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The olfactory system: the remote-sensing arm of the immune system

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Review

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Abstract

Odors may be pleasant or unpleasant and in practice, pleasant odors are attractive while unpleasant odors are repellent. However, an odor that is noxious to one species may be attractive to another. Plants, predators, and pathogens may enhance their transmission by manipulating these signals. This may be especially significant when odors attract arthropod disease vectors. Odor detection may also be important in small prey species for evasion of macropredators such as large carnivores. Conversely, pleasant odors may identify family members, parents, or sexual partners. They may also generate signals of good health or fitness and contribute to the process of mate selection. In this review, we seek to integrate these odor-driven processes into a coherent pattern of behaviors that serve to complement the innate and adaptive immune systems. It may be considered the 'behavioral immune system'.

Introduction

The antimicrobial defenses of animals are mainly the responsibility of the innate and adaptive immune systems. Both systems are activated once a pathogen enters the body. However, it has long been recognized that prevention is better than a cure. Thus, animals adopt behaviors optimized to avoid infection by avoiding others with obvious sickness. Such avoidance is not only mediated by visual and behavioral cues but also by the smell of sickness and infection. These cues collectively trigger activities that can be considered components of the behavioral immune system. As a result, animals detect, recognize, and avoid sick animals of their own species (Prugnolle *et al.*, 2009; Ferdenzi *et al.*, 2017; Murray *et al.*, 2019; Shakhar, 2019). One component of the behavioral immune system is the use of olfaction. Among its many other functions in relation to food seeking, mate selection, and hazard detection, the olfactory system also serves as a remote-sensing arm of the immune system and is a key constituent of behavioral immunity.

The olfactory system detects odors in inhaled air. It serves to detect potential food sources from both plants and other animals. Importantly, it detects pheromones from other members of the same species and thus plays a key role in mate selection and it can remotely detect potential hazards. The detection of smells is associated not only with the induction of appropriate behavioral responses but also plays a key role in both memory and emotion. As with vision, olfactory perception is built from a mosaic of signals derived from an enormous diversity of odorant receptors. These signals are integrated in such a way that the brain can obtain an overall view of the olfactory environment and respond accordingly. It has been claimed that even in an olfactory 'weak' species such as humans, the system outperforms the other senses in its discriminatory powers (Sarafoleanu *et al.*, 2009).

In human medicine, unpleasant smells were long considered a warning of health risks. For example, they warned of spoiled food or the presence of feces. The Greek physician Hippocrates around 400 BCE identified alterations in body odors as a sign of disease (Shirasu and Touhara, 2011). This concept was developed to the extent that that bad smells were believed to actually cause diseases. This 'miasma' theory was accepted by many societies, including not only Europe but also China and India. Miasmas were considered to originate from several sources, most importantly, the smell of rotting marsh vegetation and the smell of decomposing bodies.

Major pandemics such as the Black Death in the middle of the 14th century did nothing to discourage the miasma theory. Although European towns in the Middle Ages were smellier places than modern cities (or at least smelt different), contemporaries emphasized the powerful smell of plague victims. The purulent contents of ruptured buboes had a very strong odor (Tainmont, 2009). To this was added the stench of decaying bodies. It is easy to see how these smells could be equated with contagion and regarded as a health hazard. People therefore avoided the breath of plague victims. In the yellow fever outbreak in Philadelphia in 1793, Dr Benjamin Rush attributed the disease to the smell of a mound of decomposing coffee

beans in the harbor (Eisenberg, 2007). Miasma was believed by many to be the source of cholera in London in the 1850s (Bergman, 2013).

Recent studies have however confirmed that many diseases, both infectious and noninfectious may cause the release of volatile organic compounds with a characteristic odor (Shirasu and Touhara, 2011; Trivedi *et al.*, 2019). Olfactory cues may not only trigger avoidance of the sick or unhealthy, their absence may also signal good health. Therefore, the sense of olfaction is linked to the defenses of the body and olfactory signals are integrated into an animal's immune defenses. This review examines this integration and the evolutionary importance of disease avoidance through odor sensing.

Nervous and immune system interactions

The nervous and immune systems are intimately connected. Not only do they communicate directly through parasympathetic and sympathetic nerves, but the immune system is also regulated by soluble neurotransmitters. These neurotransmitters act on lymphocytes to regulate cytokine production (Chu *et al.*, 2020; Cohen *et al.*, 2020). Conversely, cytokines and chemokines modulate nervous system activities such as appetite, body temperature, and sleep behaviors (Tizard, 2008). In addition, inflammation activates sensory nerves that then relay messages to the brain. These may trigger systemic responses such as sickness behavior or very local tissue responses such as pain and itch (Trivedi *et al.*, 2019).

As a result, the immune system is affected by neural stimuli before and during exposure to pathogens. Prior to infection, as described in this review, the olfactory and visual systems provide the body with an early warning that influences an animal's behavior. Similarly, stress in its various forms influences immunity. Small bouts of stress are believed to enhance immune responses, but prolonged stress is detrimental. Stress may result from mammalian social structures and there is a relationship between social status and disease susceptibility. High levels of social stress are found in confined, crowded populations, and these tend to be the most susceptible to microbial attack. The stress effect on immunity is mediated by two major pathways. One involves the autonomic nervous system acting through adrenaline, noradrenaline, and acetylcholine, and the other involves the hypothalamic–pituitary–adrenal cortical axis acting through glucocorticoid release (Cain and Cidlowski, 2017).

Once an animal is infected, sickness behavior including fever, inappetence, and somnolence are mediated through signals emanating from the brain (Tizard, 2008). The avoidance of such sick individuals can be considered to be a component of the behavioral immune system. Neuropeptides such as the enkephalins and endorphins can influence lymphocyte activity. Many neuropeptides have a similar structure to the antimicrobial peptides so that they also have antimicrobial properties and may be involved in host defense (Chu *et al.*, 2020).

Conversely, the immune system influences nervous function. For example, several cytokines and other inflammatory mediators induce 'sickness behavior', including fever, fatigue, depressed activity, and excessive sleep (Tizard, 2008). All these are closely associated with the systemic response to infectious agents and chronic inflammation.

