expression of *Neurod1* and thus may be linked to the preferential reliance of auditory neurons on this transcription factor. One of the two fly orthologs for GATA3, Grn, has been shown to be necessary for specification and path-finding properties for subsets of neurons. In the mouse *Gata3* null mice lack all auditory neurons, but retain some vestibular neurons.[45] Because of the lack of auditory neurons conditional mutants for *Gata3* in spiral neurons will be needed to assess its later role in spiral neuron path-finding and identity.

Spiral and vestibular ganglion neurons apparently originate from distinct areas of the otocyst.[46,47] Developing from unique areas may make it easier to bestow unique identities to the vestibular and spiral ganglion neuron populations. It is also possible that neurons easily find their target because they project back to where they delaminated from. Data for this hypothesis come from the idea that hair cells and sensory neurons may have a lineal relationship.[48] This lineal relationship has been established for the chicken,[49] but it remains unclear if this has a direct effect on neuronal path finding.[47,50] However, in the absence of hair cells there seems to be initial outgrowth of fibers to the prosensory area indicating that hair cells are not necessary for initial projection of fibers.

Neurotrophins are an attractive means by which a new population of neurons could evolve. In the mammalian ear all vestibular and cochlear sensory neurons co-express *Ntrk2* and *Ntrk3*,[36] which seem to have differential effects on vestibular and cochlear fibers: vestibular innervation is more dependent on *Bdnf* and its receptor *Ntrk2*, and cochlear innervation is more dependent on *Ntf3* and its receptor *Ntrk3*. However, it was shown that differential innervation is not dependent on receptor or ligand specificity but rather on spatio-temporal expression of a specific neurotrophin resulting in differential elimination of basal or apical sensory neurons or canal cristae innervation.[36,51]

Centrally the auditory and vestibular fibers must also segregate from each other and project distinctly into the hindbrain, reaching their own information processing areas. NEUROD1 may play a role in the central projection of the auditory neurons. In its absence the cochlear projection into the hindbrain becomes disrupted and fibers do not project properly to their targets.[52]

In summary, the vertebrate ear has evolved around a conserved mechanosensory cell and separated it into distinct endorgans to extract various specific stimuli from the environment. One of these separate endorgans has been transformed into a receptor to receive sound input, and relay this information to the central nervous system. This process was achieved by co-opting existing modules from other developmental networks of the body into the ear to govern the formation of the cochlea\basilar papilla and auditory sensory neurons. The vertebrate ear can be viewed as the product of continuous alteration of an existing genetic network to govern sensory hair cell and sensory neuron development and small changes in modules that make up this network to alter the outcome of cells in the ear.

CENTRAL AUDITORY NUCLEUS

While there is consensus that auditory sensory epithelia and afferent neurons are derived from an existing population of cells, there remains controversy over what is the ontogenetic source of the auditory nuclei within the hindbrain. As outlined in the

introduction there are two separate theories as to where a new population of cells can come from. We will deal specifically here with where the auditory nucleus may be derived from: (1) Increased proliferation of the vestibular nucleus forming a redundant and therefore uncommitted neuronal population (such as stated above for sensory epithelia and afferents), or (2) Loss of an old input freeing neurons from previous functional constraints and allowing them to adapt a new function (following the example of the formation of mammalian middle ear ossicles).

Increased proliferation and formation of additional neuronal numbers has occurred in the evolution of vertebrates, as seen in both relative brain size and absolute brain size between fish, amphibians, and primates.[53] These enlargements must be the result of increased proliferation, which could result from small changes in genes regulating the proliferation of progenitor populations, or by delaying the time in which neuronal populations exit the cell cycle. This could have occurred within the vestibular nuclei within the hindbrain, although no data exists to corroborate this assumption.

The second possible scenario for the formation of an uncommitted central neuronal population may be the loss of input of a pre-existing neuronal population. The loss of input would eliminate the functional constraints acting on this population allowing them to acquire a new fate. Previously it was thought that the mechanosensory lateral line nuclei lost its input during evolution and the auditory system transformed it into a hearing nucleus. However, it has been subsequently shown that not all frogs lose the lateral line system during metamorphosis, and those that retain it have in addition an auditory nucleus.[54] Also, there is a spinal output from the mechanosensory lateral line nuclei that is not present in the auditory nucleus of any species. Thus, there is neither a coincidental loss of one nucleus and appearance of another or a similar detailed connection to support this idea.

Others have advocated that the electroreceptive sense nuclei may be transformed into the auditory nucleus of jawed vertebrates.[55,56] The loss of electroreceptive hindbrain nuclei is completed in amniotes, but within amphibians some have electroreception with specialized hindbrain nuclei but no specialized auditory system (salamanders, caecilians) whereas others (anurans) have lost the sense of electroreception but have hindbrain auditory nuclei with or without losing mechanosensory nuclei.[54,57,58] In contrast to mechanosensory nuclei, electroreceptive and auditory nuclei share a similar anatomical location within the dorsal portion of the hindbrain extending from rhombomere 2-6. In contrast, the lateral line and vestibular nuclei can extend from the cerebellum in rhombomere 1 to rhombomere 8 depending upon the species (Fig. 2). Although this idea has yet to be experimentally tested through transplantation or genetic manipulation, it is known that loss of innervation of central auditory nuclei cause them to disappear except portions that may receive a different input.[59,60] Overall, there seems to be enough plasticity in the system as transplanted Xenopus ears to the spinal cord may make afferent and efferent connections with novel targets.[61]

Recent developmental work has shed light on some of the unique genetic aspects of the auditory nuclei that may be related to its evolution. Like the inner ear the auditory nuclei also express the bHLH genes *Atoh1*[62] and *Neurod1*[63,64] and they are required for its development. This similarity in the molecular developmental network of the ear and auditory nuclei correlates well with new data showing that functional systems may be developmentally connected and require shared activation of transcription factors. It is possible that identical transcription factors govern the development of both the peripheral and central aspects of the auditory system. In concordance with this hypothesis the

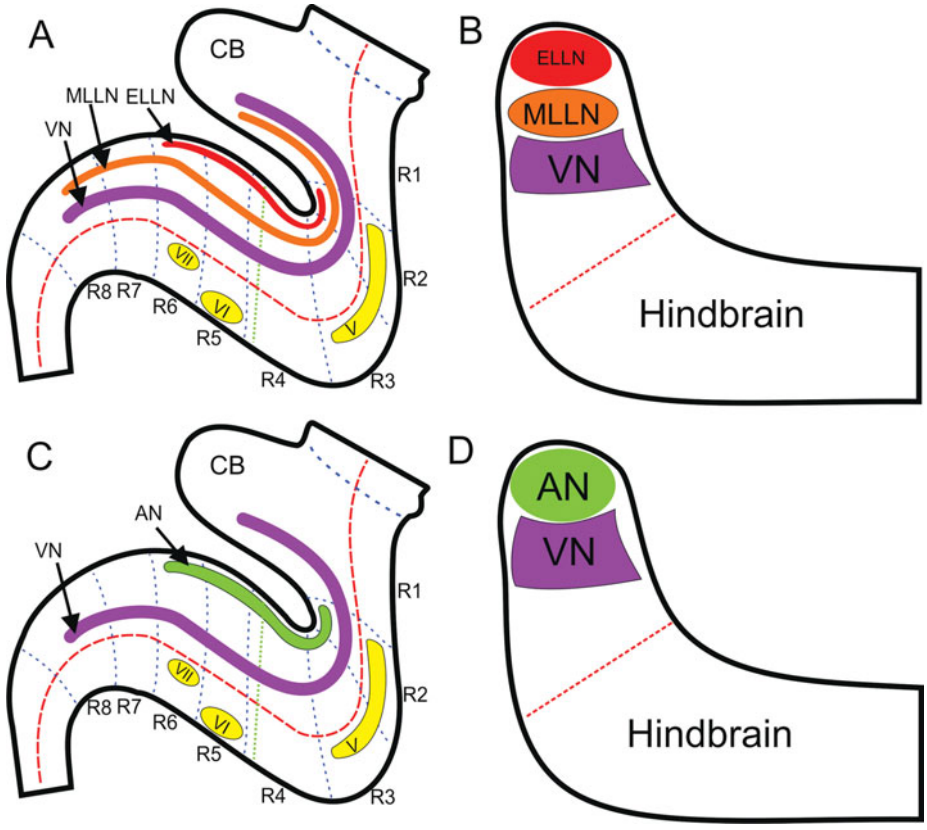

Figure 2. The transformation of the brainstem from the primitive non-auditory scheme into the derived scheme allowing the tetrapod vertebrate to hear sound pressure. A) Lateral view of primitive hindbrain. B) Coronal view of primitive hindbrain. A and B) The primitive hindbrain contains a vestibular nucleus (VN, purple) to allow the animal to detect motion and gravity. The mechanosensory lateral line nucleus (MLLN, orange) receives input from mechanosensory neuromastes of the lateral line. The electroreceptive lateral line nucleus (ELLN, red) receives input from electroreceptive ampullary organs. C) Lateral view of derived hindbrain D) Coronal view of derived hindbrain. C and D) The derived hindbrain of tetrapods have lost the MLLN and ELLN nuclei and their respective senses. There is a gain of hearing and accompanying auditory nucleus (AN). R, rhombomere; CB, cerebellum; VII, facial branchiomotor nucelus; VI, abducens nucleus; V trigeminal nucleus. Red dotted line represents the sulcus limitans; blue dotted lines mark rhombomeric boundaries; Green dotted line shows level of cross section in (B and D); yellow indicates motor nucleus. Note matching colors of various nuclei.

fly ortholog of *Atoh1, atonal,* is necessary for development of the chordotonal organ which is also used to distinguish sound.[65]

EFFERENT NEURONS TO INNER EAR

Another aspect in the evolution of hearing has been the addition of efferent fibers from the brainstem to the inner ear modulating hearing and vestibular senses. There has

been some consensus as to the ontogenetic origins of the efferent population as a whole being derived from the facial branchial motor neurons.[66,67] Recent experimental data using ear transplantation into the projection of spinal motoneurons appears to support that a newly formed ear could be innervated by the closest motoneurons, which in the case of the vertebrate ear are the facial branchial motoneurons.[61] Some of the unique genes associated with the vestibulo-cochlear efferents are *Gata2*, *Gata3*, and *Unc5* recently reviewed in ref. 68. While the role of GATA2 and UNC5C have not been assessed in relation to the vestibulo-cochlear efferents, GATA3 has been examined. While the spiral ganglion neurons do not form in the absence of GATA3, indicating its necessity in this neuronal population the absence of GATA3 in the vestibulo-cochlear efferents does not result in their absence. Part of what makes the vestibulo-cochlear efferents unique is their lack of migration into rhombomere 6 as the facial branchial motoneurons do in mammals and also their projection both contralaterally and bilaterally while the facial branchial motoneurons only project ipsilaterally. It has been shown that in the absence of GATA3 there is a severe reduction in vestibulo-cochlear efferent projection across the midline, yet there is no alteration in cell body migration.[45] Also, in *Gata3* null mice vestibulo-cochlear efferents do not project to the ear and instead reroute with the facial nerve. However, it remains to be seen if this is due to loss of GATA3 or a reduction in the target tissue. A conditional deletion of *Gata3* in the hindbrain is needed to asses these possibilities.

During development the cochlear and vestibuler efferents segregate their cell bodies from each other. The cochlear efferents migrate into the ventral portion of rhombomere 4 near the superior olive while the vestibular efferents migrate to end up dorsally. What factors mediate this segregation? The only gene that has been linked to segregation of vestibular from cochlear efferents has been *Mash1*. While both FBM and efferent precursors express MASH1 only vestibulo-cochlear efferents retain MASH1 after cell cycle exit. In the absence of MASH1 the vestibular efferents fail to migrate into their correct location and fail to project contralaterally. There is still a segregation of vestibular from cochlear efferents in the *Mash1* mutant indicating that further work is needed to identify the genetic differences between these two cell types, and how they became separate after the evolution of the cochlea.

CONCLUSION

We present here an overview of ideas related to the morphological and molecular basis of evolution of hearing and combine that with molecular data on ear development. Specifically we discuss (1) how a new sensory epithelia could be created to extract sound from the environment rather than vestibular signals; (2) how new neurons could be formed and uniquely specified to transmit this information to the hindbrain; (3) how a new hindbrain neuronal population could develop to receive this information; (4) how a new efferent population could have arisen to modulate the auditory periphery. Current data suggest that many of the necessary transcription factors for auditory and vestibular hair cells and afferent neurons are shared between the two populations. We take this as evidence for their common ontological origin. Multiplication and diversification, a well-known principle for gene duplication, is also most likely underlying the evolution of auditory sensory epithelia, cochlear afferents, cochlear nucleus and cochlear efferent neurons.

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CHAPTER 12

NEUROBIOLOGY OF SOCIABILITY

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Abstract: Sociability consists of behaviors that bring animals together and those that keep animals apart. Remarkably, while the neural circuitry that regulates these two "faces" of sociability differ from one another, two neurohormones, oxytocin (Oxt) and vasopressin (Avp), have been consistently implicated in the regulation of both. In this chapter the the structure and function of the Oxt and Avp systems, the ways in which affiliative and aggressive behavior are studied and the roles of Oxt and Avp in the regulation of sociability will be briefly reviewed. Finally, work implicating Oxt and Avp in sociability in humans, with a focus on neuropsychiatric disorders will be highlighted.

INTRODUCTION

Sociability is the tendency to seek social interactions. Navigating a social environment is not easy; for instance, the ability to discriminate a male from a female will impact the decision to fight versus mate. Yet, while the importance of engaging in normal social behavior seems obvious, our understandings of the neurobiological mechanisms underlying sociability are just now coming to light. Interestingly, it is the lack of sociability found in several neuropsychiatric disorders, such as autism and schizophrenia that has been the impetus for much of the research in this area.[1,2] To date, two neuropeptides, oxytocin (Oxt) and vasopressin (Avp), have been consistently linked with the neural regulation of sociability. With recent developments in behavioral tests to model aspects of sociability, the use of comparative studies, as well as the use of viral vectors and transgenic animals, including knockout mice, our understanding of the neural underpinnings of sociability is improving, as is our understanding of the contributions of Oxt and Avp. This chapter will

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focus on mammals and will review the behavioral components of sociability, describe the ways in which sociability is experimentally assessed, explore the contributions of Oxt and Avp to sociability and delve into some of the data on the neurobiology of altered sociability in human neuropsychiatric disorders.

SOCIABILITY IN CONTEXT

Social behavior is highly complex and varied, with some animals living in groups with complicated social structures while others are solitary and only engage in social interactions intermittently. Some of the questions researchers in this field have focused on include: Why does an animal engage in a social behavior in a specific context? What social or environment cues are required for a social exchange to occur? How does the brain regulate social interactions?

Sociability can be separated into two categories: (1) behaviors that bring animals together, such as affiliative, parental, or copulatory behaviors and (2) behaviors that separate animals, such as aggressive behaviors. This chapter will focus on the neural regulation of affiliative and aggressive behaviors; for reviews on neural regulation of parental and copulatory behaviors, please see Hammock and Young,[3] Lim and Young,[4] McCarthy and colleagues.[5]

MAJOR NEUROHORMONES IMPORTANT TO THE REGULATION OF SOCIABILITY

The first biochemicals implicated in the regulation of sociability were the gonadal steroids.[6] This hypothesis stemmed from research demonstrating that there were changes in sociability, particularly aggressive behavior, as a result of androgen manipulation, (e.g., castration or hormone replacement). There are also several species, particularly seasonal breeders, which continue to have elevated levels of aggressive behavior despite dramatic reductions in gonadal steroids.[7-11] It seems that in many species gonadal steroids may be necessary, but not sufficient, to alter sociability. Rather, the neuropeptides Oxt and Avp have been implicated in the neural regulation of sociability and specifically differences in their receptor distributions appear to be of particular importance.

The Nonapeptides: Oxytocin and Vasopressin

Oxt and Avp are both nine amino acid neuropeptides (i.e., nonapeptides) synthesized primarily in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus. Their genes sit in opposite transcriptional orientations on the chromosome as the result of the duplication of an ancestral vasotocin gene.[12,13] Both genes are composed of three exons, differ from one another by only two amino acids and are synthesized as part of a larger precursor preprohormone.[14] Since they are so structurally similar, Oxt and Avp are considered "sister" hormones though their actions both peripherally and centrally differ significantly from one another. Interestingly, Oxt and Avp are linked to several aspects of sociability and their actions appear to be fairly conserved across mammalian species.[15-21]

Oxytocin

Some of the early work on Oxt characterized its peripheral actions on the regulation of uterine contraction and milk ejection.[22,23] It is its synthesis in larger, magnocellular neurons of the PVN and SON, which project to the posterior pituitary that mediate the aforementioned actions. Oxt synthesized in the smaller, parvocellular, neurons of the PVN project centrally and mediate many of the central actions of Oxt. In mice and various vole species there have also been reports of Oxt neurons outside of the PVN.[24-26] For example, in female prairie voles (*Microtus ochrogaster*) Oxt immunoreactive (Oxt-ir) fibers that originate from both the PVN and SON have been found to innervate the nucleus accumbens (NAcc); the significance of which will be discussed later.[27]

Thus far only a single Oxt receptor (Oxtr) has been identified and it is thought to transduce all of the actions of Oxt.[28,29] The Oxtr is a member of the seven transmembrane G-protein-coupled receptor family; it is also structurally similar to the Avp receptors.[30] Identification of Oxtr expression was initially determined with receptor autoradiography using a potent ^{125}I-labeled antagonist. In rats and mice, Oxtr binding is found in several areas, including the hippocampal formation, lateral septum (LS), central amygdala (CeA), olfactory tubercle, nucleus accumbens shell, dorsal caudate-putamen, bed nucleus of the stria terminalis (BNST), medial amygdala (MeA) and ventromedial hypothalamus (VMH).[31-33]

Vasopressin

Avp's peripheral actions include the regulation of salt and water balance. Avp made in the magnocellular neurons of the PVN and SON is transported to the posterior pituitary and its release from the posterior pituitary regulates most of its peripheral actions. Centrally, Avp is also expressed in the suprachiasmiatic nucleus (SCN), BNST and MeA.[34] There are also reports of Avp immunoreactive (Avp-ir) neurons in the medial septum, LS, vertical limb of the nucleus of the diagonal band of Broca and the locus coeruleus.[35] Between the projections provided by the parvocellular vasopressinergic neurons of the PVN and the aforementioned nuclei, Avp fibers are extensive within the central nervous system.[36-39]

Avp receptors can be divided into two classes: Avp1 and Avp2 receptors (Avpr1 and Avpr2, respectively), both of which are seven transmembrane G-protein-coupled receptors that are similar in structure to the Oxtr. There are two subtypes of the Avpr1: The Avpr1a and the Avpr1b. Peripherally, the Avpr1a mediates the effects of Avp on vasoconstriction and can be found in the liver, kidney, platelets and smooth muscle.[40,41] Centrally, the Avpr1a is found in a variety of brain nuclei.[42-45] The Avpr1b was originally described in the anterior pituitary, where is prominent on the corticotrophes; though, it can also be found in the brain.[46,47] In rats, the Avpr1b has been localized to areas such as the olfactory bulb, piriform cortical layer II, LS, cerebral cortex, hippocampus, PVN, SCN, cerebellum and red nucleus,[47-51] but initial immunohistochemical and in situ hybridization histochemistry (ISHH) studies may have used antibodies and probes that lacked specificity.[52] In rats and mice however, the Avpr1b appears to be more discretely localized with prominent expression in the hippocampal field CA2 pyramidal neurons.[52] The Avpr2 is found in the periphery and is primarily expressed in the kidney; it has not been localized to the brain. Its role in the kidney is to transduce the antidiuretic effects of Avp within the renal collecting ducts.[53]

SOCIAL BEHAVIORS

On the surface, affiliative and aggressive behaviors appear to represent opposite ends of a behavioral spectrum and in fact, many of the neurotransmitters/neurohomones that regulate affiliative and aggressive behaviors are the same. However, the neuroanatomical substrates on which they act differ, suggesting that the neural circuits that underlie affiliative and aggressive behaviors differ significantly from one another. In this section, the defining features of affiliative and aggressive behaviors, how they are experimentally tested and their neural regulation will be explored.

Affiliative Behavior

Affiliative behaviors are those that include social bonding between individuals, including bonds between mates and parents with their offspring. From an evolutionary, perspective social bonds serve to reduce stress and anxiety by increasing security.[54,55] As most mammalian species are social, the formation of social bonds aids in holding groups or pairs of individuals together.

Social bonds have been studied extensively in primates and in some instances have been shown to increase evolutionary fitness.[56] For example, in a group of free-ranging baboons, females that have strong social bonds with one another live longer than those who have weaker social bonds.[57] In other mammals the direct effect of social bonding on fitness has been less studied, though a recent study in feral horses did find that in unrelated females, social bonding improved reproductive success.[58] So, it may be that for many species, social bonding has a direct benefit on fitness and that it simply has not been adequately studied across species.

The proximate cause, i.e., the neural regulation, of social bonds between male and female mammals has only been studied extensively in one species, the prairie vole.[4,18,54,59,60] Specifically, prairie voles have been used to examine the formation of the "pair bond", which is the social bond formed between males and females of a species that often implies social monogamy.[61]

The Pair Bond

Prairie voles live in extended family groups and are considered a socially monogamous species.[62] The pair bond is defined as a preference for contact with a familiar sexual partner, selective aggression towards unfamiliar conspecifics, biparental care, socially regulated reproduction and incest avoidance.[61,62] The formation of a pair bond is experimentally tested in the laboratory using a partner-preference test.[63] In this behavioral test, a male and female are paired and allowed to cohabitate. To test for the pair bond, one of the "partner" individuals is tethered to one side of a three-chambered apparatus. A novel "stranger" animal is tethered to the opposing chamber. The subject animal is permitted to explore the three chambers freely and the amount of time the subject animal spends in proximity to, or huddling with, the "partner" versus "stranger" animal is recorded over a 3-hour testing period. If the subject spends twice as much time with the "partner" animal then it is said to have formed a pair bond with that individual.[61,62,64,65]

Due to the diversity in social structures within the genus *Microtus*, comparative studies between vole species has provided significant insight into the neural regulation

of social bonding. By comparing the neurochemistry of monogamous vole species, such as the prairie or pine vole (*Microtus pinetorum*), to nonmonogamous voles, such as the montane (*Microtus montanus*) or meadow (*Microtus pennsylvanicus*) voles, scientists have had the opportunity to explore how variations in neurochemistry between highly related species can result in significant differences in social behavior. Differences in the Oxt and Avp systems between vole species has been found to contribute to their social organization.[18,60]

While there are not marked differences in Oxt and Avp immunopositive cells, or their projections, between species, there are changes in the distribution of the receptors for Oxt and Avp. Relative to nonmonogamous voles, monogamous voles have higher densities of Oxtr, as measured using Oxtr autoradiography and ISHH, in the NAcc, prefrontal cortex (PFC) and the BNST. Promiscuous voles, on the other hand, have higher Oxtr density in the LS, VMH and the cortical nucleus of the amygdala.[66-68] Evidence that the differences in the distribution of the Oxtr between species might be behaviorally meaningful comes primarily from pharmacological studies.

In female prairie voles, central infusion of an Oxtr antagonist blocks the formation of the pair bond but has no effect on sexual behavior, whereas central infusion of Oxt facilitates the pair bond in the absence of mating.[65,69,70] In the aforementioned studies the infusions were intracerebroventricular (icv), however manipulation of Oxtr signaling, using Oxtr antagonists within the NAcc, blocks formation of a partner preference following mating (Fig. 1).[71,72] This finding is supported by a recent study in which Oxtr overexpressed in the NAcc of adult female prairie voles was found to accelerate the formation of partner preference. Interestingly, the same result was not found when the Oxtr was overexpressed in the nonmonogamous meadow vole, suggesting that in a nonmonogamous species Oxtr expression within the NAcc is not sufficient to promote pair bond formation.[73]

There are also differences in the distribution of the Avpr1a between vole species. Prairie voles have a higher density of Avpr1a, as measured using receptor autoradiography and ISHH, within the MeA, accessory olfactory bulb, diagonal band, thalamus, ventral pallidum (VP) and BNST compared to montane voles.[74,75] Montane voles, on the other hand, have a higher density of Avpr1a in the medial PFC and the LS.[68,75] These differing "patterns" of Avpr1a distribution have been suggested to underlie differences in social organization between monogamous and nonmonogamous vole species. This hypothesis has been confirmed, in part, by data in pine voles and meadow voles which suggest similar, social structure-specific distributions of Avpr1a between these species.[75] Further support for this hypothesis comes from pharmacological manipulations of the Avpr1a in prairie voles. When an Avp antagonist is injected icv prior to mating, the formation of a partner preference is inhibited. Conversely, Avp infusion facilitates the formation of the partner preference.[70,76] Some of the more interesting data that supports a role for the differential distribution of the Avpr1a in the formation of social bonds comes from a study in which the prairie vole Avpr1a gene was overexpressed in the ventral forebrain of meadow voles, resulting in increases in the amount of time meadow voles spent huddled with their partners compared to controls.[77]

It has been suggested that the differences in Avpr1a distribution between species are due to changes in the regulatory region upstream of the Avpr1a promoter.[78-80] This idea is based on work demonstrating that changes in Avpr1a density within and between species can alter social behavior.[77,81,82] Hammock and colleagues[83,84] suggest

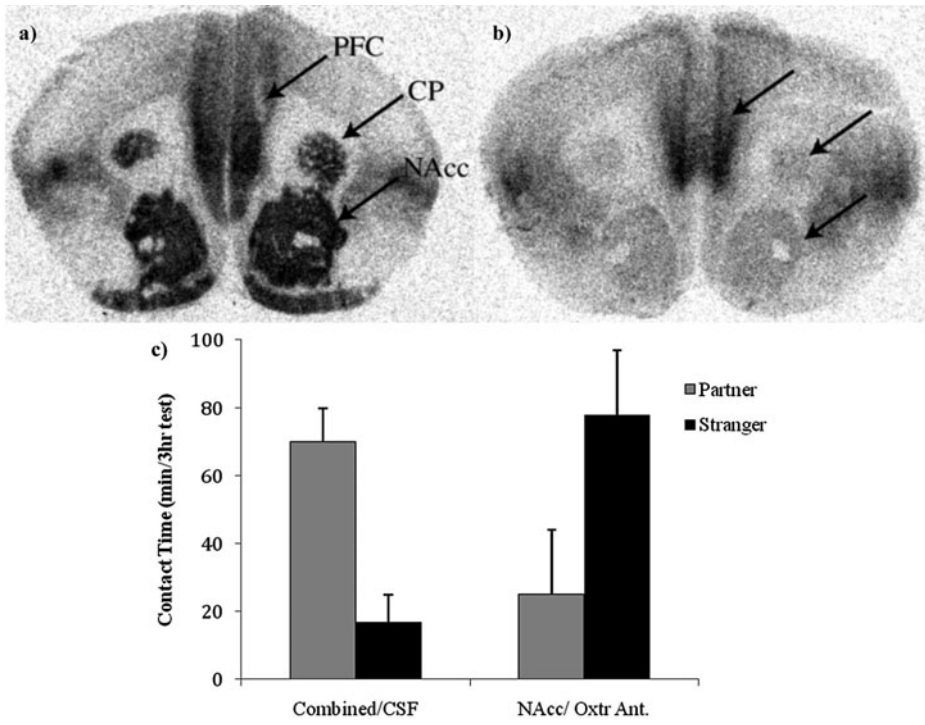

Figure 1. In female prairie voles, oxytocin receptors (Oxtr) in the nucleus acumbens (NAcc) are thought be important for the formation of partner preference. Autoradiograms illustrating Oxtr distribution between monogamous female prairie voles (A) and nonmonogamous female meadow voles (B) demonstrate that female prairie voles have increased Oxtr binding in the prefrontal cortex (PFC), the caudate putamen (CP) and the NAcc compared to female meadow voles. Further, female prairie voles given a selective Oxtr antagonist into the NAcc prior to and 12 hours into a 24 hour cohabitation period do not form a partner preference compared to females that received cerebral spinal fluid (CSF) into the PFC, CP and NAcc at the same time points (i.e., combined). (C). (A) and (B) were adapted from Hammock and Young. J Phil Trans R Soc B 2006; 361:2187-2198,[3] ©2006 with permission from The Royal Society. (C) was adapted from Young et al. Horm Behav 2001; 40:133-138,[72] ©2001 with permission from Elsevier.

that the presence or absence of a microsatellite sequence (i.e., simple sequence repeats with nonrepetitive elements) in the 5′ cis-regulatory region of the Avpr1a gene could be responsible for differences in Avpr1a density. To test this, two breeding lines of prairie voles were generated that had differing lengths of microsatellite sequence in the 5′ cis-regulatory region of the Avpr1a gene. The two breeding lines showed regional differences in the density of the Avpr1a and the breeding line with the longer microsatellite sequence tending to show more partner preference than the breeding line with the shorter microsatellite sequence.[83] However, in a study that examined individual differences in Avpr1a expression in prairie voles housed in a semi-natural setting, Avpr1a expression in the VP or LS was found to not be predictive of social or sexual fidelity. Rather, differences in Avpr1a expression in brain areas associated with spatial memory were correlated with social and sexual fidelity.[85] Further, differences in microsatellite length in field tests of prairie voles, while associated with Avpr1a density in brain areas important for pair bond formation, are not associated with differences in measures of

monogamous behavior and reproductive success.[86] Taken together, these data suggest that there are a variety of social and neurobiological factors that likely contribute to the formation of the partner preference and that changes in single gene are not sufficient to determine whether an animal is monogamous or polygamous.

Aggressive Behavior

Aggression is used by a variety of animals to develop and maintain social hierarchies, gain access to mates, protect young and defend territories. The ability to display aggression in the correct social context is critical for the survival and reproductive success of many species. Males are typically more aggressive than females, however, during pregnancy and in the postpartum period, there is often a rise in female aggression.[87,88] Our understanding of the neural regulation of aggressive behavior is fairly limited in primates, but in rodents, pharmacological tools coupled with transgenic mouse models have substantially contributed to our understanding of the neural regulation of aggression.

In rodents, the most common assessment of aggression, specifically offensive aggression, uses the resident-intruder test. Subject "resident" animals are singly housed for several weeks prior to testing; in mice this results in an increase in baseline aggression due to isolation-induced aggression. An "intruder" animal, often smaller and group housed, is then placed into the cage of the resident animal. The latency to the onset of aggression as well as the frequency and duration of aggression are common behavioral measures. To test maternal aggression a similar test is employed, only the "resident" is a postpartum female with her pups in or removed from the cage.

The role of Oxt in the neural regulation of aggression has not been examined in much depth. Though, it does appear that in females Oxt reduces nonmaternal aggression in some species and facilitates maternal aggression in others. In female Syrian hamsters (*Mesocricetus auratus*), for instance, which are more aggressive than males of the species, there is evidence that a microinjection of Oxt into the medial preoptic area-anterior hypothalamus (MPOA-AH) reduces aggression directed toward a female intruder,[89] but microinjections of Oxt, as well as Oxt antagonists, into the amygdala facilitate maternal aggression.[90,91] Female prairie voles that receive Oxt icv have decreases in male-directed aggression[92] and in rats, displays of maternal aggression can be facilitated by infusing Oxt into the amygdala[91] and reduced by lesioning or infusing Oxt antisense oligonucleotides into the PVN.[93,94] While there are mice in which Oxt and the Oxtr have been genetically disrupted, $Oxt^{-/-}$ and $Oxtr^{-/-}$ mice, respectively there are no reports of altered maternal aggression in these animals.[95,96] Overall, the actions of Oxt in the regulation of aggression in females appears to be context specific and possibly species specific.

There have been very few reports supporting a role for Oxt in the regulation of aggression in males. Studies in $Oxt^{-/-}$ mice are conflicting, with one group reporting increases in aggressive behavior[97] and another group reporting decreases in aggressive behavior.[98,99] It should be noted, though, that the $Oxt^{-/-}$ mice tested were generated by two different groups and that the increases in aggressive behavior were only found in mice that were the offspring of null mutant parents; suggesting that Oxt exposure in the prenatal environment may be important to normal displays of aggression. This possibility is supported by a report of heightened aggression in $Oxtr^{-/-}$ male mice compared to controls when tested in a resident-intruder behavioral test.[96]

Much of the work implicating Avp in the neural regulation of aggression has been completed in Syrian hamsters. As Syrian hamsters are a solitary species, they readily display aggression towards conspecifics. Further, they engage in a stereotypic type of scent marking behavior, referred to as flank marking, that is expressed at higher levels in socially dominant animals.[100] Ferris and colleagues made the serendipitous discovery that Avp injected into the MPOA-AH results in a dose-dependent increase in flank marking behavior.[101] This finding was one of the first to demonstrate that microinjection of a single neuropeptide into a specific brain region could induce a complex behavior. Avp injected into the anterior hypothalamus (AH) or ventral lateral hypothalamus (VLH) of Syrian hamsters has been found to facilitate aggressive behavior.[7,102,103] Conversely, Avp antagonists and more specifically Avpr1a antagonists, injected into the AH inhibit aggression.[102] The Avpr1b may also be important to the modulation of aggressive behavior in hamsters, as treatment with an oral Avpr1b antagonist results in decreases in aggressive behavior compared to controls.[104] It has been suggested that the neural circuit that regulates aggression in Syrian hamsters includes the AH, which has reciprocal connections with the VLH, the MeA and the BNST.[105,106]

Syrian hamsters exposed to anabolic-androgenic steroids during adolescence for at least 14 days display increased aggression in adulthood. They also have increases in Avpr-ir within the AH and injections of an Avpr1a antagonist in the AH reduces the intensity but not the onset of aggression.[107-110] There are also reports of changes in social status affecting the Avp system in hamsters. Injections of an Avp antagonist into the MPOA-AH of a dominant hamster can transiently reverse dominant/subordinate relationships, as measured by flank marking.[111] Subordinate hamsters have fewer Avp-ir cell bodies in the nucleus circularis, a structure that is found within the AH, compared to dominant hamsters.[112] In hamsters that are repeatedly defeated, there are coincident decreases in Avpr1a receptor binding within lateral portions of the VMH.[113] Similarly, in hamsters that are singly housed for several weeks and not allowed to interact with other animals, there are increases in Avpr1a binding in the AH, PVN and lateral hypothalamus, whereas socially experienced hamsters have increased Avpr1a binding within the CeA.[114] Even when Avp is used to facilitate aggression, social isolation for some period of time seems to be required.[7,106] These data suggest that, at least in hamsters, the role of Avp in the regulation of aggression can be altered by social experience.