Both the innate and adaptive immune systems respond to environmental signals. When the body is invaded by pathogens, the invaders are detected by pattern recognition receptors binding either characteristic microbial structures or by products released by damaged cells or tissues – alarmins (Kapurniotu *et al.*, 2019). The body, however, need not wait for invasion to occur. Host immunity is also modulated by volatile metabolites released by the commensal microbiota on the skin, respiratory tract, or intestine. Immune responses may either be stimulated or suppressed, depending on the nature of these microbial signals (Martin and Liras, 2019). This ability of the immune system to sense invasion, danger, and potential damage may extend far beyond the body by sensing volatile molecules acting through the olfactory system. Detection and avoidance of microbial pathogens prior to contact with sick individuals clearly offers a considerable evolutionary advantage (Kipnis, 2016).

The importance of olfaction is relatively obvious when a dead animal decomposes and the odors attract scavengers and insects while repelling others. Olfaction is also essential when an animal detects the smell of predators or of fresh blood. More subtle still, are the smells of stress, fear, or illness. Such smells can identify the sick and dying, but may also be a smell of health. The best example of this is positive mate selection based on odors associated with an individual's major histocompatibility complex (MHC) haplotype (Santos *et al.*, 2016).

The role of the microbiota

Animals emit a complex mixture of volatile organic compounds. Many of these smells originate from the microbiota, primarily by its actions on the skin, exhalation from the upper respiratory tract and the oral cavity, as well as in feces and flatus (Lize *et al.*, 2013).

Commensal bacteria also play a key role in the generation of odors from anal/scent glands and the vagina, as well as from human sebaceous and axillary apocrine glands. Antibiotic treatments that alter the intestinal microbiome may inhibit the synthesis of these odorants (Brown, 1995) and the mixture of volatile organic compounds in the odors of germ-free rats differs from the odors of conventional rats. When the above study was repeated with MHC congenic and dissimilar rats, the germ-free mice had greater difficulty discriminating between MHC congenic rats suggesting that the microbiome contributed to this discrimination. This was supported by the observation that MHC discrimination was not apparent in germ-free mice (Brown, 1995).

The MHC may affect the composition of the microbiota by directly influencing colonization or by determining the nature of early immune responses to the colonizing microorganisms. Should the intestinal microbiota be significantly disturbed by an enteric infection such as colibacillosis or cholera then the odors generated within the intestinal tract may also be significantly altered. A similar effect may result from persistent changes in diet, such as the effects of weaning.

Olfactory receptors

Mammals can detect and discriminate an enormous number of volatile substances and pheromones. This is accomplished through receptors located on sensory neurons in four chemosensory organs; the main olfactory epithelium (MOE), the vomeronasal organ (VNO), the septal organ, and the Gruenberg ganglion (Schmid *et al.*, 2010; Brechbuhl *et al.*, 2013). Odor detection in mammals is mediated by hundreds of different receptors classified into five families (Fleischer *et al.*, 2009). These are odorant receptors (ORs), VNO receptors (V1Rs and V2Rs), trace

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amine-associated receptors (TAARs), formyl peptide receptors (FPRs), and membrane-bound guanylyl cyclase (GC-D) receptors (Nilsson *et al.*, 2014). For example, individual sensory neurons in the MOE may express any of several hundred ORs, 15 TAARs, or GC-D receptors. VNO neurons express V1Rs or V2Rs and FPRs (Nilsson *et al.*, 2014).

Odorant receptors

ORs belong to the seven transmembrane receptor protein superfamily (Fleischer *et al.*, 2009). They are predominantly but not exclusively expressed on the surface of olfactory neurons. Their N-termini are exposed on the extracellular face of the cell membrane while their C-terminus is intracellular. On ligand binding, the membrane spanning domains alter their conformation, guanosine diphosphate (GDP) is converted to guanosine triphosphate (GTP) and their signal transduction pathway is activated. The signals generated are transmitted to the olfactory lobe and hence to the hypothalamus where they are integrated with other olfactory signals to influence behavior (Fleischer *et al.*, 2009).

ORs are the most abundant gene family in vertebrates and account for about 5% of the mammalian genome (Mombaerts, 2004). These OR genes lack introns and average ~1 kb in size. They are dispersed in about 55 clusters throughout the genome and are found in almost all chromosomes. As expected, OR genes are more plentiful in species with a well-developed sense of smell such as dogs and cats that have about 1500, than in primates that have about 800. Humans have 387, rats have 1284, and mice have 1194 OR genes.

OR proteins can be divided into class I receptors that detect water-borne odorants and class II receptors that bind volatile odorants. Class I receptor genes consist of four gene families while there are nine class II receptor gene families (Hughes *et al.*, 2018).

Significantly, two OR gene clusters are located on the short arm of human chromosome 6, closely associated with the MHC. This close physical association suggests these OR genes may be linked to MHC-associated behavioral traits, such as olfaction-influenced mate selection. About 50 novel OR loci (36 human, 14 murine) are located within the MHC-associated clusters. Duplications of blocks of OR genes are clearly evident and the entire OR cluster has a genomic environment very different from its neighboring regions. Extensive long-distance splicing in the 5′-untranslated regions and alternative splicing within the single coding exon of ORs have been detected (Younger *et al.*, 2001). Abundant allelic variation appears to be a general feature of human OR genes (Ehlers *et al.*, 2000). Thirteen novel OR variants have been found through direct DNA sequencing and cloning, and there may be as many as 21 OR cluster haplotypes. Two loci belonging to the telomeric cluster (OR2B8P and OR1F12) occur as either functional or non-functional alleles (Santos *et al.*, 2010*b*). This genetic variation across the olfactory receptor repertoire has been shown to affect odor perception in humans. Variation in a single human OR gene may be associated with reduced scent perception (Kacza *et al.*, 2001; Trimmer *et al.*, 2019).

Vomeronasal organ receptors

The VNO is present in the nasal cavity of amphibians and many mammals but absent in cetaceans, some bats, and some primates. The VNO is vestigial in humans (Perez-Gomez *et al.*, 2014). In mice, the VNO consists of two overlapping layers of cells (apical and basal) that express two different receptor families V1R and V2R, respectively. A subset of VNO neurons express FPRs, four of these are apical and one basal.