The modulation of aggression in rats and mice is due in part to gonadal steroid-dependent Avp projections from the BNST and the MeA to the LS.[115-117] With the LS likely regulating the emotional aspects of aggression.[118,119] Injections of Avp into the LS of rats and prairie voles can facilitate agonistic behavior.[76,120,121] In sexually naïve males, Avp injected into the AH, or overexpression of the prairie vole Avpr1a within the AH, results in increases in selective aggression (i.e., aggression directed towards novel male or female animals).[122] In mice selectively bred for either a long attack latency (LAL) or short attack latency (SAL), there is evidence of changes in Avp neurochemistry. SAL mice have fewer Avp-ir neurons in the BNST and fewer Avp-ir fibers in the LS compared to LAL mice suggesting that, within a species, less Avp within the LS may be associated with increased aggression.[123] However, monogamous California mice (*Peromyscus californicus*) have shorter attack latencies and increased Avp-ir in the BNST and LS compared the polygamous, white-footed mice (*Peromyscus leuopus*).[124] Interestingly, when California mice are cross-fostered to white-footed mice dams, they are less aggressive in adulthood than those reared by the same species and they have

less Avp-ir in the BNST and SON compared to controls.[125] The data in Peromyscus mice suggest that, similar to what has been found in hamsters, changes in the environment, in this case changes in the early postnatal period, are able to alter the Avp neurocircuity and subsequent behavior.

When mice with a genetic disruption of their *Avpr1a* were engineered, it was thought that they would provide some valuable insight into the role of the Avpr1a in the regulation of aggression. Surprisingly, Avpr1a knockout mice do not differ from wildtype controls in measures of aggression.[126] It may be that the lack of aggressive phenotype in these mice is due to developmental compensation. Mice with a disruption of the *Avpr1b* (Avpr1b$^{-/-}$ mice), on the other hand, have implicated the *Avpr1b* in the regulation of aggressive behavior. Avpr1b$^{-/-}$ mice have marked reductions of forms of "social" aggression (i.e., those forms of aggression that require the animal to interact with a conspecific), such as those measured in resident-intruder, neutral arena and maternal aggression tests and no change in predatory aggression.[127-129] When attacked, Avpr1b$^{-/-}$ mice will defend themselves but will initiate fewer "retaliatory" attacks compared to wildtype controls.[128] Even Avpr1b$^{-/-}$ mice that are crossed with a more outbred substrain of mice, *Mus musculus castaneous*, continue to have reduced aggression (Fig. 2).[130] Since the distribution of the Avpr1b in the mouse brain is fairly restricted, with prominence in the CA2 field of the hippocampus, it has been proposed that it may be important to the formation or recall of memories that have an accessory olfactory-based component.[52,127]

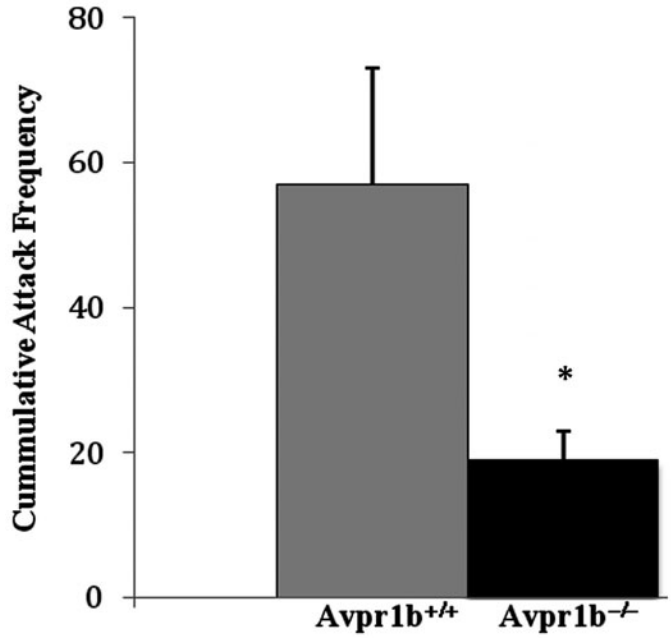

Figure 2. Even when crossed with *Mus musculus cantaneus*, male Avpr1b knockout mice (Avpr1b$^{-/-}$) have reduced aggression compared to wildtype (Avpr1b$^{+/+}$) controls; as measured by fewer attacks in a resident-intruder behavioral test. Adapted from Caldwell and Young. Physiol Behav 2009; 97:131-134,[130] ©2009 with permission from Elsevier.)

SOCIABILITY IN HUMANS

In humans there is evidence that Oxt promotes prosocial behavior. The study of prosocial behavior in humans includes testing procedures designed to measure trust, the ability to read facial expressions and the memory for socially salient information, such as faces. In most of the studies in humans, Oxt has been administered intranasally, as Oxt is thought to be able to cross the blood brain barrier using this route of delivery.[131] Intranasal administration of Oxt results in an increase in trust in humans, as measured by an individual's willingness to accept social risk during a social interaction.[132] Further, when intranasal Oxt treatment is coupled with functional magnetic resonance imaging, there is a reduction in activity in areas of the brain associated with processing fearful stimuli, such as the amygdala and some areas of the midbrain and reward feedback, such as the striatum. In individuals administered Oxt intranasally, betrayal of trust results in no change in trust behavior, whereas placebo controls decrease their trust in response to betrayal.[133] These data suggest that Oxt acting as an anxiolytic and stress-reducer is allowing for higher levels of sociability. There is also evidence that intranasal Oxt improves the ability to infer another individual's mental state, improves facial recognition memory and alters the processing of faces.[134-138]

The role of vasopressin in the regulation of social behavior in humans has not been studied as extensively as Oxt, though it is often associated with antisocial rather than prosocial behavior. In males, Avp administered intranasally results in increases in electromyogram (EMG) activity to socially neutral facial expressions. This suggests that Avp acts to bias an individual to perceive a neutral stimulus as an aggressive or threatening stimulus.[139] When administered to females, Avp decreases EMG responses to happy and angry faces, suggesting that in females, Avp acts to increase the perception of friendliness.[140] The researchers that conducted the aforementioned work suggest that the differential actions of Avp between men and women reflect differences in social strategies during socially stressful interactions.

Neuropsychiatric Disorders

Oxt and Avp have also been implicated in a variety of neuropsychiatric disorders, particularly those that are characterized by alterations in social interactions or heightened aggression, such as: Autism spectrum disorders (ASD), personality disorder and schizophrenia. In this section the contributions of Oxt and Avp to neuropsychiatric disorders will be briefly reviewed.

Autism Spectrum Disorders

ASD are characterized by repetitive behaviors, communication difficulties and abnormal sociability.[141,142] One of the reasons Oxt has been suggested to contribute to ASD is that in mice that lack Oxt, or Oxtr, there are behavioral deficits that are consistent with some of the symptoms of ASD.[95,143-149] Evidence that Oxt may have a role in ASD comes from several sources. There are reports of lower Oxt in the CSF of autistic children and reduced Oxt is correlated with impairments in social functioning.[150] There are also increases in the amount of an Oxt prohormone in the blood of autistic children, which is indicative of incomplete processing of Oxt into its biologically active form.[151] Oxt treatment in adults with ASD results in the reduction

of repetitive behaviors and improvements in emotional recognition.[152,153] Some genetic and epigenetic links between the Oxt system and ASD have also started to emerge. There are data in the Chinese Han population, Finnish families, Caucasian children and in individuals with "high-functioning" ASD suggesting that portions of the Oxtr gene may contain susceptibility loci for ASD.[154-157] Epigenetic modifications of the Oxtr gene have also been reported, with hypermethylation of the Oxtr promoter found in autistic subjects and subsequent reductions in Oxtr mRNA.[158] Though the sample size in the aforementioned study is small, the data are provocative and will likely facilitate more research in this area.

Data implicating Avp in the etiology of ASD are sparse, but there have been studies suggesting that polymorphisms of the Avpr1a may contribute to ASD.[159-161] Further, two of the polymorphisms, RS3 and RS1, have been linked to differential activation in the amygdala,[162] providing a possible neural substrate with which the Avp system may interact to mediate a genetic risk for ASD.

Personality Disorder

Personality disorder is characterized by a disconnect between an individual's behavior and cultural norms. Those diagnosed with personality disorder have impairments in at least two of the following areas: (1) cognition, (2) affectivity, (3) interpersonal functioning and (4) impulse control.[163] To date, only one study has examined changes in Oxt between individuals diagnosed with a personality disorder and healthy controls. This study found that while having a personality disorder was not correlated with cerebral spinal fluid Oxt, a life history of suicidal behavior was inversely correlated with Oxt.[164] The authors suggest that these data are consistent with the previous work in animal models which suggest that Oxt reduces aggression.[89,92-94]

Since individuals with a personality disorder are often more impulsive, which can result in increased aggression, it is not surprising that Avp has been examined in these individuals. Unfortunately, the data appear to be contradictory. A study by Coccaro and colleagues[165] found a positive correlation between Avp in the CSF of personality-disordered individuals that have a life history of aggressive behavior. Whereas another study found no differences in CSF Avp between violent offenders and controls.[166] It may be that differences in the populations studied account for the inconsistency in the findings, but it seems that more work in this area is warranted.

Schizophrenia

There are three broad categories of symptoms that characterize schizophrenia: (1) positive (e.g., hallucinations and delusion), (2) negative (e.g., anhedonia, impaired social behavior), (3) cognitive/attentional (e.g., impaired memory and executive function). Thus far, most of the work implicating a role for Oxt in aspects of schizophrenia comes from animal models.[167-169] However, in humans, while its role has remained controversial, Oxt has been linked to schizophrenia since the 1970's when it was used as an antipsychotic.[170,171] The data are mixed with regards to Oxt and schizophrenic populations, with one study reporting increases in plasma Oxt concentrations,[172] another study reporting no change,[173] and a third reporting decreases.[174] Though, similar to measures of Avp in individuals with personality disorder, these discrepancies may reflect differences in the populations of those that were studied.

Support for a potential role for Avp comes from studies indicating that treatment with neuroleptics improves psychiatric symptoms and reduces (or normalizes) Avp in blood plasma.[175,176] In studies using an animal model that lacks Avp, the Brattleboro rat, there are reports of deficits in behaviors associated with schizophrenia, specifically, social discrimination and prepulse inhibition of the startle reflex; these deficits can be rescued following treatment with antipsychotics.[177-181] It may be that Oxt and Avp only contribute to certain aspects of schizophrenia, such as the cognitive and social behavior deficits, within specific populations. Since treatment with antipsychotics often does not significantly improve the cognitive and negative symptoms associated with schizophrenia, it is important continue to investigate the neurobiology that underlies these behaviors.

CONCLUSION AND FUTURE DIRECTIONS

This is an exciting time for the neurobiology of sociability. The roles of Oxt and Avp are being explored and a more complete understanding of how these neurohormones interact with other neurotransmitter and neurohormone systems, such as dopamine and corticotropin releasing factor, are beginning to emerge.[71,182-188] There is diversity in the animal models being used, ranging from comparative studies to transgenic studies, that have revealed remarkable conservation in the roles of Oxt and Avp across species. Research examining sociability in humans is on the rise and with the use of pharmacological, genetic and imaging tools the link between the animal models of sociability and human behavior is becoming less tenuous. Further, in human neuropsychiatric disorders characterized by impaired sociability, the roles of Oxt and Avp are being elucidated and better pharmacological agents are being developed.[189-192]

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CHAPTER 13

CHANGING SENSES:
Chemosensory Signaling and Primate Evolution

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Abstract: Sensory organs provide key, and in many cases species-specific, information that allows animals to effectively forage, find mates and avoid hazards. The primary sensors for the vertebrate senses of vision, taste and smell are G-protein-coupled receptors (GPCRs) expressed by sensory receptor cells that initiate intracellular signal transduction cascades in response to activation by appropriate stimuli. The identification of sensory GPCRs and their related downstream transduction components from a variety of species has provided an essential tool for understanding the molecular evolution of sensory systems. Expansion of the number of genes encoding sensory GPCRs has, in some cases, expanded the repertoire of signals that animals detect, allowing them to occupy new niches, while in other cases evolution has favored a reduction in the repertoire of receptors and their cognate signal transduction components when these signals no longer provide a selective advantage. This review will focus on recent studies that have identified molecular changes in smell, taste and pheromone detection during primate evolution.

INTRODUCTION

Most sensory systems in animals have, at their core, a set a of signaling molecules that include sensory receptors that bind the ligand, downstream signaling molecules that amplify the signal, and ion channels that convert this biochemical process to an electrical impulse sent to the brain. The receptors for vision, taste, smell and pheromones all belong to the superfamily of G-protein-coupled receptors, with each receptor being specifically tuned to detect the appropriate sensory signal. In humans there are 4 visual receptors, 28 taste receptors and 388 odorant receptors (reviewed in ref. 1) (Fig. 1), which are

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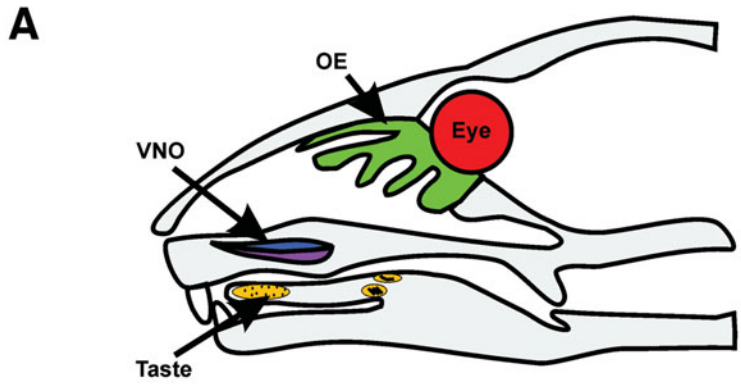

B

	Eye	OE	VNO		Taste	
					Bitter	Sweet Umami
	Opsin	OR	V1R	V2R	T2R	T1R
Mouse	3	1,037(354)	165(165)	61(148)	35(6)	3
Human	4	388(414)	2(115)	0	25(11)	3

Figure 1. Sensory receptor repertoire in mice and humans. A) Schematic diagram showing the location of the eye and the major chemosensory organs in the mouse. B) The number of functional receptors genes and pseudogenes (in parentheses) in mice and humans.[14] With kind permission from Springer Science+Business Media: Liman ER. Pflugers Arch 2006; 453(2):125-131.[91]

expressed in the eye, tongue, and olfactory epithelium, respectively. Downstream signaling molecules are structurally related to signaling molecules used throughout the body such as G protein, phospholipases, cyclases and second-messenger regulated ion channels and, in many cases, the isoforms expressed in sensory cells are not found in other parts of the body, suggesting a specialized function in sensory signaling.[2-6]

The evolutionary analysis of genes involved in sensory signaling has been an especially fruitful area of research for two reasons: (1) sensory receptors and transduction components are generally "non-essential", as seen by the viability of mice in which these genes are selectively deleted (e.g., refs. 7,8), and (2) changes in these genes can allow animals to occupy new ecological niches. For example the ion channel TRPV1 responds to capsaicin, as well as to heat and protons, all of which cause a burning sensation.[9] The capsaicin in chilies serves as a deterrent for consumption of the plant by small rodents, who are poor dispersers of its seeds. Birds have evolved a variant of TRPV1 which retains sensitivity to heat and protons, but is insensitive to capsaicin.[10] Presumably this mutation arose because it allowed birds to occupy a new niche, to the advantage of the plants whose seeds the birds disperse.[11] The identification of nearly all major sensory receptors has allowed similar inferences to be made about the evolution of other sensory systems, as will be discussed below.

GENOME MINING AND METHODS OF MOLECULAR EVOLUTION

The sequencing of the human and other genomes has provided a wealth of information on the genetic basis of sensory capabilities of different animal species (e.g., refs. 12,13). Odorant, taste and pheromone receptors, which belong to large multigene families, have been identified in many species and species-specific expansions and contractions of the repertoire of receptors have been identified (reviewed in ref. 14). Moreover, the remnants of once functional genes can be identified in the genome, providing a window on the evolutionary history of these genes and their associated functions.[15] These nonfunctional genes, called "pseudogenes", can be of two forms: Duplicated or processed (also called retrotransposed). Processed pseudogenes are intronless and are the product of viral retrotransposition of mRNA into the genome. These genes may never have been functional. In contrast nonprocessed pseudogenes are the result of a gene duplication event followed by a relaxation of selective pressure on the gene that leads to disruption in the coding sequence; in some cases duplicated genes were redundant and thus quickly eliminated from the genome, but in other cases they acquired a function only to become obsolete at a later time in evolution. By determining when these genes were "lost" we can learn about when in evolution the function they subserved no longer contributed to an animal's fitness.

Additional information about the functionality of a gene can be obtained by examining the pattern of nucleotide substitutions between genes from different species.[16-18] When the gene is functional in both species and subserves similar physiological roles, mutations that change the coding sequence will be selected against (presumably because individuals that carry these mutations are less likely to reproduce); this is purifying or negative selection. On the other hand, positive selection acts to change the amino acid sequence of a protein. For chemosensory receptors, positive selection may be exerted on regions of the receptors that bind ligands, where a change in amino acid sequence may expand the repertoire of chemosensory signals that the organism can detect. Finally, when there is no selective pressure on the gene, mutations that change the coding sequence and those that preserve it will be fixed with equal probability. This occurs when a gene serves no function and, thus, mutations in the gene do not change the fitness of the individual. Computer programs have been devised to measure and discriminate among these three scenarios: These programs measure the number of nucleotide substitutions that preserve the amino acid sequence, called synonomous substitutions and those that change it, called nonsynonymous substitutions. These values are normalized for the number of synonymous and nonsynonymous sites, giving values ds (number of synonymous substitutions/number of synonymous sites) and dn (number of nonsynonymous substitutions/number of nonsynonymous sites), respectively (also called Ks and Ka)[17,18] (see Fig. 2D). The ratio dn/ds then gives the selective pressure on the gene: If dn/ds <1, nonsynonymous (amino acid changing) substitutions are disproportionately under represented and selective pressure is purifying; if dn/ds >1, nonsynonymous substitutions are disproportionately over represented and indicative of positive selection.[16,19,20] This type of analysis becomes even more powerful when combined with the ability to reconstruct the probable sequence of ancestral genes, based on maximum likelihood methods. By comparing the sequence of living (extant) species with sequence from ancestral species one can determine the selective pressure on specific branches of the phylogenetic tree. Such an analysis was used by Messier and Stewart to identify specific lineages of primates in which the enzyme lysozyme came under positive selection[21] and a similar analysis was used to determine

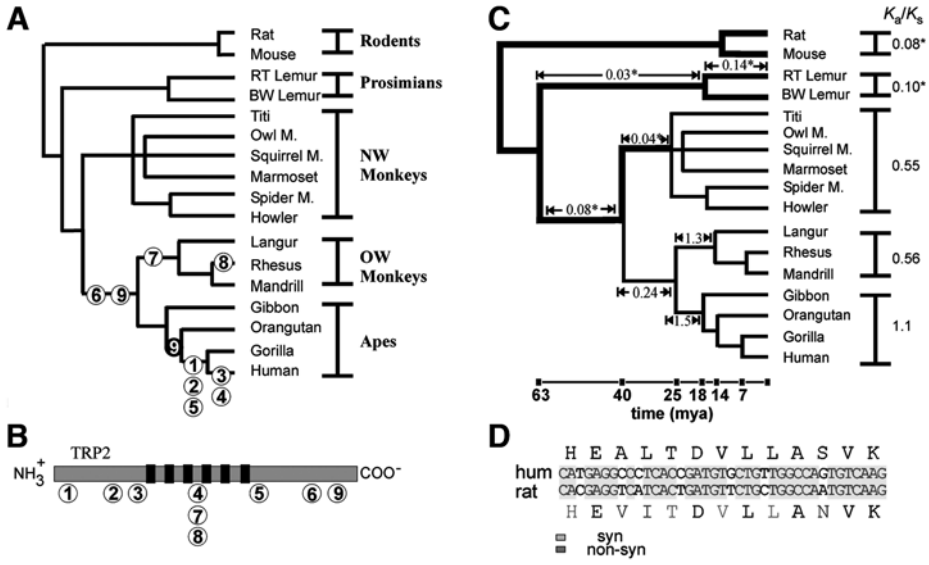

Figure 2. Evolutionary fate of the TRPC2 gene. A) Inferred time of occurrence of deleterious mutations in TRPC2 during primate phylogeny. B) A schematic representation of the TRPC2 ion channel indicating the position of each mutation. C) Rates of synonymous and nonsynonomous substitutions in the TRPC2 gene across primate phylogeny. Within group values show the average K_a/K_s ratio for all pairwise comparisons in a group. K_a/K_s values computed using predicted ancestral sequences are shown above the corresponding branch of the tree. Both types of analysis show that selective pressure was relaxed on the TRPC2 gene 25-40 mya. D) Nucleotide sequences of human and rat TRPC2, aligned and conceptually translated. Synonymous substitutions (which preserve the amino acid sequence) and nonsynonymous (which change the amino acid sequence) are shown in blue and red respectively. With kind permission from Springer Science+Business Media: Liman ER. Pflugers Arch 2006; 453(2):125-131.[91] A color version of this images is available at http://www.landesbioscience.com/curie/

that selective pressure was relaxed on vomeronasal function in primate evolution (see below and ref. 22).

TRICHROMACY IN PRIMATES

Changes in color vision during primate evolution provide a beautiful example of how changes in sensory function correlate with ecological niche.[23,24] Human, apes and old world (OW) monkeys (catarrhine primates) are trichromatic—the cone photoreceptors in our eyes contain one of three distinct photopigments, which maximally absorb blue, red or green light. The photopigments consist of a G-protein-coupled receptor, called an opsin, and a covalently bound light-absorbing chromophore, retinal, a derivative of vitamin A. The three human opsin proteins absorb different wavelengths because they "tune" the spectral sensitivity of the retinal. Rodents, new world (NW) monkeys and prosimians are mainly dichromats—their cones contain one of two color opsins, which absorb blue or green/red light maximally.

How and why did catarrhine primates evolve trichromacy? As to "how" it is clear that the green opsin gene arose as a duplication of the red opsin gene, as these genes are

nearly identical and are present in tandem array on the X chromosome.[23] This duplication occurred in the common ancestor of catarrhine primates, as can be inferred by the presence of trichromacy in OW monkeys and apes but not in most NW monkeys. Some NW monkeys have a polymorphism in their red/green opsin gene that changes the spectral sensitivity of the opsin; because the gene is on the X chromosome, which is subject to inactivation, females may possess "allelic trichromacy".[24,25] In fact the presence of this common polymorphism suggests that "allelic trichromacy" might have predated the emergence of full trichromacy.[24] In addition, one species of NW monkeys, the howler monkey, has evolved full trichromacy, in an independent duplication event.[25-27] Trichromacy in catarrhine primates most likely evolved to improve the success of foraging for ripe fruits and possibly for young shoots.[23]

LOSS OF VOMERONASAL FUNCTION IN PRIMATE EVOLUTION

Molecular analysis of sensory systems has been particularly informative in instances where it is otherwise difficult to measure function. Such is the case for the study of pheromone detection in vertebrates. There has been considerable dispute about whether humans can perceive pheromones, and if so whether it is through a nasal sensory organ called the vomeronasal organ.[28,29] The VNO in mice is a paired tubular structure located beneath the nasal cavity. In humans a small pit can be detected in the nasal cavity which some have argued may contain functional sensory cells that respond to pheromones. To determine whether the human VNO is functional is a difficult problem which has been, perhaps, best addressed with molecular tools.[22,30]

In rodents the vomeronasal organ expresses two families of GPCRs that are candidate pheromone receptors (V1Rs and V2Rs).[31-34] In mice there are 165 functional V1R genes and 61 functional V2Rs genes.[35,36] V1R-expressing cells appear to respond to small hydrophobic molecules whereas V2R-expressing cells appear to respond to peptides.[37,38] A role for V1R receptors in pheromone sensation has been substantiated by the observation that targeted deletion of one cluster of 16 receptors leads to changes in maternal aggressive and male sexual behavior.[39] Vomeronasal sensory neurons also express a unique ion channel, called TRPC2,[40] which is activated by second messengers downstream of the V1R and V2R receptors, with which it is co-expressed.[40-42] In the absence of TRPC2, male mice do not show typical pheromone-mediated male-male aggression, indicating that this ion channel is essential for normal VNO function.[7,43] Moreover when confronted with a castrated male mouse (who is not aggressive) male TRPC2 knockout mice will attempt mating, at a similar frequency with which the attempt mating with female mice, suggesting that the VNO plays a role in gender identification.[7,43]

The identification of essential components of VNO sensory transduction has made it possible to determine, solely with molecular methods, whether a particular species is likely to have a functional VNO. For humans, the preponderance of evidence shows that the VNO is vestigial; the TRPC2 gene contains four nonsense and two insertion/deletion (in/del) mutations[22,30,40,44] and most of the V1Rs are also pseudogenes.[45] By identifying mutations in the TRPC2 gene of extant species and parsimoniously assigning the time in evolution that these occurred, two groups determined that the first deleterious mutation in the gene occurred in the ancestor of OW monkeys and apes, 25-40 million years ago (mya)[22,30] (Fig. 2A,B). This first mutation was a truncation of the C terminus of the protein, and the effects of this mutation, although likely to be deleterious, not known. Additional

evidence that selection was relaxed at this time in evolution comes from an analysis of the Ka/Ks ratio along branches of primate phylogeny (Fig. 2C); this ratio is significantly less than one on branches of the evolutionary tree leading up to the ancestor of OW monkeys and apes, showing purifying selective pressure, and thereafter is not different from 1, showing that selective pressure was relaxed at this time.[22] The excellent agreement between these two types of analyses strongly supports the view that selective pressure on the TRPC2 gene was relaxed 25-40 mya.

The V1R receptor genes most likely came under relaxed selective pressure at this time, as evident by the large number of human V1R pseudogenes (~200) and presence of only 4 possibly functional genes.[45] It is not known whether these 4 receptors are functional in humans. Computational methods show that this is the number of genes expected by chance to have escaped pseudogenization, given that selective pressure was relaxed ~25 mya.[30] Moreover, none of these 4 receptors are conserved in chimps,[30] thus one would have to argue that whatever function they subserved in the common ancestor of humans and chimps was lost in modern chimps. On the flip side, these receptors have retained many of the highly conserved residues found in other V1Rs, an observation which is inconsistent with relaxed selective pressure on the genes.[45] One of these receptors appears to be expressed in the main olfactory epithelium,[46] suggesting that it might have been co-opted for a different chemosensory function. Solving this mystery will not be easy as there are no direct mouse orthologs of these genes.[47]

Together these results show that critical components of vomeronasal transduction were lost in human evolution 25-40 million years ago. Interestingly, this is the same time when trichromacy appeared, suggesting that visual signaling may have replaced pheromone signaling. Indeed catarrhine primates show prominent female sexual swelling and other sexual dimorphisms, which provide a visual signal of reproductive and social status. Thus it is likely that as primates began to rely on these signals over chemical signals, the VNO became redundant and selective pressure was relaxed on molecules it uniquely expresses.

CONTRACTION OF THE ODORANT RECEPTOR REPERTOIRE IN HUMAN EVOLUTION

In contrast to the vomeronasal organ, which may be specialized to detect nonvolatile signaling chemicals that directly regulate behavior, the olfactory epithelium plays a more general role in assessing complex chemicals in the environment, leading to odor perception. Most organisms have hundreds of distinct odorant receptors, which are encoded by intronless genes located throughout the genome.[48] Of this large repertoire of odorant receptors, each olfactory sensory neurons expresses just one,[49] allowing for the unique identification of chemicals that vary only slightly in structure. Moreover, when used in a combinatorial code, this large family of low affinity receptors can encode the identity of millions of distinct odorants.[2] Thus given the redundancy in this system, and the high sequence homology among receptors, it is not surprising that the size of the odorant receptor repertoire is highly dynamic, having undergone expansion and contractions in many different animal lineages.[50] The repertoire of functional odorant receptors in humans is considerably smaller than it is in rodents, and a large fraction of the genes are pseudogenes (~50% of the 802 human genes and 25% of the 1391 mouse genes; reviewed in ref. 14). The difference in the size of the human and mouse repertoires reflects both expansion in the murine lineage and pseudogenization in the primate lineage.[51]

The large number of human OR pseudogenes appears to be the result of two separate times in evolution at which selective pressure on these genes was relaxed. Selection pressure was relaxed first in the ancestor of OW monkeys and apes, as deduced from the observation that all OW monkeys and apes have a higher fraction of OR pseudogenes (~30%) than do NW monkeys (~18%); thus pseudogenization of the OR repertoire co-incided with the appearance of trichromacy in primates.[52] A similar deterioration in the repertoire of functional OR genes is observed in the howler monkey, a NW monkey that has independently evolved trichromacy, providing additional support for the notion that olfactory function became less important as primates developed more powerful visual systems.[52] A more recent relaxation in selective pressure on OR genes appears to have occurred specifically in the human lineage, as humans have a significantly higher percentage of OR pseudogenes as compared with other OW monkeys and apes.[53] Indeed, pseudogenization of human OR genes may be ongoing as evidenced by the presence of common polymorphisms that disrupt the coding region of the genes.[54] The reason for the increase in pseudogenization of human OR genes is not known, but may be attributed to a reduction in the use of olfactory signals as means to identify and discriminate foods.

LOSS OF SIGNALING THROUGH GC-D NEURONS EARLY IN PRIMATE EVOLUTION

The majority of olfactory sensory neurons express the canonical odorant receptors identified by Buck and Axel,[48] but at least three additional subsets of cells can be identified in the nasal epithelium.[55-59] This includes a subset that is defined by its expression of the olfactory-specific guanylyl cyclase (GC-D).[60,61] These cells have attracted interest because they project to an anatomically distinct group of interconnected glomeruli in the olfactory bulb, the necklace glomeruli, that have been implicated in the suckling response of mammals[62,63] but see.[64] Thus GC-D cells may participate in the detection of volatile signaling chemicals and regulate social interactions.

In this context, it is interesting that in humans the GC-D gene is a pseudogene[65,66] Moreover, based on bioinformatic analysis of trace-archive and genome-assembly data and sequencing of PCR amplified genomic DNA it was determined that GC-D is a pseudogene in a large number of primate species, including apes, Old World and New World monkeys and tarsier while it is intact and evolved under purifying selection in mouse, rat, dog, lemur and bushbaby. These data suggest that signaling through GC-D-expressing cells was probably compromised more than 40 million years ago, prior to the divergence of New World monkeys from Old World monkeys and apes.[66]

What chemosensory function did our ancestor lose at that time? Analysis of the olfactory system in mice shows that the GC-D cells can detect atmospheric CO_2,[67] urinary proteins,[68] and carbon disulfide (CS_2),[69] each of which may serve a specific signaling function and trigger an innate behavior. For example, CO_2 is released in the breath of conspecifics and predators and produces an innate avoidance in mice.[67] Most recently, GC-D neurons were shown to participate in the acquisition of socially transmitted food preference,[69] a phenomenon wherein food from which animals have eaten is marked by secreted CS_2 as safe for consumption.[70] The loss of GC-D signaling in primates, is consistent with a clear contribution of observational learning to food preference in humans.[71]

EVOLUTIONARY CHANGES IN TASTE SENSATION

Receptors for taste specify attraction or aversion to food[71,73] and thus changes in these receptors may be directly related to adaptive changes in foraging behavior and diet. Taste allows animals to determine the nutritive content of food prior to ingestion: Of the five identified taste modalities, three, sweet, umami, and salty, signal the presence of essential nutrients and lead to ingestive behavior. The other two modalities, bitter and sour, signal, respectively, the presence of toxins or the spoilage of food, and to most animals are aversive. The molecular basis for sour and salty tastes is not yet well understood and candidate receptor molecules await validation with genetic methods.[3-5] In contrast, receptors for sweet, bitter, and umami have been well described; a small family of 3 class C GPCRs (T1Rs) mediates sweet and umami taste and a larger family of class A GPCRs (T2Rs) mediates bitter taste.[3-5,74] Heterodimers of T1Rs form the sweet (T1R2/T1R3) and umami receptors (T1R1/T1R3).[75-77]

Taste receptors have now been identified in a large number of species, including humans and other primates. Interestingly the three sweet and umami receptors are well conserved among all land vertebrates, with the exception of cats, for which the sweet receptor T1R2 is a pseudogene.[78,79] The loss of the T1R2 gene and sweet taste perception in cats is consistent with dietary choice in these animals that are obligate carnivores. Chickens also appear to be missing a functional T1R2 gene, although the significance of this observation it not known.[79] While most species retain functional T1R receptors, it is likely that the ligand specificity of these receptors varies across species in such a way as to allow the detection of species-specific naturally occurring nutrients. For example sweet receptors of humans and mice differ in sensitivity to brazzein, a protein produced by certain African plants; this protein tastes intensely sweet to humans whereas mice are indifferent to it. The structural basis for this difference has been mapped to a cysteine-rich region in the T1R3 receptor.[80] This may represent yet another example of co-evolution of a vertebrate sensory receptor with a natural plant product.

Bitter receptor genes are present in variable numbers among different species, having undergone rapid expansion and pseudogenization.[14,81] Humans have 25 functional bitter receptor genes as compared with 35 in mice.[14,79,81,82] Three receptors have become pseudogenes specifically in the human lineage, which is a somewhat higher rate of pseudogenization than is seen in other primates.[82] It has been argued that a loss of selective pressure on bitter receptor genes in human evolution could be the result of a decreased reliance on nutrients from toxin-containing plants.[79]

Selection has acted not just to eliminate functional bitter receptor genes from the human genome, but also to change their ligand specificity. This is perhaps no where more evident than in the case of the phenythiocarbimide (PTC) taste receptor, TAS2R38.[83] In 1931 a common variation in the ability of people to taste PTC was reported which was shortly thereafter shown to be genetically determined.[84,85] Variation in PTC sensitivity was also found in chimpanzees, suggesting that the genes responsible for it became fixed prior to the divergence of the two species.[86] The stability and prevalence of this polymorphism made it a textbook example of "balancing selection", where two or more forms of the gene are maintained in the population as a result of heterozygote advantage. The genetic basis for the phenotypic variation in PTC sensitivity is now known; 3 missense mutations generate two common alleles that can explain the phenotypic variation in the human population.[87,88] Interestingly, chimpanzees do not share these alleles and instead the nontaster allele contains an interrupted reading frame.[89] Detailed analysis of human

data shows that the two common human alleles are both too divergent and too prevalent to be the result of simple genetic drift and instead reflects balancing selection. It has been speculated that the nontaster TAS2R38 allele still detects bitter chemicals, whose identity are not yet known, and thus heterozygotes have the advantage of being able to detect a larger variety of potentially toxic chemicals.[90] This is reminiscent of the visual system of NW primates where a polymorphism in the red opsin gene allows females to have allelic trichromacy (see above).[24,26] It is tempting to speculate that gene duplication in the future may result in the presence of both variants of the TAS2R38 gene on the same chromosome and that what we are observing now is an intermediate step in evolution.

CONCLUSION

The identification of a growing number of chemosensory receptors in different organisms provides a unique opportunity to understand how selective forces have shaped the evolution of sensory systems. Work in the vomeronasal system, showing that genes expressed there are nearly all pseudogenes, has provided the strongest support for the idea that this sensory modality is vestigial in humans. Other sensory systems have clearly changed during human evolution, with contractions in the repertoires of both olfactory and bitter taste receptors. The significance of these contractions are presently the subject of conjecture, and will only be understood when the cognate ligands for the pseudogenized OR and bitter receptors have been identified, and their natural occurrence in the environment understood. Moreover, changes in OR and bitter receptors are ongoing in the human population and the identification of polymorphisms in these receptors may lead to a better understanding of the variation in human food preference.