V1Rs are G-proteins that recognize volatile molecules such as pheromones (Wyatt, 2017) and sulfated steroids (Ihara *et al.*, 2013). V1Rs bind ligands in a manner similar to the ORs but are more narrowly tuned and have fewer activating ligands. V1R genes contain only a single exon and are clustered-like ORs. Rodents and marsupials have 100–300 members of the V1R gene family. The platypus has more than 800. Humans and dogs have only 5–8 functional V1R genes whose functions are unclear.

V2Rs are also G-protein-linked but unlike other olfactory receptors, V2Rs are encoded by multiple exons and form heterodimers with a partner chain of another V2R or possibly an MHC class Id molecule. A subset of mouse vomeronasal neurons co-expresses their V2R genes with H2-Mv genes, a family of nine non-classical class I MHC genes. The MHC molecule may be required to escort the V2R to the neuronal plasma membrane. Mice that lack H2-Mv have no major anatomical defects in their VNO. Their neurons can be stimulated with peptides and proteins to the same extent as neurons from wild-type mice, but require much higher ligand concentrations. Consistent with their greatly decreased vomeronasal sensory neuron (VSN) sensitivity, ΔH2Mv mice display pronounced deficits in aggressive and sexual behaviors. H2-Mv genes are not absolutely essential for the generation of physiological responses, but are required for ultrasensitive detection by a subset of these neurons (Leinders-Zufall *et al.*, 2014). Most V2R ligands are peptides including pheromones and MHC class I peptides; the same small peptides presented by MHC I and II molecules to antigen-sensitive T cells. The V2R repertoire in rodents contains about 200 genes, a large proportion of which are pseudogenes. In the dog and bovine, the V2R repertoire is completely degenerate and these species have no functional V2R genes.

Trace amine-associated receptors

TAARs are G protein-linked olfactory receptors tuned to the detection of volatile amines. TAARs are activated by amines such as 2-phenylethylamine (2-PEA), *p*-tyramine (TYR) and tryptamine (T1AM). Fifteen TAAR genes are present in mice and six in humans. TAARs mediate aversion or attraction toward volatile amines. TAAR1 is not an olfactory receptor but rather acts on neurons to regulate their excitability as a result of agonistic trace amines. The other TAARs act as olfactory receptors. TAAR4 detects 2-PEA, the odor produced in large amounts by the big cats. 2-PEA appears to be a feline pheromone and, as in other examples reported here, elicits different behaviors in predators and prey. TAAR5 detects the mouse odor, trimethylamine. Interestingly, this molecule is generated by microbial activity on molecules such as choline or L-carnitine (Bain *et al.*, 2005) and repels rats specifically.

Formyl-peptide receptors (FPR)

FPRs recognize formyl-peptides derived from bacteria. They are expressed on many cell types but predominantly on neutrophils and macrophages. FPRs are generally assumed to participate in leukocyte chemotaxis and the respiratory burst (Smole *et al.*,

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2020). Members of the FPR family are also expressed by sensory neurons in the VNO. In general, FPRs recognize ligands similar to those detected by leukocytes (Weiss and Kretschmer, 2018).

Guanylyl cyclase receptors detect three agonists, CO_2, CS_2, and natriuretic peptides (Ihara *et al.*, 2013). They have not been associated with immune functions.

Olfactory receptors on other cell types

Olfactory receptors are not restricted to the nasal epithelium. They are expressed on many different cell types. For example, TAARs are expressed on human leukocytes. The amines, 2-PEA, TYR, and T1AM, modulate blood pressure, cardiac function, brain monoaminergic systems, and olfaction-guided behavior by specifically interacting with TAARs. Blood neutrophils and T and B cells express TAAR1 and TAAR2. Both receptors are co-expressed in a subpopulation of neutrophils, where they are required for migration toward the TAAR1 ligands 2-PEA, TYR, and T1AM. The same amines, with similar potencies, trigger cytokine or Ig secretion by T or B cells. 2-PEA regulates mRNA expression of 28T cell function-related genes (Ihara *et al.*, 2013). Expression of TAAR1 and TAAR2 is increased in response to leukocyte stimulation (Bufe *et al.*, 2012; Weiss and Kretschmer, 2018). The TAAR1 ligands 2-PEA, TYR, and 3-iodothyronamine are chemoattractants for neutrophils and require the presence of both TAAR1 and TAAR2 to elicit effects (Babusyte *et al.*, 2013). The dependence on TAAR2 may be due to heterodimerization with TAAR1 (Babusyte *et al.*, 2013). TAAR1-mediated increases in interleukin-4 secretion from T-cells occur in response to either 2-PEA, TYR, or 3-iodothyronamine, while decreasing the expression of secreted phosphoprotein 1 (Babusyte *et al.*, 2013). Such responses are consistent with TAAR1-mediated differentiation of T helper cells into the Th2 phenotype and consequent B-cell activation. TAAR-mediated increases in IgE secretion by B cells have been reported in response to 2-PEA and TYR (Nelson *et al.*, 2007). TAAR1-mediated apoptosis of B cells has also been reported (Wasik *et al.*, 2012).

As noted above, members of the FPR family are also expressed on immune system cells. In mammals, FPRs are primarily expressed by immune cells, where they detect pathogenic and inflammatory chemical cues. Phylogenetic studies have indicated that only three changes in regulatory elements explain the complex expression patterns acquired by a receptor family that switched from sensing pathogens inside the organism to sensing the outside world through the nose (Dietschi *et al.*, 2017). This however raises the possibility these vomeronasal receptors may play a role in 'smelling' infection and cancer. Potent FPR-agonists are expressed by such pathogens as *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes*. Some bacteria such as *Bordetella pertussis* and *Haemophilus influenzae* produce FPR antagonists (Smole *et al.*, 2020). These are respiratory pathogens. Blockade of vomeronasal FPR may alter the contact behavior of animals and facilitate bacterial spread. Other FPR ligands include inflammatory mediators such as cathepsin G, annexin A1, and serum amyloid A. Annexin A1 is elevated in influenza-infected lungs and can bind to this virus. As a result, influenza virus may act as a FPR ligand and promote viral invasion (Smole *et al.*, 2020). The FPR ligand, serum amyloid A is especially interesting since it is an acute-phase protein elevated in inflammatory, allergic and infectious diseases (Smole *et al.*, 2020).