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CHAPTER 14

MOLECULES AND MATING:

Positive Selection and Reproductive Behaviour in Primates

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Abstract: Sexual reproduction is generally thought to be more costly than asexual reproduction. However, it does have the advantage of accelerating rates of adaptation through processes such as recombination and positive selection. Comparative studies of the human and nonhuman primate genomes have demonstrated that positive selection has played an important role in the evolutionary history of humans and other primates. To date, many dozens of genes, thought to be affected by positive selection, have been identified. In this chapter, we will focus on genes that are associated with mating behaviours and reproductive processes, concentrating on genes that are most likely to enhance reproductive success and that also show evidence of positive selection. The genes encode phenotypic features that potentially influence mate choice decisions or impact the evolution and function of genes involved in the perception and regulation of, and the response to, phenotypic signals. We will also consider genes that influence precopulatory behavioural traits in humans and nonhuman primates, such as social bonding and aggression. The evolution of post-copulatory strategies such as sperm competition and selective abortion may also evolve in the presence of intense competition and these adaptations will also be considered. Although behaviour may not be solely determined by genes, the evidence suggests that the genes discussed in this chapter have some influence on human and nonhuman primate behaviour and that positive selection on these genes results in some degree of population differentiation and diversity.

Sensing in Nature, edited by Carlos López-Larrea.

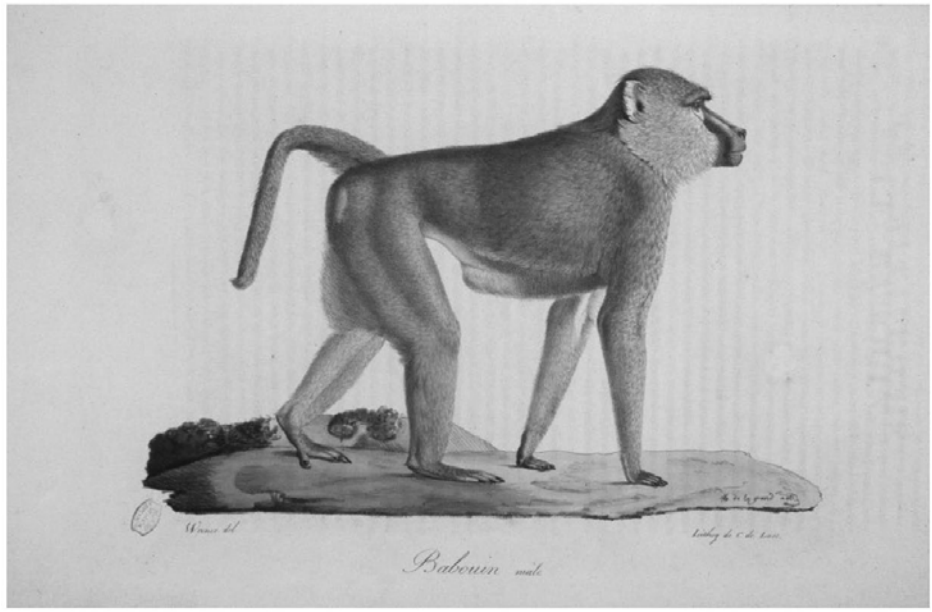
INTRODUCTION

Animal behaviour is far too complex to be solely determined by genes. Nevertheless, numerous studies have demonstrated that genes can, and do, directly influence behaviour. As a consequence, behavioural ecologists now not only study ecological and evolutionary adaptation; they also explore the ways in which genes influence natural variation in behaviour. Genes typically work in a dynamic fashion, interacting with other genes in the genome (e.g., epistasis) and in response to complex interactions with the environment. Genes can influence behaviour in several ways: some exhibit allelic variation that affects behaviour, others do not vary but change their pattern of expression within an individual over time, resulting in changes in behaviour (e.g., plasticity). Still other genes vary in function between different individuals due to reasons other than changes in a DNA sequence, such as behavioural programming or stress (i.e., epigenetics). Despite these complexities, however, genetic and genomic approaches hold great promise for elucidating not only the molecular basis of social behaviour, but also for determining the ways in which selection pressures act on genes that influence specific behaviours.

Numerous studies have demonstrated that, through selection, genes can evolve according to their effects on behaviour, even if their mechanistic roles in that behaviour are subtle and indirect. In both model and wild organisms, the effects of selection can be detected using comparative genome sequence data as well as differences in nucleotide substitution rates, amino acid codon frequencies, regulatory sequences, and gene copy number. The comparison of DNA sequences both between and within species provides valuable information for understanding the evolutionary forces affecting genetic loci. Intraspecific comparisons, in particular, reveal insight into the genetic variation within a population and also the microevolutionary forces that have shaped variation. Molecular evolution algorithms can also be used to determine whether genes have been subjected to specific selective pressures based on contrasts between the rates of synonymous and nonsynonymous nucleotide substitutions in DNA sequences. These algorithms have shown that neutral/purifying selection is frequently observed at the molecular level[1] and that stabilizing selection is common.[2] Positive selection may be observed less often, and very strong positive selection may drive alleles to fixation,[3] but positive selection has the ability to maintain high levels of phenotypic diversity when the rate of nonsynonymous substitution exceeds the rate of synonymous substitutions. Therefore, detection of positive selection and study of its consequences can provide insight on the evolution of genes, or other genomic elements, that influence behaviour.

Sexual reproduction provides numerous opportunities for positive selection to act upon individual genetic traits. Individuals that successfully mate with a partner will pass on their genetic traits to the next generation. Therefore, for sexually reproducing organisms, it is essential to find a partner that can contribute viable gamete and it is even better if their contribution enhances offspring fitness. Literature focusing on the factors contributing to reproductive success in humans and other primates is abundant. Our goal is not to discuss all of the factors that contribute to an individual's fitness, but rather to examine the relationship between genetic variation and primate reproduction and mating behaviour. In particular, we will focus on genes that are associated with mating behaviours (Fig. 1) and reproductive processes that enhance reproductive success and thus, are targets for positive selection.

Pre-copulatory mating behaviours	
Mate choice	*MHC , OR , V1R , S*
Social bonding	*OXTR, AVPR1*
Male-male competition	*MAOA , DRD2 , DRD4 , 5HTT , 5HT2A*



Post-copulatory reproductive mechanisms	
Sperm morphology	*PRM1* and *PRM2*
Sperm competition	*SEMG2, TGM4, KLK2, ACPP, MSMB*
Selective abortion	*MHC*

Figure 1. Molecules and Mating. Many genes associated with pre- and post-copulatory behaviour in primates exhibit signatures of positive selection. Genes involved in precopulatory behaviours include major histocompatibility complex (*MHC*), olfactory receptor (*OR*) and vomeronasal receptor (*V1RL*), *S* photopigment, oxytosin receptor (*OXTR*), vasopressin receptor (*AVPR*), monoamine oxidase (*MAO*), dopamine receptor D4 (*DRD4*) and D2 (DRD2), serotonin transporter (*5-HTT*) and serotonin receptor gene 2A (*5HT2A*). Genes related to post-copulatory behaviour include spermatid-associated protamin 1 and 2 (*PRM1* and *PRM2),* semenogelin II (*SEMG2*), prostate-specific transglutaminase 4 (*TGM4*), kallikrein 2 (*KLK2*), prostatic acid phosphatase (*ACPP*), beta-microseminoprotein (*MSMB*) and the *MHC* (see text for details).

MATE CHOICE

MHC Genes and Selection

One of the classic genetic systems where positive selection seems to contribute to high genetic diversity is the major histocompatibility complex (MHC). The MHC is characterized by an unprecedented level of allelic diversity.[4] This extensive polymorphism is believed to be the product of positive selection and maintained through either heterozygote advantage or frequency-dependent selection or both.

Heterozygote advantage refers to increased fitness resulting from the presence of a heterozygous genotype instead of a homozygous one. An individual with a wider variety of MHC alleles, encoding a greater diversity of MHC proteins, will be able to detect and present a greater number of pathogen types, conferring a fitness advantage on heterozygous individuals.[5] Recently, O'Connor et al[6] highlighted the importance of MHC heterozygosity in precluding simian immunodeficiency virus (SIV) in cynomolgus macaques. MHC-heterozygous individuals elicited a broader SIV-specific CD8 T lymphocyte response than MHC homozygous individuals, making it harder for the virus to evade detection. As a result, circulating viral loads were eighty times greater in MHC-homozygotes than in MHC-heterozygotes. This study eloquently demonstrates the advantage of maximising diversity within the MHC.

Frequency dependent selection describes a situation in which the fitness of a particular allele depends on its frequency within a population. The genes of the MHC encode cell surface proteins that bind peptides and present them to immune cells, initiating a cell-mediated immune response. These proteins have distinct binding properties, meaning that they each bind to peptides from particular pathogens. Pathogens that can evade the most common MHC alleles will be favoured by natural selection as they will be able to infect the greatest number of hosts and hence proliferate. Consequently, individuals with rare MHC alleles will be more resistant to pathogen infection. This fitness advantage means that the frequency of rare alleles will increase over time, shifting the selective pressures acting on the pathogen, thereby perpetuating an "evolutionary arms race".[7] Hence, the fitness advantage associated with rare alleles should promote MHC polymorphism.

Several studies have demonstrated the importance of frequency dependent selection in maintaining MHC variation in primates. Schwensow et al[8] investigated the relationship between polymorphism in the functionally important exon 2 region of class II MHC-DRB genes and parasite burden in the fat-tailed dwarf lemur (*Cheirogaleus medius*). They noted that certain rare MHC alleles, grouped together as supertypes due to similar peptide binding properties,[9] were associated with a complete absence of nematode infection. Other more common supertypes were associated with a high intensity and diversity of nematode infection. Interestingly, overall MHC heterozygosity showed no significant association with nematode burden, implying that heterozygote advantage was not responsible for the fitness advantage.[8]

Rare allele advantage is also seen in MHC genes in human populations. In humans, the MHC is also known as the human leukocyte antigens (HLA). The *HLA-DRB1*1302* allele is associated with reduced susceptibility to both malaria (*Plasmodium falciparum*[10]) and the hepatitis B virus.[11] The allele is scarce in most human populations,[10] but found in relatively high frequency in West African populations, where these diseases are prevalent. Of the individuals infected with malaria, there were a high number of heterozygotes

relative to the number of controls, suggesting that these alleles were not influenced by heterozygote advantage.[10] Instead, *HLA-DRB1*1302* homozygotes enjoyed greater disease resistance than heterozygotes, implicating a role for frequency-dependent selection.[10]

MHC-Associated Mate Choice

It is clear that maintaining diversity at the MHC can have significant fitness advantages. Primate reproductive behaviour serves to maintain this diversity, as individuals are believed to base their mate choice decisions on the MHC genotype of potential mates. As we have seen in earlier chapters, the MHC is used for cellular discrimination of "self" and "nonself", it is not unreasonable to suggest that this genetic system might be useful for detecting similarity at the level of the entire organism, which would have implications for reproductive behaviour and reproductive success. A comprehensive exposition of the mechanisms that allow individuals to discriminate others based on their MHC genotype is discussed by Ruff et al.[12] Notably, however, the varying social and ecological contexts in which primate populations live may place different selective pressures on their respective MHC genomes. In both human and nonhuman primates, a variety of context-dependent behaviours have been described and these can potentially direct the molecular evolution of the MHC according to the selective pressures on the population.[13,14]

In general, if the adaptive advantage of MHC heterozygosity is sufficient, selection should favour individuals who select mates with MHC genotypes that are highly disparate from their own. This has been observed in several primate populations. For example, research focusing on a closed, captive population of mandrills (*Mandrillus sphinx*) demonstrated how MHC dissimilarity has a significant influence on reproductive behaviour.[15] Mandrills are Old World monkeys that live in Gabon and have been studied extensively by primatologists.[16,17] One important study group lives in large multi-male, multi-female groups[18] with no immigration of individuals from outside the natal group.[15] They are polygynous and characterised by a large skew in male reproductive success.[19] Despite this skew, males whose MHC genotype was disparate from that of a given female were more likely to sire her offspring. In addition to preference for a mate with a different MHC genotype (i.e., complimentarity) females also prefer to mate with MHC-heterozygous males. Heterozygous males have increased vigour, greater resistance to pathogens and possess a greater number of rare alleles—all of which are valuable and heritable traits for offspring. Hence, females seem to employ multiple strategies when selecting an optimal mate. The lack of immigration in this particular colony may contribute to preferences for MHC-dissimilar males, ensuring genetic diversity within offspring MHC and avoiding inbreeding depression. Additionally, the population lives in a large social group in a wet climate. Both conditions increase pathogen risk.[20,21] Thus, selecting a mate with a highly polymorphic MHC can provide superior disease resistance in the offspring, while reducing exposure to local pathogens. While the polygynous social structure in mandrills living in an isolated population may seem counterproductive, other animals exhibit similar strategies. In Iberian red deer (*Cervus elaphus hisapanicus*), for example, only the most heterozygous males mate and the negative effects of diminished effective population size are significantly reduced through the presence of diverse alleles in the subsequent generation.[22] Similarly, Sauermann and colleagues[23] found that free-ranging male rhesus macaques that were heterozygous at the MHC class II locus *Mamu-DQB1* sired significantly more offspring than *Mamu-DQB1* homozygote males.

MHC-Associated Selective Abortion

MHC-disassortive mating preference has also been observed in human societies. Ober et al[24] identified MHC-related mating preference in married couples from the Hutterite community, a noncontracepting population living in North America. Ober and colleagues[24] genotyped 411 couples for five HLA loci and found a significant pattern of HLA-disassortive mating. The Hutterite community is highly endogamous and research suggests that high levels of parental HLA matching contribute to pregnancy loss and reproductive failure.[25] Although more than 40 different studies have examined the relationship between the HLA and reproductive outcome (see ref. 26), conflicting conclusions have led to controversy about the influence of the MHC on reproductive success. In general, however, a majority of the studies endorse the association between parental HLA sharing and poor reproductive outcome, many reporting that some homozygous offspring are selectively aborted post-conception. Further support for the hypothesis that MHC, or MHC-linked, genes play a role in primate reproductive success comes from a study of pigtailed macaques where MHC sharing was significantly higher among reproductively unsuccessful macaque couples when compared to successful pairs.[27]

Notably, not all studies of HLA and mate choice arrive at the same conclusions. Chaix and Donnelly[13] found evidence for HLA-based mate choice in European populations but also reported that there was no association between MHC similarity and mate choice in the West African Yoruba. This suggests that social factors may have more influence on fitness than genetic factors in some populations.[13] It is also possible that selection for MHC diversity per se may be outweighed by the selective advantage of specific MHC alleles. For example, high pathogen risk may confer a greater fitness advantage for particular MHC alleles when compared to overall MHC diversity. In these cases, mate selection may be based on "good genes" rather than on maximising MHC diversity.

Thus, under differing population parameters, there may be less pressure to maximise MHC heterozygosity. The most MHC-disparate partner may not necessarily be the best choice. For example, and in contrast to what has been found for mandrills, a study conducted on chacma baboons (*Papio ursinus*) found no support for MHC-based mate preferences. The chacma baboons are free-ranging and live in female philopatric communities in which males leave their natal group. As a consequence, there is a great deal of admixture and outbreeding, which may effectively maintain genetic diversity and, therefore, relax the pressure to select mates based on MHC genotypes. Interestingly, while there is no evidence of preference for MHC-dissimilar mates and no sign of preference for rare MHC alleles, heterozygotes seem to enjoy extended longevity.[28] Taken together, the results of this baboon study and the aforementioned mandrill research suggest that evidence supporting MHC-based mate choice may vary according to population structure and existing levels of inbreeding.

It has been suggested that an intermediate level of MHC-dissimilarity may optimise fitness.[29] Jacob et al[30] found that women preferred the odours of males with whom they shared a small number of paternally inherited MHC alleles. Selection based on an intermediate level of MHC dissimilarity may allow females to optimise the balance between the costs of inbreeding and outbreeding depression.[31] For example, while it is important to maintain heterozygosity, a preference for MHC-dissimilar mates may ultimately lead to the loss of locally adaptive genetic traits. Therefore, a trade-off that favours mate preferences based on an intermediate level of genetic dissimilarity may develop.[29] Alternatively, there might also be an immunological cost to expressing

too many MHC alleles.[7] Thymic selection means that while initially increasing the number of MHC genes will increase pathogen resistance, at some stage this will cause a decrease in T-cell repertoire.[32] Therefore, if fitness is maximised through an optimum level of MHC diversity, then selecting a mate who is somewhat dissimilar could be the best strategy.[7]

While the selective pressures exerted on a population are in part dependent on social structure, there is also strong evidence suggesting that pathogen exposure can shape the molecular evolution of a population. Nunn and Altizer[33] cite several human studies that demonstrate how adherence to MHC-associated behaviour is dependent on pathogen risk. Women perceive males with more diverse MHC genotypes as more attractive than less heterozygous males.[34] Gangestad and Buss[35] showed that across 29 cultures, the importance of physical attractiveness in a potential mate increased with pathogen risk, even when factors influencing pathogen risk (such as latitude or income) were controlled. Moreover, Low[36] analysed data from 97 cultural groups and found that women were more likely to engage in polygynous relationships in populations where innate pathogen exposure is greater. There may be numerous nongenetic reasons for polygyny to become the primary mating system in these populations. However, this pattern also fits with a scenario in which women preferably mate with a male whose MHC genotype confers the greatest level of heritable fitness. While research has shown that MHC variation is lower among populations indigenous to South America,[37] and that this may result from a bottleneck event during colonisation of the Americas, it is also possible that as these populations have been exposed to fewer pathogens, concomitantly there would be less pressure for maximal MHC diversity.[37]

Although the influence of reproductive behaviour on genomic diversity is pronounced in MHC genes, it is not restricted to these loci. The molecular evolution of many other genes associated with resistance to disease is also affected by reproduction. Sexually transmitted diseases are particularly relevant for fitness and infection can reduce fertility. Wlasiuk and Nachman[38] examined promiscuity among a diverse range of primate species in relation to the rate of protein evolution in immune genes such as a chemokine receptor (CCR5) and toll-like receptor (TLR4 and 5) genes. The authors found that the rate of protein evolution increased with the promiscuity of the species and the size of the population. They suggest that reproductive behaviour and mating systems are driving forces behind the molecular evolution of populations.[38]

Signalling MHC Genotypes

As described by Ruff et al[12] mate choice behaviours are mediated by various phenotypic signals. Chemical signals, known as pheromones, have a particularly important role in advertising MHC profile to conspecifics. Currently, relatively little is known about the role of pheromones in primates. While a substantial body of behavioural studies have implicated pheromones in the reproductive behaviour of several primate species (e.g., mandrills; humans), in general, anthropoid evolution is characterised by the retrogression of olfactory function. This is supported by morphological data such as the decrease in the size of the olfactory epithelium and the volume of the olfactory bulb.[41] However, several recent molecular studies suggest that the evolution of primate olfaction and pheromone perception may be more convoluted than the morphological evidence suggests (see refs. 42, 43). Future research focusing on additional primate species will undoubtedly improve our understanding of olfaction and MHC-based signals.

The olfactory receptor (OR) genes are one of the largest known multigene families and encode the primary receptors of the main olfactory epithelium.[44] While the vomeronasal organ is generally associated with pheromone perception, Barton[42] demonstrated that the main olfactory system might also play a role in the perception of specific pheromones and therefore could also play a role in MHC-associated mate choice. Primates in general have a far smaller OR gene repertoire than mice. Humans are thought to have a functional OR gene repertoire three times smaller than that of mice.[45] Moreover, an increase in the rate of accumulation of pseudogenes has been demonstrated in the lineages between New World monkeys and Old World monkeys to great apes.[46] The pseudogenisation of the human OR region is particularly pronounced. Studies have indicated that humans accumulate pseudogenes 4.3 times faster than other great apes[47] and that the human OR region consists of approximately 70% pseudogenes; by far the greatest proportion found in any primate.[46] Gilad et al[48] showed that this OR deterioration coincided with the transition to obligate trichromatic vision and that the most precipitous leap occurred between human and nonhuman primates.[47] This suggests that relaxed selection, afforded through increased dependency on vision, was one impetus for the retrogression of olfaction in primates.

Notably, however, recent research indicates that the molecular evolution of primate olfaction has not taken such a direct trajectory. For example, Dong et al[43] have shown that the proportion of pseudogenes vary between primate clades, but contraction and expansion events (known as birth-and-death effects) in the olfactory gene repertoire throughout anthropoid evolution means that the number of intact functional OR genes in any primate species genome does not vary significantly between clades. This implies that selective pressures on the olfactory gene repertoire of different extant and ancestral species vary, so only certain OR genes will be under positive selection. Indeed, analyses of expansive regions of the human and chimpanzee genomes has shown that olfactory genes display some of the strongest signatures of positive selection of all genes that have been examined.[49,50]

Further support for the argument that functional adaptation leads to positive selection on olfactory genes comes from two publications. Both studies compared the OR regions of humans and chimpanzees and found human-specific signatures of positive selection at particular OR genes.[47,51] Specifically, Go and Nimura[51] found that the OR gene *HsOR7.6.15* displayed 13 nonsynonymous substitutions, one of which is known to affect the ligand-binding site. The authors suggest that functional variation might confer a fitness advantage and therefore would have been selectively advantageous.

In addition to these signs of selection in OR region genes, the vomeronasal receptor genes (V1R) also show signs of varying selection throughout anthropoid evolution. These selective pressures vary between species rather than correlate directly with behavioural complexity. Mundy and Cook[52] showed that *V1RL1* became a pseudogene multiple times throughout anthropoid evolution. This gene has an intact open reading frame in six species analysed, including humans, gorillas, pygmy marmosets and three species of howler monkey. However, other species that shared a close phylogenetic relationship with these species possess a pseudogene. Mundy and Cook[52] also found that *V1RL1-4* exhibited significant signs of positive selection, indicating species-specific pheromone functioning. These findings support the idea that while primate olfaction exhibits a general trend of decline, the molecular evolution of the system is highly convoluted and dependent on the ecological constraints and behavioural repertoire of particular species.

The acquisition of obligate trichromatic vision in catarrhine species, such as baboons, macaques, apes and humans, is often believed to co-occur with the deterioration of olfactory function. This is based on the concept that trichromatic vision emerged at approximately

the same time as reliance on olfaction begins to decline.[48] However, as discussed, the suggestion that sensory perception may correlate directly with the increasing complexity and visual requirements of primate species may be somewhat misguided.

While the opsin (S photopigment) gene allowing for vision of blue light is located on chromosome 7,[53] the gene necessary for full trichromatic vision (L photopigment) is present on the X chromosome. In many platyrrhine (New World monkey) species this X-linked opsin gene has two alleles, one for red vision and one for green. As such, all male and homozygous female New World monkeys have dichromatic vision. However, heterozygous females have all three opsin alleles and therefore have full trichromatic vision.[54] In catarrhines (Old World monkeys, apes and humans), this opsin gene was duplicated and resulted in relaxed selective pressures on the duplicate. This allowed the duplicate gene to mutate outside the parameters of the evolutionary constraints that were placed on the parent gene. Hence, new genotypes led to perception of a wider range of the visual light spectrum. Surridge et al[55] suggest that it is unlikely that this obligate trichromacy could have evolved in such a way in the absence of positive selection.

Bright colour for sexual signalling is widely used in primate mate choice and colour vision should be highly advantageous. However, phylogenetic analysis shows that trichromatic vision evolved prior to colourful sexual integument.[56] It is believed that trichromatic vision first provided an advantage in foraging efficacy, either allowing for better detection of fruits[57,58] or young leaves.[59,60] Trichromacy also provides the added advantage of detection of predators with yellow coloration.[61] Ultimately however, colour has become an important mediator of mate choice decisions in many primates. It is well known that females of many Old World monkey species advertise their sexual receptivity with exaggerated sexual swellings (see ref. 62). Chimpanzee females also have conspicuous sexual swellings during the middle stages of the ovarian cycle.[63] Recently, it has been shown that size and shape of sexual swellings in female chacma baboons provide a signal of receptivity and also an indication of quality and possession of "good genes" for disease resistance (i.e., optimal MHC alleles).

Interestingly, coloration signals are not restricted to female sexual swellings. It appears that trichromatic colour vision provides advantages to females as well. Setchell et al[64] observed that facial coloration in female mandrills was associated with reproductive state and condition and may also influence male mate choice decisions. Female facial colour signals information concerning age, parity and reproductive status, which may be useful for both male and female conspecifics. Waitt[65] showed that female rhesus macaques display preference for males with more vibrant red sexual skin. This coloration is caused by an increase in skin blood perfusion, induced by increased testosterone concentrations.[66] Interestingly, Gerald et al[67] recently reported that female vervet monkeys pay attention to variation in male scrotal coloration but preliminary research suggests that coloration differences alone do not explain female patterns of affiliative behaviour.

Despite the importance of colour in guiding the reproductive behaviour of higher primates, olfaction is not necessarily obviated as a result. For example, Setchell et al [107] have recently shown that mandrills, who have elaborate colourful sexual integuments and trichromatic vision, also rely on scent to mediate MHC related reproductive behaviour. Moreover, howler monkeys have developed trichromatic vision but also retain high vomeronasal function[68] Considering the numerous advantages that come with both trichromatic vision and pheromonal detection, including, but not limited to, recognition of sexual signals and detection of social status, it is unsurprising to find evidence of positive selection in genes related to both of these traits.

COMPETITION FOR MATES

Male-Male Aggression

Behaviours associated with social dominance can influence the outcome of competition for critical resources such as mates and these in turn determine an individual's evolutionary fitness. In the past decade, research focusing on genes involved in the production or metabolism of neurotransmitters such as dopamine, serotonin and norepinephrine, has shown how small changes or even deletions in a DNA sequence can lead to variation in levels of aggression and other behaviours associated with social dominance. In many primate societies, the intense competition for sexually receptive females leads to escalated levels of aggression among males and the ability to dominate in male–male competitions has major consequences for an individual's reproductive success. Like other primates, men compete for reproductive opportunity, with the competition taking the form, at least in some societies, of violent conflict between individuals or between kin groups.[69] Therefore, genes that enhance competitive access to mating partners should be positively selected in both humans and nonhuman primates.

Numerous studies have shown that neurotransmitter compounds influence aggressive behaviour. Monoamine oxidase (MAO) is an enzyme, found in the outer membrane of mitochondria, that degrades biogenic amines and is responsible for the destruction of neurotransmitter compounds in the synaptic cleft. The *MAOA* gene encodes monoamine oxidase A, which is involved in the breakdown of neurotransmitters, including norepinephrine and serotonin. Various polymorphisms of the *MAOA* gene have been associated with antisocial behaviour and the presence of deleterious mutations or deletions in the *MAOA* gene has been linked to aggression in both mice[70] and humans.[71] Recent studies have found a correlation between the low-activity form of *MAOA* and aggression in observational and survey-based studies of humans. Only about one-third of people in Western populations have this form of *MAOA*. By comparison, low-activity *MAOA* has been reported to be more frequent (approximately two-thirds of people surveyed) in some populations with a history of warfare. These findings led to the controversial suggestion that *MAOA* should be called the "warrior gene".[72] Other molecular genetic studies of MAOA polymorphisms in humans have identified similar patterns of nucleotide substitution that suggest DNA sequence diversity in modern humans could be the consequence of positive selection during our evolutionary history.[73,74]

While the same signatures of positive selection are lacking in nonhuman primates,[73] *MAOA* variants have been identified in monkeys and apes and some polymorphisms are thought to influence aggressive and impulsive behaviours in nonhuman primates.[75] A survey of single nucleotide polymorphsims (SNPs) in *MAOA* genes in rhesus and longtailed macaques found different variants largely fixed in different rhesus populations and a skewed distribution of one variant in the longtailed macaque populations.[76] The authors suggest selective pressure may favour different *MAOA* alleles in varying macaque populations or environments. If male reproductive success can be attributed to male competitive abilities, and the most aggressively successful males are able to control access to fertile females, then these traits may be favoured through sexual selection in polygynous primates.

Genetic variation that alters molecular structure of a cell surface receptor can also result in behavioural variation. Two genes that are known for their involvement in the regulation of the dopaminergic system are the dopamine receptor D4 (*DRD4*) gene and

the dopamine receptor D2 (*DRD2*) gene. *DRD4* is one of the most variable genes known, consisting of a large number of DNA sequences that differ slightly from individual to individual. The brain chemical dopamine plays a role in attentiveness and activity and the 4R (4 variable number tandem repeat or VNTR) allele is thought to have been the most common throughout most of human prehistory. This ancestral allele has the fewest amino–acid-changing variants, implying strong purifying selection.[77] However, another variant, known as the 7R allele, exhibits suboptimal dopamine signalling in comparison with the 4R allele and has an unusual geographic distribution, with low frequency in Asian populations but high frequency in the Americas.[78,77] The 7R allele is associated with the personality trait of novelty-seeking and recent evidence suggests that it originated as a rare mutational event that eventually increased to a high frequency in human populations through positive selection.[77] While novelty-seeking may be a risky behaviour in modern society, some degree of risk-taking behaviour may have been a path to reproductive success in past human societies (for reviews, see refs. 79, 80). Therefore, sexual selection, influenced by the local cultural norms and by the response to behaviours affected by this variant, may have contributed to the unusual *DRD4* allelic distribution we see today.[81]

The dopamine receptor D2, or *DRD2*, gene also modulates emotional behaviour and this gene has been studied extensively in humans. Multiple polymorphisms, as well as alternative splicing, have been described for *DRD2*. Several variants have been detected and their distribution across human population has prompted speculation about their origin.[82] A 1998 worldwide survey of *DRD2* types suggested that modern variation was probably the result of genetic drift.[82] Interestingly, however, studies of the genetic variants and their associated behaviours have found that certain *DRD2* alleles are found in individuals that tend to have low offspring investment and those that exhibit high mating effort.[83,84] A significant association has been found for *DRD2* alleles and sexual behaviour in both sexes, but the relationship appears to be stronger in men than women.[83] Given dopamine's role in emotion and arousal, these findings suggest a facilitative role for dopamine in sexual motivation, which may have a long and intertwined evolutionary history in humans. To date, little comparative research has been undertaken in nonhuman primates. The *DRD2* gene has been identified in rhesus and green monkeys, but research on the relationship between *DRD2* polymorphism and behaviour is currently lacking.

Serotonin, another neurotransmitter, is also thought to play a significant biological role in human behaviour, including impulsive behaviour which is suggested to occur due to a dysfunction of neurotransmission in the central nervous system. A number of genes involved in serotonin production and metabolism have been identified and studied. These include the neurotransmitter serotonin or 5-Hydroxytryptamine (*5-HT*), the serotonin transporter gene (*5-HTT*) and the serotonin receptor gene 2A (*5HT2A*). Serotonin functions as a neurotransmitter in the nervous systems of both simple and complex animals and recent studies involving the serotonin transporter gene *5-HTT* have identified genetic polymorphisms that are related to specific types of behaviour. The *5HTT* gene contains a 44 base pair VNTR in its promoter. Individuals with a long repeat show greater basal activity than the short variant.[85,86] Contrastingly, presence of the short allele is associated with elevated impulsivity and stress reactivity. A study by Hamer[87] reported a possible association between the serotonin promoter polymorphism and human sexual behaviour. This study found a statistically significant association between serotonin and frequency of sexual activity among men; males that possess at least one copy of the short variant reported that they had sex more frequently than men with other *5HTT* alleles.

Several studies have reported associations between *5HT2A* receptor gene polymorphisms and impulsive behaviour.[88,89] While earlier findings proved controversial, a study by Nomura[90] found a correlation between a *5HT2A* receptor gene polymorphism and impulsive behaviour in healthy male and female subjects using a behavioural task test. Further behavioural studies focusing on *5HT2A* receptor gene polymorphisms and receptor function are now needed to clarify the complex relationships between impulsive behaviours and their effect on reproductive success.

Interestingly, studies of serotonin and behaviour in both humans and nonhuman primates show parallel results. A number of studies focusing on rhesus macaques have shown that serotonin is significantly correlated with sociosexual behaviours that include frequency of consortships per hour and number of heterosexual mounts per hour during the mating season. In contrast, during the nonmating season high levels of the neurotransmitter were correlated with grooming activities.[91] In light of the fact that there is intense competition for sexually-receptive females only during the mating season, the observation that higher serotonin results in escalated aggression in rhesus macaques suggests that maintenance of these traits may be the result of positive selection and/or female mate choice.

Social Bonding

Evolutionary theory predicts that aggression and competition should be less important behaviours for females of any species. Therefore, it is unsurprising that scientific study has been more limited with respect to female sexual behaviour and neurotransmitters. Instead, and perhaps in light of the discovery of plausible candidate genes for reproductive behaviour, such as the vasopressin receptor gene, genetic variation resulting in hormone differences and reproductive behaviour in females and males has been a field of growing interest. Oxytocin is a hormone that acts as a neurotransmitter in the brain. It plays a key role in female reproductive processes, such as uterine contraction and lactation.[92] It has also been implicated in social interactions including pair bonding and maternal behaviours. Polymorphisms in the oxytocin receptor (*OXTR*) gene are particularly interesting because of the well-known involvement of oxytocin and *OXTR* in intimate, familial, social and maternal attachment.[93] Research focusing on *OXTR* polymorphisms has found that women that are homozygous for the long VNTR genotype have a tendency to avoid oral contraception and to give birth at a younger age than women with other *OXTR* polymorphisms. These results suggest that women who experience early pregnancy and childbirth may also exhibit partner preference and pair-bonding at an early age. These behaviours may have been selectively advantageous for women during early human evolution.

Vasopressin is another hormone that has been associated with reproductive behaviour in humans. While the hormone is involved in regulation of water retention, several microsatellite polymorphisms have been identified in the promoter region of the human vasopressin receptor (*AVPR1A*) gene.[94] High levels of *AVPRIA* expression in the brain have been associated with phenotypes related to reproductive behaviour, including the process of attachment that characterises parental and intimate bonds.[93,95] Similar to mammals such as the prairie vole, humans with high *AVPR1A* expression are generally characterized by a reproductive behavioural pattern of monogamous pair-bonding and long-term nurturance of offspring.[93] Researchers examining the relationship between *AVPR1A* in men and women report a positive association between early age of first sexual intercourse and certain polymorphisms in the *AVPR1A* gene.[96] Homozygous males and

females with the long *AVPR1A* polymorphism were significantly more likely to have sex before age 15 than heterozygotes or individuals with any other *AVPR1A* genotype. Prichard and colleagues[96] argue that their results are best explained by balancing selection occurring in human populations since polymorphisms conferring an advantageous life history trait, such as reproductive fitness, would become fixed in the population, resulting in zero genetic variation observed for that trait. However, the results of a study by Fink et al[97] suggest that this may not be the case as the level of nonsynonymous substitutions in the prarie vole *AVPR1* gene exceeds those found in related hormone receptors with similar functions.

In primates, sexual behaviours are complex and simultaneously influenced by multiple genes as well as biological, psychological and socio-contextual factors over time. Thus, identification of any single gene responsible for behaviour remains too simple, especially given the complex relationship between genotype and phenotype. Despite this, research has shown how genetic differences among individuals contribute to behavioural variation. Since genetic differences are necessary for selection to produce a response, disentangling the genetic and environmental influences on any trait is an important step towards understanding its evolution. However, since behavioural traits are influenced by other individuals (e.g., aggression gives rise to more aggression) an interesting complication can arise in that differences between individuals will be due not only to genes and the environment, but potentially also to genes in a particular environment. These results suggest that genes do influence primate behaviour and that, in many cases, genetic variability could be the consequence of natural selection.