Odors and avoidance behavior

Signals from the ORs are carried to the olfactory bulb and then to the cerebral cortex and the limbic system. Presumably, the signal to the cortex triggers scent awareness whereas signals to the limbic system generate reflexive emotional and memory responses. Information regarding olfactory odors is stored in long-term memory and also emotional memory.

Pleasant and unpleasant odors activate different regions of the brain. Imaging by positron emission tomography or magnetic resonance imaging (MRI) demonstrates that the regions of the brain activated by odors differ according to their degree of pleasantness (hedonistic values) (Rolls *et al.*, 2003; Zarzo, 2011). A mixture of pleasant and unpleasant odors induce complex responses in many different brain regions (Grabenhorst *et al.*, 2007) with regional specialization based on the odorant hedonistic values (Fulbright *et al.*, 1998). For example, H_2S affects brain regions responsive to unpleasant odors. Presumably, the identification and avoidance of odors associated with unsafe food or air (or possibly disease) is an important skill (Schiffman and Williams, 2005).

One strategy to prevent infection (and predation) is to simply avoid pathogens and predators. Animals must be aware of and evade micro- and macro-predators to survive. The smell of a diseased or dead animal or the odor of a predator is powerfully repellent for a prey species. Biologically speaking, being eaten by a predator has the same consequences as dying from microbial invasion – micropredation. It is unsurprising therefore that the olfactory system is employed to detect and avoid both macro- and micro-predators.

The smells of sickness and infection

Social investigation is a key element in olfactory communication, which involves close approaches to conspecifics. When animals such as rodents, cats, and dogs meet, they undertake an initial olfactory investigation of each other. Environmental scent marking serves a similar function (Aulik *et al.*, 2012; Soso and Koziel, 2017). They obtain information regarding the spectrum of body odors emanating from the urine, scent glands, feces, and the mouth. This may provide information regarding gender, reproductive status, social status, relatedness, and health. The information gained determines either avoidance or further approach and essentially prevents unwanted surprises. Notably, odorant signals may provide information regarding the health status of others. Release of illness-related odors from another individual may inhibit further social investigation by healthy conspecifics (Penn and Potts, 1998*c*).

Physicians have long sought to diagnose diseases based on body odors. Some infections such as *Clostridium perfringens*-mediated gangrene generate obvious repellent smells. Other characteristic smells have been linked to plague, smallpox, yellow fever, typhoid, and diphtheria (Penn and Potts, 1998*c*; Tainmont, 2009). For centuries, the miasma theory, based on the detection of obnoxious scents, was the prevalent explanation for the cause of many infectious diseases (Karamanou *et al.*, 2012).

Innate immune responses, specifically inflammation and the acute-phase response, influence the release of odor cues, perhaps mediated by formylpeptide receptors, and provide information about health (bacterial or parasitic infection, stress, and tissue damage) (Wyatt, 2017). Inflammatory processes also have a profound impact on social behavior, through a direct effect on the sick individual, such as reduced social interaction, and anhedonia.

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This dual impact of acute illness on both odor and behavior can minimize pathogen transmission between individuals (Arakawa *et al*., 2011).

Such disease avoidance has been demonstrated in humans. Innate immunity transiently activated by injection of volunteers with bacterial lipopolysaccharide (LPS)-induced significant discomfort and malaise in the recipients. Body odor samples (worn white T-shirts) were taken from after LPS-injection or saline-injection. The shirts and appropriate facial photographs were subsequently presented to a separate group of participants who smelled them and rated their liking of the odor during fMRI scanning. Faces were less attractive to the participants when in the presence of sickness, and sick body odors. Sickness status, indicated by odor and facial photographs resulted in increased neural activation of odor- and face-perception networks, respectively. Taken together, these results suggested that multiple integrated neural mechanisms are involved in the detection of disease cues (Regenbogen *et al*., 2017). Primates are vision-oriented animals and in general have fewer functional olfactory receptors when compared to other mammals; perhaps, primates rely more on visual cues than olfactory ones when detecting sickness/infectivity (Niimura *et al*., 2018).

In many species, females sense a complex mixture of male odors that provide information regarding their suitability as mates. These scents inform not only gender and sexual activity but also dominance status and food consumption. Odors can provide an effective indication of an individual's health status and especially parasite load. Selection of mates with low parasite burdens provides some assurance those individuals are genetically capable of developing resistance to parasites or infections (Penn and Potts, 1998*c*; Kavaliers *et al*., 2005*a*).

Detection of micropredators

Bacteria metabolize organic media to generate volatile organic compounds such as alcohols, terpenes, and aliphatic acids. These volatiles are released by the body in exhaled air, in body fluids, and directly by the surface microbiota. The composition of this mixture will depend upon the specific bacteria or parasites involved. Many of these volatiles will be detected and trigger avoidance behavior by conspecifics (Shirasu and Touhara, 2011).

Numerous studies have indicated that mice avoid sick conspecifics. Reduced attractiveness has been demonstrated in animals infected with the helminths *Heligmosomoides polygyrus*, *Taenia crassiceps*, *Trichinella spiralis*, and *Hymenolepis diminuta*. This effect is not simply due to the smell of worms because avoidance can also be provoked by protozoa such as *Eimeria vermiformis*, *Babesia microti*, and *Plasmodium chabaudi*; by bacteria such as *Salmonella enterica* Typhimurium; by viruses such as influenza, mouse mammary tumor, and tick-borne encephalitis viruses; as well as by the louse *Polyplax serrate* (Kavaliers *et al*., 2005*a*).

For example, female mice can distinguish between uninfected males and males infected with the nematode *H. polygyrus*. The urine of infected males was as attractive as that of water controls, suggesting the odor of infected mice simply loses its attractiveness (Penn and Potts, 1998*c*). This odor-based parasite avoidance behavior can be disrupted by deletions of the oxytocin gene and the genes for estrogen receptors (Kavaliers *et al*., 2004; Kavaliers *et al*., 2006). It is interesting to note these same gene knock-outs do not influence predator (cat) aversion behavior in mice, implying a different pathway for macropredator avoidance (Kavaliers *et al*., 2005*b*).