Sperm Morphology

Male mating success can also be enhanced post-copulation and research on reproductive proteins in primates provides numerous examples of how adaptive evolution has influenced molecular evolution in seminal fluid factors and proteins in the female reproductive tract. Mating systems and related behaviours determine the intensity of sperm competition. Polyandry (i.e., female promiscuity) leads to especially fierce sperm competition. Sperm competition is a form of sexual selection whereby ejaculates of different males compete to fertilize ova.[98] The phenomena is widespread in animals, but has been especially well studied in primates. Differences in the midpiece volume of sperm and other morphological features have been associated with differential mating success.[99] Strong evidence for positive selection has been reported for spermatid-associated protein genes, such as protamine 1 and 2 (*PRM1* and *PRM2*), in humans and other primates. *PRM1* and *2* are thought to influence sperm morphology and recent study of their DNA sequences have shown that the *PRM1* gene has had a particularly high rate of nonsynonymous substitution particularly in humans, chimpanzees and gorillas.[100]

Sperm Competition

The use of molecular genetic techniques has also made it possible to determine whether selective pressures have had major consequences for DNA sequence polymorphism in genes that encode seminal proteins. Semenogelin II is a main structural component of semen coagulum and is encoded by the gene *SEMG2*. A number of studies have generated coding DNA sequences for *SEMG2* in nonhuman primates to evaluate the relationship between the molecular evolution of the gene and differences in mating systems.

Dorus and coworkers[101] discovered accelerated protein evolution in the common chimpanzee lineage during the period following human-chimpanzee divergence and in the lineage from the catarrhine ancestor to crab-eating macaques. In these species, mating systems are multimale and multifemale and *SEMG2* sequence evolution is greatest in species with high numbers of male partners during the female periovulatory period in species where males have large testes and in species with rapid rates of semen coagulation. Although the precise function of *SEMG2* remains undefined, the pattern of rapid evolution suggests that the gene may directly influence the biochemical dynamics of semen coagulation.

In many primate species, semen coagulation results in the formation of a firm copulatory plug which can effectively enhance mating success. Four prostate-specific candidate genes (*TGM4, KLK2, PSA*, and *ACPP*) participate in coagulation and there is evidence of significant positive selection for amino acid change in three, *TGM4, KLK2*, and *ACPP*.[102] Prostate-specific transglutaminase 4 (*TGM4*) forms both semen coagulum and copulatory plugs using its transglutaminase (TG) domain. Although *TGM4* has lost its function in gorillas and gibbons, resulting in loss of sperm coagulation, comparative studies of chimpanzees and humans demonstrate a strong signature of positive selection. It is suggested that TGM4 proteins may protect sperm from immune attack in the reproductive tract by altering the sperm surface and suppressing immune response against sperm. Thus, maintenance of this protein in polygynous species could enhance overall fitness. Beta-microseminoprotein (*MSMB*) sequences also show evidence of high numbers of nonsynonymous substitution, suggesting positive selection. Like *TGM4*, *MSMB* could be important in immune response as it is a main immunoglobulin binding factor in human seminal plasma.[103,104]

Kallikrein 2 (*KLK2*) has a known role in seminal fluid dynamics. *KLK2* activates prostate-specific antigen (PSA), a protease that breaks down semen coagulum. Genetic study shows that *KLK2* has lost its function in the rhesus macaque, gorillas and lesser apes, resulting in reduced ability to dissolve semen coagulum. However, like *TGM4, KLK2* shows significant evidence of positive selection in many primate species. The gene encoding prostatic acid phosphatase (ACPP) also shows significant variation in nonsynonymous substitution rates, particularly in the chimpanzee and rhesus macaque lineages. PSA, which is important in copulatory plug dissolution, does not have a high rate of nonsynonymous substitution but it also shows significant variation in selective pressure during its evolution.[105]

While the extremely high rates of nonsynonymous substitution for the genes involved in semen coagulation could be due to either positive selection or perhaps a reduction in functional constraint, the aforementioned seminal fluid proteins show dynamic evolutionary histories, significant positive selection and variable selective pressures between primate lineages. Multiple instances of loss of function also suggest differences in selective pressure. Seminal protein adaptation could result from several types of pressure, including sexual selection, pathogen response or even co-evolution with other proteins. For the most part, however, it seems likely that sexual selection, namely sperm competition, has had a major impact on the molecular evolution of proteins that aid in successful reproduction.

The rapid rates of evolution seen in reproductive tissues could be explained by a number of factors, such as female choice, self versus nonself recognition, or even meiotic drive and immune defence. Distinguishing between these different processes is often very difficult, as many of the explanatory hypotheses make similar predictions. Furthermore, many hypotheses may not be mutually exclusive. Therefore, it is possible that female

choice, inbreeding avoidance and immune function may all be driving these high rates of protein evolution. Many immune function genes are expressed in reproductive tissues and it is essential that sexually reproducing organisms avoid sexually transmitted disease.

Sexual selection has been shown to result in behavioural and physical adaptations. In polygynous primates sexual selection can take the form of mate choice but it does not necessarily end with copulation. Post-copulatory sperm competition and selective abortion can also influence reproductive success.

CONCLUSION

Most behavioural traits are at least partly heritable and ultimately influence life-history traits. They are, therefore, potential targets of selection. As discussed in this chapter, there is good evidence for genetic influences on precopulatory behavioural traits that include mate choice and aggression in humans and nonhuman primates. We have also seen how phenotypic features that are useful for mate choice decisions, such as bright colour, physical appearance or olfactory stimuli, impact the evolution and function of genes involved in the perception and regulation of, and the response to, these signals. Furthermore, the evolution of post-copulatory strategies such as sperm competition and selective abortion may also result in the presence of intense sexual selection. In all of these cases, there is considerable evidence that the adaptive function of behaviours, and the signals that trigger them, are under strong selection.

In this chapter we have seen that molecular genetic study of the variation underlying differences in behavior is frequently used to infer past selection. Most of the tests that detect the effects of selection on nucleotide sequence divergence rely on contrasts between the rates of synonymous and nonsynonymous nucleotide substitutions in DNA sequences. When the rate of nonsynonymous substitution exceeds the neutral rate (measured as the rate of synonymous substitutions), then positive selection for divergence, or diversifying selection, is usually invoked. We have seen that this is one of the primary ways in which evolutionary biologists investigate the ways in which natural selection works on behavioural traits. Although there are limitations to a DNA sequence-based approach to detect selection,[106] a large number of recent studies suggest that modern behavioural traits and physical features in primates, especially humans, are the consequence of selective pressures in the past.

Positive selection has clearly played a critical role in the evolution of humans and other primates. Many of the phenotypes and behaviours that are frequently used to define the primate order, including complex social organisation and behavioural flexibility, may also be the result of positive selection. Additionally, many aspects of primate biology that are of great interest to primatologists and evolutionary biologists, such as host–pathogen interactions, reproduction, dietary adaptation and physical appearance, are the product of varying levels of positive selection. Further research on genetic factors related to these features can only improve our understanding of the relationship between molecular evolution and behavioural traits. Furthermore, since some genes affecting related behaviors are also probably physically linked, attention to the possibility of gene co-evolution and the relative magnitude of genetic influences on any trait will be required for future studies into genes and behaviours. Together, this information will be essential for the development and testing of evolutionary models and also for a full understanding of the adaptive function of primate behaviours.

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CHAPTER 15

IMMUNE SYSTEMS EVOLUTION

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Abstract: Animals and plants have a complex and effective immune system that protect them from invading microorganisms. The mechanisms of immunity are evolutionarily selected throughout host-pathogen interaction to be tolerant to self-antigens and to recognize nonself molecular patterns. Plants and animals share a germ line encoded diversity of receptors capable of nonself recognition. Somatic rearranging of immunological receptors emerges at early stages of vertebrate evolution, allowing these animals to generate an almost unlimited diversity of receptors. Nevertheless, this recombinational system came with a high price: The potential for self-reactivity. In this chapter we will discuss the differences and the striking similarities of the immune mechanisms across different taxa in the context of evolution and the selective pressures that favoured the development of the adaptive immune system and the lymphoid organs.

INTRODUCTION

Microorganisms represent a constant threat to metazoans. Therefore, the development of mechanisms able to fight the invasion of microorganisms has been an essential factor for the evolution of the different species over billions of years. Most multicellular organisms have some type of immune response to pathogens that threaten their lives. In fact, it is estimated that a 98% of multicellular animal species have acquired evolutionary mechanisms that we shall call here "innate immunity" to respond to aggression from various infectious agents.

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Table 1. Comparison between innate and acquired immune systems

Characteristics	Innate Immunity	Acquired Immunity
Receptors	Germ line (Toll and TLRs)	Encoded in different gene segments which re-order (Igs, TCR)
Expression	Nonclonal (constitutive)	Clonal (inducible)
Effectors	Peptides, melanin	Igs and T cells
Response	Immediate	Not immediate
Recognize	PAMP (Pathogen associated molecular pattern)	Structural components (proteins, peptides, etc.)
Complement systems	C3, Bf (equinoderms), MBL pathway (elasmobraches)	Acquisition of classic pathway (prochordates)
Evolutionary origin	Metazoans	Cartilaginous fishes
Self/nonself recognition	Throughout the repertoire established in germ line	Variable in somatic cells

Some of these mechanisms have been preserved throughout evolution, allowing us to establish common features from plants through to higher vertebrates (Table 1). Their main characteristics are: (a) ability to recognise invasive agents and distinguish between infectious agents and the host; (b) the use of receptors encoded in the germ line that recognize repeated patterns of molecular structures that are expressed on the surface of microorganisms and are absent in eukaryotic cells; and (c) the induction of genes that, due to this recognition, encode antimicrobial cationic peptides that work by damaging the cell membranes of the microorganisms.

The adaptive immune system suddenly emerged in metazoans. Probably such a complex system was necessary for vertebrates to reach their current level of development. This group of animals has a slower growth and it takes longer for them to reach reproductive maturity than invertebrates. Therefore, they have a higher risk of acquiring infections. In addition, vertebrates have new eating habits based on more varied diets that increase the contact with different pathogens. Therefore a more complex immune system is necessary, one that is even further developed in more complex animals, with more developed circulatory systems and blood cells specialized in immune defence.

ORIGIN AND EVOLUTION OF INNATE DEFENCE SYSTEM

One type of innate defence is the production of peptides and small proteins able to damage infectious agents. As we will see later, the generation of these peptides is induced by the activation of membrane receptors of host cells able to recognize specific structures of the pathogenic agents. These peptides act on the physical properties of the pathogens such as the microbial-membrane charge density, preventing the development of resistance. Antimicrobial peptides are widely distributed in nature. They have been described in insects and higher species such as amphibians and mammals. In fact, in these animals, these peptides are produced by cells inside the intestine and the respiratory and urogenital tracts.[1-3] One of the most studied models, the Drosophila, produces up to six different peptides with specific activity against fungi, gram-negative and gram-positive bacteria. The promoters of the genes encoding peptides have motifs related to elements of the NF-κB response,[4-5] as will be mentioned below (see Table 2).

Table 2. LRR receptors involved with the innate immune response

Species	Receptors	Ligands	Activation	Effectors	Activity
				Peptides	
				Drosomicin	Fungicide
	Toll	Spatzle	DIF/Dorsal	Metchnokowin	"
Drosophila				Defensins	Gram +
	Imd	?	Relish	Diptericin	Gram –
				Attacin	"
				Drosocin	"
				Cecropin	"
	TLRs			**Immune response genes**	
	TLR2/ TLR6(X)	Lipoproteins	NF-κB	IL-8, RANTES, defensins	
		Peptidoglycans		MIP-1, B7 coreceptor	
Human	TLR5	Flagellin	"	IP-10, RANTES	
	TLR9	CpG	"	MIP-1, defensins	
	TLR4	LPS	"	Cytokine production and coreceptors induction	
Plants	**LRR (NB)**				**Resistance**
Arabidopsis	FLS2	Flagellin	MAPK	PR genes	*P.Syringae*
Rice	Xa21	avr?	"	"	*X. oryzae*
Tomato	Cf-9	avr-9	"	"	*C. fulvum*
	Prf (NB)	avr-Po	CDPK	"	*?*

MAPK—Mitogen Activation Protein Kinase, TLR—Toll-like Receptors, CDPK—Calcium Dependent Protein Kinase, LRR—Leucine Rich Receptors.

Most of these peptides, in particular the group called defensins, are composed of a group of protease-resistant molecules (3-5 KD) with 3-4 disulphide bridges. In eukaryotes, four defensin families which have activity against gram-positive bacteria have been described. Defensins behave in a very different way to conventional antibiotics. They generally act by destroying parts of the membranes of pathogens or interrupting cell activation signals.[6,7] Another group is represented by a group of peptides with gram-negative activity, among which *Cecropin, Diptericin, Drosocin and Metchnikowin* are very important. Other peptides such as *Drosomicin* have fungal activity.[8]

The innate immune response has the ability to distinguish between self and nonself molecular structures. This is carried out by a group of membrane and/or soluble receptors that recognize a specific pathogen-associated molecular pattern (PAMP) and that has no cross-reactivity against host cells. For example, most of these molecules recognize carbohydrate structures associated with bacterial cell walls, yeasts and protozoa essential for the organism. Specifically, PAMPs are vital for their virulence and/or survival and hence have been preserved in the main groups of microorganisms. The most important are Lipopolysaccharides of gram-negative bacteria (LPS), fungi mannose and some of the double-stranded RNA viruses. The identification, as explained below in the Table 2, relies on using specific receptors that recognize these molecular patterns.

This process of recognition is not: (a) as flexible as in adaptive systems; (b) does not detect small differences between similar antigens; and (c) does not change through mechanisms such as gene re-arrangement of the recognition receptors. This system has developed due to the evolutionary relationship between hosts and pathogens.[9,10]

There are additional mechanisms of innate immunity which emerged later in the evolutionary process that do not involve a recognition of the PAMP, protecting host cells from the destructive effects of the response. Of paramount importance among these is the activation of the alternative complement pathway, in which a wide range of proteins prevent the activation of the complement on the surface of the host cells. However, cells of foreign organisms do not have these inhibitor proteins on their surface and are therefore lysated by the activation of the lytic phase of the complement pathway.

A group of receptors encoded in the germ line, called pattern recognition receptors or PRR, have the ability to recognize PAMPs structures. These receptors are structurally and functionally heterogeneous, having been preserved in evolutionary distant organisms such as Drosophila and humans.[11]

The most important receptors have a C-type lectin domain, receptors with cysteine-rich domains and leucine-rich repeats (LRR). Drosophila Toll receptors are a good example and homologous receptors are found in the mammalian immune response, the toll-like receptors (TLR). These receptors belong to the LRR group (extracellular domain) and have a cytoplasmic domain similar to the IL-1R receptor referred to as the Toll/IL-1R (TIR) domain. This domain interacts with various "adaptive" intracytoplasmic proteins that have domains involved with apoptosis and cell activation (Fig. 1).[12] Equivalent systems exist in the immune response of plants conferring resistance to microbes infection.[13] PRR may be soluble, expressed on the surface of the cells or intracellularly. They act as signal receptors that trigger induction of the transcription of a variety of genes that participate in the immune response such as antimicrobial peptides and cytokines in vertebrates.[14]

TOLL RECEPTORS AND IMMUNE RESPONSE IN DROSOPHILA

Immune mechanisms in Drosophila merit especial attention, given that it is one of the models in which the response has been most closely studied.

In Drosophila, the process of defence has different phases. (1) The surface of the epithelium in the body provides a first line of defence. The epidermis (digestive and genital tracts cells) of the trachea and the malpighian tubules produce antimicrobial peptides that inhibit microbial growth. (2) The cellular and humoral response takes place in the cavity of the organism called the hemocele. The peptides are produced by the fat body (equivalent to the human liver) and are secreted into the hemolymphatic system.[15]

Humoral reactions also involve several proteolytic cascades. (a) The melanization cascade in which the generation of intermediate toxic factors of oxygen culminates in the production of melanin in those areas close to the microorganisms with bacteriostatic, fungicidal and antiviral activity. This system, probably an evolutionary precursor of the complement system, is the main defence mechanism in invertebrates, especially in insects and crustaceans. Drosophila and Anopheles have additional mechanisms, the equivalent of a complement-like cascade, which may contribute to the opsonisation of microorganisms. (b) Zymogens involved with coagulation also play a role in the induction of peptides by the fat body.[16,17]

As shown in Figure 1, there are two different PRR receptors, Toll and Imd, involved with the production of antimicrobial peptides. It is worth emphasizing that an organism such as Drosophila, which is devoid of an adaptive immune system, is able to detect an infection and also determine the type of the infection. The detection of a response against fungi and bacteria occurs through soluble receptors that recognize specific patterns that

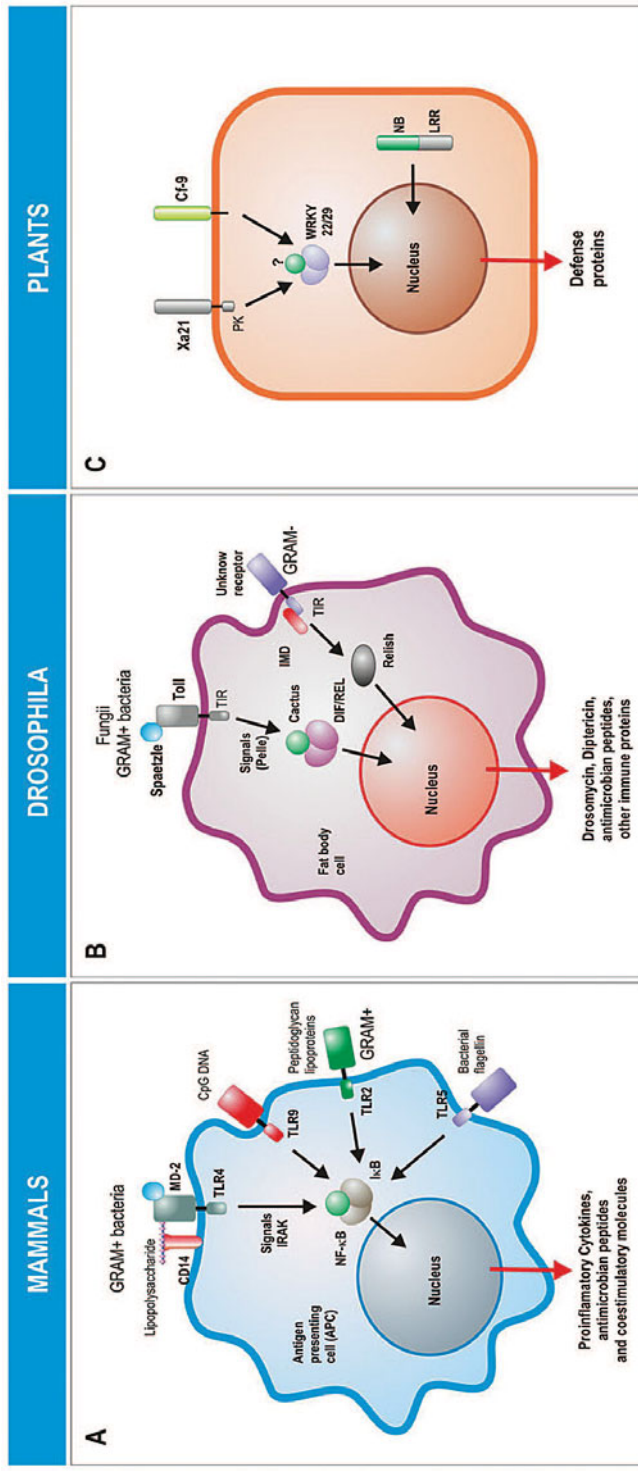

Figure 1. Mechanisms involved with the generation of the innate immune response: Preservation of activation pathways of the genes in the innate immune response in mammals, insects and plants.

lead, via the zymogene cascade, to proteolytic cleavage of a cytokine-like polypeptide that interacts directly with Toll. The extracellular part of this process involves four serine proteases acting in series and that finally act on a protein called Spatzle. The proteolysis of this protein triggers the production of a fragment able to act on the Toll receptor.[18]

To date, eight different Toll genes have been identified (Table 2). The various Toll receptors seem to be specialized in the recognition of different pathogen groups inducing specific responses through the secretion of effector molecules such as drosomycin or attacin, depending on the type of infection. The activation of Toll receptors triggers the activation of a homologue pathway to NF-κB. Several related adaptor proteins have been identified. Among them, it is worth mentioning Tube and Pelle in particular. The signal transduction as a consequence of the activation of these proteins leads to the activation of transcription factors homologue to the NF-κB family such as DIF and Dorsal. This factor forms a complex with a protein called Cactus (similar to I-κB) from which it separates. Protein DIF plays an essential role in the antifungal and anti-Gram-positive response during the adult period of the Drosophila. This process, as previously mentioned, is very similar to that which occurs in the activation of NF-κB in mammals. The dissociation triggers the release of DIF or Dorsal, their translocation to the nucleus and the activation of immune response genes.[15]

There is also an independent pathway in Toll-like receptors, called "Imd", the activity of which is not yet well understood but it is known to lead to the production of the peptide called *Diptericin,* involved in the response to gram-negative infections. The Imd signalling pathway is suggestive in some respects of TNFα receptor signalling in vertebrates.[19]

TOLL-LIKE RECEPTORS IN MAMMALS

As we have described, PRR receptors have emerged in the early stages of evolution, having been preserved in evolutionary distant organisms such as Drosophila and humans. There are genes homologous to Toll in mammals called TLRs. Specifically, it has been observed that there is homology between the cytoplasmic region and the IL-1 receptors, while the extracellular domains of TLRs and IL-1 are different.[20,21] To date, more than 10 different TLRs have been described in human, with a wide spectrum of microbial responses through NF-κB activation (Fig. 1).

However, there are differences in the activation pathways of Toll and TLR between insects and mammals. (1) In the first place, TLR recognizes PAMP microbial determinants through their association with coreceptors (CD14) and/or adaptor proteins. (2) On the other hand and in addition to antimicrobial peptide generation, the transcription of genes involved in the adaptive immune response is carried out as a consequence of the activation of the NF-κB pathway.[22] We are referring here to genes encoding pro-inflammatory molecules (cytokines, chemokines, etc.). Therefore, it is important to point out that in vertebrates there is an interconnection between the innate and acquired immunity functions. The same is the case with the functions of the complement system, as we will describe later and the evolutionary acquisition of acquired immunity.

The various TLR receptors are expressed differentially in the immune system cells and seem to be specialized in the recognition of different stimuli (Table 2). For example, TLR4 is activated when faced with peptidoglycans and Lipopeptides (LPS). This receptor mediates the response against PAMPs derived from *M. tuberculosis, Borrelia b*., etc. On the other hand, the TLR5 receptor recognizes flagellin, a protein

derived from flagellated bacteria. Another example is the TLR9 receptor that recognizes unmethylated CpG dinucleotides of bacterial origin able to stimulate human lymphocytes and to induce Th1 cytokines. All of them induce the degradation of I-κB and the activation of NF-κB.[22]

IMMUNE RESPONSE MECHANISMS IN PLANTS

A potential battery of pathogens, including fungi, bacteria, nematodes and insects, interfere with photosynthesis. In addition, viruses use the host's replication mechanism for their own benefit. Plants have developed mechanisms similar to those described in the innate immune response of animals.

One of the most powerful weapons in plants response is a mechanism called HR (hyper response). The HR mechanism works quickly and consists in cell death of host cells (apoptosis) where the infection occurs. This mechanism blocks pathogens access to the sources of nutrition and thereby limits their proliferation. In addition, signals produced by dead cells are involved in the induction of a wide variety of defence-related genes. Several physiological changes involved with cellular attack occur as a consequence of the infection. Among them are the production of reactive oxygen species (ROS), changes in intracellular pH as a consequence of ions flow, cell-wall hardening in places near the infection, release of molecules involved with the second signals, such as nitric oxide (NO) and synthesis of proteins and antimicrobial peptides (PR). PR proteins, such as glucanases and chitinases, have antibacterial and anti-fungicidal properties. Another immune mechanism, referred to as SAR (systemic acquired resistance), is characterized by the induction of a large number of PR proteins. There is substantial evidence of the relationship between production and accumulation of salicylic acid and resistance to infections.[23,24]

The pathogen-plant interaction, especially those involving biotropic parasites (virus), are governed by specific interactions between genes of virulence (avr) and alleles of plants corresponding to genes of resistance (R). This simple model explains the resistance to diseases or the potential development of a specific infection. The model explains how the R products recognize avr dependent signals and activate signalling transduction mechanisms that culminate with the activation of defence mechanisms. R genes are polymorphic and avr recognition depends on the genetic variability of the plant. In the case that the host or pathogen lacks the corresponding R or avr gene, plant-microbe interaction leads to disease.[25]

The majority of R genes use LRR receptors similar to Toll and TLRs. As in the case of mammals and Drosophila, they use innate immune response receptors coupled to cellular signals with apoptotic domains, kinase cascades and effector pathways transcriptionally activated. The existence of different types of cytoplasm and membrane domains suggest that R proteins are specialized in detecting ligands on the cells surface and some of them detect internal ligands.[25] The existence of polymorphism in these regions may indicate an ability to recognise different pathogens (Fig. 1). In addition, there is a group of similar intracellular proteins (NB-LRR) that also plays a role in the immune response of plants. The existence of genes similar to NB-LRR in mammals (called Nod) implies that they too have an intracellular receptor system with the same function as the R genes (Table 2).[26] All this evidence demonstrates that the innate immune response has evolved from ancestral immune systems.

THE COMPLEMENT SYSTEM IN BOTH INNATE AND ACQUIRED IMMUNITY

The complement system, a key subsystem in innate immunity, seems to be limited to Deuterostomia (Echinodermata and Chordata). Complement activation pathways can be induced directly or indirectly through microorganisms resulting in the opsonization through phagocytosis or the formation of pores on the surface of the pathogen through the creation of a membrane attack complex (MAC). The different evolutionary stages that have generated these functions have developed over millions of years (Fig. 2).

There are three different complement activation pathways which differ in the way in which proteolysis of the C3 component is induced: (a) the classical pathway, which requires the creation of antibodies and the first component C1; (b) the alternative pathway, which is activated by microorganisms; or (c) the lectin pathway, with activation of MBL-associated serine proteases (MASP) able to activate C3-convertase.

The evolutionary history of the complement system can be traced from echinoderms, which have a vestigial system similar to the alternative pathway. In fact, sea urchins have been found to have two components homologous of C3 and B factor (SpBf. SpC3) that participate in functions involved with opsonisation through specific nonlymphoid phagocytic cells.[27] This ancestral system seems to: (a) have the capacity to generate a C3-convertase similar to the alternative complement pathway and (b) enable phagocytosis of pathogens coated with C3, probably through specific receptors. However, components C4 and C5 have not been identified earlier than elasmobranches. From prochordata (such as ascidia) and in agnate fish, new factors emerge involved with the lectin pathway (MBL).[28] This pathway has been evolutionary efficient in the recognition of microbial surfaces

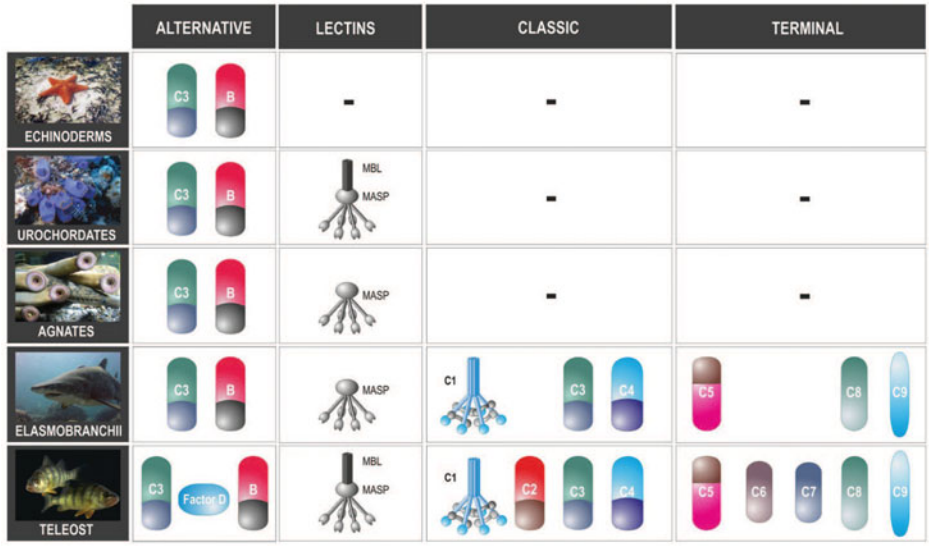

Figure 2. Evolution of the complement system. The first complement factors described (B and C3 factors) in echinoderms have been involved with opsonisation functions. During the period of emergence of the adaptive systems (elasmobranches) the complexity of the complement system increases with the appearance of the classical pathway and the terminal factors (MAC) with lytic activity.

of various pathogens with high content of repetitive mannose and N-acetylglucosamine residues. Here too the final aim is related to the opsonisation of foreign particles.

The appearance of cartilaginous fishes (elasmobranches) marked a milestone in the evolution of the immune system, given that all the features of adaptive immunity appear at this stage. The existence of high biological complexity brought with it the development of additional functions with duplication of the components and complement activation pathways. From elasmobranches onwards, two new features can be observed: (a) the appearance of the so-called classical pathway, able to be activated through antigen-antibody complexes as a consequence of the appearance of immunoglobulins; and (b) the generation of proteins of the membrane attack complex (MAC), able to lysate foreign microorganisms.[29] As we can see, both systems (innate and acquired) co-exist in vertebrates and are closely linked and interrelated.

SELF AND NONSELF RECOGNITION MECHANISMS IN MULTICELLULAR ORGANISMS

Immune systems of multicellular organisms are an integral part of the homeostatic system with various functions. On the one hand, they protect living organisms from infectious agents through the generation of an innate immune response. On the other hand, they prevent from the possible loss of individual integrity threatened by parasitism and potential fusion with nongenetically related organisms. During metazoan evolution, identification systems have been developed, making it possible to distinguish between "self" and "nonself". This feat is performed through systems for recognition of highly polymorphic structures (akin to histocompatibility) that in some senses are like the adaptive systems involved with the MHC described in higher vertebrates.

Compatibility systems developed through evolution can act on different levels: (a) on germ cells, avoiding fertilization with genetically similar gametes, hence the name Self- Incompatibility systems (SI), of which there are many examples in the Plant Kingdom and (b) on somatic cells, through the development of allorecognition systems which enable invertebrates to distinguish between individuals or cells when they form colonies.

Self-incompatibility systems that control sexual reproduction in fungi and angiosperms avoid self-pollination, thereby increasing heterozygosity. The so-called S-locus determines the compatibility of plants and 30-50 different alleles have been described. These correspond to glycoproteins and membrane receptors with enzymatic activity expressed in the stigma and on the pollen surface. Self-fertilization is avoided through biochemical mechanisms of rejection induced by the activity of cytotoxic RNAsa generated when the S alleles of the gametes are identical.[30]

Sea sponges are a classic model for the study of the aforementioned compatibility systems. The majority of the knowledge we currently have about the mechanisms that allow the sponge to preserve its identity come from the experimental transplants and the study of processes of rejection. From these it can be deduced that: (a) sponges have the capacity to reject allograft and fuse autologous grafts; (b) there is an ancestral system of cell memory as demonstrated by the acceleration of rejection with second and subsequent grafts; (c) the molecules description (AF) and surface receptors (AR) are involved with intercellular aggregation, the molecules involved with the adhesion mechanisms behaving as a histocompatibility system, with polymorphic domains related to immunoglobulins; and (d) molecular structures similar to cytokines (AIF-1) and which are inducible during

allograft response have been identified.[31,32] Note that some of these mechanisms (described above) are reminiscent of functions related to those of the adaptive immune systems.

Similarly, an autosomal and codominant genetic system has been described in tunicates; it is highly polymorphic (called Fu/HC) and controls the reactions of fusion and rejection in the formation of colonies. The match of just one allele between two members of a colony is sufficient to enable aggregation and fusion between them. This same genetic system seems to be involved in the fertilization of tunicates increasing the heterozygosity in a similar way to in fungi and plants, as mentioned earlier.[33]

There is no evidence of a common precursor that may have been the source of the appearance of both compatibility systems and the MHC of vertebrates. The independent appearance of these systems with similar functions suggests the existence of mechanisms for convergent evolution.

ADAPTIVE IMMUNE SYSTEMS EMERGE DURING THE FIRST EVOLUTIONARY STAGES OF VERTEBRATES

Currently available data indicates that adaptive immunity emerged on our planet during the earliest evolutionary stages of vertebrates and specifically with the appearance of gnathostomata (vertebrates with jaw) (Fig. 3).