Several mechanisms may be involved in parasite scent recognition. Secondary changes in the intestinal microbiota may alter the composition of volatiles generated in the gut. Infection and inflammation may trigger odor changes as described above. Increased expression of MHC genes during infection may signal that the immune defenses are activated. MHC genes influence the concentrations of urinary volatile acids that also serve as sexual cues (Singer *et al*., 1997). In humans, assigning negative bias to an odor prior to an exposure results in the reporting of significantly more health-related symptoms following exposure, suggesting these symptoms are not mediated by the odor directly, but rather by an individual's cognitive associations between odor and health (Greenberg *et al*., 2013).

Some examples

Gas chromatography/mass spectrometry has made it possible to identify the volatile metabolites of *Mycobacterium tuberculosis*. Four distinctive volatile markers, methyl phenylacetate, methyl *p*-anisate, methyl nicotinate, and *o*-phenylanisole are produced by *M. tuberculosis* and *M. bovis* (Syhre and Chambers, 2008). Rats can identify these mycobacteria by smell with a high sensitivity and specificity.

It is perhaps appropriate that the term malaria means 'bad air'. Malaria parasites affect the attractiveness of their hosts to their mosquito (*Anopheles* spp.) vectors by altering body odor (Lacroix *et al*., 2005). Infected individuals attract more mosquitos, increase mosquito–host contacts and enhance transmission. Analysis shows there are quantitative differences in the composition of skin volatiles between infected and healthy children. *Plasmodium*-infected individuals produce more aldehydes, heptanal, octanal, and nonanal compared to non-infected individuals. Heptanal is especially effective at increasing the attractiveness of human scent to *Anopheles* mosquitos (Lacroix *et al*., 2005; Robinson *et al*., 2018). A similar process has been reported in Leishmaniasis where hamsters infected with *Leishmania infantum* were more attractive to female sandflies (*Lutzomyia longipalpis*) than are uninfected hamsters (O'Shea *et al*., 2002).

The feces of cholera patients have a characteristic sweetish odor due to the presence of dimethyl sulfide and *p*-menth-1-en-8-ol (Probert *et al*., 2009). Historically, physicians believed that infections that cause diseases such as diphtheria, scarlet fever, smallpox, typhoid, and yellow fever had characteristic odors (Shirasu and Touhara, 2011).

In addition to the smell of disease, many mammals avoid dead and decomposing animals. The volatile organic compounds associated with decomposition are generated by the microbiota as they break down the proteins of the corpse. For example, cadaverine is a diamine, (1,5-diaminopentane) ($NH_2(CH_2)_5NH_2$), produced by the microbial decomposition of animal tissues. It is also produced in small quantities in living animals and is partially responsible for the distinctive odors of urine. Putrescine, (1,4-diaminobutane) $NH_2(CH_2)_4NH_2$ is related to cadaverine, and is also produced by the breakdown of amino acids in living and dead organisms. The two compounds are largely responsible for the foul odor of decaying flesh.

Detection of macropredators

Early detection of macropredator-derived odors is essential if an animal is not to be eaten. The scent of blood is one of the most fundamental and survival-relevant of these olfactory signals. Its

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association with injury, danger, death, and nutrition ensures that its scent activates fundamental responses such as predatory approach behavior or prey-like withdrawal behavior, or both (Nilsson *et al.*, 2014). The smell of blood attracts predators to wounded prey while triggering avoidance and increased vigilance in prey species. Lipid peroxidation of blood generates *trans*-4,5-epoxy-(*E*)-2-decenal (E2D) that appears to be the predominant blood odor. Studies on captive predators showed that they respond to odorized wooden logs in a manner identical to their response to whole blood (Nilsson *et al.*, 2014). E2D will attract wolves while eliciting avoidance responses in mice and even in humans. A single invariant chemical cue guides behavior in both predator and prey. Interestingly, the perceptual thresholds for this odor are unimodally distributed for both sexes, with women being more sensitive. Furthermore, both women and men's emotional responses to the scent of blood divide strongly into positive and negative responses. For women, this split is related to the phase of their menstrual cycle and the use of oral contraception (Moran *et al.*, 2015).

Other volatile molecules are commonly produced by carnivores and elicit rodent avoidance behavior. The most important appears to be urinary 2-PEA (Trimmer *et al.*, 2019). Another is 2,5-dihydro-2,4,5-trimethylthiazole in fox feces. Both compounds induce the release of adrenocorticotrophic hormone (ACTH) or corticosterone in prey species reflecting an alarm response (Ihara *et al.*, 2013). 2-Propylthietane is a Gruenberg ganglion activator secreted by the stoat (*Mustela erminea*) anal gland (Brechbuhl *et al.*, 2013). Three sensory neuron groups, the VNO, the Gruenberg ganglion, and subsets of TAARs within the MOE have been implicated in predator odor aversion (Perez-Gomez *et al.*, 2015). The mammalian olfactory system has evolved multiple parallel mechanisms to detect predator odors that converge within the brain (the ventromedial hypothalamus) to induce a common behavioral response (Perez-Gomez *et al.*, 2015).

The smells of danger and fear

Within the body, microbial invasion results in the release of cell breakdown products, often called alarmins (Yang *et al.*, 2017; Kapurniotu *et al.*, 2019). These alarmins trigger innate immune responses. A similar process occurs outside the animal body. Alarm pheromones secreted by threatened or injured conspecifics have been identified in mice (Brechbuhl *et al.*, 2008). These molecules, generally heterocyclic sulfur or nitrogen-containing compounds are produced by predators (kairomones) and by stressed prey (alarm pheromones). One pheromone molecule that has been well-characterized is 2-*sec*-butyl-4,5-dihydroxythiazole (Lize *et al.*, 2013). Its structure is remarkably similar to some of the predator volatiles described above and it is specifically recognized by neurons in the Gruenberg ganglion. Sulfated sterols are secreted by mice subjected to restraint stress in their urine (Ihara *et al.*, 2013). These sulfated steroids are recognized by V1Rs in the VNO (Stein *et al.*, 2016).