It has been speculated that the adaptive immune system emerged with the insertion of a RAG trasposon into the germ line of a vertebrate ancestor, in the V region of a gene similar

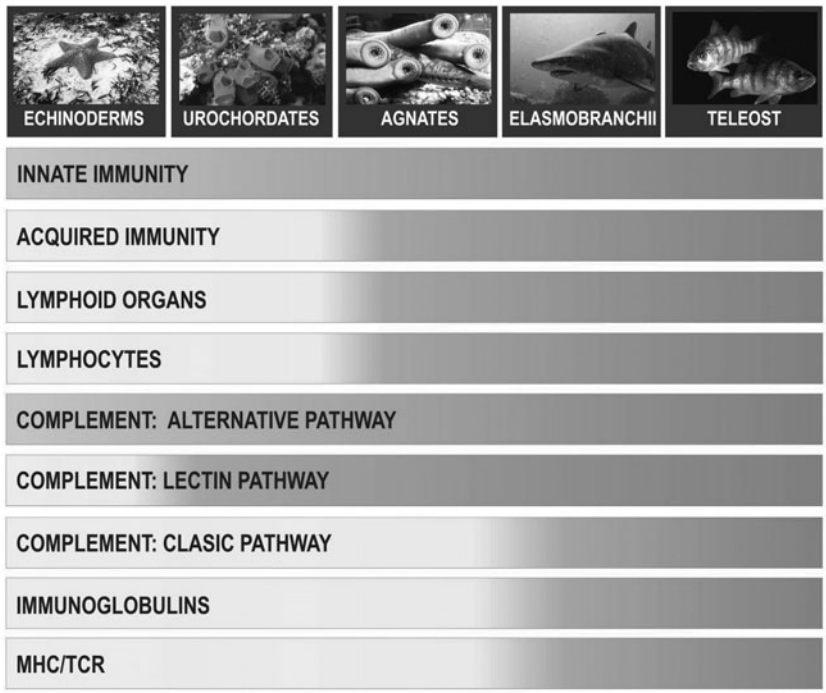

Figure 3. General phylogeny of the immune system.

to those of immunoglobulins and T-cell receptors. This trasposon RAG would presumably have been formed by RAG1 and RAG2 genes (that would act as a transposase) flanked by recombination signals. The split of genes RAG1 and RAG2 would leave the original gene interrupted and with the recombination signals inserted. This would generate a gene with a structure analogous to that of immunoglobulins and T-cell receptors. Subsequent duplications or even new insertions could have resulted in genes with a similar configuration to the current genes. The sudden appearance of innate immunity requires that several molecules such as transcription factors and receptors, which might not be involved with immunological defence, acquire new functions inside the adaptive immune system.[34]

Immunoglobulins are already present in elasmobranchs (Fig. 4) which produce IgM-type antibodies. However, this group of animals does not produce any other type of

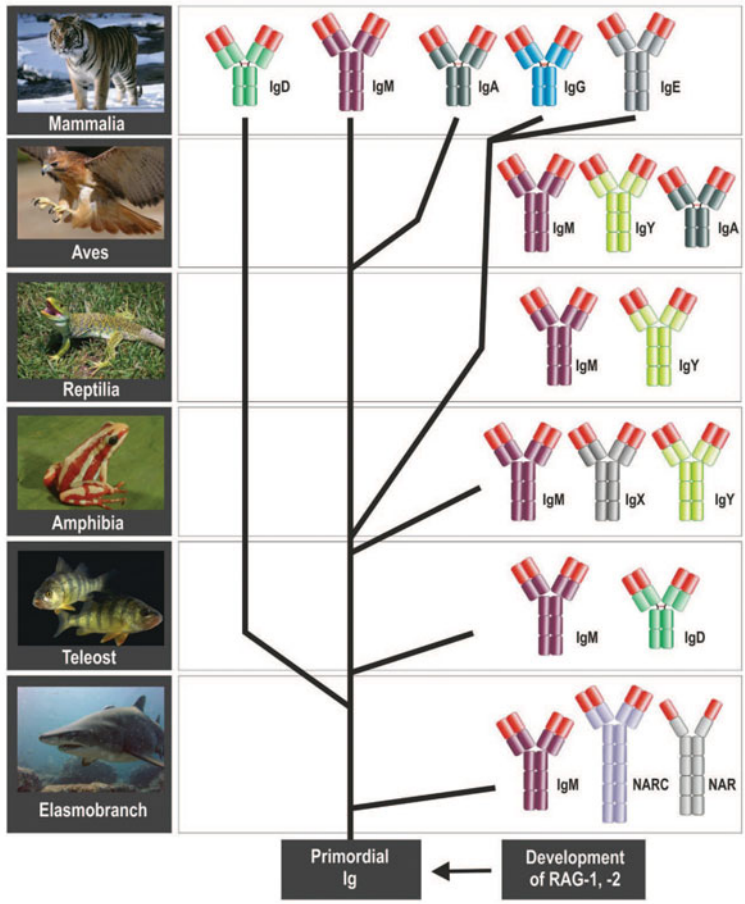

Figure 4. Evolution of immunoglobulins. Diagram of evolutionary development of the different Igs types in vertebrates IgNARC and IgNAR identified in cartilaginous fishes. These are candidates for the ancestral antibodies from which the other types have developed. It is important to mention the early appearance of IgM (Elasmobranches) and IgD (teleosts). IgY founded in amphibians, reptiles and birds may be the precursor of IgG and IgE in mammals.

immunoglobulin. In teleosts appears for first time a new kind of immunoglobulin, similar to IgD, allowing the simultaneous expression of more than one type of immunoglobulin. IgA first appears in birds, while IgG and IgE probably came from an IgY that appeared in amphibians. TCRs are already present in elasmobranchs, which also have all the antigenic receptors found in mammals (TCR$\alpha\beta$ and TCR$\gamma\delta$).[35]

The MHC also appeared in vertebrates although its origin may be an ancestral MHC already present in invertebrates. This hypothesis is supported by the presence of regions homologous to MHC in invertebrates and the existence of three "MHC-type" regions in the human genome located on chromosome 1, 9 and 19. Almost 40 HLA genes have one, two or three homologous genes located in these regions. To explain this, the so-called 2R hypothesis has been proposed; it suggests that there have been two rounds of duplication of the whole genome after the appearance of urochordata and before the appearance of vertebrates.[36] Therefore, these duplications may have happened before the appearance of a real MHC. In this ancestral MHC, at the same time as the T-cell receptor genes were created, class I and II molecules appeared. At this point, other genes located in MHC were recruited by the immune system to participate in the antigenic presentation and their proximity probably allowed them to evolve together.[37]

EVOLUTION OF LYMPHOID ORGANS

Lymphocytes are produced and matured in lymphoid organs. This group of organs includes primary lymphoid tissues (bone marrow and thymus) involved in the generation of the primary repertory of antigen-specific receptors in lymphoid cells and secondary organs (lymph nodes with germinal centres, spleen and lymphoid tissues of the mucosa), colonized by mature effectors cells, allowing the interplay between them and the regulation of immune response locally.

Jawless vertebrates do not have well defined lymphoid tissues, neither B nor T population. The first jawless vertebrates appeared 500 millions year ago and diverged into the actual jawed and jawless vertebrates. In lampreys hematopoietic sites change their location throughout the different stages of development. Thus, the primary lymphoid sites in larval stage are the typhlosole and the nephric fold. After metamorphosis, in the adult stage, the hematopoietic activity is found mostly in the supraneural body which is formed by adipose cells derived from fibroblasts in the connective tissue surrounding the spinal cord and the meningeal tissue. Therefore, agnates do not have a real lymphoid tissue (except a tissue associated to the mucosa with no germinal centres). They do not have real lymphocytes either but cells analogous to these, without TCR, MHC or immunoglobulins in their membrane, although they express receptors called VLRs (variable lymphocyte receptors) capable of antigen binding similar to monoclonal antibodies. The VLRs are created by various LRR motifs encoded by genes that through somatic recombination produce different receptors in the different "lymphocytes", a system that is reminiscent of adaptive systems. The VRL system in lamprey generates receptor diversity by gene conversion and therefore it is not as powerful as the VDJ recombination system.[38-39]

The emergence of lymphoid organs and adaptive immunity during evolution took place simultaneously in jawed vertebrates. The cartilaginous fish are the oldest organisms with distinguishable T and B populations. They have spleen and thymus (although not bone marrow) and more developed gut-associated lymphoid tissue (GALT). B-cell poiesis takes place in liver, kidney and spleen. In teleost B poiesis happens mainly in

Table 3. Lymphoid organs in vertebrates

	Galt	Thymus	Spleen	Head Kidney	Bone Marrow	Lymph Nodes
Agnatha	+	–	–	–	–	–
Elasmobranches	+	+	+	–	–	–
Teleostei	+	+	+	+	–	–
Anura	+	+	+	–	+	–
Aves	+	+	+	–	+	+?
Mammalia	+	+	+	–	+	+

the kidney, while in reptiles a bone marrow where B-lymphocytes develop appears for the first time. All types of lymphoid tissue are found in birds and mammals. In these two groups lymph nodes and spleen have germinal centres. In mammals, B-lymphocytes are produced in the bone marrow and they also complete their maturation there. In birds, immature cells, precursors of B-lymphocytes, migrate to a characteristic organ, Bursa of Fabricius, to complete their maturation. On the other hand, T-lymphocytes precursor cells migrate, in both birds and mammals, from the bone marrow to the thymus in order to finish their maturation. Therefore, B-cell poiesis can happen in a broad array of anatomic and organic locations in jawed vertebrates, although T-cell maturation seems to be linked only to the thymus (Table 3).[35] Interestingly, the thymus is the first lymphoid organ that evolved in jawed vertebrates, but the selective forces that favoured the evolution of this organ are largely unknown. It has been proposed that apparition of thymus in vertebrates is functionally related to the development of the jaw and the acquisition of new feeding habits that could damage the foregut. Nevertheless, this is unlikely since the thymus is a primary lymphoid organ committed to lymphocyte T maturation and not for local immune regulation. More likely, the thymus evolved to address the threat of self reactivity derived from the development of VDJ recombination system given that the thymus is the only organ capable of T-cell maturation in vertebrates. On the other hand, the generation of primary lymphoid organs during development is initially independent of hematopoietic cells. It is possible that the pharyngeal region in jawed vertebrates could provide a suitable area for the development of thymus. Indeed, the gut tube has originated during evolution numerous organs such as pancreas, thyroid and liver and therefore provides an epithelial primordium capable of organ development throughout the evolutionary history of vertebrates.[40]

In conclusion, it is likely that the risk of self-reactivity derived from the adaptive immune system generated the selection pressure that favoured the evolution of lymphoid organs in jawed vertebrates.

CONCLUSION

Microorganisms represent a threat to the survival of all the living organisms. The generation of immune response mechanisms (called innate) has been crucial for the survival of all types of multicellular organisms. They were established in very early evolutionary stages and they are characterized by: (a) the selective discrimination between self and infectious agent molecular structures and (b) effector systems able to produce responses

against infectious agents. These tasks are carried out through a variety of mechanisms among which are the ones involved with the opsonisation (such as the complement system), the signalling of infectious particles and the mechanisms for recognition of the molecular structure of different infectious agents. These are based on the recognition of common and preserved structures of the pathogens by specific membrane receptors (LRR). As a consequence of the activation, antimicrobial peptides are generated through mechanisms similar to those involved in the activation of NF-κB. Related receptors have been found in distant organisms such as Drosophila (Toll receptors), humans (TLRs receptors) and plants. The appearance of adaptive immune systems has occurred during the first evolutionary stages of vertebrates and because of the arranged acquisition of different gene related systems (Igs, MHC, TCR), mechanisms of generation of antigenic diversity (RAG genes) and lymphoid organs. The emergence of these adaptive systems has involved the acquisition of additional functions of the innate system components involved with the acquired immune response.

ACKNOWLEDGMENTS

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CHAPTER 16

INVOLVEMENT OF SIRTUINS IN LIFE-SPAN AND AGING RELATED DISEASES

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Abstract: Since healthy aging remains one of the ideals of modern society, both, the identification of the underlying molecular mechanisms and interventions regarding the aging process are of considerable interest. Among the mechanisms currently being considered, the sirtuin family of histone deacetylases have been implicated to play a crucial role during the aging process both due to their requirement of NAD^+ as a cofactor for enzymatic activity, which determines a crucial link between sirtuins and the energy dependent regulation of gene transcription and their versatile target substrates mainly consisting of key regulators of metabolic, stress and cell cycle control. This chapter summarizes current evidences linking sirtuins to aging and outlines their potential as promising therapeutic targets for the treatment of age-related diseases.

INTRODUCTION: AGING, AGE-ASSOCIATED DISEASES AND CALORIE RESTRICTION (CR)

Cellular senescence and organism aging is characterized by a progressive loss of physiological functions and metabolic processes which is often accompanied by age-associated diseases, such as neuronal degeneration e.g., Alzheimer's or Parkinson's disease, metabolic disorders such as Type II diabetes or cancer.[1,2] Due to biological complexities, we still lack a complete picture of the molecular mechanisms related to aging, cellular senescence and longevity particularly with regard to humans. There are several factors associated with the rate of aging among them increased genomic instability, the metabolic control, changes of gene expression patterns and the production of reactive

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oxygen species (ROS).[3,4] As mitochondria constitute the major production site of ROS, this organelle most likely plays a key role in life-span and aging. Interestingly, around 20% of mitochondrial proteins are suggested to be posttranslationally modified by reversible acetylation, especially those involved in life-span and metabolism.[5]

As healthy aging remains one of the ideals of modern society, both, the identification of the underlying molecular mechanisms and interventions regarding the aging process are of considerable interest. Up to now, calorie restriction (CR), a phenomenon first described in 1930s by McCay and coworkers where an organism is provided with at least 20% fewer calories below *ad libitum* level, constitutes the most robust and reproducible way of extending health and longevity.[6] CR has not only been shown to increase the median and maximum lifespan of a variety of organisms,[7-11] but is also associated with a decreased incidence or delayed rate of age-related diseases as demonstrated in several rodent studies.[10,11] Furthermore, the beneficial effects of CR are related to lower circulating insulin levels and an increased insulin sensitivity, thereby reducing the predisposition to diabetes as well as other metabolic disorders which is also associated with life span extension based on experiments in animal models.[12,13] Another key feature of CR is a lowered core body temperature that results in a reduced and more efficient energy expenditure which is related to an increased life span as well.[14,15] Despite the lack of long-term studies, there is also emerging evidence that CR might constitute a life span extending mechanisms for humans. Studies on dogs,[16] cows,[17] and nonhuman primates revealed that many of the physiological responses in these organisms resemble those observed in rodents on CR.[18,19] Importantly, the National Institute of Aging initiated short-term human CR studies (6-12 months) at Washington University, Tufts University and the Pennington Center at Louisiana State which already confirm, albeit on a preliminary basis, reduced plasma insulin levels and body temperature that are key features of the CR response repeatedly observed in animal studies.[20,21] In consideration of the already demonstrated positive effect on human health,[22,23] these data are the first evidence that humans might indeed benefit from CR not only due to disease protection but also in terms of increased survival.

AGING AND AGE-RELATED DISEASES—THE SIRTUIN CONNECTION

Members of the sirtuin family of histone deacetylases (class III histone deacetylases (HDACs)), termed after their homology to the yeast protein silent information regulator 2 (Sir2), are widely distributed in all three domains of life. So far, seven human sirtuins have been identified (SIRT1-SIRT7). Based on phylogenetic analysis they are further grouped into four subclasses.[24-26] The main characteristic feature, distinguishing sirtuins from the remaining histone deacetylases, is their unique enzymatic mechanism. Unlike class I, II and IV HDACs, which are Zn^{2+}-dependent hydrolases, sirtuins possess a unique NAD-dependent protein deacetylase (SIRT1, SIRT2, SIRT3, SIRT5) and in some cases a secondary ADP- ribosyltransferase activity (SIRT4 and SIRT6).[27,28] They have been implicated in a variety of cellular processes such as heterochromatin silencing, differentiation, metabolism, neuronal protection, apoptosis and cell survival due to their ability to deacetylate both histone and numerous nonhistone targets.[29]

Several signal transduction pathways have been linked to the lifespan extending capacity of calorie restriction (CR). Among them the sirtuin family of histone deacetylases has been suggested to play a central role in this process. This is mostly due to their

requirement of NAD^+ as a cofactor for enzymatic activity, which determines a crucial link between sirtuins and the energy dependent regulation of gene transcription. Indeed, studies in lower organisms such as Saccharomyces cerevisiae, Drosophila melanogaster and Caenorhabditis elegans demonstrated that overexpression or hyperactivity of yeast Sir2 and its orthologs is connected to a prolonged life span.[30] The emerging evidence that the human sirtuins may play a crucial role in life span and the age-related diseases as well is discussed below in detail (Table 1).

SIRT1

SIRT1, the closest human homolog to yeast Sir2, is the best studied sirtuin family member with regard to life-span and age-related diseases. It has been implicated to play a crucial role during the aging process for several reasons.[26,31-34] First, SIRT1 is downregulated in senescent cells[35] and during aging.[36] Secondly, calorie restriction induces SIRT1 expression in mammalian cells and humans thereby promoting cell survival,[37] whereas SIRT1 knockout mice fail to display a phenotype of CR.[38] Consistent with this observation, phenotypes of *sirt1*-over expressing mice partially display phenotypes of its calorie-restricted counterparts.[39] In addition, SIRT1 is involved in the upregulation mitochondrial biogenesis due to its capability to deacetylate and thus activate the peroxisome proliferation activating receptor (PPAR)-gamma co-activator-1α (PGC-1α),[2,40] which stimulates mitochondrial activity and subsequently increases glucose metabolism, which in turn improves insulin sensitivity.[41] The regulation of the mitochondrial biogenesis and metabolism is widely accepted as a key component in the regulation of lifespan and aging.[42] Furthermore, SIRT1 has not only been demonstrated to mimic calorie restriction, but also to possess a neuroprotective function. The Resveratrol-mediated activation promotes the SIRT1 induced resistance to axonal degeneration[43] and there is emerging evidence that SIRT1 protects neurons from apoptosis[44] and is involved in preventing neurodegeneration in models of Alzheimer disease and amyotrophic lateral sclerosis.[45] Interestingly, pharmacological activation of SIRT1 recapitulates many of the results generated by knockout or transgenic over expression of SIRT1 in mice.

The hallmark activator of SIRT1 is resveratrol (3,4,5 trihydroxystilbene). Analysis in no-mammalian organisms revealed that a treatment with resveratrol extends the life span through a direct activation of SIRT1[46,47] by increasing its substrate binding affinity.[48] Furthermore, it retards cellular senescence in human diploid fibroblasts.[49] Several lines of evidence indicate that resveratrol possesses a SIRT1-mediated life extending capacity in mammals as well. In a study of Baur and coworkers resveratrol treatment has been demonstrated to improve the health and longevity of mice on a high-calorie diet.[50] Although both high-calorie fed mice suffered from obesity, the group receiving resveratrol lived significantly longer and exhibited the typical molecular changes associated with longer lifespan including increased insulin sensitivity, reduced insulin-like growth factor-1 (IGF-I) levels, increased peroxisome proliferator-activated receptor-gamma co-activator 1alpha (PGC-1á) activity and increased mitochondrial number.

Lately, three novel highly-specific SIRT1 activating compounds (SRT1460, SRT1720 and SRT2183) have been identified by a high-throughput fluorescence polarization assay followed by a high-throughput mass spectrometry assay.[51] These activators are structurally unrelated to resveratrol and exhibit nanomolar to low micromolar potency towards SIRT1 in vitro (resveratrol EC_{50} = 46,2 μM, maximal activation 201%; SRT1720 EC_{50} = 0,16 μM,

Table 1. Evidences implicating the involvement of sirtuins in life span and age-related diseases

Sirtuin	Size	Cellular Localization	Summary of Evidences	Proposed Mechanism
SIRT1	82 kD	Nuclear	• SIRT1 is involved in mitochondrial biogenesis[36]	Activation of PGC-1α activity[36]
			• SIRT1 overexpression (mice, cell lines) or pharmacological activation mimics calorie restriction[33,34,45]	Needs to be determined
			• SIRT1 overexpression shows neuroprotective characteristics[38-40]	Deactylation mediated activation of PGC-1α and inhibition of p53 activity[38-40]
SIRT2	42 kD	Cytoplasmic	• Target for the therapeutic invention of neurodegenerative disorders[53]	Inhibition of SIRT2 activity promotes the formation of less toxic α-synclein aggregates[53]
			• SIRT2 overexpression inhibits adipocyte differentiation whereas SIRT2 knockdown promotes adipogenesis[51]	Deacetylation-mediated activation of forkhead transcription factor 1 (FOXO1)[51]
SIRT3	44 kD	Mitochondrial	• Increased SIRT3 expression upon CR[56]	Activation of mitochondria-related genes such as PGC-1α and uncoupling protein 1 (UCP1)
			• G477T polymorphism is associated with the survivalship of elderly males[59]	Needs to determined
			• VNTR polymorphism correlates with increase survival rates of males >90 years[58]	Needs to be determined
SIRT4	35 kD	Mitochondrial	• High insulin secretion of pancreatic islets isolated from SIRT4$^{-/-}$ mice[60]	Repression of glutamate dehydrogenase (GDH) activity by ADP-ribosylation[60]
			• SIRT4 knockdown enhances and SIRT4 overexpression suppresses insulin secretion in INS-1E cells in response to glucose[61]	Interaction with insulin-degrading enzyme (IDE) and ATP/ADP translocase 2 und 3 (ANT2/3)[61]
SIRT5	34 kD	Mitochondrial	• SIRT5$^{-/-}$ mice show elevated blood ammonia during fasting	Reduced deacetylation-mediated activation of Carbamoyl phosphatase synthetase 1 (CPS1)[64]
SIRT6	39 kD	Nuclear	• SIRT6$^{-/-}$ mice exhibit a premature ageing phenotype and genomic instability[66]	Defects in base excision repair (BER) mechanism[66]
SIRT7	48 kD	Nuclear (nucleolar)	• SIRT7 knockdown in U2OS cells leads to increased apoptosis resulting in reduced cell survival[68]	Reduction of RNA polymerase I mediated transcription[68]
			• SIRT7$^{-/-}$ mice possess a shorter lifespan accompanied with enhanced cardiac hypertrophy[70]	Increased p53 activity promoting cardiomyocyte apoptosis[70]

maximal activation 780%; SRT2183 EC_{50} = 0,36 μM, maximal activation 296%; SRT1460 EC_{50} = 2,9 μM, maximal activation 447%). Importantly, the compound SRT1720 has not only been proven to be useful in activating SIRT1 in vitro, but also in three different in vivo models which displayed the characteristic changes of calorie restriction. In diet-induced obese (DIO) as well as genetically obese mice ($Lep^{ob/ob}$), the treatment with SRT1720 significantly improved the insulin sensitivity, decreased the plasma glucose levels and increased the mitochondrial biogenesis. Consistent with these results, the glucose homeostasis and insulin sensitivity in adipose tissue, skeletal muscle and liver was markedly improved Zucker fa/fa rats, a genetically obese rodent model. Taken together SIRT1 activation by SRT1720 seems to mimic the effects of calorie restriction on the metabolic and mitochondrial function and therefore constitutes a promising drug for the treatment of age-related diseases such as Type 2 diabetes.

Consistent with the observations that different activators of SIRT1 successfully mimic the beneficial effects of calorie restriction, the specific inhibition of SIRT1 activity by sirtinol, a cell permeable 2-hydroxy-1-napthaldehyde derivate, induced senescence-like growth arrest in human endothelial and cancer cells as demonstrated by increased histone H3 lysine 14 (H3K14) and histone H4 lysine 16 (H4K16) as well as p53 acetylation levels, accompanied by an attenuated DNA synthesis, an increased SA-ß-gal activity as well as senescence-like morphological changes.[52,53] This is further supporting the idea that SIRT1 activating compounds might be useful as promising treatment strategy for aging or age-related diseases.

SIRT2

SIRT2 is a predominantly cytoplasmic protein that is involved in cell-cycle regulation, adipocyte differentiation and the oxidative stress response.[54-57] Interestingly, the yeast ortholog of SIRT2, *Hst2*, can function in parallel to SIR2 in certain strains with regard to lifespan extension and rDNA silencing.[58] Most recently, one study on SIRT2 reported the therapeutic utility of inhibitors for the treatment of neurodegenerative diseases such as Parkinson's disease.[59] Using three different Parkinson's disease models, Outeiro et al showed that human neuroglioma cells were rescued from α-synclein-mediated toxicity when treated with AGK2 probably due to the formation of less toxic α-synclein aggregates. Although AGK2 exhibited >14 fold selective inhibition of SIRT2 relative to SIRT1, further work will be needed to establish the mechanisms of inhibition.

SIRT3

SIRT3 was the first protein shown to be localized to the mitochondrial matrix of mammalian cells.[60-62] There are several lines of evidence implicating a close connection between this member of the sirtuin family and the regulation of the cellular energy metabolism therefore affecting the aging process. First, SIRT3 is highly expressed in brown adipose tissue and this expression is further increased upon CR.[63] Second, two independent studies demonstrated that the mitochondrial form of acetyl CoA synthetase 2 (ACS2) is a target of SIRT3 and is activated upon deacetylation.[61,64] ACS2 catalyzes the formation from acetate into acetyl CoA which is an intermediate of the TCA cycle

and is required for cholesterol and fatty acid synthesis as well. Therefore, it is most likely that SIRT3 plays a key role in the regulation of the entry of carbons from acetate into the central metabolism. Furthermore, analyses of SIRT3-related polymorphisms based on human population studies implicated a direct link between SIRT3 and aging.[65,66] First, the G477T transversion, while not affecting the amino acid sequence, has been demonstrated to associate with survivalship of elderly males.[66] Second, the loss of enhancer activity due to a variable number of tandem repeats (VNTR) polymorphism within intron 5 of *sirt3* has been correlated to increased survival rates of males >90 years.[65] Although these findings need to be validated in larger samples, they further strengthen the rationale that the expression of SIRT3 may promote longevity in humans.

SIRT4

SIRT4 is another mitochondrial protein that demonstrates a strong ADP-ribosyltransferase activity but lacks almost any deacetylase activity.[60,67,68] Several key regulators of the cellular metabolic status have been identified to be regulated by SIRT4. First, the activity of glutamate dehydrogenase (GDH), a mitochondrial enzyme involved in the conversion of glutamate to α-ketoglutarate, is repressed by SIRT4-mediated ADP-ribosylation which constitutes an important mechanism for the regulated amino acid-stimulated insulin secretion.[67] Consistently, SIRT4 knockout mice are viable but pancreatic islets isolated from these mice secrete higher levels of insulin, revealing a role for SIRT4 in down regulating insulin secretion through repression of GDH activity.[67] The loss of SIRT4 could therefore contribute to diabetes due to higher insulin levels that are known to increase the predisposition to both diabetes and other metabolic disorders.[13] The observation that both insulin-degrading enzymes and adenine nucleotide transporters have been shown to be substrates of SIRT4 as well further strengthens the idea that SIRT4 plays a direct role in maintaining physiological insulin levels in response to glucose.[69]

SIRT5

SIRT5 has been the least characterized sirtuin for several years. Its is described as a mitochondrial protein[60] with a weak deacetylase activity.[70] Most recently, two mitochondrial substrates have been identified. First, cytochrome c, a key regulator of oxidative metabolism and apoptosis initiation, has been demonstrated to be deacetylated by SIRT5 in vitro.[71] Despite the colocalization of both proteins within the mitochondrial intermembrane space, the physiological relevance of this deacetylation still needs to be shown in vivo. Second, Nakagawa and coworkers most recently identified carbamoyl phosphatase synthetase 1 (CPS1), which catalyses the first step of the urea cycle, as a substrate of SIRT5 being activated by SIRT5-mediated deacetylation both in vitro and in vivo.[72] Interestingly, increased CPS1 activity during calorie restriction (CR) has been correlated to hypo-acetylation and an 50% increase in mitochondrial NAD^+ pointing to key role of SIRT5 in the up-regulation of the urea cycle for ammonia disposal.

SIRT6

SIRT6 is a nuclear protein that possesses both a weak deacetylase and strong ADP-ribosyltransferase activity.[73,74] Studies on SIRT6-deficient knockout mice implicated that SIRT6 fundamentally influences the aging process since these mice display genomic instability and premature aging symptoms and die several weeks after birth.[75] These observations are ascribed to a deficiency in a specific DNA repair mechanism, the base excision repair (BER). Consistent with these results, SIRT6$^{-/-}$ MEFs exhibit an impaired proliferation and enhanced sensitivity to DNA-damaging agents resulting in multiple chromosomal defects (fragmentation, detached chromosomes, gaps and translocations) due to a defect in BER as well.[75] Interestingly, the proper regulation of genomic stability is widely accepted to protect against tumor formation and aging.[76]

SIRT7

The protein SIRT7 localizes to the nucleolus of human cells.[60,77,78] So far, neither a deacetylase nor an ADP-ribosyltransferase activity has been detected. Nevertheless, several lines of evidence indicate an involvement of SIRT7 in life span extension. First, knockdown of SIRT7 expression in human cells induces apoptosis, indicating that SIRT7 is required for cell survival.[77] This observation is based on the perception that SIRT7 constitutes a positive regulator of RNA polymerase I transcription and therefore of the ribosome biogenesis. Second, analysis of SIRT7-deficient mice showed a shortened lifespan with enhanced inflammatory cardiomyopathy compared to wild-type mice. In such mice, decreased SIRT7 levels lead to an increase p53-activity subsequently resulting in enhance cardiomyocyte apoptosis.[79]

CONCLUSION

Since healthy aging remains one of the ideals of modern society, both the identification of the underlying molecular mechanisms and interventions regarding the aging process are of considerable interest. The fundamental role that the sirtuins play in cellular metabolic control indicated that they present important determinants of whole-body metabolism constituting potential therapeutic targets for many chronic diseases associated with metabolic dysfunction such as Type II diabetes. Likewise, potential applications of the sirtuins in neuronal cell survival and response to stress and cell-cycle control hint to eventual importance of this gene family in the pathogenesis and treatment of age-related diseases such as neurodegenerative diseases and cancer. Up to now, research focussed on the involvement of SIRT1 in lifespan extension and age-related diseases. But there is emerging evidence that the remaining sirtuins significantly contribute to molecular mechanisms of aging as well. While we discussed the different sirtuin family members separately for reasons of clarity, their physiological functions are most likely to be interconnected. Future investigations regarding the concerted interplay of the different sirtuins will not only contribute to a more detailed understanding of the aging process, but might also lead to the development of therapeutic drugs for the treatment of age-related diseases.

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CHAPTER 17

MOLECULAR DIVERSITY OF Dscam AND SELF-RECOGNITION

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Abstract: Cell recognition requires interactions through molecules located on cell surface. The insect homolog of Down syndrome cell adhesion molecule (Dscam) manifests huge molecular diversity in its extracellular domain. High-affinity Dscam-Dscam interactions only occur between isoforms that carry identical extracellular domains. Homophilic Dscam signaling can, thus, vary in strength depending on the compositions of Dscams present on the opposing cell surfaces. Dscam abundantly exists in the developing nervous system and governs arborization and proper elaboration of neurites. Notably, individual neurons may stochastically and dynamically express a small subset of Dscam isoforms such that any given neurite can be endowed with a unique repertoire of Dscams. This allows individual neurites to recognize their sister branches. Self-recognition leads to self-repulsion, ensuring divergent migration of sister processes. By contrast, weak homophilic Dscam interactions may promote fasciculation of neurites that express analogous, but not identical, Dscams. Differential Dscam binding may provide graded cell recognition that in turn governs complex neuronal morphogenesis.

INTRODUCTION

Cell-cell recognition underlies the development, maintains the integrity and regulates the physiology of multi-cellular organisms. Cells interact with one another to acquire specific fates, undergo proper differentiation and dynamically modulate each other's behaviors. In regards to the wiring of neural circuitry, cell recognition regulates growth cone migration to control neurite extension, fasciculation versus defasciculation, pathfinding and target selection.[1,2] Cell recognition also governs neurite elaboration for the acquisition of complex neuronal architectures and proper tiling of an entire receptive field. Following

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target selection, intimate cell-cell interactions promote synaptogenesis and functional maturation of synapses.[3] Dynamic changes in cell recognition may further underlie neural plasticity. Multiple mechanisms of cell recognition probably act in concert to support brain development and function. Further, novel mechanisms of cell-cell recognition may arise to drive brain evolution from simple to higher organisms.

In the complex central nervous system (CNS), distinct neurons are wired in stereotyped patterns. Selective and dynamic cell-cell recognition governs the acquisition of different projections by distinct neurons. First, neurons expressing different cell recognition molecules or the same cell recognition molecules at different levels may interact differentially with the environment, to establish different trajectories and synapse with specific targets.[4] Cell recognition molecules involved in differential neuronal morphogenesis often exist as ligand/receptor pairs. Known examples include diverse pairs of semaphorin/neuropilin[5] and ephrin/Eph receptor.[6] Second, neurons utilize specific cell recognition molecules to support neurite extension, arborization versus synaptogenesis. The diverse members of cadherin-related neuronal receptors or protocadherins have been implicated in regulating neuronal morphogenesis from neurite outgrowth to synapse formation.[7] In contrast, neurexins and neuroligins are selectively involved in maturation of distinct synapses.[8] Differential expression of various cell recognition molecules has the potential for shaping the morphology and connectome characteristic of each neuron type.

Proper wiring of neural circuits further requires neurons of the same type to recognize each other for coordinated morphogenesis. Such homotypic interactions may promote adhesion (Fig. 1A) or lead to repulsion (Fig. 1B), depending on the nature of their downstream signaling.[9] Homotypic adhesive interactions between opposing axons facilitate neurite extension along specific paths, a process commonly referred to as fasciculation (Fig. 1A). Various homophilic cell adhesion molecules including NCAM/Fasciclin II have been shown to mediate neurite fasciculation.[10] By contrast, homotypic repulsive interactions repel neurites of the same type to ensure effective non-overlapping coverage of a large receptive field by multiple neurons, a phenomenon called tiling (Fig. 1B). In

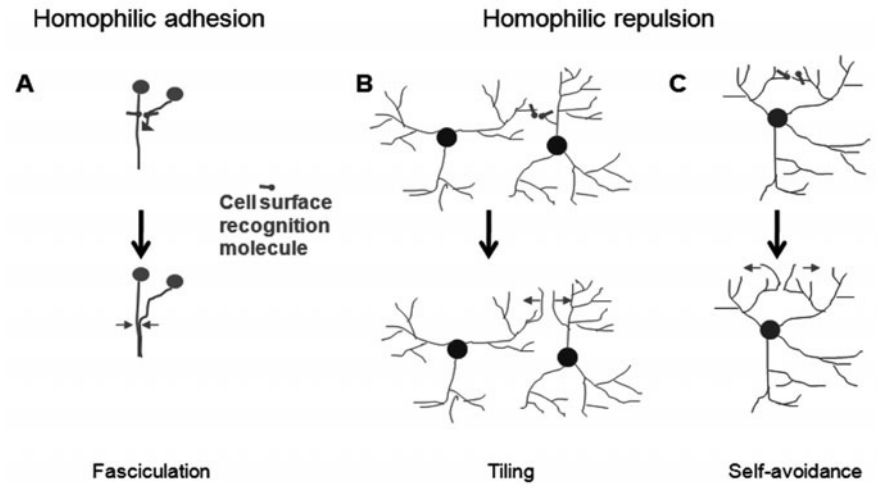

Figure 1. Homophilic interactions between cell surface molecules on the opposing membranes mediate diverse processes of neuronal wiring. This schematic illustration was modified from Hattori et al, 2008.[9]

theory, the inhibitory interaction can be realized by contact-dependent retraction. Genetic analysis of dendritic tiling among a particular class of cuticle sensory neurons of *Drosophila* has led to identification of various genes required for neuronal tiling.[11-13] However, no cell surface protein or secreted molecule has been implicated in regulating the dendritic tiling, leaving the origin of the repulsive homotypic cell-cell interactions undetermined.

Moreover, neurites derived from the same cell need to recognize each other for proper segregation, an essential step for a single neuron to co-innervate multiple sites. This requires discrimination of self from nonself, given that limiting the sister neurite interactions to each individual cell would be critical for fellow neurons not to perturb each other's neurite elaboration. Self-recognition leads to self-avoidance, which ensures extension of sister branches along separate paths to target multiple objects. A homophilic repulsive mechanism could, in principle, mediate self-avoidance (Fig. 1C). However, some sophisticated system would be needed to generate a unique molecular tag for every neuron in the brain or at least for each of the neurons that potentially meet. Intriguingly, the insect homolog of Down syndrome cell adhesion molecule (Dscam) exists in numerous distinct isoforms[14] that can vary in the homophilic interaction domains. *Drosophila* Dscam is broadly expressed in the developing CNS and selectively governs neurite arborization. Determining the roles of Dscam in neural development and its mechanisms of action has revealed a novel molecular mechanism for self-recognition, which will be the subject of our discussion in the chapter here.