Odors and attraction behavior

It is a good strategy to avoid others who may possibly be suffering from infectious or parasitic diseases, but this behavior cannot be taken to extremes. Animals must get close to mate. Mammals rely on olfactory signals to convey information including gender, reproductive status, individual identity, and health status.

As microbiota act as major sources of volatiles, they can modify the scent of an individual and affect both mate choice and kin recognition (Lize *et al.*, 2013). This has been extensively demonstrated in insects such as *Drosophila*, termites, and social wasps and likely occurs to a certain extent in vertebrates.

Scents play key roles in mediating reproductive behavior, both by recognition of the opposite sex and in assessing suitability as potential mates. The mechanisms that underlie female attraction to male scents involve an interaction between the main and vomeronasal olfactory systems to recognize four major types of scent. The most obvious of these are scents generated by the exocrine scent glands. A second family, the major urinary proteins (MUPs), provides a unique genetic identity that underlies individual recognition, assessment of male competitive ability, and kin recognition. The third family is encoded by the MHC. Familiar mates are recognized through MHC peptides and possibly by specific receptors.

The fourth system involves pheromones. Pheromones promote sexual maturity, induce ovulation, and reduce postpartum anestrus in rodents, pigs, and ruminants. By coordinating this information from all four sources, conspecifics can recognize and react to the scent of others and rapidly identify them without requiring physical contact. Key information that induces attraction to a male's scent is also mediated by non-volatile components detected through the vomeronasal system (Hurst, 2009).

Major urinary proteins

Mammals excrete multiple volatile MUPs that play a role in mate choice. MUPs are a family of proteins belonging to the lipocalin family encoded by 21 genes in mice (Ihara *et al.*, 2013). They are synthesized in the liver and their production is regulated by testosterone and growth hormone. MUPs can also bind small volatile urinary molecules and so may act as odorant signal carriers as well as signaling on their own.

Exocrine scent glands

In many mammals, specific scent glands convey sexual messages (Ihara *et al.*, 2013). Changes in these messages may result from underlying variation in the microbiota of these glands. Examination of the microbiota in the scent glands of wild hyenas has revealed that the structure of these microbial populations affects the volatile fatty acid profiles of scent secretions. Microbiota profiles differed between spotted and striped hyenas, and varied with sex and reproductive state (Theis *et al.*, 2012; Theis *et al.*, 2013).

Mate selection and the MHC

In addition to the well-recognized functions of MHC gene products in antigen presentation, the MHC also plays a role in the recognition and determination of mate suitability and social behaviors by influencing olfactory cues. MHC-mediated mating preferences have been recognized since 1976 (Yamazaki *et al.*, 1976) and have been confirmed in fish, birds and reptiles, and many mammals (Wittzell *et al.*, 1998; Wedekind and Penn, 2000; Sin *et al.*, 2015; Galaverni *et al.*, 2016). MHC gene polymorphism appears to determine a specific scent signature, which, when decoded by the olfactory system, may encourage (or discourage) pair formation. In general, mammals can detect the MHC haplotype of their congeners, and subsequently

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preferentially mate with individuals of a different haplotype and avoiding mating with others of an identical haplotype (Capittini *et al.*, 2008). Lower heterozygosity at the MHC is a possible marker for lower immune competence. It is well recognized that excessive homozygosity has detrimental effects on health and survival and is best avoided. In humans, this avoidance is in large part cultural. However, it is also mediated through olfactory cues. In effect, odor plays a potential role in mate selection. They thus maximize hybrid vigor (heterosis), the improved physical and physiological characteristics that occur as a result of hybridization in the F1 generation (Burke and Arnold, 2001).

Mice

Mice can discriminate odors from other mice that differ in only a single MHC haplotype (Penn and Potts, 1998*a*). Mice can also discriminate between two MHC haplotypes differing only in their peptide-binding site (Carroll *et al.*, 2002). The mechanisms of this discrimination are clearly olfactory as shown by cross-fostering studies. Thus, if mice are removed from their mother at birth and raised by a mother of a different MHC haplotype, their MHC preferences change and they will avoid mating with males of the foster family haplotype. These mice learn to recognize the MHC-associated odors associated with their own family, a process called familial imprinting (Penn and Potts, 1998*b*). Other MHC-regulated behaviors include kin recognition and pregnancy blockage after a changeover in dominant males (Slev *et al.*, 2006). Specific olfactory cues associated with the MHC and its genetic background can also be identified by rats (Eggert *et al.*, 1996; Penn and Potts, 1998*a*).

MHC class I peptides in urine clearly influence mouse behaviors. Adding new novel peptides to a male's urine greatly increased the levels of pregnancy block in females (Leinders-Zufall *et al.*, 2004). Peptides from a MHC dissimilar mouse added to urine from an MHC-similar mouse greatly increase its attractiveness to others (Spehr *et al.*, 2006).

Humans

Despite having only a vestigial VNO, humans can also evaluate MHC peptides in body odor (Milinski *et al.*, 2013). Human volunteers recognized the supplementation of their body odor by MHC peptides and preferred 'self' to 'non-self' peptides when asked to decide whether the modified odor smelled 'like themselves' or 'like their favorite perfume'. Functional magnetic resonance imaging indicated that 'self'-peptides specifically activated a region in the right middle frontal cortex. MHC-associated mate choice in humans is based on three broadly different aspects including odor preferences, facial preferences, and actual mate choice surveys. As in mice, most odor-based studies demonstrate dissociative preferences, although there is much variation in the strength and nature of the effects. In contrast, research on facial attractiveness indicates a preference for MHC-similar individuals. Olfactory and visual channels may work in a complementary fashion to achieve an optimal level of genetic variability (Havlicek and Roberts, 2009). Evidence suggests that MHC-dependent mate choice favors mates with dissimilar, diverse or specific genotypes non-exclusively (Spehr *et al.*, 2006).

Given that MHC homozygosity is likely to result in lower immune competence and is a marker for greater uncertainty in the future, then it may be expected to also result in 'faster' sexual strategies (Murray and Schaller, 2017). Remarkably, women who were more homozygous at the MHC were more favorable to short-term mating and began their sexual activity a year earlier than heterozygous women (Murray and Schaller, 2017).

Other MHC signals

MHC-linked signals can also affect male reproductive strategies. For example, stallions individually exposed over a period of 4 weeks, either to an unfamiliar 'MHC-similar' mare and then to an unfamiliar 'MHC-dissimilar' mare, or vice versa demonstrated changes in blood testosterone and sperm quality and quantity. Peripheral blood testosterone levels were determined weekly. Three ejaculates each were collected in the week after exposure to both mares and mean sperm number and sperm velocity were assessed. The highest testosterone levels developed when stallions were kept close to MHC-dissimilar mares and were significantly lower in stallions kept adjacent to MHC-similar mares. Mean sperm number per ejaculate was positively correlated with testosterone levels and was also affected by the order of presentation of mares: sperm numbers were higher if MHC-dissimilar mares were presented last than if MHC-similar mares were presented last. MHC signals, presumably olfactory in origin, also influence testosterone secretion and semen characteristics in horses (Setchell and Huchard, 2010; Burger *et al.*, 2015). MHC-encoded proteins may also guide spermatozoa along chemical gradients to their target, the oocyte (Ziegler *et al.*, 2010).

Mechanisms

Superficially, no obvious link exists between MHC molecules that act as peptide receptors in adaptive immunity and olfactory receptors that bind volatile ligands in the nose. However, such links do exist. One is the close proximity of a cluster of OR genes to the class I region of the MHC. The other is the fact that they share similar ligands.

Individual recognition is an important component of behaviors, such as mate choice and maternal bonding, and vital for reproductive success. Several types of chemosensory signals of individuality are influenced by the highly polymorphic families of MHC proteins or MUPs. Both can bind small peptides and may influence the chemosensory profile of an individual in biological fluids such as urine, skin secretions, or saliva. Moreover, these proteins, or peptides associated with them, can act on the VNO where they potentially interact directly with the vomeronasal receptors. This is particularly interesting given the expression of MHC Ib proteins by the V2R class of vomeronasal receptor and the highly selective responses of accessory olfactory bulb (AOB) mitral cells to strain identity. These findings are consistent with the role of the vomeronasal system in mediating individual discrimination that allows mate recognition in the context of the pregnancy block. This is hypothesized to involve a selective increase in the inhibitory control of mitral cells in the AOB at the first level of processing of the vomeronasal stimulus (Brennan, 2004).

It is possible to alter recognition of mouse MHC-associated odor by bone marrow allografts (Eggert *et al.*, 1989). As a result, the odors of the urine change! This implies that odor recognition is affected by the cells generated from bone marrow stem cells – essentially leukocytes. Many leukocytes express TAARs and FPRs as well as MHC molecules. Thus TAAR2 is found on blood polymorphonuclear phagocytes (PMNs), T cells, and B

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cells (Babusyte *et al*., 2013). This receptor modifies numerous T and B cell functions. A stem cell allograft could alter the TAAR profile of the recipient and thus possibly how it senses smells.

OR-MHC linkage

Almost all vertebrates appear to have one or more clusters of genes encoding ORs in close physical linkage to the MHC class I region. It has been suggested that these MHC-linked OR genes are involved in MHC-influenced mate choice. In addition, several olfactory receptor genes or pseudogenes are located within the class I region of the human MHC. At least one of these genes is intact, appears to encode an mRNA, and is homologous to a previously reported murine olfactory receptor (Fan *et al*., 1995).

A systematic comparison of DNA and protein sequences of ORs from the genomes of human, chimpanzee, gorilla, orangutan, rhesus macaque, mouse, rat, dog, cat, cow, pig, horse, elephant, opossum, frog, and zebra fish reveal a pan-vertebrate conservation of the evolutionarily conserved MHC-OR linkage. Each of the taxa studied (primates, rodents, ungulates, carnivores, proboscids, marsupials, amphibians, and teleosts) showed a typical architecture of MHC-linked OR genes. This conserved linkage between distinct OR genes and the MHC supports the concept that some alleles may function in a concerted fashion during mate selection (Santos *et al*., 2010*a*).

On the contrary, linkage disequilibrium studies between these OR genes and the MHC genes reveal reduced recombination but suggest this physical association is not so strong as to preclude assortment at the population level. Thus, it is likely insufficient to support selection of specific HLA-OR haplotypes (Thompson *et al*., 2010; da Silva *et al*., 2013).

It is, of course, possible that there are no gene interactions linking the MHC and the OR genes. Balanced polymorphisms may simply reinforce each other. Thus, the two gene clusters, while in close physical proximity may still have independent effects on fitness. The closeness of one locus to another under balancing selection may well permit the other to be retained indefinitely even when unlinked (Tennessen, 2018). As a result, it cannot be assumed that the ORs and the MHC are functionally linked.

Apart from MHC class I molecules, the products of several other genes within the extended MHC are candidates for involvement in mate choice, especially because the respective loci are subject to long-range linkage disequilibrium. Among these loci are polymorphic OR genes that are expressed not only in the olfactory epithelium, but also within male reproductive tissues (Kang *et al*., 2015).

MHC-derived peptides

The class I and II proteins of the MHC are heterodimeric cell surface receptors. They bind peptides derived from degraded antigens and present these peptides to antigen receptors (TCRs) on either helper or effector T cells. The antigen-binding sites of class I and class II molecules while structurally different, are optimized to bind and present nonapeptides. The peptides bound to MHC class I molecules are recognized by $CD8^+$ effector T cells. Conversely, peptides displayed by MHC class II molecules are recognized by $CD4^+$ helper T cells (Konig *et al*., 1992).

Peptides released from the MHC and subsequently excreted in the urine represent the diversity of peptide antigens stimulating the immune system through MHC-binding. These MHC-derived peptides are present in extremely low concentrations in mouse urine, but are recognized by olfactory receptors. Urine contains abundant peptides that differ between mouse strains as a result of single-nucleotide variations or complex polymorphisms. Thus, urinary peptides represent a sampling of the expressed genome. This peptide population may contain sufficient information for individual recognition (Overath *et al*., 2014).