IDENTIFICATION OF *DROSOPHILA* Dscam AND MOLECULAR DIVERSITY OF INSECT DSCAMS

Drosophila Dscam was first identified as a phosphotyrosine-containing protein via co-immunoprecipitation with Dock, an SH3/SH2 adaptor protein.[14-16] Dock may regulate cytoskeleton dynamics through the Pak serine/threonine protein kinase.[17] Although Dscam might activate Dock/Pak to govern the axon pathfinding of larval photoreceptors in the Bolwig's nerve,[14] it later becomes clear that Dscam is minimally involved in axon guidance[18] and the role of Dock/Pak in Dscam signaling remains to be clarified. Interestingly, Dscam was subsequently re-uncovered from an unbiased forward genetic mosaic screen, searching for genes controlling various aspects of neural development and morphogenesis in the *Drosophila* olfactory learning and memory center, the mushroom bodies (MBs).[19] Dscam controls divergent migration of sister axonal branches in the MBs, providing initial evidence for the involvement of Dscam in self-avoidance (see below).

Regardless of its physiological function, *Drosophila Dscam* immediately catches many scientists' attentions because of its potential for encoding thousands of distinct isoforms. Dscam was predicted as a cell surface protein, containing a putative signal peptide, ten immunoglobulin (Ig) domains and six fibronectin Type III (FNIII) domains in the extracellular portion, a transmembrane domain and the Dock-interacting cytoplasmic domain (Fig. 2).[14] There are three variable extracellular Ig domains encoded by blocks of alternative exons: 12 alternative exon 4s encode the first half of Ig2, 48 alternative exon 6s encode the first half of Ig3 and 33 alternative exon 9s encode Ig7. Besides, there are two alternative Dscam transmembrane domains encoded by exon 17.1 and exon 17.2, respectively. In addition, four endodomain variants exist due to presence or absence of exon 19 or 23.[20] Splicing at each of the exon blocks is independent of splicing at the other blocks and thereby these alternative choices of variable exons in *Dscam* could

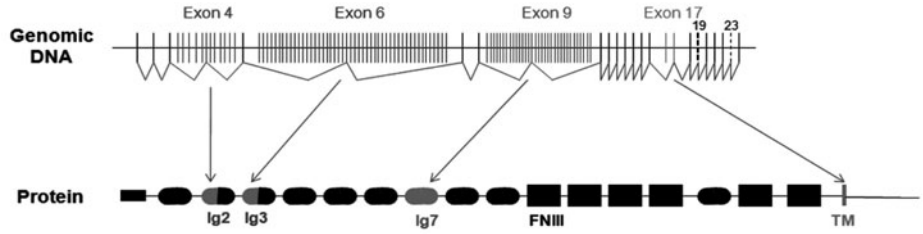

Figure 2. Tens of thousands of Dscam isoforms are generated through alternative splicing on multiple exon alternatives (exons 4, 6, 9 and 17). The alternative splicing exons encode variable regions on Ig2, Ig3, Ig7 and transmembrane domains of Dscam isoforms, respectively. Additional variants in the Dscam cytoplasmic domain, are generated through skipping of exon 19 and/or 23.[20] This schematic illustration was modified from Schmucker et al, 2000.[14]

potentially generate up to 152,064 distinct isoforms. Here we selectively concern its huge molecular diversity in the extracellular domain, not only because of the presence of 19,008 possible ectodomains but also due to the ectodomain variants determining isoform-specific Dscam-Dscam homophilic interactions (see below).

The three variable exon clusters encoding the variable Ig domains of the Dscam ectodomain exist in all the sequenced insect genomes.[21,22] Notably, comparative genomics reveals that exon 4s, 6s and 9s undergo distinct patterns of evolution.[23] Although the most similar pairs of exon 4s may exist in a single species, most exon 4s within one species are orthologous to exon 4s of other species, suggesting a common ancestor for divergent exon 4s. About exon 6s, while similarities of exon 6s exist between species, there are large blocks of highly similar exons within each species, suggesting independent expansions of ancestral exon 6s through evolution. By contrast, there is little similarity between exon 9s of different species and large blocks of homologous exon 9s exist within each species. Staggered homologous recombination may underlie the exon duplication by which an intron and its downstream exon were replicated. This pattern of exon duplication could initiate a cascade of duplication events while preserving the feature of the mutually exclusive splicing. New variants can thus be added within a given species. These phenomena imply that the molecular diversity of Dscam matters more than any specific Dscam variant, since its molecular diversity continues to increase such that no particular Dscam isoform has been conserved in its entirety through evolution.

GENETIC EVIDENCE FOR Dscam-MEDIATED SELF AVOIDANCE

Dscam's involvement in self avoidance was initially revealed by its requirement for the divergent segregation of axonal sister branches in the mushroom body (MB).[19] The MB is the olfactory learning and memory center in the insect brains, functionally equivalent to the olfactory cortex in mammals.[24] One MB derives from four indistinguishable neuroblasts that deposit two postmitotic neurons through an intermediate precursor following each self-renewal asymmetric cell division.[25,26] During development, MB axons transverse through the brain in a large bundle called peduncle.[27] At the end of the peduncle, most axons bifurcate into two sister branches. These two sister branches migrate along different pathways, one dorsally and the other medially, where they fasciculate and extend with

other MB axon branches. Notably, the sister axonal branches at the peduncle terminus no longer reliably segregate from each other in *Dscam* mutant MB neurons (Fig. 3A).[19] Further, despite presence of only two divergent MB sub-bundles, mutant axons often give rise to three or more axonal branches that may extend side by side along the same path. Failure in the divergent segregation of sister branches suggests defects in self-avoidance. The acquisition of supernumerary branches in the axons with defective self-avoidance implies that self-avoidance may control how many axonal branches from a single MB neuron are allowed and indirectly govern the pattern of bifurcation according to the available axonal paths that lead to discrete targets.

Interestingly, Dscam mediates proper segregation of sister branches in diverse neurons of the *Drosophila* brain. Antennal lobe (AL) projection neurons (PNs) relay the olfactory information from olfactory receptor neurons to MB neurons and the lateral horn (LH).[28] Loss of Dscam in single PNs leads to reduced dendritic fields in the AL and clumped axonal processes within their target zones where branching occurs.[29] These defects in neurite elaboration specifically occur as neurites arborize, consistent with the involvement of Dscam in sister branch segregation. Further, defects in PN axons as well as dendrites reveal roles of Dscam in both dendritic and axonal branching (Fig. 3B). Notably, Dscams carrying the exon17.1-encoding transmembrane domain are targeted to dendrites and govern dendritic elaboration, whereas exon 17.2-containing Dscams are enriched in axons and control axonal arborization.[30] Analogous neurite elaboration defects are observed in the neurons that arborize extensively in the ellipsoid body (EB), one of the four neuropils in the central complex residing at the midline of the adult brain.[19] Two patterns of EB axon arborization exist: some establish circular trajectories and the axons form repeated discrete arbors inward along the entire circle (Fig. 3C-I) while others project the axons to the EB center before branching into multiple processes that radiate outward and end with non-overlapping arborization around the EB (Fig. 3C-II). In both types, loss of Dscam selectively blocks axon arborization within the EB. Mutant axons consistently stall with clumps of branches (Fig. 3C), possibly due to defects in sister branch segregation again.

Dscam is not only required in the CNS but also mediates self-avoidance in the peripheral nervous system. Through late embryonic and larval development, four classes of dendritic arborization (da) neurons, class I-IV, elaborate dendrites continuously in

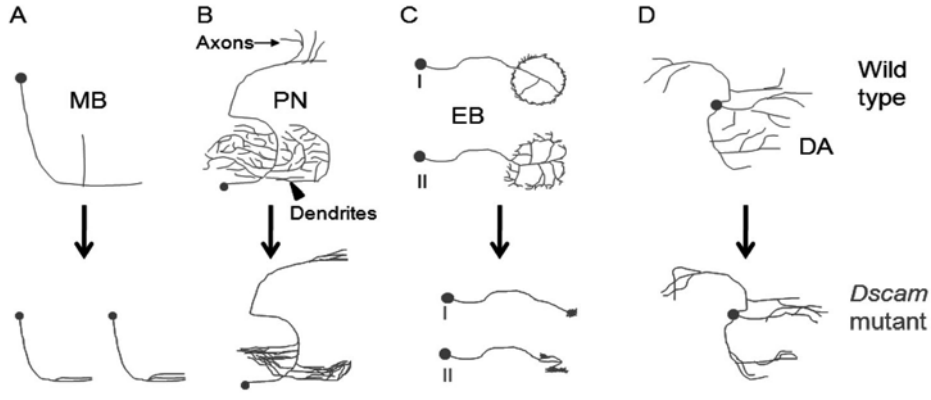

Figure 3. Dscam regulates self avoidance of mushroom body (MB) neurons (A),[19] projection neurons (PN) (B),[29] ellipsoid body (EB) neurons (C)[19] and dendritic arborization (DA) neurons (D).[32-34]

two-dimensional patterns within the larval body wall.[31] Dendrites of different classes of da neurons spread in overlapping manners. In contrast, dendrites within a given class avoid each other and jointly tile the body wall. Effective dentritic tiling requires intercellular as well as intracellular neurite-neurite repulsion. Notably, loss of Dscam selectively affects the intracellular neurite segregation (Fig. 3D).[32-34] Mutant dendrites aberrantly cross their sister processes while staying apart from neurites of neighboring cells. This phenotype is again consistent with involvement of Dscam in sister branch segregation.

The Dscam-mediated neurite repulsion should only occur between neurites derived from the same cell, since fellow neurons often need to extend branches along a common path. Interestingly, unrelated neurites aberrantly repel from each other when a single-isoform Dscam transgene is ectopically expressed between them. Such gain-of-function phenotypes were first detected following ectopic expression of Dscam in a subset of midline-crossing interneurons as well as the midline glia (Fig. 4B).[35] When they co-express the same Dscam transgene, the interneurons selectively avoid the midline glia and fail to extend processes across the midline. Abnormal neurite repulsion is also observed between dendrites of different neurons that express the same Dscam transgene (Fig. 4C).[32-34] Distinct classes of dendritic arborization (da) neurons exhibit overlapping dendritic fields. However, when a single Dscam isoform is broadly expressed in da neurons, inter-class dendritic recognition and avoidance become evident as dendritic fields of distinct da neurons no longer overlap. In another case, the wild-type PN neurons, DA1 and VA1d, normally have their dendrites elaborate side by side in neighboring AL glomeruli. When a single Dscam isoform is ectopically expressed in both DA1 and VA1d PNs, the dendrites of VA1d neurons are shifted ventrally and get separated by other glomeruli from the DA1 glomerulus (Fig. 4D).[29] These repulsive neurite-neurite interactions probably occur

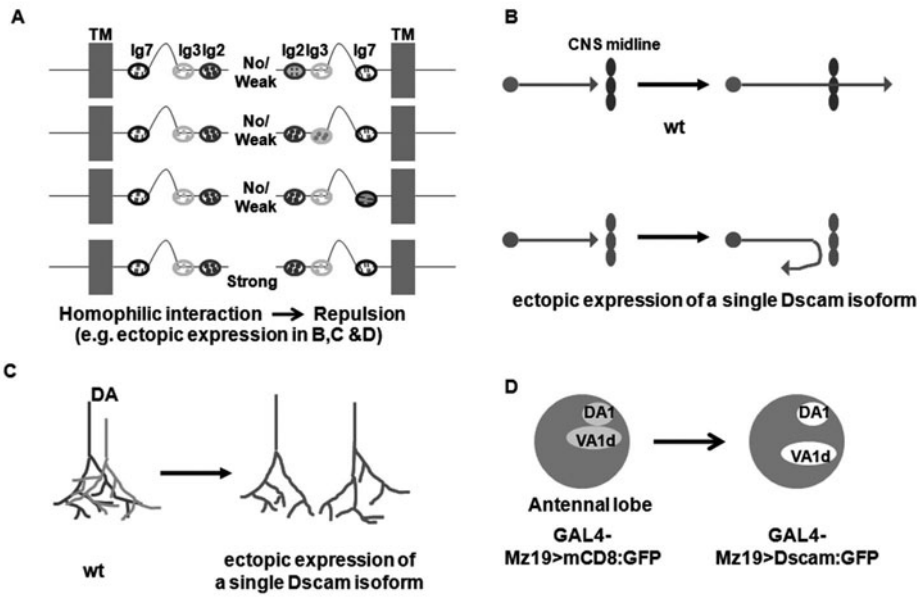

Figure 4. Homophilic Dscam-Dscam binding results in repulsion of neurites, as exemplified in neurites of embryonic neurons (B),[35] dendritic arborization (DA) neurons (C)[32-34] and PNs (D).[29]

through homophilic Dscam binding between opposing cell surfaces, providing indirect evidence for the involvement of Dscam-dependent, contact-mediated repulsion in the regulation of sister branch formation and segregation.

STOCHASTIC EXPRESSION OF DISTINCT Dscam ISOFORMS IN INDIVIDUAL NEURONS

The promiscuous Dscam-mediated neurite repulsion occurs when a single-isoform Dscam transgene is ectopically expressed in neighboring neurons. These gain-of-function effects imply that individual neurons are endogenously tagged with distinct Dscams, limiting the mutual repulsive interactions to the neurites derived from the same cell. The huge repertoire of Dscam isoforms does have the potential for conferring every neuron with a unique molecular identity to render divergent segregation of sister branches in each neuron while allowing comigration of unrelated neurites. But do individual neurons even of the same type express distinct Dscam isoforms? And if so, what molecular mechanisms can ensure expression of a unique subset of Dscam isoforms in each neuron?

First, analysis of *Dscam* transcripts by RT-PCR has revealed expression of distinct *Dscams* in individual neurons.[36,37] Different exon alternatives are differentially utilized in different neuron types. Further, single-cell RT-PCR documents that individual cells express different combinations of *Dscam* isoforms. It was estimated that each neuron might express 14-50 distinct *Dscam* transcripts chosen from the spectrum of thousands of splice variants characteristic of its neuron type. Dynamic and stochastic, though biased, expression of multiple *Dscam* splice variants apparently occurs in each neuron. Stochastic selection of a small number of *Dscam* isoforms from a huge collection of combinatorial exons can randomly generate numerous unique *Dscam* ensembles, such that only neurites derived from the same neuron are endowed with the same Dscams.

Second, analysis of *Dscam* genomic sequences across different insect species provides clues about the mechanisms underlying the stochastic mutually exclusive selection of distinct exon alternatives. In the cluster of exon 6s, there exist two conserved elements—the docking site and the selector sequence.[38] The docking site is located in the intronic region between the constant exon 5 and exon 6.1; the selector sequence is found in the intronic region upstream of each exon 6 variant. Further, each selector sequence is complementary to a portion of the docking site. Pairing of them would juxtapose one exon alternative to the upstream constitutive exon and only one selector sequence may pair with the docking site. In addition, an RNAi screen in *Drosophila* S2 cells has led to identification of the heterogeneous nuclear ribonucleoprotein (hnRNP) hrp36 as a negative regulator that binds to all exon 6s to prevent inclusion of multiple exon 6 alternatives.[39] The pairing of the selector sequence with the docking site may selectively eliminate hrp36 from the juxtaposed exon 6 alternative, thus ensuring inclusion of only one exon 6 variant in each *Dscam* transcript (Fig. 5). The docking site could pair with different selector sequences by chance, leading to dynamic stochastic expression of various exon 6s in each neuron.

About the exon 4 variants, an evolutionarily conserved RNA secondary structure, termed inclusion Stem (iStem), exists in the intron between exon 3 and exon 4.1.[40] Loss of iStem results in frequent skipping of the entire cluster of 12 exon 4s, but does not affect the inclusion of any particular exon 4. And no conserved sequences could be uncovered in the intervals between different exon 4s to account for their mutually exclusive alternative

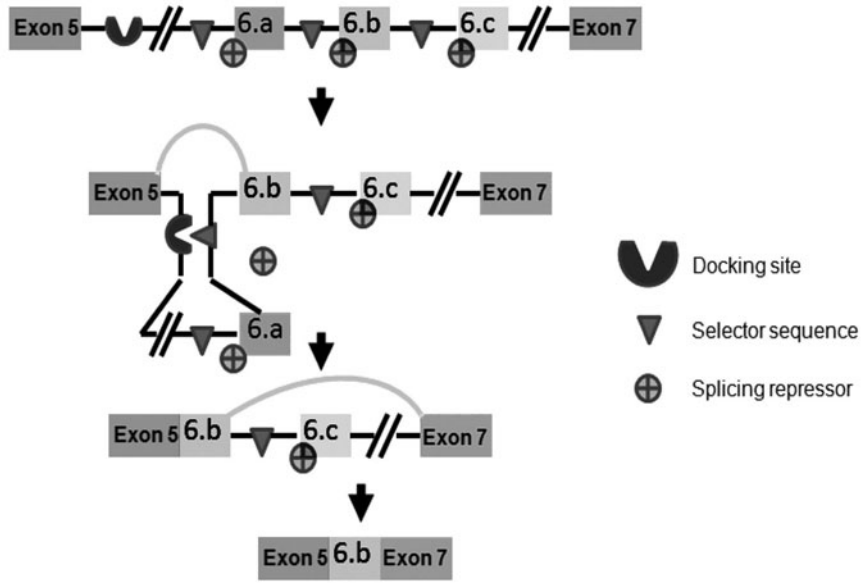

Figure 5. Model for the mechanism of mutually exclusive alternative splicing in exon 6. Only three consecutive exons in exon 6 cluster are shown (a can be 1-46). The splicing suppressor normally prevents the splicing events. However, when a selector binds to the docking site, the splicing repressor is dissociated from one of the exon 6 alternatives, allowing the mutually exclusive splicing. This schematic illustration was modified from Graveley BR, 2005.[38]

splicing. No conserved sequences or secondary structures could be identified in the exon 9 cluster either. Novel mechanisms of alternative splicing are probably involved in the stochastic selection of exon 4s and exon 9s. Independent stochastic selection of one exon variant from many alternatives in the three variable regions ultimately yields Dscams of numerous possibilities regardless of cell types. This mechanism cleverly confers each neuron with a unique yet dynamic molecular identity essential for proper wiring of complex nervous systems.

BIOPHYSICAL BASIS OF Dscam ISOFORM-SPECIFIC HOMOPHILIC INTERACTIONS

Dscam-mediated repulsive interactions normally occur between neurites that derive from the same cell and thus express an identical set of Dscams. And ectopic expression of a single Dscam isoform elicits promiscuous repulsive interactions among unrelated neurites. These phenomena implicate isoform-sepcific Dscam-Dscam homophilic interactions as the molecular basis of neuronal self-recognition. The extracellular segment of *Drosophila* Dscam carries ten Ig and six FN domains (Fig. 2). The variable exon 4, 6 and 9 encode the Ig domain 2, 3 and 7, respectively. The restriction of repulsive interactions to neurites expressing identical Dscams further suggests that Dscam-Dscam homophilic binding primarily occurs through the Ig domain 2, 3 and 7 and that mismatch in any of the variable Ig domains can drastically diminish its binding affinity.

The above predictions are validated by a series of in-vitro binding assays, showing that Dscam isoforms display isoform-specific homophilic binding.[35,41] Each isoform exclusively binds to itself with high affinity. Even closely related isoforms carrying analogous sequences in the variable regions bind poorly to each other. All three variable Ig domains play decisive roles in determining the binding specificity. High-throughput in-vitro binding among 95% (>18,000) of Dscam ectodomain variants further reveals that when two of the three variable Ig domains are kept the same between opposing Dscam isoforms, the effects of a mismatched 3rd variable Ig domain on the binding affinity remain constant regardless of the actual identity of the other variable Ig domains. This argues that each variable Ig domain only interacts with its counterpart in opposing Dscams and that the three variable Ig domains act as independent modules for self-binding.

However, to achieve high-affinity binding between opposing Dscams requires self-binding in all three variable Ig domains. Crystal structure analyses together with biochemical studies for the N-terminal eight Ig domains of Dscam (termed as $Dscam_{1\text{-}8}$), which includes all three variable Ig domains, provide some biophysical basis for the modular strategy of isoform-specific homophilic interactions (Fig. 6).[42,43] First, each $Dscam_{1\text{-}8}$ molecule in a homophilic dimer adopts a S-shape configuration: the domains Ig1-Ig4 in a horseshoe shape locate at the top half of the "S" and the domains Ig5-Ig8 reside at the bottom half of the "S". Second, anti-parallel pairwise matching occurs in the self-binding of Ig2, Ig3 and Ig7. Any mismatch in the variable Ig domains may produce enough strain to uncouple the entire anti-parallel pairing. Although further structural analysis is necessary to provide additional details, this model illustrates how the remarkable binding specificity of numerous Dscam isoforms can be achieved through self-binding of three independent Ig domains.

REQUIREMENT OF Dscam MOLECULAR DIVERSITY FOR CONCURRENT MORPHOGENESIS OF FELLOW NEURONS

Thus a single Dscam gene can encode many cell surface proteins that selectively bind to Dscams carrying the same ectodomain. And the presence of thousands of ectodomain variants makes it possible for each neuron to express a unique set of Dscams simply by chance without involvement of any cell-fate-dependent gene expression. However, how many choices of affinity-defining ectodomains are actually needed to confer individual neurons in a complex brain a unique identity such that only neurites derived from the same cell express the same subset of Dscams and repel each other following isoform-specific Dscam-Dscam homophilic interactions?

As to a lone neuron, a single-ectodomain-containing Dscam is sufficient to mediate self-avoidance among neurites of the same origin. This was first evidenced by the observation that a single-isoform Dscam transgene can effectively rescue the sister-branch segregation defects in isolated *Dscam* mutant neurons.[20,37] The dispensability of Dscam ectodomain diversity in lone neurons was further demonstrated in the mosaic brains where no loss-of-Dscam phenotypes could be detected in the sparsely labeled neurons born with only one functional Dscam gene encoding only one possible ectodomain.[44] But to support neurite arborization involving multiple neurons requires Dscams with different ectodomains. Induction of a single-isoform Dscam transgene in multiple local neurons is detrimental and aberrantly makes fellow neurites repel from each other and suppress further extension regardless of their origin (Fig. 4).[19,29,32-35] Analogous neurite

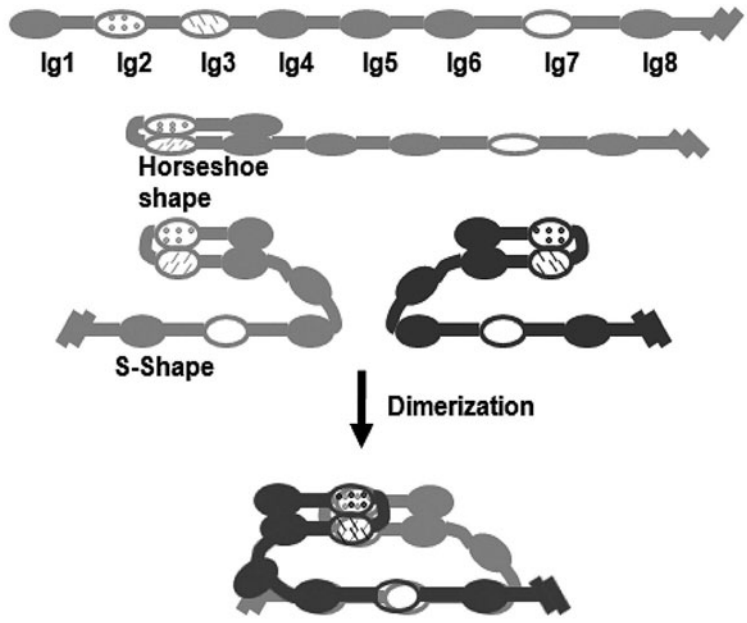

Figure 6. Structural basis for Dscam-Dscam homophilic interaction. The $Dscam_{1\text{-}8}$ crystal structure shows a dimer of two S-shaped Dscam monomers with direct opposing contact between Ig2, Ig3 and Ig7 variable domains. Electron micrographs on $Dscam_{1\text{-}8}$ further show that, though the first four Ig domains constitute a compact horseshoe structure, the remaining four Ig domains are relatively flexible. This schematic illustration was modified from Schmucker et al, 2009.[53]

elaboration defects exist in the organisms heterozygous for a *Dscam* allele encoding only one possible ectodomain. Various ectodomains were tested in the experiments with single-ectodomain-containing Dscam transgenes or alleles. They show no difference in the rescue efficiency or the elicitation of gain-of-function phenotypes.[20,37,44] These observations argue that the actual identity of Dscams in any particular neuron is not critical as long as each neuron expresses a unique set of Dscams.

However, most endogenous Dscams may encode more ectodomain variants than what's needed for expression of Dscams with different binding specificities in individual neurons to restrict self-avoidance (mediated through isoform-specific Dscam homophilic interactions) to neurites of the same origin. Some redundancy among various ectodomains was first suggested through analysis of various *Drosophila Dscam* small deficiency lines.[20,32] Loss of subsets of alternative exon 4s does not affect organism viability or neurite elaboration of most model neurons. This is true even when nine of the twelve exon 4s were deleted, reducing the choices of Dscam ectodomains from 19,008 to around 5,000.

A series of *Dscam* knock-in mutants were further created to determine how much Dscam diversity is required to achieve self versus nonself discrimination.[45] The numbers of Dscam ectodomain variants are reduced to different degrees. Dscam encoding 1, 12, 24, 576 or 1152 possible ectodomains is not sufficient and elicits qualitatively comparable abnormal neurite elaboration phenotypes in diverse model neurons. By contrast, no defect in neurite elaboration could be detected in the organisms carrying about 5000 Dscam

ectodomain variants. The same conclusions are obtained regardless of the actual identity of the remaining ectodomains. Taken together, presence of thousands of Dscam ectodomain choices, about one quarter of what's encoded by the wild-type gene, is necessary for individual neurons in the *Drosophila* brain to discriminate self from others.

CONCLUSION AND OTHER Dscam FUNCTIONS

Self-recognition for individual neurons in the complex nervous system is a daunting task and has been long neglected until the identification of *Drosophila* Dscam. Dscam governs neurite branching and elaboration by ensuring divergent segregation of sister branches while allowing comigration of unrelated neurites. Intriguingly, the *Drosophila Dscam* encodes tens of thousands of cell surface proteins with distinct ectodomains that exhibit isoform-specific homophilic interactions. Without Dscam, sister branches no longer repel from each other and fail to innervate multiple targets. By contrast, presence of identical Dscams in fellow neurons elicits repulsive interactions among unrelated neurites and aberrantly suppresses each other's neurite extension/arborization. These phenomena imply that each neuron expresses a unique set of Dscams, which is made possible by dynamic stochastic expression of tens of Dscams, chosen from thousands of distinct possibilities, in each neuron. This simple yet clever mechanism does not require acquisition of distinct fates by individual neurons. However, it entails evolution of multiple sophisticated features in the ancestor insect Dscam gene.

First, the Dscam ectodomain carries three variable Ig domains besides seven constant Ig structures. Mismatch in any of the variable Ig domains potently inhibits homophilic binding. Second, the three variable Ig domains are respectively encoded by three independent blocks of exons. Every exon alternative in each block specifies a unique amino acid sequence that supports high-affinity homophilic binding while preventing promiscuous Dscam-Dscam interactions. Third, only one exon alternative from each exon block exists in the mature transcripts. While cell fate may bias the selection of certain exon variants, the mutually exclusive alternative splicing within each exon block occurs rather stochastically. This permits random assortment of multiple choices at three independent loci, underlying the dynamic expression of distinct Dscams even among cells of the same type. Fourth, DNA duplication and divergence have taken place to expand the repertoire of the mutually exclusive *Dscam* exons through evolution. The generation of enough Dscam ectodomain diversity is critical for wiring of complex neural circuits and may drive insect brain evolution from simple to higher organisms.

Besides mediating self-avoidance among neurites of the same origin, *Drosophila* Dscam may govern additional aspects of neurite elaboration. Given the stochastic yet cell-type-biased incorporation of specific exon alternatives, neurons of the same type may express less divergent Dscams and undergo weak Dscam-mediated neurite-neurite interactions. Such interactions have been suggested to result in attraction rather than repulsion, as proper fasciculation of MB neurites of different origins requires Dscam.[37] Cell-type-dependent expression profiles of Dscams might also help pattern neurite elaboration characteristic of specific neuron types. The affinity of Dscam homophilic interactions could vary depending on levels of expression or complexities of isoform compositions, which might elicit different intensities of neurite-neurite repulsion and help govern the geometry of neurite arborization. In addition, Dscam may govern neuronal morphogenesis through interacting with netrin, a well-known axon guidance cue.[46-48]

Further, Dscam2, another Dscam gene in the *Drosophila*, was found to be required for tiling of L1 neurons, a subset of neurons in the visual system.[49] Outside the nervous system, *Drosophila* Dscam is selectively expressed in immune-competent cells and required for mounting immune responses to various pathogens.[50] The huge binding specificities displayed by the numerous Dscam ectodomains may facilitate recognition of diverse microbes and constitute a novel mechanism of innate immunity in insects.[51]

Do analogous mechanisms provide self-recognition for individual neurons in vertebrates? Vertebrate DSCAM shares significant homology with insect Dscam in the extracellular domain and has been implicated in neurite tiling and self-avoidance of certain neurons.[52] However, in the mouse genome there exist only two DSCAM genes that lack the molecular diversity reminiscent of insect Dscams, making it unclear how the vertebrate nervous system achieves the more complex neural recognition. One possibility is through combinatorial action with other cell surface receptors, such as cadherins, protocadherins and neurexins which could provide diverse receptor specificities.[53] Interestingly, the recent genetic and biochemical studies in the mouse commissural axons have showed that DSCAM could mediate axonal growth through forming heterodimeric complexes with DCC.[46,48] Future studies on receptor complex formation between DSCAM and other mammalian cell adhesion molecules might provide insights into how cell recognition is achieved in higher organisms. To identify additional neuron surface proteins with huge molecular diversity should also shed light on the delicate cell-cell interactions in the complex brain.

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CHAPTER 18

THE NEURAL BASIS OF SEMANTIC AND EPISODIC FORMS OF SELF-KNOWLEDGE:

Insights from Functional Neuroimaging

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Abstract: Throughout evolution, hominids have developed greater capacity to think about themselves in abstract and symbolic ways. This process has reached its apex in humans with the construction of a concept of self as a distinct entity with a personal history. This chapter provides a review of recent functional neuroimaging studies that have investigated the neural correlates of such "higher-level" aspects of the human self, focusing in particular on processes that allow individuals to consciously represent and reflect on their own personal attributes (semantic forms of self-knowledge) and experiences (episodic forms of self-knowledge). These studies point to the medial prefrontal cortex (MPFC) as a key neural structure for processing various kinds of self-referential information. We speculate that the MPFC may mediate dynamic processes that appraise and code the self-relatedness or self-relevance of information. This brain region may thus play a key role in creating the mental model of the self that is displayed in our mind at a given moment.

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INTRODUCTION

> *One great splitting of the whole universe into two halves is made by each of us; and for each of us almost all of the interest attaches to one of the halves; but we all draw the line of division between them in a different place.*
> *—William James, 1890, p. 289*

The great splitting William James refers to is the division of the world into "me" and "not-me".[1] Such division between self and nonself is implemented in many systems, at various levels of complexity; simple living organisms and even some robots,[2] have some sort of self-models that allow them to distinguish between themselves and the external environment. Yet, among all known systems, it is undoubtedly in human beings that the sense of self has reached the highest level of refinement. The human sense of self comprises multiple facets or levels, from the consciousness of oneself as an agent and immediate subject of experience to the construction of a concept of oneself as a distinct entity with a personal history.[3-6] Although a complete understanding of the brain mechanisms that support these multiple dimensions of self is still currently out of reach, research in social cognitive neuroscience[7] has made important progress in identifying the brain regions that are involved in representing and reflecting on different types of self-referential information.

In this chapter, we review recent studies that have used functional neuroimaging techniques such as functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) to investigate the neural correlates of "higher-level" self-referential processes. By higher-level self-referential processes, we mean processes that allow an organism to consciously represent and reflect on its own attributes and experiences, what Damasio has called the "autobiographical self".[5] We address more specifically functional neuroimaging studies that have investigated the neural basis of semantic and episodic forms of self-knowledge. Our aim is not to provide an exhaustive review of all existing studies but rather to illustrate how functional neuroimaging has helped to better understand the brain mechanisms that contribute to creating the self/nonself distinction that William James referred to. It will be suggested, in particular, that the medial prefrontal cortex (MPFC)[a] may play a key role in implementing this process.

REPRESENTING ONE'S PERSONAL CHARACTERISTICS: SEMANTIC FORMS OF SELF-KNOWLEDGE

In comparison with simpler, unreflective forms of self-awareness, the ability to think about oneself in abstract and symbolic ways is thought to have emerged late in evolution (perhaps in the Middle-Upper Paleolithic transition, between 40,000

[a] In this chapter, we use MPFC as a broad term to refer to the medial portion of the superior frontal gyrus, orbitofrontal gyrus and rostral anterior cingulate cortex (Brodmann's areas 9, 10, 11 and 32). We use this general term because in many studies of self-referential processing, activations along the medial part of the prefrontal cortex is quite extensive, encompassing several anatomically distinct subregions. It is likely, however, that different subregions within the MPFC support distinct functions in relation to self-referential processing (see ref. 8), though this issue remains to be investigated in detail.

and 60,000 years ago, where we find instances of technological advances, art, body adornment and ritualistic burial) and it may be specifically human.[9] Humans are able to consciously represent and reflect on their own personal attributes, such as their abilities and skills (e.g., "I can play guitar"), social roles (e.g., "I am a father"), psychological characteristics (e.g., "I am a shy person") and preferences (e.g., "I like red wine"). This collection of information about ourselves (which is not necessarily accurate) constitutes the self-concept, a complex knowledge structure stored in long-term memory that includes abstract, summary representations of our own personal characteristics.[10] Those self-representations are semantic in nature in the sense that they have been abstracted from multiple experiences and can be accessed without the need to remember any specific past event.[11,12]

The experimental paradigm that has been most frequently used to study the neural correlates of semantic self-knowledge consists of asking participants to represent and reflect on their own psychological traits. Kelley et al, for example, measured brain activity using fMRI while participants made different types of judgments on trait adjectives (e.g., polite, dependable, daring, talkative).[13] In one condition, participants had to judge whether or not the adjectives described their own psychological traits (self condition), whereas in a second condition they had to judge whether or not the adjectives described the traits of George W. Bush (other condition). In a third condition, participants performed a shallow processing task consisting of judging whether the adjectives were printed in uppercase letters (case condition). The results showed that the two semantic judgments (i.e., the self and other conditions) were associated with greater activity in the left inferior frontal cortex and the anterior cingulate cortex relative to nonsemantic judgments (i.e., the case condition). More interestingly, there were also differences in activations between the two types of semantic judgments, with judgments about the self leading to greater activation in the MPFC than judgments about the other. An increased activation in the MPFC when reflecting on one's own traits (compared to the traits of others or to making semantic judgments) has also been observed in several subsequent studies[14-26] (see Fig. 1 for an illustration of the MPFC activations detected in different studies).

In functional brain imaging studies, successful assignment of a specific cognitive process to the detected brain activation depends on the appropriate contrasting of task conditions. Because early studies of self-referential processing used a public figure rather than a personally well-known person for the comparison condition, it has been argued that the brain activations observed when making judgments about the self versus others may reflect differences in the amount and complexity of retrieved knowledge and/or differences in affective response rather than the self versus other distinction per se.[27] To address this issue, Heatherton et al[28] used a similar task as Kelley et al[13] but the other-referential condition involved a personally familiar other (i.e., one's best friend) rather than a public figure. They found that the same MPFC region previously identified as more activated when thinking about the self versus a nonclose other was also more activated when thinking about the self versus an intimate other. Similar results have been obtained in most reports,[15,29,30] although some studies failed to detect differential activity in the MPFC when contrasting judgments referring to the self with judgments referring to a close other.[31,32] The reasons for these divergent findings remain unclear but a possible explanation would be that the difference in MPFC activity when contrasting self and close other conditions depends on the perceived similarity or overlap between oneself and close others, which in turn varies across individuals and situations. We will return to this point when discussing the possible function of the MPFC.

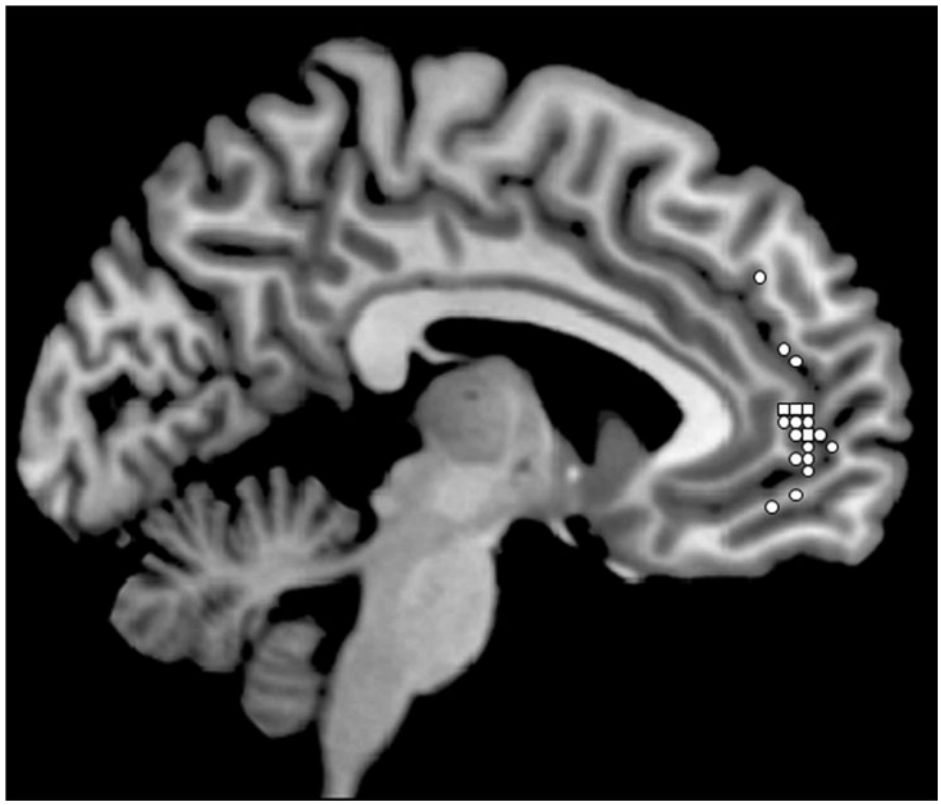

Figure 1. MPFC activations associated with semantic forms of self-knowledge. White circles represent the locations of peak MPFC activations detected when reflecting on one's own psychological traits relative to reflecting on the traits of others or making semantic judgements.[13-23,25,26,29,30] White squares represent the locations of peak MPFC activations detected when reflecting on one's own promotion goals.[38-41]

The self-concept comprises multiple self-representations. People may hold distinct views of themselves across different contexts (e.g., in different relationships).[33] Furthermore, an important part of our self-concept concerns our beliefs about how we are seen by others. Although our own beliefs about ourselves and about how we are seen by others reciprocally influence each other, these two types of self-representations do not necessarily match.[34] In a recent fMRI study,[15] we investigated whether representing and reflecting on these two types of self-representations recruit similar brain regions. To do so, we adapted the task used by Kelley et al by including four types of judgments. The first two conditions were similar to previous studies and required participants to judge the extent to which trait adjectives described their own personality (e.g., "Are you impatient?") or the personality of a close friend (e.g., "Is Caroline impatient?"). In the other two conditions, participants were asked to estimate how their friend would judge the adjectives, with the target of the judgments again being either the self (e.g., "According to Caroline, are you impatient?") or the other person (e.g., "According to Caroline, is she impatient?"). We found that the MPFC was more activated when making judgments about the self than when making judgments about the other, both

when participants made judgments from their own perspective and when they made judgments from the perspective of their friend. Interestingly, we also found that patients with Alzheimer disease presented less accurate self-representations than healthy older adults and did not recruit the MPFC when making judgments about themselves (both when making the judgments from their own perspective and from the perspective of their relatives).[35] These findings thus suggest that the MPFC may play an important role not only in representing and reflecting on our own self-beliefs, but also in thinking about how we are seen by others.

Besides representations of personal traits, the self-concept also includes knowledge about personal goals (i.e., future states of the self that one strives to achieve or avoid), which plays a key role in guiding and motivating behavior.[36,37] The neural correlates of reflection on personal goals has been recently investigated by Johnson et al.[38] During f MRI scanning, participants were instructed to think about their hopes and aspirations, about their duties and obligations and about nonself-relevant topics (e.g., polar bear fishing). The results showed that some regions of the MPFC and posterior cingulate/ precuneus were more activated when thinking about personal goals than when thinking about nonself-relevant topics. There were also distinct activations as a function of the type of personal goals considered: A region of MPFC showed greater activation when thinking about hopes and aspirations, whereas a posterior medial region showed greater activation when thinking about duties and obligations. Subsequent studies have detected similar activations in the MPFC when reflecting on personal goals, especially promotion goals (i.e., things the individual would like to achieve)[39-41] (see the location of squares on Fig. 1).

In summary, since the advent of functional brain imaging techniques, a growing interest has been devoted to the study of the neural correlates of semantic forms of self-knowledge. Current data indicate that representing and reflecting on various kinds of self-knowledge (such as our own beliefs about our psychological traits, our beliefs about how we are seen by others and our personal goals) typically induce greater activity in the MPFC compared to representing and reflecting on other individuals or processing the general (nonpersonal) meaning of the stimuli.

REPRESENTING ONESELF IN TIME: EPISODIC FORMS OF SELF-KNOWLEDGE

A fascinating achievement of the human mind is its ability to temporarily disengage from the immediate environment to mentally revisit past experiences or imagine future ones.[42-44] Such "mental time travels" are associated with the subjective feeling of "re-experiencing" the past or "pre-experiencing" the future (e.g., "seeing" in one's mind the location where a past event took place and the persons and objects that were present, remembering what one thought and felt at that time and so forth)[45-48] and may thus play a key role in providing the individual with a sense of personal continuity through time.[44,49] Although semantic self-knowledge is represented separately from (and thus can be accessed independently of) episodic memory,[12] representations of specific past and future experiences can constrain and nourish our beliefs about ourselves.[50] For example, the belief that "I am a shy person" may be fostered by my memory of feeling uncomfortable and behaving awkwardly the last time I met new people at a party. An important function of mental time travel may therefore be to provide "episodic forms of

self-knowledge", that is, representations of specific personal experiences that support and constrain more abstract representations of the self.

The neural correlates of episodic forms of self-knowledge have been mainly investigated in studies of autobiographical memory (for a review, see refs. 51-53). In many of these studies, participants were asked to recall memories of specific past experiences (i.e., events that were specifically located in place and time and that lasted less than a day) in response to a series of cue words (e.g., vacation, dress). This autobiographical retrieval condition is typically compared to the retrieval of nonpersonal information (e.g., retrieving nonpersonal semantic knowledge or recalling stimuli that have been learned in the laboratory before the scanning session). Recent meta-analyses indicate that autobiographical memory retrieval relies on a specific set of brain regions that includes the MPFC, regions in medial and lateral temporal cortices, the posterior cingulate/retrosplenial cortex and the inferior parietal lobe.[54-56] Notably, recent studies have shown that similar brain regions are also associated with the imagination of specific future events,[57-63] suggesting that common processes are involved in representing specific past and future events.[42,43]

Autobiographical memory and future thinking involve multiple component processes,[42,43,64,65] and it is likely that different processes depend on distinct brain areas within the network described above. In a recent fMRI study,[66] we sought to isolate the brain regions that are specifically related to self-referential processes when representing specific future events. We asked participants to imagine future events that were relevant to their personal goals (personal future events; e.g., getting married next summer) and future events that were plausible and could be vividly imagined but were unrelated to their personal goals (nonpersonal future events; e.g., taking a pottery lesson next summer), as determined by individualized prescan interviews. As a control task, participants were asked to imagine routine activities (e.g., taking a shower), which involved the construction of mental representations of complex scenes but lacked the process of projecting oneself into the future. In line with other studies of episodic future thinking,[57-63] a network of brain regions that included the MPFC, the posterior cingulate cortex (PCC), the inferior parietal lobe and the lateral temporal lobe was more activated when participants imagined personal future events (i.e., future events that were related to their personal goals) than when they imagined routine activities. Our main interest was then to directly contrast the imagination of personal and nonpersonal future events in order to isolate the brain regions that support personal goal processing during episodic future thought. This comparison revealed greater activation in the MPFC and PCC when imaging personal future events relative to nonpersonal future events. Importantly, these two types of future events were matched for vividness and temporal distance, suggesting that differences in brain activation cannot be accounted by these factors alone. The findings thus suggest that the MPFC and PCC may play a specific role in personal goal processing during episodic future thought.

In the same study, we also sought to investigate whether common brain regions were implicated in self-referential processing across different functional domains. To this end, we isolated the brain regions that were associated with semantic forms of self-knowledge in the same participants, using a task that involved making judgments about one's own psychological traits (see the section on the semantic forms of self-knowledge). We then looked at the overlap between brain activations related to this task and brain activations associated with self-referential processing in the episodic

domain (i.e., imaging personal versus nonpersonal future events). Brain activations associated with the two tasks overlapped in the MPFC and PCC. These findings suggest that semantic and episodic forms of self-knowledge may engage common self-referential processes, which may in part be supported by the MPFC and PCC. In line with this view, a meta-analysis of functional neuroimaging studies has revealed that cortical midline structures (i.e., the MPFC and the PCC/precuneus) were involved in processing self-referential information across multiple cognitive domains and sensory modalities (e.g., the recognition of one's own body and actions, self-face recognition and the representation of one's own traits).[67]

SELF-REFERENTIAL PROCESSING AND THE "DEFAULT NETWORK"

The brain regions that are most frequently engaged during self-referential tasks (i.e., the MPFC and PCC) are part of the brain's "default network", a network of areas that show decreased activity during a wide range of demanding cognitive tasks relative to passive resting or viewing states.[68-71] The precise function of the default network remains to be investigated in detail, but an interesting possibility is that this network mediates a number of processes that are ongoing during resting states and attenuated when resources are temporarily re-allocated to the processing of a particular task.[72,73] The specific processes that are ongoing during resting states are probably manifold (e.g., the monitoring of external environment and body state, autobiographical retrieval, future thinking) and it is likely that different brain areas within the default network are involved in distinct processes.

In a PET study, we sought to investigate whether self-referential processes occur during resting states and to determine whether common brain regions are engaged during resting states and intentional self-reflection.[14] To this end, scans were acquired while participants were asked to simply relax and not think in a systematic way (rest scans) or to focus their mental activity on specific topics pertaining either to the self (i.e., thinking about one's own traits) or other topics (i.e., thinking about the traits of another person or about social issues). Importantly, subjects' activity during the reflective tasks was exclusively internal (i.e., no stimuli were presented during the scans and no responses were required), so that the reflective and rest scans were closely matched in this respect. Immediately after each scan, subjects were asked to verbally report the thoughts, images, feelings, sensations and memories that they experienced during the scan and they also rated different dimensions of their subjective experience using rating scales (e.g., the amount of self-referential thoughts, the amount of thoughts about other individuals). The data revealed that participants spontaneously experienced a substantial amount of self-referential thoughts during rest scans. We then investigated brain regions that were commonly activated during rest and intentional self-referential processing relative to the other tasks and found common activation in the MPFC. Furthermore, across all conditions, we found that the degree of activity in MPFC correlated with self-reported amount of self-referential thoughts; on the other hand, there was no correlation with the amount of thoughts concerning others. These findings thus suggest that some sort of self-referential processes spontaneously occur during so-called resting conditions and that such processes are associated with MPFC activity.

A POTENTIAL ROLE OF THE MPFC IN SELF-REFERENTIAL PROCESSING

As we have seen, activation of the MPFC has been repeatedly observed in association with tasks that require to process information in reference to oneself. There is currently no consensual view, however, regarding the precise nature of the cognitive processes that are supported by this brain region. While it is beyond the scope of this chapter to provide a comprehensive review of the various processes that have been linked to the MPFC, it is important to note that the question of whether or not this brain region plays a specific role in self-referential processing is debated. Some authors have argued that although the MPFC supports processes that are recruited when one is considering information about the self (e.g., when making judgments about one's personal characteristics), the nature of these processes may have nothing to do with the self per se and may instead consist of nonself-specific processes, such as inferential processing and memory retrieval, for example.[27,74] On the other hand, other researchers have suggested that the MPFC may play some specific role in self-referential processing.[8,28,29,67,75,76] It has been suggested, in particular, that the MPFC may support supramodal processes that, explicitly or implicitly, appraise and code the self-relatedness or self-relevance of multiple sources of information.[8,67,76] According to this view, the MPFC may mediate dynamic processes that locate external stimuli and internal representations on a continuum of personal relevance. In line with this hypothesis, there is evidence that activity in the MPFC increases linearly with increased ratings of self-relevance of stimuli.[75,77] A recent study by Moran et al[78] further suggests that the MPFC signals the personal relevance of incoming information even in the absence of explicit requirements for self-reflection (i.e., during passive viewing conditions).

We have recently speculated that by processing degrees of self-relevance or self-relatedness, the MPFC might sustain the process of identifying oneself with versus distancing oneself from particular mental contents (e.g., thoughts, opinions, preferences), which would therefore be regarded as "me" (or "mine") versus "not-me" (or "not-mine").[29] The MPFC might thus contribute to the great splitting of the universe made by each of us that William James referred to more than a century ago.[1] Of course, we agree that the creation of our sense of self involves multiple nonself-specific processes, such as memory and reasoning processes.[27,74] These nonself-specific processes rely on multiple brain regions, including medial and lateral temporal cortices, the posterior cingulate cortex and the lateral prefrontal cortex, which are involved in acquiring, retrieving and using information (whether it be about the self, others or the world). Our suggestion, however, is that the MPFC might play a role in processing the self-relatedness or self-relevance of information that is represented in other "high-level" or "low-level" brain regions. Representations that elicit high activity in the MPFC might be those that constitute the mental model of the self that is displayed in our mind at a given moment ("the working self").[10] In this section, we discuss evidence that we think supports this hypothesis.

If the MPFC is involved in processing self-relevance or self-relatedness, then factors that diminish the perceived degree of self-relatedness of information should modulate neural activity in the MPFC accordingly. Recent studies that have examined the effects of temporal perspective on the neural correlates of self-referential processing suggest that this is indeed the case. Some philosophers have suggested that a person is a succession

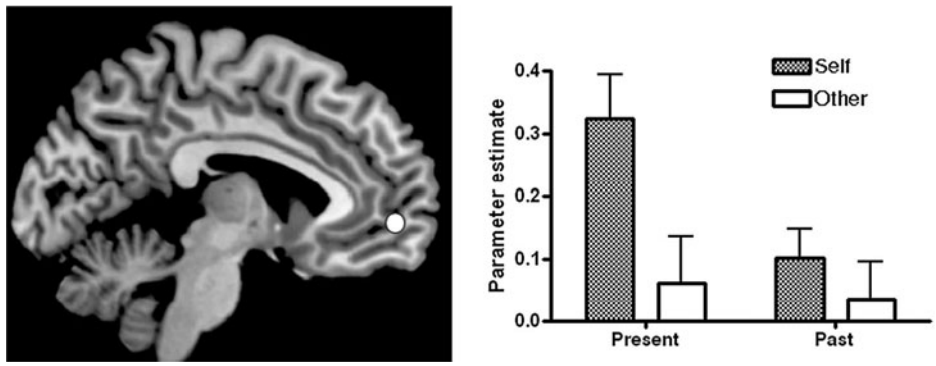

Figure 2. The effect of temporal perspective on MPFC activity when thinking about oneself and others. The left panel shows a region of MPFC in which activity was modulated by adopting different temporal perspectives on the self and others. As can be seen on the right panel, the MPFC was recruited to a greater extent when reflecting on the present self than when reflecting on the past self and when reflecting on the other person; thinking about the past self and thinking about the other person were associated with similar levels of activity. Adapted from reference 29.

of overlapping but different selves,[79] and research in social psychology has revealed that when people feel they have changed, they tend to distance themselves from psychologically remote former selves, which are then regarded as "other persons".[80-82] Someone may, for example, have a mental model of who she was five years ago (e.g., knowing that she was shy) but may no longer identify herself with that model. Although she can recognize that the person to whom the mental model refers to was herself, she no longer identifies that model as "me" (i.e., her current self) because she feels she has changed (e.g., she feels she is not shy anymore). We recently found that activity in the MPFC is sensitive to these effects of temporal perspective.[29] Specifically, we asked participants to reflect on their own psychological traits and those of an intimate other, for both the present life period (i.e., at college) and a past life period (i.e., high school years) that involved significant personal changes. The MPFC was commonly recruited by the four reflective tasks (reflecting on the present self, past self, present other and past other), relative to a control condition (making valence judgments), suggesting that this brain region may play a general role in reflecting on mental states and psychological characteristics.[83] Importantly, however, the degree of activity in MPFC also varied significantly according to the target of reflection, this region being recruited to a greater extent when reflecting on the present self than when reflecting on the past self and when reflecting on the other person; thinking about the past self and thinking about the other person were associated with similar levels of activity in the MPFC (see Fig. 2). This study thus demonstrates that increasing the psychological distance of self-representations leads to diminished activity in the MPFC, such that the degree of activity associated with thinking about oneself is similar to the degree of activity associated with thinking about others.

In a subsequent study,[84] we replicated and extended these findings, by showing that differences in MPFC activity when thinking about current versus temporally distant selves are symmetrical between the past and the future. Specifically, participants showed higher activity in the MPFC when making judgments about their present self than when making judgments about themselves five years ago or five years from now; there was no difference between past and future selves. Importantly, these findings could not simply

be accounted for in terms of task difficulty or memory retrieval, suggesting that the critical factor that underlies the effects of temporal perspective on MPFC activity relates to feelings of connectedness to self-representations. Research has further showed that individual differences in perceived connectedness to future selves and its neural signature (i.e., MPFC activity) hold important implications for decision making, such as choosing whether to save for the future or spend in the present.[85,86]

Other studies suggest that the degree of MPFC activity when thinking about others depends on the perceived similarity or degree of overlap between oneself and the other person under consideration. As we have seen, although judgments about oneself versus a nonclose other have been repeatedly associated with greater activation in MPFC, the studies that have directly compared judgments about the self versus a close other have provided mixed results (see the section on semantic forms of self-knowledge). These findings can be seen as supporting the view that the MPFC is not involved in self-referential processing per se but instead supports nonself-specific processes (e.g., familiarity processing, memory retrieval), which would be engaged to different extents when making judgments about oneself, close others and nonclose others.[27,74] The modulation of MPFC activity as a function of closeness with others can be interpreted differently, however. As already noted by William James,[1] people's identities include not only elements that are unambiguously part of them (e.g., their body and mental states) but also outer aspects of their lives, such as their family, friends and possessions. Extensive research has indeed shown that people treat the resources, perspectives and identities of close others as their own (think, for example, about how you react when a loved one is mistreated) and these effects depend on the degree to which the individual has included the other person in the self.[87] The fact that the magnitude of activation in the MPFC is more similar between the self and close others than between the self and nonclose others may thus reflect differences in degrees of inclusion of others in the sense of self.

Recent studies that have explored cultural differences in the neural correlates of self-referential processing are consistent with this interpretation. East Asian cultures promote collectivistic self-views more than Western cultures, emphasizing the interconnectedness rather than separateness between the self and close others.[88] Zhu et al measured brain activity using fMRI while Western and Chinese participants judged trait adjectives in reference to the self, their mother or a public person.[30] The results showed that the MPFC was more activated in the self condition than in the public person condition for both Western and Chinese participants. The comparison between mother and public figure differed between groups, however, with MPFC being more activated for the close versus public other in Chinese but not Western subjects. Furthermore, Western participants showed increased activations in the MPFC when thinking about the self versus their mother, whereas there was no difference between self and mother in Chinese participants. This study therefore demonstrates that differences in MPFC activity between self and a close other is modulated by cultural differences in the degree of inclusion of intimate others in the sense of self.

There is also evidence that inferring the mental states of unfamiliar individuals that are perceived as similar to the self engages the MPFC more than inferring the mental states of unfamiliar individuals that are perceived as dissimilar.[19,89,90] For example, Mitchell et al[90] had participants read descriptions of an unfamiliar individual whose social and political views were similar to their own views and descriptions of another unfamiliar individual whose social and political views were dissimilar. Then, during fMRI scanning, participants had to infer the opinions, likes and dislikes of these two target persons. The results showed that the

ventral MPFC was more engaged during judgments of the individual who was perceived as similar to the self than during judgments of the individual who was perceived as dissimilar. Furthermore, correlation analyses revealed that the more participants considered themselves similar to the "similar" other, the greater the difference in ventral MPFC activation during judgments of similar versus dissimilar other. These findings clearly demonstrate that the extent to which the MPFC is engaged when thinking about others depends on the degree of perceived similarity of the other person to oneself.

According to the view defended here, the MPFC may not be involved in making fixed and rigid self/nonself distinctions, but may instead mediate dynamic processes that locate information on a continuum of self-relevance or self-relatedness. The distinction between self and nonself may thus be a matter of degree and what is regarded as the "self" may vary across time and situations, depending on what information one identifies with at a given moment (i.e., what one includes in the currently activated self-concept).[10] This hypothesis predicts that, depending on contextual factors, the same information could be included in versus excluded from the current mental model of the self and this should be reflected in MPFC activity.

A recent study suggests that this is indeed the case, showing that priming cultural values of individualism versus collectivism in bicultural individuals induces increased activity in the MPFC for culturally congruent self-judgments.[91] Behavioral studies have shown that people of individualistic cultures tend to think about themselves using general self-descriptions (e.g., I am honest), whereas people from collectivistic cultures tend to think about themselves using more contextual self-descriptions (e.g., when talking to my mother, I am honest). Research has also shown that when primed to orient more toward either an individualistic or collectivistic schema, people will think about themselves in a way that is consistent with the cultural schema temporarily brought to mind. Chiao et al[91] investigated whether neural activity when making self-referential judgments would be influenced by such cultural priming. Participants were Asian-Americans living in the United States who identified themselves as bicultural as assessed by a questionnaire. They were asked to make judgments about general self-descriptions, contextual self-descriptions and a control task (judgments about font style). Before doing those tasks, half of the participants received priming procedures designed to activate individualistic cultural schemas (e.g., thinking about what make them different from their family and friends), whereas the other half received priming procedures designed to activate collectivistic cultural schemas (e.g., thinking about what they have in common with their family and friends). The authors found a significant interaction between priming conditions and types of self-judgments in the MPFC. Specifically, participants primed with individualism showed greater activation in the MPFC for general relative to contextual self-descriptions, whereas individuals primed with collectivism showed greater activation in the same regions for contextual relative to general self-descriptions. Furthermore, across all participants, the degree of cultural priming of individualistic or collectivistic values was associated with the degree of MPFC response to general or contextual self-descriptions, respectively. This study thus suggests that the response of the MPFC to a particular self-description depends on whether or not this information is congruent with the self-concept activated at a given moment.

In summary, recent functional neuroimaging studies have shown that a) the degree of activity in the MPFC when thinking about oneself diminishes when the psychological distance of self-representations increases, b) the degree of activity in the MPFC when thinking about others depends on the extent to which the other person is included in one's sense of self and c) the response of the MPFC to particular self-definitions depends

on whether or not these definitions are congruent with temporarily activated cultural values. Overall, these findings are consistent with the hypothesis that the MPFC may mediate dynamic processes that locate information on a continuum of self-relevance or self-relatedness. Information that is located at the upper end of this continuum may be incorporated in the mental model of the self that is displayed in our mind at a given moment, thereby being subjectively considered to be part of "me" or "mine".

CONCLUSION

Throughout evolution, hominids have developed greater capacity to think about the self in abstract and symbolic ways. This process has reached its apex in humans with the construction of a concept of oneself as a distinct entity with a personal history. Humans are able to consciously represent and reflect on their own personal attributes (e.g., their abilities, social roles, psychological characteristics and preferences) and frequently engage in mental time travels to mentally revisit their past experiences or imagine future ones. Recent studies that have used functional neuroimaging techniques to investigate the neural correlates of these self-referential processes point to the MPFC as a critical neural structure for processing both semantic and episodic forms of self-knowledge. A key function of this brain region may be to appraise and code the self-relatedness or self-relevance of information. Mental representations (e.g., traits, opinions, preferences, experiences) may be located on a continuum of self-relatedness, depending on the degree of activity they elicit in MPFC. Information that is located at the upper end of this continuum may be incorporated in the mental model of the self that is currently displayed in our mind, thereby being subjectively considered as "me" or "mine". The MPFC may thus implement dynamic neural processes that contribute to the division of the world into "me" and "not-me" that each of us subjectively experience. Recent studies that have examined the effects of cultural values and temporal perspectives on the neural correlates of self-referential processing are consistent with this proposal. The neuroscience of self-referential processing is still in its infancy, however, and additional investigations are needed to develop a full understanding of this aspect of human experience.

ACKNOWLEDGMENTS

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CHAPTER 19

HALLMARKS OF CONSCIOUSNESS

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Abstract: Consciousness, ranging from the primary, or perceptual, level to high levels that include a sense of self, can be identified in various organisms by a set of hallmarks that include behavioral, neural and phenomenal and/or informational. Behavioral hallmarks include those that indicate high cognitive abilities, such behavioral flexibility, verbal abilities, episodic memories, theory of mind, object constancy, transitive inference and multistability, all of which have been demonstrated in birds as well as in primates. Neural hallmarks include the thalamocortical model for mammals and similar circuitry in some nonmammalian taxa. Informational hallmarks include sensorimotor awareness, as provided by somatosensory and/or lateral line systems, which may form the basis for the sense of self and distinguishing self from nonself, as well as other sensory information, such as the richness and quantity of color and form information obtained by the visual system. The comparative method reveals a correlation of these different types of hallmarks with each other in their degree of development, which thus may be indicative of the level of consciousness present in a particular species.

INTRODUCTION

In this volume, a very wide range of systems that enable an organism to discriminate between self and nonself and to sense external signals has been discussed, ranging from bacterial sensory and communication systems to immune responses in metazoans to sensory systems and sociability in vertebrates and the self-recognition systems of primates. The topic of consciousness—particularly what it is, how we recognize it to be present and how it is generated—has only recently been recognized as a legitimate avenue of inquiry for neuroscience. This final chapter will consider how consciousness

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evolved, how it may be related to the perception of self versus nonself, and what its hallmarks are.

My own conjectures about consciousness focus on neural systems and do not include either nonneural biological systems of self and nonself recognition, such as immune responses, or the question of machine consciousness. Nor do I think that single-celled organisms are capable of consciousness, although some have many of the components of neurons. For example, the paramecium *Stentor* responds to a variety of sensory stimuli, but the response is limited to intracellular contractility that results in propulsion through the medium, and the stimuli themselves are not complex in their content.[1,2] Thus, my view is that consciousness is a biologically generated phenomenon that requires, at minimum, a population of cells that has the ability to detect sensory stimuli, such as light, sound, or chemicals, and to communicate among each other—i.e., a set of neurons within a multicellular organism.

Taking a comparative view of extant nervous systems allows us to reconstruct a lot of information about their evolutionary history. In this chapter, I will focus on the vertebrate radiation and, as a number of the chapters in this volume speak to, consciousness itself and the ability to distinguish self from nonself may both have arisen quite early and/or multiple times across cephalopod and arthropod invertebrates as well as in vertebrates.[3]

The many advances in understanding the functions of neurons at the molecular level have provided the basis for appreciating the role of ion channels and associated intracellular mechanisms in producing consciousness, along with corresponding insights into the neurotransmitter systems involved and molecular mechanisms for the action of anesthetics and of the sleep cycle that alter it.[4] From this work along with evidence from neurological diseases, trauma cases, and a broad range of experimental studies, we now know that it is the activity of neurons that generates all that we experience as consciousness.[3] What we do not know is how the neurons do it, and this question is at once both one of the most mystifying and challenging ones confronted by modern neuroscience as well as one of the most pivotal for understanding ourselves.

Defining Consciousness

Most discussions of consciousness begin with a definition of it, and I will do so here as well, because it seems that one is invariably challenged to do so. Defining it often evokes the argument that consciousness is subjective and thus cannot cross the bridge to objective analysis. Many recent papers have nonetheless provided solid and credible definitions, and among the most useful from the scientific standpoint are relatively brief and functionally grounded ones. For example, Tononi[5] states that "Consciousness is everything we experience... ." The use of the plural "we" is important, as it implies that, at least among neurologically normal humans, one's own experience of consciousness is either identical or very similar to that of other individuals. If this is so, the impediment of the "subjective" philosophical argument is removed. Perhaps even more simply, I have adopted the somewhat flippant definition that consciousness is everything you experience, which effectively dismisses the "subjective" objection altogether. Somewhat more specifically, I define consciousness as "the biological phenomenon of aware, subjective experience that is generated by the activity of neurons and includes present stimuli, thoughts and/or feelings or remembered, imagined, or anticipated ones."[6] Consciousness thus includes that which one experiences when fully awake and alert as well as during rapid eye movement sleep.

The hypothesis that the experience of consciousness is identical or highly similar across individuals is strongly supported by the argument of parsimony. A commonly used example of a conscious experience is that of the subjective visual experience evoked by looking at a stimulus that emits a wavelength of about 675 nanometers, i.e., within the range that we agree to refer to as the color red. Is your experience of seeing that color the same as mine, or is it significantly different? Let us consider the world human population, which is currently over six and one-half billion individuals. If we all have different experiences of "red," there thus would be the potential for more than six billion different experiences, which immediately strikes one as not credible, because it is highly unparsimonious. The parsimonious hypothesis, in contrast, is that our experiences of "red" are very much alike and, with more than six billion individuals experiencing it, essentially identical.

Levels of Consciousness and Cognition

Most definitions of consciousness include the specification of a number of levels, from "primary" or "perceptual," referring to the subjective perception of stimuli, to "higher-order," the latter including a sense of one's own self and the awareness of being aware.[7,8] I view these as all being on a continuum and suspect that once we understand how perceptual consciousness is generated, we will understand the entire range that it comprises. I use the term "higher-level" consciousness to encompass subjective perception and some cognitive functions but not necessarily including self-awareness or being aware that one is aware.[9]

Identifying the neural correlates of consciousness (NCC) is the focus of a number of studies but is based on the mammalian neural model that focuses on the thalamocortical circuit.[7,10,11] Since higher-level cognition is correlated with a high level of consciousness in humans, it is parsimonious to hypothesize that such a correlation holds for nonhuman species as well, as these abilities likely co-evolved.[12] Many cognitive functions (or their components) are unconscious, and consciousness, even well above elementary levels, cannot be ruled out where cognitive functions have not yet been observed. Nonetheless, the neural bases for consciousness and cognition may considerably overlap.[6] Thus, since birds exhibit high levels of cognition, as will be discussed below, comparison of avian and mammalian neural features can be used to test the thalamocortical circuit model.[6,13] If the model is accurate, most if not all of its neural features would be expected to be shared. The model can then be tested further by comparisons of neural circuitry in non-amniote taxa, particularly those that exhibit some level of cognitive behavior.

Hallmarks of Consciousness

In the quest to develop a more complete model for the neural basis for consciousness that comprises the features that are both necessary and sufficient for it,[5] we need to specify its hallmarks. Using the normal human model as a starting point, Edelman and Seth[8] discuss benchmarks of consciousness at the behavioral, neural and phenomenal levels, the latter including a sensorimotor scene, first-person perspective, and emotions. They explore the possibility of consciousness in other animals, including birds and cephalopod molluscs, using the behavioral and neural benchmarks.

In previous papers on this topic,[6,9,14] I have likewise focused on the behavioral and neural hallmarks of consciousness in a comparative perspective. In this chapter, I also

will consider the latter and an additional, third category, that of informational hallmarks. As discussed below, Tononi[5,15] has recently posited that consciousness is integrated information and thus the amount of sensory information available to an animal may be correlated with the level of consciousness. This informational category partially overlaps the phenomenal level discussed by Edelman and Seth[8] in terms of the sensorimotor scene and its relation to a first-person sense of self. We can use the comparative method to explore whether the behavioral, neural and informational hallmarks are consistent with each other, i.e., whether the degree of elaboration of the neural features is consistent with the amount of sensory information available to the animal and with the level of behavioral complexity and flexibility that can be observed. As Edelman and Seth[8] also have discussed, to the extent that these hallmarks are consistent with each other, their combined presence may be taken to infer consciousness and its approximate, proportional level.

BEHAVIORAL HALLMARKS

Besides mammals, birds appear to have the most elaborate cognitive behaviors and will thus be focused on here. As noted, though, some degree of cognitive ability may be present in non-amniote species as well and this generally correlates with higher brain-body ratios and elaboration of the forebrain. Rial et al[3] discuss a variety of behaviors that one can look for across animal taxa, including anticipatory behavior, such as increases in body temperature and heart rate in anticipation of a noxious stimulus; the capability to rank different sensations; detour behavior, a cognitive ability that involves reaching a goal by going around an obstacle, during which time sensory contact with the goal object is lost, indicating a working memory function; and play.

The Complex Behaviors of Birds

In addition to having many physiological/behavioral similarities to mammals, including homeothermy, habitual bipedalism, grasping ability, extended parental care of young, and similarities of sleep physiology, reviewed by Butler,[6] birds are highly cognitive. The pioneering work of Pepperberg[16] with African grey parrots and recent, new findings with members of the corvid (crow) family are examples of what might be referred to as "species-sensitive" paradigms—i.e., studies that are designed to be considerate of and take advantage of a given animal's natural behavioral repertoire in order to allow responses to experimental questions that the animal is capable of giving in the experimental context.

African gray parrots are outstanding in their cognitive abilities, being capable of acquiring a large verbal vocabulary, counting to at least the number seven, having a zero-like concept, understanding the concept of "same" versus "different," understanding relative concepts (bigger versus smaller), distinguishing shapes, colors, and materials, and being able to form categorical classes. Further, they exhibit many other cognitive abilities, including working memory, as evinced by a high level of Piagetian object constancy.[16] In humans, working memory involves prefrontal cortex (PFC)[17] and has been postulated by Baars[18] to require consciousness. As is clearly apparent from many of the various behavioral findings to date in birds, this posited association of working memory and consciousness is of crucial significance.