Peptides dissociated from MHC molecules can be detected by the cells within the VNO (Leinders-Zufall *et al*., 2004). MHC-dependent peptides occurring at very low concentrations in mouse urine can be detected by the VNO and single amino-acid variations among peptides can be discriminated (Sturm *et al*., 2013). The VNO system detects relatively non-volatile chemosensory cues following direct contact. The VNO can respond highly selectively to non-volatile peptide ligands associated with the MHC, acting on V2Rs.

Mouse neurons expressing the vomeronasal receptor gene V2R1b detected MHC peptides at subpicomolar concentrations and exhibited combinatorial activation with overlapping specificities. In a single cell, peptide responsiveness was broad, but highly specific. Peptides differing by a single amino-acid residue could be distinguished. The MHC peptide presentation system may have co-evolved with the peptide recognition systems expressed by T cells and VSNs (Leinders-Zufall *et al*., 2009).

Neurons in the VNO bind peptides in a manner similar to MHC molecules (Leinders-Zufall *et al*., 2004; Leinders-Zufall *et al*., 2009). Short synthetic peptides known to bind to mouse MHC will activate V2R neurons. A given VSN can detect a range of peptides, even those associated with different haplotypes, and the peptide responses are exquisitely sensitive and specific. Some VSNs have (half-maximal effective concentration) EC_{50} values of $\sim 10^{-14}$ M. Any given peptide activates VSNs with heterogeneous peptide specificities. The specificity of the response to any specific peptide is unchanged over a wide range of peptide concentrations. Importantly, VSN receptors appear to be more specific than MHCs and may be able to discriminate a single amino acid substitution among peptides. Peptide recognition combines features of both the MHC and TCRs (Leinders-Zufall *et al*., 2009). Minor changes in the sequence of these peptides involving their anchor residues of the binding site resulted in a failure to stimulate the neurons. MHC peptides were subsequently found to be bound by MOE sensory neurons, activating them at subnanomolar concentrations. There were, however, some differences observed between the responses of the two organs. The signal transduction pathway was different. Changes in the anchor peptides reduced but did not abolish signaling and MOE recognition did not influence pregnancy blockage.

Leinders-Zufall *et al*. (2004) also demonstrated that mouse VNO receptors could detect MHC peptides at subpicomolar concentrations with combinatorial activation by peptides of overlapping specificities. Recognition was broad but very specific for a given peptide and mice could distinguish between peptides differing in a single amino acid (Leinders-Zufall *et al*., 2009).

TAARs also play a role in mammalian MHC-dependent mate choice. Female mate choice in bats (*Saccopteryx bilineata*) is based on MHC alleles but linked to variations in chemosensory TAARs with TAAR3-heterozygous females being more likely to choose MHC-diverse males (Santos *et al*., 2016).

Olfactory learning mechanisms, such as with increased noradrenaline, associated with mating, and with neonatal development, share features resulting in changes at the level of the olfactory bulb. These changes are likely to refine the pattern of activity in response to the learned odor, enhancing its

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discrimination from those of similar odors. Information from the main olfactory and VNO systems is integrated at the level of the corticomedial amygdala. Evidence suggests this region plays an important role in the learning and recognition of social chemosignals (Kwak *et al.*, 2008).

The profiles of some volatile compounds released by the body also show an MHC association. A small number of specific compounds as well as the profile of some ubiquitous volatiles act as MHC-associated odor cues. It may be that the pattern of volatiles other than specific odors serves as cues (Robinson *et al.*, 2018). Note that the MHC also influences volatile signals (Singer *et al.*, 1997) present as a mixture of volatile carboxylic acids whose composition varies based on the MHC. Ether extracts worked. No compounds were unique to any specific MHC, but their ratios were characteristic of MHC haplotypes. Eight peaks seemed to determine the phenotype. One was phenylacetic acid.

MUP-derived peptides

The studies on non-volatile MHC class I peptides described above raise questions about the importance of volatile MHC signals. MHC genes, however, can also influence the generation of volatile odorants, as odor type can be detected from a distance. Numerous volatile odorants that differ in abundance between mice of different MHC genotypes can be detected in mouse urine. In addition, urine samples from MHC-different mice evoke distinct odor-induced activity maps in the main olfactory bulbs. These volatiles have been tested for their ability to mediate chemosensory discrimination of MHC genotypes. Mice trained to discriminate between unadulterated urinary signals of the congenic mice generalize the discrimination, without reward or training, to the buffer solution containing the peptide-free urinary volatiles such as increased noradrenaline. Volatile signals, perhaps together with non-volatile ones, can also mediate behavioral discriminations of mouse MHC genotypes (Kwak *et al.*, 2009).

Dietary-derived peptides

Mice trained to discriminate urines from mice that differed both in diet and MHC type generally found the diet odor more salient. When mice were trained to discriminate among mice with only MHC differences (but on the same diet), they also recognized the MHC difference when tested with urines from mice on a different diet. Thus, MHC odor profiles can persist despite large dietary variation. Chemical analyses of urinary volatile organic compounds confirm this observation. Although there are clear dietary effects on urinary volatile profiles of mice, they do not obscure MHC effects (Winternitz *et al.*, 2017).

The benefits of MHC-related mate selection

MHC-associated mate choice is thought to provide offspring a fitness advantage through increased heterozygosity, to reduce susceptibility to infectious diseases. Avoidance of inbreeding coupled with the production of increased MHC diversity (more infectious agents may be identified) would reduce susceptibility to infectious diseases and increase the fitness of offspring. The extremely high variability in MHC genes in vertebrates is assumed to be a consequence of parasite-driven selection and mate preferences based on promotion of offspring heterozygosity at the MHC, and overall inbreeding avoidance (Boehm, 2006).

Conclusion

There are multiple, complex links between the olfactory and immune systems. The interaction between olfactory receptors, the MHC, and T cell antigen receptors together influence behaviors that promote the evasion of pathogens and predators, as well as influence the selection of possible mates based on genetic suitability and health. It makes sense to consider that the role of the olfactory system is to detect both noxious and pleasurable odors and to modify animal behavior accordingly. In this respect, the olfactory system may be considered an important component of the behavioral immune system. Why wait for microbial invasion when it can be avoided simply by staying away from the sick, dying, and dead?

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