Higher-level cognitive abilities also have been demonstrated in other avian species. Working memory abilities in scrub jays,[19-21] which are corvids, include episodic memory (remembering past events and which event occurred before another) and theory of mind—the attribution of one's own mental state and future behavior to another individual.[19,22] Episodic memory also has been demonstrated in pigeons[23] and hummingbirds.[24] Transitive inference, another working memory task, which involves ranking something or oneself in relation to others through serial comparisons, has been demonstrated in pigeons, great tits,[25] and pinyon jays.[26] Likewise, multistability, in which the perception of an ambiguous figure is stable for several moments before switching to the alternative interpretation, has been demonstrated in pigeons.[27] Play behavior has been convincingly documented in ravens.[28] These and other higher-level cognitive and possibly conscious[18] abilities in birds rival and even exceed those of most mammals. For further discussion of avian cognition and its relationship to neural features, precluded here due to space limitations, the reader is referred to Butler et al,[14] Butler and Cotterill,[9] Emery,[21] Århem et al,[29] Butler[6,13] and Kirsch et al.[30]

The Seemingly Less Complex Behaviors of Reptiles and Amphibians

The literature on any behaviors of reptiles or amphibians that might be considered cognitive is very sparse. Studies of more basic behaviors have been done, such as a simple, visual discrimination task for turtles by Bass[31] and of territorial and other social behaviors in lizards by Greenberg.[32] Also, as Rial et al[3] discuss, hunting chameleons exhibit detour behavior and green iguanas have the capability to rank different sensory experiences, while amphibians are incapable of doing so. Also common to amniotes is play behavior,[3] which has been documented not only in birds[28] but also in reptiles.[33] However, no studies that even hint at more complex behaviors such as those discussed above for birds exist. While the behavioral repertoires of both reptiles and amphibians appear to be quite limited in general terms, the potential remains for future studies that are designed in a more species-sensitive way to yield some surprises. Crocodiles, for example, might be expected to have more in the way of cognitive capabilities than some other reptiles. As discussed below, a recent study reveals a surprisingly high level of cognitive ability in a species of fish, and similarly inventive experimental designs may do likewise in at least some species of reptiles.

New Insights into the Behavioral Abilities of Teleost Fishes

The work of Salas, Rodriguez, Broglio, and their colleagues has revealed roles for areas of the telencephalic pallium in goldfish in learning and spatial memory and in fear conditioning.[34] Although one could argue that such abilities do not necessarily require consciousness, working memory tasks do.[18] A recent study by Fernald and his colleagues[35] is intriguing in this regard. This study demonstrated that male cichlid fishes (*Astatotilapia burtoni*) are capable of determining their own rank in the social hierarchy by successively observing interactions between pairs of other fishes. This appears to be fully comparable to the behavior of birds who use transitive inference to infer their social ranks, as discussed above, an ability that requires working memory.

NEURAL HALLMARKS

Neural hallmarks of consciousness are both physiological and anatomical. The latter will be focused on here because, with only a few exceptions, most of the physiological data is limited to mammals. Some data for nonmammalian species on sleep states and their physiological correlates as well as on the physiological behavior of thalamic neurons will be noted in the context of the anatomical comparison.

Neural Comparisons across Amniotes

Forebrain evolution across vertebrates has been remarkably similar in terms of preservation of its components and remarkably diverse in the degree to which various components are elaborated, i.e., exhibit increased cell proliferation and/or migration of neuron cell bodies. Elaboration of both diencephalic and telencephalic (Fig. 1) cell groups has occurred independently multiple times within different vertebrate taxa, including some cartilaginous fishes, some bony fishes and, among tetrapods, particularly birds and mammals.[36,37] In comparison to mammals, birds also exhibit extensive forebrain elaboration, and they have the highest brain-body ratios of all nonmammalian vertebrate taxa.[38,39] Larger brain-body ratios have been linked to behavioral flexibility and longer survival in birds, such as shown by Sol et al.[40] The elaborated pallia of birds and mammals exhibit both similarities and differences in neural features, so comparison of them may help to formulate hypotheses about the neural bases for their shared behavioral abilities. These hypotheses can then be tested by examining other vertebrate taxa, because, in addition to birds, many cartilaginous fishes and some ray-finned fishes also exhibit markedly high brain-body ratios.

In mammals, as shown in Figure 2, the thalamocortical (i.e., thalamopallial) system, including reciprocal, glutamatergic, palliopallial and palliothalamopallial projections with involvement of local-circuit pallial GABAergic neurons and a GABAergic thalamic reticular nucleus (TRN), is the core of the NCC model.[7,10,11] Still not widely appreciated is that birds have remarkably similar circuitry, as reviewed by Butler and Cotterill[9] and Butler[13] and as shown in Figure 2. In the avian pallium, densely spiny glutamatergic pyramidal projection neurons are present[41,42] as is the other important component of mammalian palliopallial circuitry, intrinsic GABAergic neurons.[43-46] Likewise, glutamatergic thalamic nuclei[42] are involved with reciprocal projection loops to the pallium,[9,13] although the reciprocal projections to the thalamus are less extensive than they are in mammals. In mammals, the behavior of the cortically-projecting, dorsal thalamic neurons has been characterized: with depolarization of the cells, they generate tonic, repetitive, single-spiking behavior and, following sufficiently sustained hyperpolarization, a high-frequency burst of action potentials.[47,48] Perhaps not surprisingly due to the similarities in the thalamopallial circuitry, dorsal thalamic neurons in birds behave in the same way.[47,49]

Also similar between birds and mammals are the more diffuse pallial inputs from extrathalamic ascending systems, including serotoninergic inputs from the raphe nuclei, noradrenergic inputs from the locus coeruleus, dopaminergic inputs from the substantia nigra and ventral tegmental area, and cholinergic inputs from nucleus basalis, as well as the nonspecific thalamic inputs relayed from the reticular formation through intralaminar nuclei in mammals and their homologues in birds.[37,50,51] Further, higher-order association areas, included in the NCC concept, also are best developed in birds among nonmammals. An association region of the avian pallium, the nidopallium caudolaterale, compares to mammalian PFC for its involvement in working memory tasks and executive control of

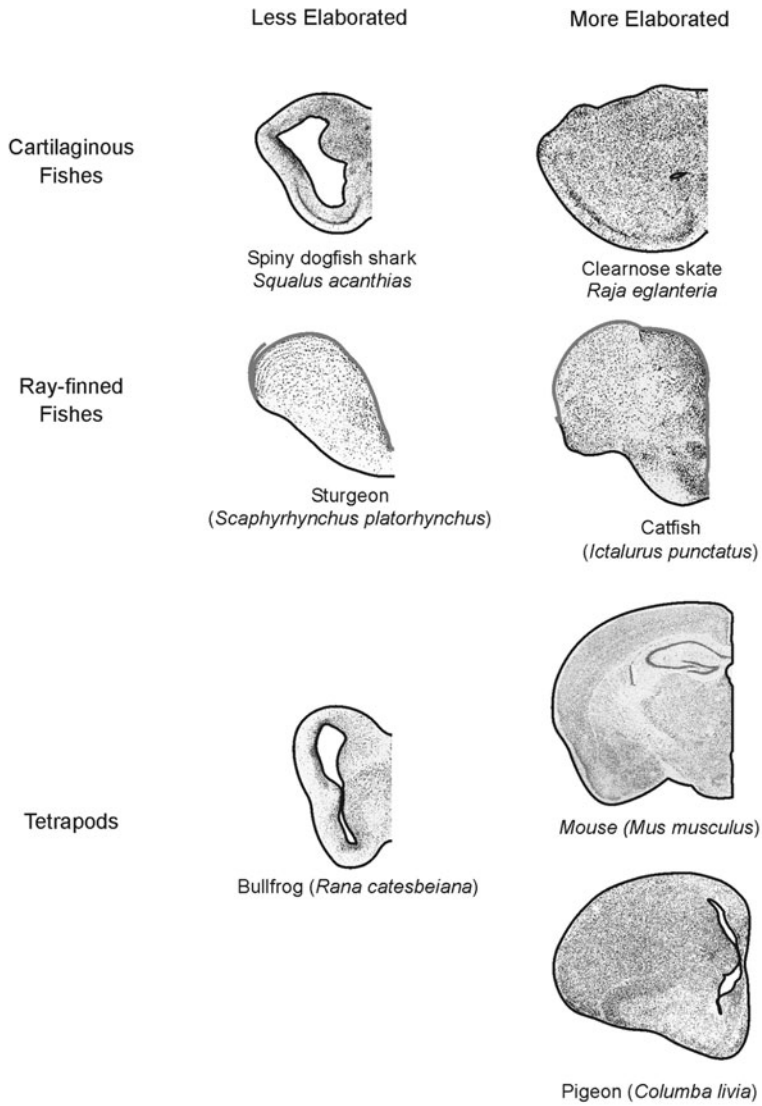

Figure 1. Nissl-stained transverse left hemisections through the telencephalon from different vertebrate taxa showing different degrees of elaboration.

behavior, and it also has hodological and electrophysiological similarities to PFC.[30,52] Likewise, encephalographic patterns during slow-wave sleep, rapid eye movement sleep and waking are similar in birds and mammals, even to the degree that both birds and mammals exhibit sleep homeostasis such that the level of slow-wave activity during slow-wave sleep is regulated according to the length of prior time spent awake or asleep.[53]

However, two major salient differences between the pallia of birds (and other reptiles) and mammals have long been thought to exist. The first is in terms of cytoarchitecture,

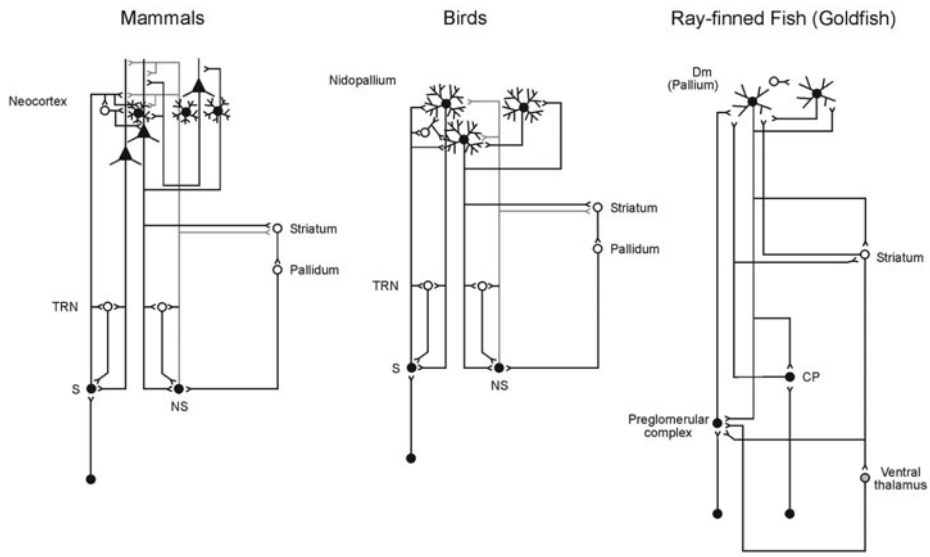

Figure 2. Comparison of thalamocortical circuitry in mammals with comparable circuitry in birds and, to a lesser extent, in a teleost fish. For mammals and birds, glutamatergic neurons (indicated in with filled black circles or triangles for cell bodies) in both specific (sensory relay) nuclei, S and nonspecific (intralaminar) nuclei, NS, of the dorsal thalamus are shown projecting to part of the pallium (neocortex in mammals and nidopallium in birds). (The axonal processes of the NS neurons are indicated by gray lines). In mammals, both glutamatergic pyramidal neurons (with triangular cell bodies) and the smaller, glutamatergic granule cells of layer IV (with smaller, circular cell bodies) are shown, whereas in birds, only the large, multipolar neurons are represented. All of these neurons have heavily spiny dendrites. In both mammals and birds, there are intrinsic GABAergic neurons (represented with open circles for cell bodies) present in the pallium, reciprocal glutamatergic projections from the pallium to the thalamus and glutamatergic collateral projections to the GABAergic neurons of the thalamic reticular nucleus, TRN, which projects back into the dorsal thalamic nuclei. Corticostriatopallidothalamic projections are also shown that involve the GABAergic neurons of the basal ganglia (striatum and pallidum). Ray-finned fishes have some but not all elements present in amniotes. In the diagram of circuitry in the goldfish, a teleost, the glutamatergic neurons of the central posterior nucleus, CP, of the dorsal thalamus are shown projecting to the pallium. Fish also have a more caudal set of diencephalic nuclei, the preglomerular nuclear complex, that relay information to the pallium, as shown. As in amniotes, intrinsic GABAergic neurons are present in the pallium. The glutamatergic neurons of the pallium project reciprocally back to CP but not directly to the preglomerular nuclear complex. The pallium also projects to the striatum, which projects to the ventral thalamus, but the neurotransmitter for the neurons of the latter, which project to the preglomerular nuclear complex has not been identified. Thus, whether fish have palliothalamic or palliopreglomerular circuitry that involves a TRN-like system is not known.

as shown in Figure 3, which compares a higher-magnification wedge of mammalian neocortex to the avian pallium. Although the part of the avian pallium, the hyperpallium, also known as the Wulst, is laminated, it is not laminated in the way that neocortex is. The distribution of the progenitor cells that produce the various cell populations of the Wulst is unlike that of neocortex and independently evolved.[54] Most of the avian pallium, including the mesopallium and nidopallium, does not exhibit any lamination of its neuronal populations.

The second salient difference has been held to be in the dendritic architecture of the mammalian pyramidal neurons, which have apical dendrites that are distinctively longer than the rest of the dendrites of the cell and that are oriented perpendicularly to

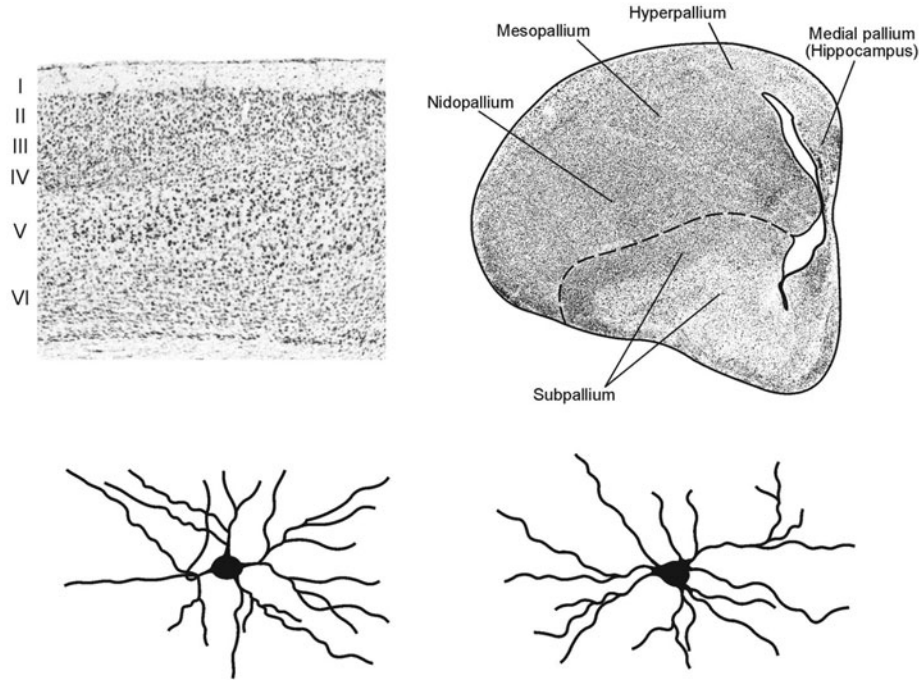

Figure 3. Nissl-stained photomicrograph on the upper left through the mouse neocortex from the atlas by Slotnick and Leonard,[97] with the layers indicated by Roman numerals and Nissl-stained photomicrograph on the upper right through the pigeon telencephalon. Three of the four main pallial regions—hyperpallium, mesopallium and nidopallium—are visible at this level, with the medial, hippocampal pallium also present. The dashed line indicates the pallial-subpallial boundary. Drawings of multipolar neurons are compared below, drawn from a published photomicrograph by Elston et al[56] showing the main branches of the basal dendritic arbor of a pyramidal cell from anterior cingulate cortex of macaque monkey on the lower left and from a published photomicrograph by Tömböl[41] of a multipolar cell from caudal mesopallium of chicken on the lower right. Dendrites of both these neurons are densely spiny, although the spines are not included here. The number of dendritic extrusions from the soma is variable for both avian and mammalian cells. From this perspective, the dendritic arbors of the mammalian pyramidal neuron and the avian multipolar neuron look remarkably similar.

the cortical layers. The pyramidal cell-type is not entirely unique to mammals, as the dorsal cortex of reptiles contains glutamatergic neurons that have similar, vertically oriented, apical dendrites; however, the reptilian cells are small to medium in size and not characteristic of most of the pallium. In most of the reptilian pallium and in the entire pallium in birds, the glutamatergic neurons have a multipolar dendritic array rather than a typically pyramidal one.[41,55]

The mammalian pyramidal neuron may be much more similar to the pallial neurons of reptiles and birds than previously appreciated, however. Recent work on pyramidal neuron morphology by Elston and his colleagues[56] has revealed that dendritic tree complexity and density of spines on these distinctively mammalian neurons tend to be correlated with higher-level association cortices and cognitive abilities. This work also allows a new perspective gained by viewing the basal dendrites of the pyramidal neurons from tangentially cut sections. While neocortical lamination is unique, the morphology of

the basal dendritic array of pyramidal cells is substantially more similar to the dendritic array of the large multipolar neurons of the avian pallium[41] than previously realized. In fact, the similarity of these two dendritic arbors is striking. Figure 3 shows drawings of pallial neurons, one a basilar view of a mammalian pyramidal cell and the other an avian pallial multipolar neuron. The avian neuron is from an association area adjacent to the nidopallium caudolaterale. Neurons in the latter region and other pallial areas are similar.[41] The only significant difference between the avian and mammalian glutamatergic pallial neurons thus appears to be the lack of an apical dendrite in the avian neuron. Given the high level of cognitive processing that some birds exhibit and, by correlation, the implied high level of consciousness, apical dendrites may be a neural feature that is not necessary to support these processes.

As noted above, large, densely spiny, multipolar neurons also are present in a large part of the pallium of reptiles, in a structure called the dorsal ventricular ridge,[55] which contains areas comparable to the mesopallium and nidopallium of birds. These neurons thus may have been present in stem amniotes and homologous to neocortical pyramidal neurons. Reptiles, however, lack some of the circuit features of avian and mammalian brains that involve these neurons, having a more modest TRN and most species lacking palliothalamic projections.[6,13] As discussed above, reptiles also appear to lack higher-level cognitive abilities and, unlike either birds or mammals, reptiles do not exhibit slow-wave sleep.[53] These large, densely spiny, multipolar neurons thus may be a necessary but not, in and of themselves, sufficient neural feature for consciousness.

From this perspective, one might now think of avian and reptilian pallial multipolar cells as being highly similar to mammalian pyramidal cells, both in terms of geometry, with the only exception being a single, longer dendrite in the latter and in terms of information-processing capabilities. Multiple inputs to the dendritic arrays of these cells may be extremely similar in their effects on the cell soma, for distinguishing inputs from different sources at different times and for recognizing and integrating inputs from multiple sources that arrive simultaneously. Such integrative properties, as part of the thalamopallial loop circuitry that is particularly well developed in birds and mammals,[13] could contribute to the binding phenomenon that is proposed to be at the root of conscious perceptions,[10,57] which thus could be a shared ability of both birds and mammals and possibly of some other relatively large-brained taxa as well.

This observation suggests the hypothesis that large, multipolar pallial neurons with densely spiny dendrites, but not necessarily apical dendrites, are one of the multiple neural features required for supporting mental functions for higher-level cognition and perhaps also for higher-level consciousness. This hypothesis is consistent with the findings of Elston and his coworkers[56] on the correlation of pyramidal cell complexity with higher-level association cortices and across primates ranked by cognitive ability. It supports the mammalian model of NCC[7,10] with only the exceptions being the apical dendrite of the pyramidal cells and their layered array of inputs. It will be testable by future studies of neural features of birds and other vertebrate groups and of behavioral abilities across additional taxa, including reptiles, designed in species-sensitive ways.

Testing the Amniote Model

Thalamopallial circuitry like that present in birds and mammals is only partially present in ray-finned fishes, such as the goldfish, *Carassius auratus*, which is a teleost (Fig. 3). The dorsal thalamus of ray-finned fishes is very small in comparison to that of amniotes.[37]

It contains only three nuclei, of which the rostral-most one, nucleus anterior, receives retinal projections while the other two, the central posterior and dorsal posterior nuclei, receive their predominant input from the midbrain roof. The latter two nuclei project to the pallium, but a more caudal part of the diencephalon, the preglomerular nuclear complex, which also receives midbrain roof inputs, serves as the major sensory system relay to the pallium.[58,59] Both the central posterior nucleus and the preglomerular nuclear complex receive reciprocal projections from the pallium,[58,59] but these projections are quite sparse in comparison to the palliodiencephalic projections in amniotes. Ray-finned fishes have intrinsic, GABAergic neurons within the pallium, but whether or not they have a thalamic reticular nucleus-like pathway that provides GABAergic input to the dorsal thalamus and/or preglomerular nuclear complex remains to be determined. Intriguingly, however, electrophysiological recordings from the dorsal thalamus in goldfish revealed the same behaviors as observed in mammalian and avian dorsal thalamic neurons, noted above.[47]

The cytoarchitecture of the pallium in teleosts resembles the avian condition in that it is not laminated-like mammalian neocortex, but it gives the impression of being less well developed than the nidopallial and related areas of the avian pallium that receive ascending thalamic inputs and/or palliopallial association connections. The teleost pallial neurons have spiny dendritic trees. A quantitative comparison of them with the pallial neurons in amniotes has not been made, but from Golgi tracings[60] in the teleost, *Sebastiscus marmoratus*, they appear to be somewhat less branched and somewhat less spiny than their amniote counterparts.

Thus, the basis elements of the amniote thalamopallial model appear to be represented in the teleost forebrain but in a less elaborate version. Diencephalic sensory relay nuclei with projections to the pallium, multipolar pallial neurons with at least moderately spiny dendrites, associational palliopallial connections, intrinsic pallial GABAergic neurons, and reciprocal pallial glutamatergic projections to the diencephalon are present. The electrophysiological behavior of the diencephalic neurons resembles that in amniotes and this version of the thalamopallial (and preglomerulopallial) circuit is sufficient to support hippocampal-like and amygdala-like learning functions as well as the apparently more cognitive behavior of transitive inference, as discussed above.

INFORMATIONAL HALLMARKS

Tononi[5,15] recently has argued in his Integrated Information Theory (IIT), that "consciousness is integrated information," i.e., a mechanism for being able to access large amounts of information in a single instant and thus to experience "an integrated whole." It would seem that identity of consciousness with integrated information errs on the side of over-simplifying consciousness, for although it makes intuitive sense that consciousness involves integrated information, this equation does not illuminate how the subjective experience of consciousness is produced: that is, although we know that consciousness, as we experience it, is produced by neural activity, particularly by the fluxes of ions across a membrane, we do not yet know how it is produced by these processes. The latter issue is also an impediment to postulating that a nonbiological machine could be conscious, as Tononi[5,15] does "to the extent that [the machine] is capable of generating integrated information." Considering a simpler but similar biological action illuminates the problem: although we know that both consciousness and muscle contractions are the result of biological processes and, further, that the muscle contractions are produced by

actin and myosin myofilaments sliding along each other, no computer yet designed can produce a muscle-like contraction. It is thus specious to suppose that any nonbiological system, such as a computer, that is incapable of doing something as simple as producing a muscle-like contraction could nevertheless produce consciousness.

Nonetheless, the posited relationship of consciousness to integrated information of Tononi's[5,15] IIT allows us to form and test hypotheses for multicellular organisms. A productive line of inquiry may be developed from observations of the perceptive capabilities of sensory systems in a variety of species. Sensory capabilities vary markedly across vertebrates and invertebrates alike and determine the amount of sensory information available to the organism for potential analysis and integration.

The idea that consciousness involves integrated information implies a range that is bounded: (1) on the low end such that the level of consciousness possible in a given organism will be limited by the quantity and quality of information that its sensory systems provide, no matter how well it can integrate that information and (2) on the high end such that the level of consciousness present in another organism that is provided with greater quantity and quality of sensory information and is able to integrate that information will be correspondingly greater. In other words, the level of consciousness in a given species should be proportional to the quantity and quality of sensory information available to it and the degree to which it can integrate that information. This idea is fully consistent with the idea that consciousness evolved in relation to sensation, as Rial et al[3] discuss and see Århem et al[29] and Baars.[61] The quantity of sensory information as posited here would include, for example, the number of sensory receptors in proportion to body area for the somatosensory system or to the size of the visual field for the visual system; the quality of sensory information would include the number of different sensations, such as fine versus deep touch, pain, temperature discrimination, and so on for the somatosensory system or color and fine form discriminations for the visual system. One would thus expect species that have the better developed sensory peripheries, i.e., greater information-capturing capacities, to also have a greater degree of elaboration of the brain in terms of internal complexity and brain-body ratios, as well as more flexible and elaborate cognitive abilities, because the advantages afforded by increased sensory information could only be selected for if they conferred increased evolutionary fitness. Being able to integrate the sensory information would be an advantage to the organism and selected for in this way.

Information and the Visual System: Early Evolution of Consciousness?

Available information on the evolution of color and form vision is consistent with this view. As briefly surveyed here, such visual system enhancements tend to correlate with larger brain-body ratios. Of course, other sensory system enhancements, such as of the olfactory system in hagfish,[62] also correlate with higher brain-body ratios, as reviewed by Nieuwenhuys,[51] and in most taxa, enhancements of multiple sensory systems have occurred. As Nieuwenhuys et al[51] note in regard to teleosts, those "with more than one highly developed sensory system often have proportionally large brains." An examination of the visual system in this context illustrates this relationship between the enhanced sensory periphery and brain elaboration, the latter providing the substrate for integrating the information in an adaptively advantageous manner.

Color vision and image-enhancing adaptations, such as increased retinal ganglion cell density and multifocal optical systems, have been gained and/or retained multiple

times across both invertebrate and vertebrate taxa and to varying degrees. Color vision derives from having multiple opsins with differing spectral sensitivities and, in some taxa, a variety of additional filtering devises, such as colored oil droplets in the receptor cells that filter the light before it even reaches the opsin pigment.[63] Although most mammals are dichromatic, having two cone opsins, the catarrhine primates, which include Old World monkeys, apes, and humans, are trichromatic.[64,65] The number of opsins varies considerably across vertebrates, however, with the catarrhine primates having an intermediate number in comparison to other clades.

Color vision arose very early in vertebrate history.[63,66-69] Among cyclostomes (jawless vertebrates), hagfishes lack image-forming eyes, having no lens, and the eyes lie beneath an opaque epithelial sheet.[63] Hagfishes have at least two types of retinal receptor cells, distinguished by morphologically different outer segments and at least one opsin,[70] but the type of opsin has not been identified. In contrast, lampreys have well-formed eyes with lenses and multiple opsins, allowing for color vision. Northern hemisphere lampreys (*Petromyzon* and *Lampetra*) have only two opsins and thus dichromatic vision at best. In contrast, the Southern hemisphere lamprey *Geotria australis* has five opsins, allowing for pentachromatic vision.[63,68,71]

Since lampreys also have multifocal lenses, as revealed by photoretinoscopy,[71] their color vision is also well-focused. The combination of pentachromacy and a multifocal lens in *G. australis* allows for a substantially greater amount of visual information to be transmitted centrally to the rest of the brain, predicting a higher brain-body ratio for *G. australis* than for Northern hemisphere lampreys. Information on forebrain anatomy is available for Northern hemisphere lampreys,[62] but thalamopallial circuitry such as that present in ray-finned fishes has not been identified. Brain-body data[72-74] place Northern hemisphere lampreys at the lowest range for all vertebrates, at the lower end of the cyclostome range and with all cyclostomes falling well below the other vertebrate taxa. Neither anatomical nor brain-body data for *G. australis* are yet available, but, based on their substantially more elaborate visual capabilities, one would predict the forebrain to be more elaborate in its cytoarchitecture and circuitry and the brain-body ratio for *G. australis* to be significantly higher than for *Lampetra* and *Petromyzon*. As argued for above, for the increased sensory information to be of adaptive value, it would have to be integrated to some extent and, as per Tononi's[5,15] IIT, would indicate the presence of at least visual sensory consciousness in this species. If the basic anatomical elements of the thalamocortical circuit of mammals and comparable thalamopallial circuitry of birds and their physiological behaviors, as discussed above, are necessary for the production of consciousness, these elements and their behavioral characteristics should thus also be identifiable in the forebrain of *G. australis*. Future experimental investigations of this species may be able to answer this question.

Among jawed fishes, as Bowmaker[67] and Collin et al[63] discuss, some cartilaginous fishes have three cone opsins, allowing for trichromatic vision, while others have fewer. As noted above, the brain-body ratios of cartilaginous fishes almost completely overlap those of amniotes and, like ray-finned fishes, they have multiple ascending sensory systems that reach the pallium via the dorsal thalamus and preglomerular nuclear complex (or its homologue), as well as palliopallial association connections.[37,51] Other details of the thalamopallial circuitry in cartilaginous fishes, such as whether reciprocal palliothalamic (or palliopreglomerular) projections and/or a thalamic reticular nucleus are present, are not known, however.

Within teleosts, multiple opsin genes are present and the opsin pigments in the photoreceptor cells tend to vary in correlation with the most abundant wavelengths in the species's habitat; in the black bream, *Acanthopagrus butcheri*, this correlation has been shown to occur because of regulation of opsin gene expression by the environmental light.[75] Opsin genes duplications have occurred in various taxa, including in some species of cichlids, which have up to seven cone opsin genes. In any one species, only three tend to be expressed, however, allowing for trichromatic vision.[76,77] Among teleosts, cichlids are among those with highly elaborated telencephalons.[78]

Within the sarcopterygian radiation that gave rise to land vertebrates, the Australian lungfish, *Neoceratodus forsteri*, has four cone opsins and may thus have tetrachromatic vision.[79,80] However, *N. forsteri* has low spatial resolving power[79,81,82] and thus relatively poor form vision. In line with this level of visual function, the Australian lungfish is more active during the dark phase of the diurnal cyclc,[83] the telencephalon of all three genera of lungfishes is relatively modest in its development,[51,84] and their brain-body ratios are about the same as for amphibians—overlapping part of the range for bony fishes and well below the ranges for cartilaginous fishes and amniotes.[37,51]

In the amniote radiation, catarrhine primates have well-developed trichromatic vision, as noted above. Birds stand out among amniotes, however, as they have tetrachromatic or even pentachromatic vision.[67,85,86] Some birds also have exceptionally good spatial resolution,[87] so their visual capabilities are highly developed. Correspondingly, brain-body ratios in birds range up to very high levels, overlapping the lower range for primates.[37,38,51]

From this sampling of visual system adaptations across the vertebrate taxa, the relationship between the degree of development of the visual periphery for information-capture and elaboration of the rest of the brain is clearly a positive one in most cases. The brain-body ratio of the Southern hemisphere lamprey, *G. australis*, will be of interest in this regard, as it is predicted to be higher than that for the Northern hemisphere lamprey genera.

Information and the Somatosensory System: Evolution of the Sense of Self

The aspect of consciousness that involves having a sense of self is often claimed as one of the few characteristics that distinguishes hominids—humans and great apes—from other animals. As Griffin[88] has discussed, however, many animals are probably aware of their own bodies and have an understanding that when they run or climb or chase something, they are doing those things. Integrated information from the somatosensory system for amniotes and the lateral line system for fishes may provide for this type of self-perception, which would be consistent with the IIT proposed by Tononi.[5,15] It would seem that the use of the word "I" by humans, allowed for by our language capability, is simply a label for that basic body awareness and does not rise to the level of a qualitative difference.

Various animal behaviors are consistent with having awareness of self. Griffin[88] recounts that grizzly bears "try to avoid leaving tracks, indicating that they realize that their tracks may be followed by hunters." Scrub jays, which are members of the corvid family as discussed above, try to conceal their bodies behind leaves when observing another jay who is caching food, and the jays who are in the act of caching first look around at their surroundings to see whether another jay is watching them. Studies on scrub jays,[19,22] as noted above, have strongly indicated that these jays have an understanding of their own selves in terms of both being observed and trying to be covert when observing others.

Griffin[88] also discusses the mirror self-recognition (MSR) paradigm, which is alleged to be a legitimate test for a sense of self. He notes the findings that most animals react to their mirror image as if it were another animal or fail to pay attention to it at all. The MSR test is not a "species-sensitive" paradigm for most nonprimates, however. For the mirror studies, the responses of hominid primates that entail using their limbs to reach for parts of their bodies, supposedly indicating a sense of self-awareness, are part of their natural repertoire. It is not natural, however, for a cat or dog, for example, to use their paws for exploratory behavior of either their selves or of external entities. They are much more likely to use olfactory cues for exploration or for greeting or other social behaviors.

With understanding of these constraints and using inventive, more species-sensitive experimental designs, several recent studies have demonstrated mirror self-recognition in several diverse taxa—in dolphins by Reiss and Marino,[89] in elephants by Plotnick et al[90,91] and in magpies by Prior et al.[92] Plotnick et al[90] noted that for the mammalian species of apes, dolphins, and elephants, this level of cognitive ability is most likely related to complex sociality and co-operation, which also are characteristic for many species of birds. Among mammals, both dolphins and elephants have large, complex brains,[93,94] and among birds, magpies are members of the corvid family and have relatively large brain-body ratios.[38] Thus, as better experimental designs are developed, we may be able to identify evidence for the sense of self in additional species. As addressed throughout this book, the sensory information that is a prerequisite for self-recognition is widely available across the animal kingdom.

CONCLUSION

As Griffin[88] discusses, scientific consideration of the subjective experiences of nonhuman animals has long been considered taboo. Fortunately, more and more attention is now being focused on this subject. The hazard of anthropomorphism also has long been thrown in the way of these inquiries, but even this unavoidable tendency is now being addressed and appropriately dealt with, as discussed by Mitchell et al.[95] In this regard, it is important to turn back to Lloyd Morgan's[96] canon, written in 1900, which has long been quoted out of context and thereby extensively misinterpreted and misused. Lloyd Morgan wrote (p. 209) that "we should not interpret animal behaviour as the outcome of higher mental processes, if it can be fairly explained as due to the operation of those which stand lower in the psychological scale of development." By itself, this statement has been interpreted as an inviolable interdiction against any anthropomorphic interpretations of the behavior of nonhuman animals, leading to an isolationist view of human behavior and the conceit that the human "mind" is unique. In the very next sentence, however, Lloyd Morgan went on to state: "To this it may be added—lest the range of the principle be misunderstood—that the canon by no means excludes the interpretation of a particular act as the outcome of the higher mental processes, if we already have independent evidence of their occurrence in the agent." In other words, it is legitimate to draw on scientifically rigorous, objective evidence to establish that some nonhuman species do use higher mental processes. The spate of recent studies on a variety of mammalian and avian species does just that and strongly supports the common sense, reasoned approach taken by Griffin and others.[88,95] Taken with the qualification that Lloyd Morgan[96] added to his canon, these studies usher in a new era of research that will hopefully soon overcome the long impeded progress in understanding animals' minds and thus the phenomenon of consciousness itself.

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